**A REAPPRAISAL OF MESENCHYMAL-TO-EPITHELIAL
TRANSITION WITHIN THE ENDOMETRIUM**

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by
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LIST OF ABBREVIATIONS

Amhr2	Anti-Müllerian Hormone Receptor 2
β-gal	Beta-galactosidase
BrdU	Bromodeoxyuridine
CE	Coelomic Epithelium
CHD1	Epithelial cadherin, E-cadherin
CSPG4	Chondroitin Sulfate Proteoglycan 4
DAPI	4',6-diaminino-2-phenylindole
DNA	Deoxyribonucleic Acid
E ()	Embryonic Day ()
E-cadherin	Epithelial cadherin, CHD1
EpCAM	Epithelial Cell Adhesion Molecule
EMT	Epithelial-to-Mesenchymal Transition
ESR1	Estrogen Receptor 1
EYFP	Enhanced Yellow Fluorescent Protein
E2	Estrogen
FOXA2	Forkhead Box A2
FRT	Female Reproductive Tract

GE	Glandular Epithelium
GFP	Green Fluorescent Protein
H2B	Histone Molecule H2B
IGFBPs	Insulin-Like Growth Factor Binding Proteins
IGFBP-1	Insulin-Like Growth Factor Binding Protein-1
Ki67	Marker of Proliferation Ki67
KO	Knockout
LE	Luminal Epithelium
LHX1	LIM Class Homeo-Domain Transcription Factor
LIF	Leukemia Inhibitory Factor
MD	Müllerian Duct
MDE	Müllerian Duct Epithelium
MDM	Müllerian Duct Mesenchyme
MET	Mesenchymal-to-Epithelial Transition
mRNA	Messenger Ribonucleic Acid
N-cadherin	Mesenchymal Cadherin; CDH2
PDGFR β	Platelet Derived Growth Factor Receptor Beta
P ()	Postnatal Day ()

PGR	Progesterone Receptor
PRL	Prolactin
P4	Progesterone
RU486	Mifepristone; Progesterone Receptor Antagonist
SEM	Scanning Electron Microscopy
SM22 α	Smooth Muscle Protein 22-Alpha
TEM	Transmission Electron Microscopy
TRE	Tetracycline Response Element
tTA	Tetracycline Transactivator
TUNEL	Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling
WNT	Wingless-Type MMTV Integration Site
WT	Wild-type
X-gal	5-Bromo-4-Chloro-3-Indolyl- β -D-Galactopyranoside
[¹²⁵ I]IdUrd	5-[¹²⁵ I]iodo-2'1-deoxyuridine

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Abstract

A controversial topic, mesenchymal-epithelial transition (MET) is thought to be a mechanism involved in regeneration of the uterine epithelial layer following pregnancy and menstruation. Little is known about this process though, requiring further exploration. Previously, MET was thought to only occur as a damage-repair mechanism following parturition and menses-like events in mouse models. However, in the current study we hypothesized that MET would also occur in other endometrial epithelialization events as a mechanism of homeostatic epithelial turnover. To identify mesenchymal-derived cells within the adult uterine epithelium, an *Amhr2-Cre; Rosa26-EYFP* reporter mouse line was used. Mice were staged by vaginal cytology prior to the isolation of uterine epithelial and stromal cells, which were then stained with EpCAM to identify epithelial cells and analyzed by flow cytometry. EpCAM⁺YFP⁺ cells were identified in all stages of the estrous cycle except diestrus, indicating a proportion of epithelial cells were derived from the stroma (i.e., mesenchyme). Up to 80% of the uterine epithelia was EpCAM⁺YFP⁺ during estrogen-dominant stages (proestrus and estrus) of the cycle, with negligible amounts found during progesterone-dominant stages (metestrus and diestrus), suggesting this population may be responsive to estrogen. Uteri were also evaluated direct fluorescence and immunofluorescence in tissue sections. Immunofluorescence for EpCAM, forkhead box protein A2 (FOXA2), Ki67, estrogen receptor alpha (ESR1), and progesterone receptor (PGR) was performed to assess epithelial characteristics and potential functionality. To further investigate the role of MET

in epithelialization and assess the temporal origin of mesenchymal-derived epithelial cells, we evaluated key postnatal (P) developmental time points using *Amhr2-Cre; Rosa26-tTA; H2B-GFP* reporter mice. Flow cytometry data indicated that MET may initially occur immediately after birth at P 0.5, with results varying from negligible amounts (0.21%) to approximately 82%. Similar results were found at P 3, but with decreasing variation; the highest EpCAM⁺GFP⁺ population representing approximately 50% of the epithelium. Between P 3 and early adenogenesis at P 8, this population decreased to average less than 2%. By the completion of adenogenesis initiation at P 14, approximately 10% of epithelial cells were EpCAM⁺GFP⁺, suggesting MET may occur during adenogenesis initiation and is maintained through P 21. Together, these results suggest that MET may be a more ubiquitous mechanism of epithelialization than originally thought and is likely hormone regulated. This research will help elucidate the role of MET in uterine epithelialization with potential for insights into dysregulation of MET in diseases such as endometrial adenocarcinoma and endometriosis.

CHAPTER 1: REVIEW OF LITERATURE

1.1 Introduction

Occurring monthly, women shed the innermost uterine layer, the endometrium, approximately 400 to 500 times during their reproductive lifespan. With the ability to not only respond to hormonal changes during the estrous/menstrual cycle, but to rapidly regenerate and repair itself following parturition, and in higher primates, menses, the uterus continually demonstrates its highly dynamic capacity. Although not every species sheds the endometrium as women do, this layer still responds to the synchronized increase and decrease of ovarian steroid hormones, estrogen and progesterone, resulting in cellular turnover through proliferation and apoptosis. This cellular turnover occurs in two different cell types present within the endometrium: mesenchymal, stromal cells capable of migration in the stromal layer, and the epithelial cells within the static epithelial layer, where damage and epithelialization occur.

Epithelialization events occur during adenogenesis, in which a single layer of luminal epithelial cells invaginates into the stroma to form endometrial glands—beginning in gestation (humans) or soon after birth (mice)—and in adulthood during estrus, following menses and parturition. Uterine epithelial regeneration subsequent to menstruation and parturition is aided by multiple processes to ensure rapid repair of the tissue. Likely, one of these mechanisms involves the proliferation of epithelial stem cells to regenerate epithelia in damaged areas. These putative epithelial stem cells are thought to reside within the uterine epithelial glands, and historically, outgrowth of residual basal epithelial glands to

repopulate the luminal epithelium is the most widely accepted theory of epithelialization in women (Ferenczy, 1976). Originally suggested in 1967 (Baggish), recent evidence supports an additional mechanism, mesenchymal-to-epithelial transition (MET), contributes to endometrial epithelialization after tissue damage, though little is known about this mechanism (Gary et al, 2009, Huang et al, 2012, Patterson et al, 2013, Cousins et al, 2014).

MET is the process of a mesenchymal cell differentiating into an epithelial cell. Along with its reverse process, epithelial-to-mesenchymal transition (EMT), these cellular mechanisms are important during embryonic development, but contribute to disease and disorders in adults, such as cancer and endometriosis (Banyard and Bielenberg, 2016, Bartley et al, 2014, Matsuzaki and Darcha, 2011, Wilson et al, 2020). However, a reevaluation of menstrual epithelialization with histological techniques and scanning electron microscopy showed possible contribution of stromal cells to the luminal epithelium, suggesting MET may occur in this normal physiological event (Gary et al, 2009). More recently, studies have additionally suggested MET occurs within the endometrium following parturition in mice (Huang et al, 2012, Patterson et al, 2013), and support the likelihood of MET occurring in women following the shedding of the functional layer during menstruation demonstrated through the use of menses-like mouse models (Patterson et al, 2013, Cousins et al, 2014). Dual-staining with stromal and epithelial cell markers—vimentin and pancytokeratin, respectively—have also revealed cells co-expressing these markers and undergoing locational changes during the first 96 hours of menses-like endometrial regeneration, possibly

identifying mesenchymal-epithelial transitional cells (Patterson et al, 2013, Cousins et al, 2014). Although research has begun to elucidate the contribution of MET during the first few days of epithelial regeneration/repair, studies addressing the potential of MET as a mechanism in uterine epithelialization events outside of menses-like endometrial repair and following parturition do not exist. Additional studies during postnatal epithelialization events including adenogenesis and during the estrous cycle are necessary to address this gap in knowledge and are the focus of this thesis.

1.2 Uterine Structure and Histoarchitecture

Human and mouse uteri are both comprised of three tissue layers. The outermost portion of the uterus is the serosa, which lines the female reproductive tract and is contiguous with the broad ligament to support the tract within the body cavity. Spanning the uterus, the region closest to the broad ligament is termed the mesometrial pole, with the opposing side being the anti-mesometrial pole. Moving inward from the serosal layer is the myometrium, which is smooth muscle. Lastly, the inner-most layer is the endometrium consisting of stroma and epithelia. The epithelia can be further classified into the luminal epithelium (LE), lining the lumen of the uterus with a single layer of cells, and glandular epithelium (GE), extending into the stromal compartment. Morphologically, endometrial epithelial cells are polygonal in shape in contrast to underlying stromal cells which are spindle-like in shape. In women and higher primates that undergo menstruation, the endometrium is partitioned into the functionalis and basalis layers (**Fig. 1A**). The functionalis is the upper two-thirds of the endometrium and

Figure 1. Uterine Histoarchitecture and Proposed Cellular Mechanisms of Uterine Involution. **(A)** Comparison of human and mouse uterine histoarchitecture. The human endometrium is partitioned into functionalis and basalis layers. Although mouse endometrium do not have specified functionalis/basalis layers, they demonstrate similar characteristics including luminal epithelium (LE) and glandular epithelium (GE) extending into the stroma. **(B)** Legend for illustrated uterine histoarchitecture. **(C)** Schematic of proposed cellular mechanisms of uterine involution including 1.) replication of GE and LE, 2.) endogenous epithelial, stromal, and myometrial stem cells, 3.) mesenchymal-to-epithelial transition (MET) and 4.) exogenous bone marrow-derived cells. From Spooner et al, 2021. Reproduction 161, 3; 10.1530/REP-20-0425

is shed during menses. The lower one-third, closest to the myometrium, is the basalis, which remains during menses and serves as the seed for regenerating the functionalis (Padykula et al, 1989). Although not classified as such, mice have similar layers to the functionalis and basalis (**Fig. 1A**). The functionalis-like layer in mice is not shed during the estrous cycle, instead, there are cyclic changes in cellular proliferation and apoptosis resulting in waves of endometrial growth and regression (Wood et al, 2007). The functionalis-like layer is shed postpartum, however, and is then regenerated by the lower basalis-like layer. Additionally, the myometrium is comprised of two distinct muscle layers with fibers of opposing direction. These layers are the outer longitudinal and inner circular, which work together for contractile purposes during labor and parturition. The myometrium and endometrium of both species are also inundated with vasculature, lymphatics, and various leukocyte populations. Despite some differences in the histological make up of uteri between mice and humans, the two species share many qualities pertaining to the endometrium, making mice a reasonable animal model to use when addressing questions that would otherwise be unethical to perform in women.

1.3 Uterine Development

1.3.1 Müllerian Duct Development

Mesenchymal-to-epithelial transition (MET) and its counterpart, epithelial-to-mesenchymal transition (EMT), are fundamental during embryonic developmental processes such as gastrulation, neural crest development and organogenesis (Handyside, 1980, Ziomek and Johnson, 1980, Fleming, 1987,

Linask et al, 1997, Wiggan et al, 2002, Wiggan et al, 2006, Anani et al, 2014, Jackson et al, 2017.) Of importance though, is how MET affects development of the urogenital tract, specifically the formation and differentiation of the Müllerian ducts that subsequently give rise to majority of the adult female reproductive tract (FRT).

The FRT is composed of oviducts, uterus, cervix, and vagina. These tubular structures are derived from the Müllerian (paramesonephric) ducts with the exception of the lower vagina, which originates from the urogenital sinus. The male reproductive tract forms similarly via the Wolffian (mesonephric) ducts, which develop prior to the Müllerian ducts. Both Müllerian and Wolffian ducts form from the intermediate mesoderm and are both initially present in male and female embryos (Jacob et al, 1991, Kobayashi and Behringer, 2003, Spencer et al, 2005). Although Müllerian ducts develop similarly between various mammalian species as seen with Gruenwald's research using human and chick embryos (1941), a major difference in the fully formed FRT is the length of the uterine horns compared to the size of the uterine body. This variation is dependent on the degree of Müllerian duct fusion during development (Kobayashi and Behringer, 2003). Adult uteri are therefore classified based on the prominence of uterine horns and body including duplex (marsupials), simplex (higher primates), and bicornuate (bovine and equine) as a subset of examples.

In both sexes, the Wolffian ducts are completely developed in mice by embryonic day 10.5 (E 10.5), approximately a day before the Müllerian ducts begin developing on E 11.5. Müllerian duct development occurs in three stages:

initiation, invagination, and elongation (**Fig. 2**). Müllerian ducts were observed to derive from the coelomic epithelium (CE) which overlies the urogenital ridge (Gruenwald, 1941), but the mechanism remained poorly understood for several decades. Over 60 years later, studies have provided evidence to support this finding (Orvis and Behringer, 2007, Guioli et al, 2007) as well as develop the current model—CE cells that commit to the Müllerian duct epithelium (MDE) are located between the pronephros and mesonephros in a transitional area and are LHX1⁺ (LIM class homeo-domain transcription factor). The initiation stage begins with specification of LHX1⁺ CE cells at E 11.5, however, it is unknown what leads to expression of LHX1 (Kobayashi et al, 2004, Orvis and Behringer, 2007). LHX1⁺ cells then invaginate caudally towards the Wolffian duct (Kobayashi et al, 2004). Global deletion of *Lhx1* is embryonic lethal due to failure to establish maternal-fetal placental connections, and female embryos lacked Müllerian duct-derived structures (Kobayashi et al, 2004). Generation of MDE-specific *Lhx1* knockout (KO) mice was possible with use of *Wnt7a*-Cre mice (Huang et al, 2014). A time-lapse study using the epithelial-specific KO of *Lhx1* indicated that initiation and invagination occurred but developing Müllerian ducts did not elongate suggesting the mesoepithelial nature of invaginating cells may be affected. Epithelium was absent in adults except in shortened oviducts, and uterine mesenchyme remained undifferentiated due to the lack of interaction with epithelia and consequential mesenchymal-epithelial signaling (Huang et al, 2014).

Wingless-type MMTV integration site family members (*Wnts*) have been

Figure 2. Stages of Müllerian Duct Development. The Müllerian duct is derived from the coelomic epithelium (CE, brown cells). The first stage of Müllerian duct development is initiation of LXH1^+ CE (yellow) cells. LXH1^+ CE cells invaginate to form Müllerian duct epithelium (MDE). Elongation of the Müllerian duct occurs by proliferation of the growing tip (blue cells) until the Müllerian duct contacts and fuses with the urogenital sinus.

shown to demonstrate a critical role in Müllerian duct development and MET. A subset of *Wnts*—*Wnt4*, *Wnt5a*, and *Wnt7a*—are required for Müllerian duct differentiation and development (Vainio et al, 1999, Mericskay et al, 2004, Parr and McMahon, 1998). *Wnt4* is expressed in mesenchyme beginning at E 11 in both males and females (Vainio et al, 1999), but is expressed in both epithelia and mesenchymal cells postnatally (Hayashi et al, 2010). Specified LHX1⁺ cells invaginated caudally towards the Wolffian duct in response to *Wnt4* signaling from the mesenchyme (Kobayashi et al, 2004). Therefore, global deletion of *Wnt4* prevented Müllerian duct formation by inhibiting invagination of MDE and mesenchymal-epithelial interactions. Also expressed in mesenchyme, *Wnt9b* acts upstream of *Wnt4* and like ablation of *Wnt4*, *Wnt9b*-null mice lack reproductive structures derived from Wolffian or Müllerian ducts in males or females due to disrupted mesenchymal-epithelial interactions (Carroll et al, 2005). MDE-specific, *Wnt7a* expression was seen at E 11.5 (Prunskaitė-Hyyryläinen et al, 2016), becoming restricted to LE in the postnatal uterus (Miller and Sassoon, 1998, Hayashi et al, 2010). *Wnt7a*-null mice demonstrated abnormal morphology of the female reproductive tract and in both epithelial and mesenchymal differentiation. Histologically, female reproductive tracts of these mice showed disorganized myometrial layers, shallow stroma, lacked uterine glands, and demonstrated stratified epithelium in the uterus (Miller and Sassoon, 1998, Parr and McMahon, 1998). Similar to *Wnt4*, *Wnt5a* was expressed in Müllerian duct mesenchyme (MDM) by E 15.5 (St-Jean et al, 2019), and its expression in the stroma was critical for uterine gland formation postnatally

(Mericskay et al, 2004). *Wnt5a*-null mice also displayed abnormal FRT morphology but differed from *Wnt7a* mutant mice. *Wnt5a*-mutant mice developed normal oviduct and uterine horns, but posterior structures—cervix and upper vagina—were absent (Mericskay et al, 2004). Taken together, these studies indicate that proper spatio-temporal Wnt signaling is required for development, patterning, and maturation of the FRT.

The tip of the Müllerian duct continues to invaginate until it contacts the Wolffian duct at E 12.5, then begins to elongate next to the Wolffian duct with only a basement membrane separating the two structures (Orvis and Behringer, 2007, Guioli et al, 2007). This process of invagination and elongation with close association to the Wolffian ducts were also noted in both human and chick embryos by Gruenwald (1941). Because of the intimate contact between the elongating Müllerian ducts and the Wolffian ducts, the question arose whether the Wolffian duct epithelium contributed to the MDE. Using a Wolffian duct-specific Cre mouse line (*Hoxb7-Cre*), fate mapping was used to address this question. In *Hoxb7-Cre; Rosa26-LacZ* mice, MDE elongation was not aided by contribution of cells from the Wolffian duct, as no cells of the Müllerian duct expressed *LacZ*. Additionally, an *in vitro* urogenital ridge recombinant explant culture assay was developed by cutting the urogenital ridge of wild-type (WT) and *Lim1^{l2/+}* (LHX1; fate mapping of LHX1-derived cells reported by *LacZ* expression) into rostral and caudal halves and recombining *Lim1^{l2/+}*rostral/WT caudal sections. The elongating Müllerian duct was *LacZ* positive, demonstrating elongation occurred by proliferation of the growing tip instead of continual

contribution from CE or input of Wolffian duct cells, which were *LacZ* negative. Elongation in mice was complete by E 13.5 as the ducts contacted and fused with the urogenital sinus (Orvis and Behringer, 2007, Arango et al, 2008).

During elongation, MDE cells are mesoepithelial as they express both mesenchymal and epithelial markers in a distinct rostral to caudal pattern. At E 12.5, epithelial marker, pancytokeratin, was weakly expressed across the growing MDE, but E-cadherin (CDH1) was absent. Simultaneously, mesenchymal marker, vimentin, was strongly expressed in the growing tip of the MDE, but showed weak expression located at the rostral end. Upon completion of elongation at E 13.5, pancytokeratin expression remained and CDH1 expression became expressed within the apical region of the rostral cells; this was coupled with diminished vimentin expression throughout the duct, as well. Despite substantially weak expression of epithelial markers at this developmental point, the acquisition of apical/basal polarity, lumen, and simple columnar morphology of the cells rostral to growing tip demonstrate those of epithelial characteristics. The mesoepithelial transitional state of MDE cells is likely necessary for the cells at the growing tip to migrate, a mesenchymal characteristic, while the cells at the rostral region become stationary and engage in cell interactions through expression of CHD1, an epithelial quality. For the remainder of gestation, there were increased expression of additional epithelial markers while mesenchymal markers were no longer seen within the epithelium (Orvis and Behringer, 2007), becoming restricted to the respective regions of stroma and myometrium of the fetal and adult uteri (Orvis and Behringer, 2007,

Ni et al, 2018). This suggests that the mesoepithelial cells complete the process of MET and fully differentiate into an epithelial cell type by birth.

1.3.2 Overview of Postnatal Uterine Maturation

At birth (postnatal day 0.5), the mouse uterus is an aglandular, single layered epithelial tube surrounded by undifferentiated mesenchyme (**Fig. 3A**). From birth to postnatal day (P) 5, the mesenchyme begins to differentiate into stromal cells nearest the epithelium and the outer smooth muscle layers of the myometrium (Brody and Cunha, 1989). By P 10, distinct stromal, and circular and longitudinal smooth muscle layers are easily identifiable (Brody and Cunha, 1989, Cunha et al, 1989). Although the epithelium is undifferentiated at birth, scanning electron microscopy (SEM) revealed the presence of two morphologically distinct cell types: cells with small, round and convex surfaces and cells with larger, flat surfaces, both having microvilli (Plapinger, 1982). One of the earliest postnatal epithelialization events is the formation of endometrial glands, termed adenogenesis (discussed in detail below). Adenogenesis begins around P 5 and budding glands are visible at P 8 (Vue et al, 2018, Vue and Behringer, 2020) (**Fig. 3B**). By P 15, the histoarchitecture of the uterus resembles that of the adult with distinct, differentiated epithelial, stromal, and myometrial layers (Hu et al, 2004) (**Fig. 3C**). Interestingly, the two cell types identified by SEM are no longer morphologically distinct but rather only differ by size (Plapinger, 1982). Peripubertal uteri at P 21 continue with glandular morphogenesis, and the uterus is similar to the adult, although smaller in size (Vue et al, 2018) (**Fig. 3D**).

Figure 3. Uterine cross-sections during postnatal maturation. Nuclei were stained using 4',6-diaminino-2-phenylindole (DAPI). **(A)** Postnatal day (P) 0.5, epithelium consists of a single layer of luminal epithelium at birth; **(B)** P 8, during glandular formation (adenogenesis). Uterine epithelia demonstrate bud- and teardrop-shaped glands; **(C)** P 14, uterine histoarchitecture represents that of the adult, but glandular morphogenesis continues during this time point; **(D)** P 21; the uterus represents that of the adult, though smaller in size.

Proliferation of uterine cells and subsequent growth are independent of ovarian hormones neonatally (Ogasawara et al, 1983, Bigsby and Cunha, 1985, Branham et al, 1995). No difference in labeling of proliferative cells by 5-^[125I]iodo-2'-deoxyuridine (^[125I]IdUrd) or uterine weight occurred between intact and ovariectomized neonatal mice, supporting that neither estrogen nor progesterone are required for normal uterine growth postnatally until approximately P 25 (Ogasawara et al, 1983). When both groups were given exogenous estradiol, uterine weight and ^[125I]IdUrd uptake significantly increased compared to control groups but again there was no difference between intact and ovariectomized mice. Additionally, mice that were ovariectomized on P 0 or P 25 and allowed to age to P 40 were able to respond to exogenous estradiol. Uterine growth, measured by weight and ^[125I]IdUrd labeling, did not differ between the two groups, indicating the absence of ovaries and corresponding hormones beginning at P 0 or P 25 did not affect the ability of the adult uterus to respond to estrogen administration. Proliferative activity in response to administration of progesterone differs from estrogen in the postnatal uterus in that it was cell type specific. Mice given progesterone between P 2 and P 10 revealed increased stromal proliferation while inhibiting epithelial proliferation (Bigsby and Cunha, 1985, Filant et al, 2012), and epithelial proliferation inhibition was found 18 hours following administration in mice aged P 5 (Bigsby and Cunha, 1985). Furthermore, administration of estradiol or progesterone to neonates delayed adenogenesis and reduced the number of glands present in the adult (Stewart et al, 2011, Dhakal et al, 2015), but adults lacked uterine glands completely with

progesterone administration during P 3 through P 9 (Cooke et al, 2012). These studies also demonstrate that although ovarian hormones are unnecessary for postnatal uterine maturation, the postnatal uterus is able to respond to exogenous estrogen and progesterone, and administration during key postnatal events, like adenogenesis initiation, negatively affect the adult uterus. It is not until increased hormone production and feedback signaling through the hypothalamic-pituitary-gonadal axis and initiation of the onset of puberty around P 30 (Swerdlow and Odell, 1975, DiVall and DiBlasi, 2018, Day et al., 1987, Safranski et al, 1993, Piekarski et al, 2017) that cyclic cellular changes in response to ovarian hormones occur.

1.3.3 Adenogenesis

Having a crucial role in the maintenance of pregnancy, uterine glands and their secretions are pertinent for the establishment and maintenance of pregnancy (Kelleher et al, 2019). Adenogenesis, occurs postnatally in rodents (described above) and begins during gestation in humans (Grey et al, 2001 and Cooke et al, 2013). Initial studies documenting the timeline of adenogenesis in mice indicated that at P 3, the uterine epithelium was still undifferentiated. But, by P 6, there was budding from the luminal epithelium into the stromal compartment indicating adenogenesis had begun (Hu et al, 2004). More recently, 3D analysis was performed at select times between P 0 and P 21 revealing a more detailed timeline of key events in adenogenesis. According to these studies, adenogenesis began as early as P4 with initiation of budding from the luminal epithelium, and by P 8 there was a mixture of bud-shaped and

teardrop-shaped glands (Vue et al, 2018, Vue and Behringer, 2020). With these analyses, identification of the uterine rail was possible—a region of epithelium that runs along the mesometrial pole that does not form glands. By P 15, initial gland formation was complete and was followed by morphogenesis of these glands (Vue et al, 2018). During morphogenesis, glands first elongate, then become sinuous in morphology around P 21, and finally branch as they continue to mature into adulthood (Vue et al, 2018).

Adenogenesis has also been evaluated by expression of forkhead box protein A2 (*Foxa2*). *Foxa2* was shown to be expressed in uterine glands of various species, including mice and humans, although the extent of knowledge regarding *Foxa2* in humans is lacking due to ethical considerations (Villacorte et al, 2013). In mice, detection of *Foxa2* mRNA by *in situ* hybridization began as early as P 3 (just prior to bud initiation) and was localized to the cytoplasm of the undifferentiated LE, and interestingly, was also present in the cytoplasm of differentiating mesenchymal cells. This pattern of expression continued to be observed through P 15 in both cell types. However, at P 9, when both bud- and teardrop-shaped glands were present, *Foxa2* was nuclear, and shortly thereafter at P 12 and P 15, Ki67 expression increased in the epithelia as an indicator of proliferation. By P 28, *Foxa2* expression was solely restricted to the nuclei of GE and continued as such throughout adulthood (Dunlap et al, 2011).

As with Müllerian duct development and differentiation, *Wnts* are necessary for gland formation in the postnatal mouse. Global deletion of *Wnt7a* embryonically resulted in an abnormal anteroposterior phenotype of the FRT

such that morphologically and histologically, proximal structures represented distal structures (e.g., oviducts represented uterine characteristics), and a glandless uterus in the adult (Miller and Sassoon, 1998, Parr and McMahon, 1998). However, the effect of deleting *Wnt7a* postnatally on the developing uterus was unclear. To investigate the roles of *Wnt7a* and *Foxa2* in the postnatal uterus, specifically their effects on adenogenesis, these genes were conditionally ablated using *Pgr-Cre* mice (Dunlap et al, 2011, Jeong et al, 2010). At P 3, preceding adenogenesis initiation, PGR was expressed in the uterine epithelia and stroma by P 7 (Franco et al, 2008, Kurita et al, 2001) thus genes of interest were deleted beginning at these times points. Both conditional deletions of *Wnt7a* and *FOXA2* in the neonate resulted in glandless uteri, suggesting that they are important to the process of adenogenesis (Dunlap et al, 2011, Jeong et al, 2010, Kelleher et al, 2017). As adults, mice lacking uterine glands were infertile due to implantation failure, reduced decidual response and decreased or lack of expression of genes required for uterine receptivity and implantation (Dunlap et al, 2011, Jeong et al, 2010, Kelleher et al, 2017).

As mentioned previously, *Wnt5a*-null mice also demonstrated abnormal anteroposterior phenotype. Like *Wnt7a*-null mice, mice lacking *Wnt5a* were unable to form glands (Mericskay et al, 2004). Interestingly, Mericskay and others (2004) utilized tissue recombination with *Wnt5a*-null and WT mice where *Wnt5a*-null epithelia were recombined with WT mesenchyme, and vice versa. Glands did not form in recombined WT epithelia/*Wnt5a*-null stroma, but adenogenesis was rescued in *Wnt5a*-null epithelia/WT stroma tissue. This would

suggest that WNT5a is required in the stroma and acts to signal to the epithelia to initiate adenogenesis. The study also showed *Wnt7a* was restricted to the luminal epithelium postnatally and was not found in GE. With this, they hypothesized that *Wnt5a* was required within the stroma to downregulate *Wnt7a* in regions of the LE that form glands (Mericskay et al, 2004). This study further supports that epithelial-mesenchymal interactions are necessary for correct postnatal development in addition to embryonic development.

Information relating to the development of the FRT in humans is limited, though in 1973, some light was shed on the development of the uterus from late fetal life through late adolescence (Valdes-Dapena). Gross anatomy demonstrated little growth of the uterine body during late gestation and early life, but within this time, morphological changes occurred. Although majority of adenogenesis occurred postnatally in humans (Valdes-Dapena, 1973, Grey et al, 2001), initiation occurred during gestation: no glands were seen at 23 weeks of gestation via histological sections, but budding was seen by 26 weeks of gestation (Valdes-Dapena, 1973). Adenogenesis continued, resulting in elongated glands that were torturous in nature, and representative of adult glands, by puberty (Valdes-Dapena, 1973).

1.4 Uterine Cycles

1.4.1 Menstrual Cycle

The menstrual cycle has been standardized to 28 days and is divided into three phases: menses, proliferative and secretory. The first day of menses is designated day 1 of the menstrual cycle and ensues with the shedding of the

functionalis lasting 2-7 days (Padykula et al, 1989). The proliferative phase follows menstruation up to day 14 and is characterized by epithelial and stromal proliferation in response to increased circulating estradiol levels. This proliferative response causes the endometrium to thicken substantially from 1-4 mm to 11 mm (Nalaboff et al, 2001), and simple uterine glands become tortuous extending the length of the endometrium (Rock and Bartlett, 1937, Garry et al, 2010). Serum estrogen levels are dominant during the proliferative phase leading up to ovulation on day 14, at which time they decline rapidly. Progesterone levels are low but rising during the proliferative phase and dominate in the post-ovulatory, secretory phase of the menstrual cycle from days 14-28 (Sherman and Korenman, 1975, Apter et al, 1978). Ciliated and secretory epithelial cell populations are not static throughout the menstrual cycle, with ciliated cells primarily located around glandular openings following menses and increasing in number throughout the LE at the end of the proliferative phase. In the secretory phase, secretory cells within the epithelia developed mature microvilli, giant mitochondria, glycogen particles and ribosomes. They then acquired the ability to produce secretions seen by cytoplasmic budding and secretory droplets with scanning electron microscopy (Ferenczy and Richart, 1973). Under progesterone stimulation, the endometrium also continues to thicken until reaching its peak measurement of 16mm during the mid-secretory phase (Nalaboff et al, 2001). This thickening is due to distention of glands caused by increased secretory activity of GE (Hempstock et al, 2004), stromal edema and stromal decidualization (Teacher, 1930).

Spontaneous decidualization of stromal cells in humans is induced by progesterone, which as previously stated, is the dominant ovarian steroidal hormone at this time (Daly et al, 1983, Brosens et al, 1999). This has been shown *in vitro* utilizing isolated stromal cells from patient endometrial samples collected during the proliferative phase, cultured and treated with estradiol, progesterone, or a combination of estradiol and progesterone. In treatments of progesterone alone or with the addition to estradiol, decidualization occurred, but decidualization was delayed with addition of estradiol (Daly et al, 1983). Decidual cells become enlarged and rounded in shape, and gain gap junctions, and are described as being epithelial-like (Kerns and Lala, 1983). These specialized cells must produce factors including leukemia inhibitory factor (LIF), prolactin (PRL), and insulin-like growth factor binding protein-1 (IGFBP-1), among others to be competent decidua and support an implanting embryo (reviewed by Vinketova et al, 2016). Without implantation of an embryo during the window of receptivity in the secretory phase, the decidua is shed during menses and the endometrium undergoes regeneration thus continuing the cycle.

1.4.2 Estrous Cycle

In mice, the estrous cycle lasts approximately 4 to 5 days and is composed of 4 stages—proestrus, estrus, metestrus, and diestrus. As seen in the menstrual cycle, ovarian steroids, estrogen and progesterone, are dominant hormones during different stages of the estrous cycle. Proestrus and estrus stages correspond to the estrogen-dominant follicular phase of the ovary, while

metestrus and diestrus are progesterone-dominant stages corresponding to the ovarian luteal phase (Walmer et al, 1992, Wood et al, 2007).

Prior to ovulation, there is increased proliferation during proestrus in response to elevated serum estrogen concentrations: Wood and colleagues used immunohistochemistry to determine proliferative cells with PCNA and apoptotic nuclei with *in situ* DNA end labeling. The authors noted a trend of high luminal epithelial proliferation and stromal proliferation correlated with high serum estrogen concentrations during proestrus or estrus. In contrast, the greatest amount of apoptosis was noted in metestrus with the LE demonstrating a percentage of apoptotic nuclei approximately ten times greater than the GE or stroma (2007). Unlike women, mice do not demonstrate decidualization in the absence of pregnancy but do express similar decidual markers like prolactin (Monice et al, 2001). Because there is no decidualization, the endometrium is not shed as seen during the menstrual cycle, however, there is coordinated apoptosis and proliferation resulting in growth and reabsorption of endometrial tissue throughout the cycle (Wood et al, 2007).

Cycle stages can be determined by circulating serum concentrations, visual assessment of the vaginal opening, or vaginal cytology. Most commonly used, vaginal cytology shows varying proportions of nucleated epithelial cells, cornified epithelial cells, and leukocytes are indicative of different cycle stages. Proestrus largely consists of nucleated epithelial cells, estrus distinctively shows cornified epithelial cells, metestrus has all three types of cells and diestrus predominantly consists of leukocytes (Byers et al, 2012, Caligioni, 2009,

Ekambaram et al, 2016, Ajayi and Akhigbe, 2020). It is important to note that although these four stages are referenced throughout the scientific community, they are not absolute—transitional stages occur between each stage, easily seen with the short estrous cycle length in rodents.

1.5 Uterine Repair

1.5.1 Epithelialization During Menses

As mentioned previously, the luteal phase of the menstrual cycle is influenced by dominant levels of progesterone followed by a substantial decrease in the steroid hormone nearing the end of the luteal phase due to regression of the corpus luteum. It is widely accepted this sharp decline in progesterone induces multiple pathways leading to menstruation in women (Slayden and Brenner, 2004, Patel et al, 2014). Progesterone loss results in vascular changes, promotion of inflammatory response through chemokines, and increased expression of matrix metalloproteinases (MMP) thus contributing to tissue destruction and subsequent shedding of the functionalis (Rock and Bartlett 1937, Critchley et al, 1994, Jones et al, 1997, Milne et al, 1999, Salamonsen et al, 1997). In response to the upregulation of inflammatory factors, an initial increase in leukocyte number, and their migration from the stroma to clear debris from the sloughing tissue is noted immediately prior to menses and during the first few days (Rock and Bartlett, 1937, Ludwig and Spornitz, 1991, Garry et al, 2010). Concomitantly with tissue degradation due to necrosis, is an increase in the number of apoptotic cells within the functionalis in menstruating primates and humans (Xu et al, 2007). With sloughing of the functionalis layer, tissue repair

and regeneration ensue. Of significance is epithelialization of the endometrium: in the degenerated state, the endometrium is essentially an extensive wound and rapid epithelialization is needed to prevent infection and continued hemorrhage. Epithelial repair occurs through the rise of novel epithelial cells in the form of islands over the denuded area and from the mouths of the uterine gland stumps in the thin basalis layer. Using SEM, mature ciliated cells were visualized in residual gland stumps during early epithelialization, but ciliogenesis in *de novo* epithelial cells did not occur until days 7 and 9 (Ludwig and Metzger, 1976, Ludwig and Spronitz, 1991). Repair and epithelialization occur concurrently with the shedding of the endometrium beginning on day 2, and novel surface epithelium covers over 2/3 of the denuded area by day 4 of the menstrual cycle (Garry et al, 2009, Ferenczy, 1976, Ludwig and Metzger, 1976, Ludwig and Spornitz, 1991).

Menses-like mouse models have been critical in studying endometrial tissue regeneration that mimics menses in women. Timed mating with vasectomized males induces pseudopregnancy in female mice and maintains increased progesterone levels. Day 0.5 begins at observation of a vaginal plug indicating the female was mated. On Day 4, mice are anesthetized for sesame oil injection into the lumen of the uterine horns to induce decidualization. Mifepristone (RU486), a progesterone receptor antagonist, is administered subcutaneously on days 7.5 and 8.5 (4 and 5 days after sesame oil injection) to induce shedding of the endometrium. This can also be induced by ovariectomy to remove progesterone stimulus on the decidua. Endometrial shedding in the

menses-like model simulates menses in women in that the shedding occurs progressively, initiates an immune response, and results in rapid repair (Finn and Pope, 1984, Brasted et al, 2003, Xu et al, 2007, Cousins et al, 2014). An alternative method to the use of timed mating with vasectomized males is ovariectomizing and giving coordinated injections of estrogen and progesterone to prime the uterus. In place of progesterone injections, a progesterone implant can be placed subcutaneously and removed following decidualization to induce endometrial shedding (Cousins et al, 2014).

1.5.2 Epithelialization Postpartum

To maintain a successful pregnancy, the stroma must differentiate into decidual cells, and without proper decidualization, the uterus is unable to sustain and support a pregnancy in both women and mice (Kara et al, 2007). Decidua provide protection to the embryo from being recognized as foreign by the maternal immune system, and to the mother by limiting the amount of invasion that occurs (Verma et al, 2005, Hiby et al, 1997). Production of prolactin (PRL), leukemia inhibitory factor (LIF), and insulin-like growth factor-binding proteins (IGFBPs) to support an implanting embryo and developing fetus also occur (Rosenberg et al, 1980, Bryant-Greenwood et al, 1987, Golander et al, 1988, Sawai et al, 1997, Rutanen et al, 1986, Clemmons et al, 1990). Both mice and humans have intrusive implantations in which embryonic trophoblasts displace the LE and invade into the decidualized stromal cells of the endometrium by destroying the basement membrane (Schlafke and Enders, 1975, Bischof and Campana, 1996, Li et al, 2015). Although they differ slightly in the extent of

invasion, structure, and number of trophoblast layers, both species exhibit a hemochorial placenta—the most intimate relationship between mother and fetus. Women demonstrate greater amounts of remodeling due to invasion occurring through the stroma to the myometrium and including remodeling of spiral arteries (as reviewed by Schmidt et al, 2015, Rossant and Cross, 2001).

As with menses, the process following parturition in which the uterus returns to its pre-gravid state, termed uterine involution, requires an extensive amount of remodeling and epithelialization. Endometrial regeneration and myometrial remodeling are required to return the uterus to its original state and size before pregnancy. In 1953, Sharman observed the appearance of glands lined with cuboidal epithelial cells in the non-placental regions in women 4 days postpartum. By day 7 postpartum, the non-placental site had distinct LE and was almost devoid of decidual cells within the stroma. At the placental site, blood clots and leukocytes predominated early on, but similar repair as the non-placental site began with few glands comprised of squamous shaped epithelium and a fibrous covering over the denuded stroma. By Day 14, epithelialization of the entire endometrium was complete, and this was followed by stromal repair and expansion (Sharman, 1953).

In postpartum mouse models, proliferative and apoptotic cells have been identified within the endometrium following damage using bromodeoxyuridine (BrdU) (Huang et al, 2012 and Cousins et al, 2014) and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assays, respectfully (Huang et al, 2012). Few stromal and LE cells were positive for

BrdU at the time of progesterone withdrawal but increased in both layers by 4 hours after withdrawal. At 24 hours following progesterone withdrawal, BrdU labeling was detected in cells lining the basal glands with immunohistochemistry (Cousins et al, 2014). Not surprisingly, a shift from increased apoptosis to proliferation occurred following parturition as endometrial repair continued (Huang et al, 2012). Specifically, more cells located within the mesometrial pole were shown to express BrdU, indicating the mesometrial pole undergoes greater proliferation than the anti-mesometrial pole (Huang et al, 2012). Since implantation occurs within the mesometrial pole, this likely is indicative of the required rapid repair that must take place within this region of the uterus postpartum.

Hypothesized uterine repair mechanisms contributing to epithelialization following menses and parturition include: 1.) replication of residual GE and LE, 2.) uterine stem cells (epithelial, stromal and myometrial, 3.) exogenous bone marrow-derived cells, and 4.) mesenchymal-to-epithelial transition (MET) (**Fig. 1B and C**). The focus of this thesis is MET during uterine epithelization and differentiation events in postnatal and adult uteri.

1.6 Mesenchymal-to-Epithelial Transition (MET) in Health and Disease

Mesenchymal-to-epithelial transition (MET) is the change of a mesenchymal cell type to an epithelial cell type—regarding the uterus, MET is the transition of a stromal cell into either a luminal or glandular epithelial cell. Mesenchymal cell types are known for their migratory and invasive properties. These cells are spindle-like in shape, demonstrate little-to-no apical-basal

polarity, and do not have strong cell-to-cell adhesions thus aiding their migratory capability. Conversely, epithelial cell types are stationary, with strong cell-to-cell adhesions through cell adhesion molecules on the cell surface, tight junctions, and integrins which interact with components of the basement membrane allowing for greater stability. Epithelial cells also demonstrate apical-basal polarity and are polygonal in shape. During MET, mesenchymal cells differentiate into epithelial cells through the acquisition of epithelial characteristics and loss of mesenchymal characteristics; vice versa is seen in the reverse process, EMT (as reviewed in Thiery and Sleeman, 2006). MET and EMT are critical processes that take place embryonically during gastrulation, neural crest development and organogenesis (Thiery, 2002, Thiery et al, 2009) and are also implicated in uterine diseases. Evidence is growing to support a role for MET in non-pathological endometrial events, specifically epithelial repair postpartum and/or during menstruation.

1.6.1 MET and EMT in Disease and Disorders

Despite MET and EMT being critical to physiologically normal processes during embryonic development, when these mechanisms are exploited and dysregulated, diseases and disorders occur. MET has been hypothesized to be a contributing mechanism to uterine cancer and endometriosis, but it is still unclear how it is dysregulated.

Uterine cancer has the 4th highest rate of new cases and 6th highest rate of deaths in the United States (CDC, 2017), and like other cancers, it can metastasize if not caught in early stages. To produce a secondary tumor away

from the primary site, cancer cells must travel to the new site and microenvironmental conditions must be favorable. Cancer cells may use EMT in which they lose epithelial characteristics, including the downregulation of epithelial cadherin (E-cadherin), to invade and travel through the lymphatic and vascular systems before the reverse process, MET, is required to set up a secondary tumor at a novel site (Banyard and Bielenberg, 2016).

Occurring in approximately 10% of women (Buck Louis et al, 2011), it has long been hypothesized that endometriotic lesions are a result of retrograde menstruation (Sampson, 1927a, Sampson, 1927b). A recent study developed a mouse model to address this theory. This menses-like mouse model was utilized to induce shedding of the endometrium: an incision was made at the utero-tubule junction in conjunction with a salpingectomy to allow endometrial fluid and tissue access to the peritoneal cavity. Over 50% of mice showed visible lesions, adding credibility to retrograde menstruation contributing to endometriosis in an *in vivo* model (Wilson et al, 2020). Interestingly, 90% of women experience retrograde menstruation, but only a subset of those women develop and suffer from endometriosis (Halme et al, 1984). This begs the question: what causes endometriotic lesions in some women and not others with retrograde menstruation? The answer has been elusive, but some scientists speculate that MET and EMT may be occurring concurrent with retrograde menstruation. Like with metastatic cancer, it is suggested that EMT occurs, allowing shed endometrial epithelial cells to become mesenchymal-like, thus allowing them to migrate to a secondary site outside of the uterine cavity due to retrograde

menstruation and re-differentiate through MET (Bartley et al, 2014, Matsuzaki and Darcha, 2011, Wilson et al, 2020). Endometrial tissue samples from women diagnosed with endometriosis show a decrease in E-cadherin with an increase in mesenchymal-cadherin, N-cadherin, by immunohistochemistry on lesion sections (Bartley et al, 2014, Matsuzaki and Darcha, 2011). Bartley and colleagues (2014) showed in addition to the altered expressions of E-cadherin and N-cadherin, transcription factors associated with EMT, Twist, Snail and Slug, were increased in tumors via immunohistochemical and mRNA level analyses. These findings support EMT, and likely its counterpart MET, as mechanisms exploited in endometriosis.

1.6.2 MET in Uterine Epithelialization

MET was hypothesized as a mechanism of uterine epithelialization in the 1960s. Baggish and colleagues used various staining techniques including hematoxylin and eosin (H&E) and acridine orange fluorescence to investigate endometrial mitotic cells during menses (1967). A subset of surface epithelial cells demonstrated similar characteristics with “indifferent” cells within the stromal-epithelial junction with H&E staining, such as ill-defined morphology, intense nuclear stain, and little cytoplasm similar to adjacent stromal cells. Intense nuclear staining with acridine orange fluorescence were seen in stromal cells and some “indifferent” epithelial cells within the stromal-epithelial junction, indicating they were actively proliferating. In contrast, mature surface epithelial cells showed minimal nuclear staining, suggesting these cells were inactive. Baggish and others speculated that these mature, inactive epithelial cells were

unlikely to contribute to the expansion of new surface epithelial progeny. With stromal cells and the subset of epithelial cells both proliferating at the stroma-epithelial border, this begged the question whether the stroma and epithelia could be derived from the same stem cell—a reasonable theory considering the process of growth and differentiation during embryonic development (1967).

Following this research, studies using scanning/transmission electron microscopy (SEM/TEM) concluded that residual glandular stumps protruding from the denuded surface, as well as persistent LE in the cornua and isthmus regions of the uterus, were the source of proliferating and migrating epithelial cells that reformed the LE. Many years later using SEM, hysteroscopy, and histology, a reappraisal of endometrial repair in the uterus was performed during menses (Garry et al, 2009). In the early stages of repair, single cells and small patches of *de novo* LE cells were frequently found to be isolated and without direct contact with protruding gland stumps. The new epithelial cells were cuboidal in shape and lacked microvilli unlike the mature, microvilli columnar cells around the glands. Following fusion of the new islands with each other, they grew toward the remaining cells surrounding the basal glands to form a continuous epithelial lining that covered the underlying remodeled stroma (Garry et al, 2009). These results suggested that it was unlikely that the mature, differentiated cells surrounding the glands would be able to give rise to the new, morphologically different epithelial cells seen, but rather the isolated singlets or small clusters originated from the stroma presumably by MET (Garry et al, 2009).

A number of scientists used lineage tracing mouse models, direct fluorescence, immunofluorescence and immunohistochemistry to investigate MET as a uterine repair mechanism and to identify putative transitional cells. In nulliparous mice, pancytokeratin was specific to epithelia. Following decidualization and progesterone removal in menses-like mouse models, cells co-expressing pancytokeratin (epithelial marker) and vimentin (stromal marker) were observed in the stromal compartment at 12 hours (Cousins et al, 2014) and up to 48 hours (Patterson et al, 2013) suggesting identification of mesenchymal-epithelial transitional cells. The co-expressing cells were initially located at the border of the endometrial stroma and myometrium, a proposed mesenchymal stem cell niche for the stroma (Cervelló et al, 2007, DePace et al, 1981), and advanced towards the luminal epithelialization zone over time (Patterson et al, 2013). In two studies, lineage tracing was performed using Anti-Müllerian Hormone Receptor Type 2 (*Amhr2*)-Cre mice to drive expression of either EYFP (*Amhr2-Cre;Rosa26-Eyfp*) or LacZ (*Amhr2-Cre;Rosa26-LacZ*) reporters (Huang et al, 2012, Patterson et al, 2013). The *Amhr2* promoter is active only in the mesenchymal cells of the uterus (*i.e.*, stroma and myometrium), therefore reporter expression was restricted to stromal and myometrial cells of the developing and adult uteri. Following completed endometrial regeneration postpartum or using a menses-like model, *EYFP*⁺ or *LacZ*⁺ mesenchyme-derived cells were identified in the GE and LE. These studies concluded that stromal cells underwent MET as a mechanism to repair the epithelium, and that this mechanism is reserved for damage/repair processes such as that seen following

menstruation or pregnancy, but not during the minimal turnover that occurs during the estrous cycle. These mesenchyme-derived epithelial cells were shown to persist up to 2 months postpartum (Huang et al, 2012).

Underpinning these studies is the assumption that the *Amhr2* promoter is only active in the mesenchyme and not in the epithelia to be able to conclude that reporter-positive epithelial cells are derived from the stroma. *Amhr2* promoter activity has been investigated using *Amhr2-LacZ* mice; *LacZ* expression is only seen when *Amhr2* promoter is active and is detected by β -galactosidase staining. At E 13.5, E 14.5, E 15.5, and E 18.5, β -galactosidase expression was seen only in the Müllerian duct mesenchyme and was more robust in the anti-mesometrial mesenchyme (Arango et al, 2008). In mice aged P 14, P 25, 6 weeks and 6 months, *LacZ* expression in postnatal tissue was not identified in LE or GE and was restricted to the myometrium in adults. Additionally, *Amhr2-LacZ* mice collected at 3 days postpartum or 2 days following progesterone removal (ovariectomy) in a menses-like model demonstrated no *LacZ* expression in the epithelia during endometrial regeneration (Patterson et al, 2013). These data indicate that no *Amhr2* promoter activity occurs within epithelia embryonically, postnatally or in adulthood, thus supporting the use and validity of *Amhr2*-lineage tracing models when studying MET within the uterus.

A recent study evaluated the contribution of putative stromal stem/progenitor cells to epithelial repair using a menses-like mouse model (Yin et al, 2019). SM22 α^+ cells were identified as a subpopulation of AMHR2 $^+$ cells and co-expressed CD34 in the repairing zone of the endometrium. SM22 α marks

perivascular pericytes, a putative mesenchymal stem cell population, while AMHR2 is a well-known marker of endometrial mesenchyme and CD34 is expressed by various types of stem/progenitor cells (Arango et al, 2008, Cervelló et al, 2011). SM22 α ⁺ stromal-derived cells appeared to populate the epithelium and were CDH1⁺ (epithelial marker) as epithelial repair progressed. To further validate this finding, SM22 α ;mT/mG reporter mice were used to track the fate of SM22 α -expressing stromal cells and their progeny during endometrial repair. During the first 36 hours, SM22 α GFP⁺ stromal cells increased drastically, followed by an increase in GFP⁺ cells found in the epithelium after 72 hours of repair. Within the epithelium, stromal-derived GFP⁺ cells remained even after repair was completed by 96 hours supporting their contribution to epithelialization following damage. Interestingly, this study also demonstrated a subset of stromal cells transition to epithelial cells when treated with estrogen for 10 days *in vitro* (Yin et al, 2019). Results from this research not only supports MET as a mechanism of epithelial repair but begins to address Baggish's theory that one progenitor population potentially contributes to both stromal and epithelial lineages. Additionally, it suggests that estrogen may play a role in the transition of mesenchyme to epithelia, however further validation is required.

Using multiple lineage tracing models with mesenchyme-specific Cre reporters (*Amhr2*, *Cspg4*, *Pdgfr β*) that are initially expressed during Müllerian duct development, it was suggested that resulting labeled epithelia in the postnatal and adult uteri are maintained from embryonic epithelial labeling and are not derived from the stroma by MET (Ghosh et al, 2020). All models

discussed use *LacZ* reporters, and of importance, one of the models was *Amhr2*-*Cre* driven. The investigators identified *LacZ*⁺ epithelial cells in adult nulliparous mice that had not undergone endometrial damage and repair in contrast to prior reports (Huang et al, 2012, Patterson et al, 2013). To determine the origin of these cells, *Amhr2* expression (e.g., promoter activity) was examined embryonically by *in situ* hybridization. As previously described, the MDE is derived from invaginating CE and so, *Amhr2* promoter activity was evaluated in the CE, MDE and MDM, in E 15.5 embryos. The MDM was positive for *Amhr2*, the MDE was negative, and the overlying CE was positive. It was concluded that because CE gives rise to the first MDE cells at E 11.5, that *Amhr2*⁺ CE cells contributing to the MDE must be the source of *LacZ*⁺ epithelial cells in adult reporter mice. However, the *Amhr2* promoter is not active until E 12.5 after the contribution of CE cells to the MDE (Arango et al, 2008). As detailed previously, after initial invagination of the CE, the MDE elongates of its own accord and does not receive additional input from the CE (Orvis and Behringer, 2007, Arango et al, 2008). This would explain why the investigators did not observe *LacZ* expression in the MDE. Therefore, it is very unlikely that *LacZ*⁺ epithelial cells in the adult are derived from *LacZ*⁺ MDE in the embryo. Using another mesenchyme-specific marker, *Cspg4* promoter activity was found grossly in the Müllerian duct, although MDE or MDM were not specified. At P 1, promoter activity within the stroma was restricted to stromal perivascular cells and maintained through adulthood (Ghosh et al, 2020). Immunostaining demonstrated X-gal expression in uterine epithelia at P 35 and P 56 in *Cspg4*^{Cre}-

LacZ^{fl/+} mice with increased expression in LE and GE following tamoxifen administration (Ghosh et al, 2020), but with promotor activity specific to mesenchyme, this may indicate MET postnatally and responsiveness to estrogen despite alternate interpretation. Using a third mesenchyme-specific marker, *Pdgfr β ^{rtTa}; tetO^{Cre}; R26-LacZ^{fl/+}* mice were generated and labeling of *Pdgfr β ⁺* cells was controlled with doxycycline administration to encompass Müllerian duct E 8.5-P 1 development (Ghosh et al, 2020). Adult mesenchyme and epithelia, specifically uterine GE, expressed LacZ, indicating progeny of embryonically labeled cells. Again, it is unknown if cells labeled during this period were within MDE or restricted to MDM. A second period of labeling was induced in adults for 3 days, then these mice were chased for a year before collection, and X-gal positive cells were identified within the uterine epithelia (Ghosh et al, 2020). Since these models are lineage tracing or fate mapping, and promotor activity has been shown to be restricted to mesenchymal cells in two mouse models, it is still unclear whether labeled epithelial cells are maintained from embryonic origin as suggested or arise at later times from labeled mesenchyme via MET outside of the context of postpartum or menses-like epithelial repair.

CHAPTER 2: A REAPPRAISAL OF MESENCHYMAL-TO-EPITHELIAL TRANSITION WITHIN THE ENDOMETRIUM

2.1 Introduction

The uterus is a dynamic organ that not only responds to hormonal changes throughout the estrous/menstrual cycle but is also able to regenerate and repair itself rapidly following parturition, and in higher primates, during menses. A woman will undergo approximately 400 to 500 damage/repair cycles (i.e. the menstrual cycle) throughout her reproductive lifetime. Although not every species sheds the innermost layer of the uterus, the endometrium, as women do, this layer still responds to the synchronized increase and decrease of ovarian steroid hormones, estrogen and progesterone, resulting in cellular turnover through proliferation and apoptosis. Within the endometrium, two phenotypically different cell types are present: the underlying stromal layer contains mesenchymal, stromal cells capable of migration, and the static epithelial layer, luminal (LE) and glandular (GE), where epithelialization occur.

Uterine epithelial regeneration is aided by multiple processes to ensure swift repair of the tissue. One of these processes likely involves stem cells of the epithelium that proliferate to regenerate the epithelium over damaged areas. These putative epithelial stem cells are thought to reside in the epithelial glands of the uterus (Ferenczy, 1976) with subsequent research both supporting this as well as putting it into question (Padykula et al, 1989, Gary et al, 2009, Jin et al, 2019). It is possible that another stem cell population within the epithelium contributes to the regeneration of the epithelium, but this has yet to be definitively shown. More recently, evidence suggests the mechanism, mesenchymal-to-

epithelial transition (MET), contributes to endometrial epithelialization following tissue damage, though little is known about this mechanism (Huang et al, 2012; Patterson et al, 2013; Cousins et al 2014; Yin et al, 2019).

MET and its counterpart, epithelial-to-mesenchymal transition (EMT) are cellular mechanisms important in embryonic development but may become dysregulated and contribute to diseases like cancer and endometriosis (Banyard and Bielenberg, 2016, Bartley et al, 2014, Matsuzaki and Darcha, 2011, Wilson et al, 2020). Recent studies have demonstrated that MET occurs within the endometrium following parturition and induced endometrial shedding in mice (Huang et al, 2012, Patterson et al, 2013, Cousins et al, 2014). It is probable that MET occurs monthly in women following the shedding of the functional layer of the endometrium, although human studies are limited in this area. Historically, the most widely accepted theory of endometrial epithelialization in women is regrowth of residual basal epithelial glands to repopulate the LE layer (Ferenczy, 1976). However, in 2009, histological techniques and scanning electron microscopy (SEM) were utilized to reevaluate menstrual epithelialization, revealing possible contribution of stromal cells to the LE, presumably through MET (Garry et al). Utilizing mouse models, several studies were conducted in the next few years that supported previous findings. Dual-staining identified cells co-expressing stromal and epithelial cell markers—vimentin and pancytokeratin, respectively—and undergoing locational changes over the first 96 hours of menses-like endometrial regeneration, possibly identifying mesenchymal-epithelial transitional cells (Patterson et al, 2013, Cousins et al, 2014). While

previous research has focused on the first few days of repair/regeneration, information and characterization of mesenchymal-derived cells following these foundational hours when epithelial regeneration is complete and throughout the estrous cycle do not currently exist.

In addition to epithelialization events in the adult—during estrus, following menses and parturition—epithelialization occurs during adenogenesis, in which a single layer of LE cells invaginates into the stroma to form endometrial glands. This process begins during late gestation in humans and after birth in mice at approximately postnatal day (P) 5. By P 15, the adult uterine histoarchitecture is established and the uterus continues to increase in size until reaching sexual maturity at approximately 5-6 weeks of age (Hu et al, 2014, Vue et al, 2018). Because epithelialization events are not confined to the adult, it is possible that MET also occurs prior to adulthood. Recently, it was suggested that the putative mesenchymal-derived cells seen within the uterus are maintained from embryonic origin and that MET does not occur in the adult uterus (Ghosh et al, 2020). Due to these gaps in knowledge, the current study aims to: 1.) identify the temporal origin of these cells 2.) investigate the contribution of MET to epithelialization events postnatally (adenogenesis) and in the nulliparous adult (during the estrous cycle) and 3.) assess the longevity of mesenchymal-derived epithelial cells following postpartum endometrial epithelial repair. We hypothesize that MET contributes to additional epithelialization events outside of menses-like and postpartum regeneration, including adenogenesis and during the estrous cycle as homeostatic epithelial turnover.

2.2 Materials and Methods

Animals

All protocols involving animal experiments were approved by Animal Care and Use Committee at University of Missouri. *Amhr2-Cre* mice (Arango et al, 2008) were obtained from the Mutant Mouse Resource and Research Center (MMRCC), *Rosa26-Stop^{fl/fl}-EYFP*, *Rosa26-Stop^{fl/fl}-tTA*, and *TRE-H2B-GFP* (Fuchs and Horsley, 2012) mice were obtained from Jackson Laboratories.

Fate-mapping Studies

Amhr2-Cre mice were crossed to *Rosa26-Stop^{fl/fl}-EYFP* mice to generate double transgenic reporter females (*Amhr2-Cre; Rosa26-EYFP*) and littermate controls (*Amhr2-WT; Rosa26-Stop^{fl/fl}-EYFP*). In cells with *Amhr2* promotor activity, Cre-mediated excision of the loxP floxed stop codon in the *Rosa26* promotor allow constitutive expression of EYFP (**Fig. 1**). Uteri from *Amhr2-Cre; Rosa26-EYFP* and control females were collected at 8 weeks or greater in nulliparous mice (n=25) and after one pregnancy (n=20) at each stage of the estrous cycle (nulliparous: proestrus n=7, estrus n=4, metestrus n=10, diestrus n=4, postpartum: proestrus n=4, estrus n=4, metestrus n= 5, diestrus n=8). Uteri were also collected from *Amhr2-Cre; Rosa26-EYFP* females at postnatal day 21 (n=4). To generate triple transgenic females (*Amhr2-Cre; Rosa26-tTA; H2B-GFP*), *Rosa26-Stop^{fl/fl}-tTA* mice were crossed with *TRE-H2B-GFP* reporter mice to produce homozygous, double transgenic mice (*Rosa26-Stop^{fl/fl}-tTA; H2B-GFP*) before a final cross with *Amhr2-Cre* mice (**Fig. 2**). *Rosa26-Stop^{fl/fl}-tTA; H2B-GFP* mice served as controls. This tet-off system allows for constitutive expression of

Figure 1. Schematic diagram of generating *Amhr2-Cre*; *Rosa26-EYFP* female mice and resulting constitutive EYFP expression. *Amhr2-Cre* mice were crossed with *Rosa26-Stop^{fl/fl}-EYFP* mice. In cells with *Amhr2* promoter activity, there is Cre-mediated excision of the loxP floxed stop codon in the *Rosa26* promoter. With this stop codon cleaved out, EYFP is constitutively expressed in cells with *Amhr2* promoter activity.

Figure 2. Schematic diagram of generating *Amhr2-Cre*; *Rosa26-tTA*; *H2B-GFP* female mice and resulting H2B-GFP expression. Triple transgenic females were produced crossing *Amhr2-Cre*, *Rosa26-Stop^{fl/fl}-tTA* and *TRE-H2B-GFP* reporter mice. Under *Amhr2* promoter activity, Cre-mediated excision of the loxP floxed stop codon in the *Rosa26* promoter allows expression of tetracycline transactivator (tTA), which binds to its response element (TRE). In the absence of doxycycline, this tet-off system allows for constitutive expression of H2B-GFP in cells with *Amhr2* promoter activity.

H2B-GFP in the absence of doxycycline administration specific to cells with *Amhr2* promotor activity due to Cre-mediated excision of the loxP floxed stop codon in the *Rosa26* promotor and subsequent binding of tTA to TRE. Uteri were collected at P 0.5 (n=5), P 3 (n=4), P 8 (n=6), P 14 (n=6), and P21 (n=5).

Estrous Cycle Staging

Cycle staging was ascertained using vaginal lavage method with 40 μ L of 10% trypan blue/PBS solution. Stage was determined microscopically according to proportions of cornified epithelial cells, nucleated epithelial cells, and leukocytes as seen by (Caligioni, 2009, Ajayi and Akhigbe, 2020), and verified by gross visualization of the uterus (Croy et al, 2014).

Uterine Epithelial Cell Isolation and Flow Cytometry

Uterine epithelial cell isolation was performed using a modified protocol (De Clercq et al, 2017). Uterine horns were collected from *Amhr2-Cre; Rosa26-EYFP* females aged 8 weeks or older that were either nulliparous or had 1 pregnancy, and from *Amhr2-Cre; Rosa26-tTA; H2B-GFP* at PND 0.5, 3, 8, 14 and 21. At time of collection, uterine horns were cut longitudinally to expose the lumen, cut cross-sectionally at 2-4 mm apart to expose uterine glands and placed in 15 mL conical tubes with 10 mL of HBSS+ (Hank's Balanced Salt Solution with antibiotic/antimycotics; Gibco) with 1 mL of 2.5% Trypsin (reconstituted; Sigma; Cat: T7409-1G). Adult uteri underwent a series of digestions: 4°C oscillating at 50-70 rpm for 1 hr, RT without oscillation for 40 min, 37°C without oscillation for 25 min. Postnatal uteri underwent a similar series of digestions: RT without oscillation for 12 min (P 0.5), 17 min (P 3), 20 min (P 8), 25 min (P 14), or 30 min

(P 21), 37°C without oscillation for 5 min (P 0.5), 10 min (P 3, P 8), 15 min (P 14), or 20 min (P 21). All contents were poured through 100 µm nylon mesh filters into 50 mL conical tubes and uteri were then transferred into Mouse Endometrial Epithelial Cell (MEEC) media for 5 min. Uteri were transferred into 2.5 mL of HBSS+ in 15 mL conical tubes and vortexed vigorously for 10 sec because contents were poured through filters. Transfer of tissue into MEEC media, vortex and filtration were repeated 3 times before tubes and petri dishes were rinsed and poured through filter. Uteri were rinsed on filter with 1 mL HBSS+ before discarded. 50 mL conical tubes were centrifuged at 500 xg for 5 min before supernatant were aspirated. Cell pellets from adult uteri underwent red blood cell lysis with 2 mL AKC lysis buffer (Gibco, Grand Island, NY; cat # A10492-01) for 5 min, then 20 mL 1x phosphate-buffered saline (PBS) was added before centrifugation at 300 xg for 5 min and aspiration of supernatants. Cells were resuspended before transferring into Eppendorf tubes. Tubes were rinsed with 1 mL HBSS+ before added to Eppendorf tubes and centrifuged at 300 xg for 5 min. Supernatants were aspirated and pellets were resuspended in 1 mL PBS working buffer (De Clercq et al, 2017) and incubated for 10 min. Tubes were centrifuged at 300 xg for 5 min, aspirated and stained with 10 µL of EpCAM (APC rat anti-mouse CD326; BD Biosciences; PLACE; cat # 563478) in 90 µL of PBS working buffer for 15 min. 1 mL of PBS working buffer was added and tubes were centrifuged at 300 xg for 5 min before aspiration and cells were washed with 1 mL of PBS working solution. Following centrifugation at 300 xg for 5 min and aspiration of supernatants, cells were resuspended in 30 µL of PBS working

solution before being analyzed by FlowSight (Amnis; IDEAS Version 6.2; **Fig S1**).

Gelatin Embedding and Frozen Tissue Preparation

EYFP-expressing, GFP-expressing and control uteri were fixed using 8% PFA for 30 min at 4°C before washed 3 times with 1x PBS and incubated in 15% sucrose buffered in PBS overnight. Samples were incubated in gelatin (7.5% gelatin in PBS; Gelatin from porcine skin; Sigma Life Science; cat # G2500) for 1 hr at 37°C, embedded in gelatin, and frozen at -50°C to -65°C in 2-Methylbutane cooled by liquid nitrogen, and stored at -80°C until cryo-sectioning. Tissues were cryo-sectioned at 5-8 µm and thaw mounted. Gelatin was removed in 37°C PBS before mounting with a proportion of slides undergoing immunohistochemistry, but all samples were stained with 4',6-diaminino-2-phenylindole (DAPI) prior to mounting with aqueous mounting media (Fluoro-gel with TES; Electron Microscopy Sciences, Hartfield, PA; cat # 17985-30) and viewed using fluorescent microscopy (Leica 5500 microscope; LAS X version: 3.7.4.23463).

Immunohistochemistry and Indirect Immunofluorescence

Uteri from EYFP-expressing and GFP-expressing uteri were obtained to perform IHC by fluorescence. Postnatal tissue (P 0.5, P 3, P 8, P 14, and P 21; n=4-6 per time point) and adult tissue (nulliparous or postpartum in proestrus, estrus, metestrus, or diestrus; n=3-4 per cycle stage) were collected, fixed in 8% PFA for 30 min at 4°C, processed for gelatin imbedding and freezing and sectioned at 5-8 µm. Following thaw mounting and removal of gelatin in 37°C PBS, immunofluorescence for EpCAM (Purified rat anti-mouse CD326; BD

Biosciences, 1:100; PLACE; cat # 552370), Ki67 (rabbit monoclonal; 1:100; Invitrogen, Rockford, IL; cat # MA5-14520), estrogen receptor α (ESR1; 1:100; Santa Cruz Biotechnology; cat # A0716, discontinued), progesterone receptor (PGR; rabbit monoclonal 1:100; Invitrogen; cat # MA5-14505), and FOXA2 (rabbit monoclonal 1:100; Abcam, Cambridge, MA; cat # ab108422) were performed. To verify EYFP and GFP expression, tissues were stained using an anti-GFP antibody (Rabbit polyclonal 1:1000; Invitrogen, Eugene, OR; cat # A-11122). Briefly, PBS working buffer (PBS, 10% BSA, 10% NGS, and Triton) was applied to block tissue for 1 hr at RT before incubation in primary antibodies; EpCAM, Ki67 and GFP antibodies were applied for 1 hr at RT, and ESR1, PGR, and FOXA2 were applied and tissue incubated overnight at 4°C following blocking. Tissues were washed thrice for 10 min each before incubation in secondary antibodies for 40 min in dark (Anti-rat IgG (H+L) Alexa Fluor 555 Conjugate, 1:1000, Cell Signaling Technology, cat # 4417; Alexa Fluor 568 goat anti-rabbit (H+L), 1:1000, Invitrogen, Eugene OR, cat # A11036). After two, 10 min washes, tissues were incubated in DAPI (300nM) for 5 min and washed twice, as before. After incubation in DI water for 5 minutes, tissues were mounted with aqueous mounting solution (Fluoro-gel with TES; Electron Microscopy Sciences, Hartfield, PA; cat # 17985-30) and stored at 4°C in dark. Fluorescent imaging was performed using a Leica 5500 microscope (LAS X version: 3.7.4.23463).

Blood Serum Collection and ELISAs

Amhr2-Cre; Rosa-EYFP females aged P 21 were anesthetized prior to

submandibular bleed and collected in Eppendorf tubes. Samples were allowed to coagulate for 30 min to 1 hr before centrifugation at 4°C for 10 min. Blood serum was removed and transferred into a clean Eppendorf tube and stored at 20°C until use. Mouse/rat estradiol and progesterone ELISA kits (Calbiotech; cat #s ES180S-100 and PG362S, respectively) were used to assess blood serum estradiol and progesterone concentrations and read using a Synergy HT multi-detection microplate reader (BioTek).

Statistical Analyses

All statistical analyses were performed using GraphPad Prism version 9.1.2 (226) for Windows, GraphPad Software, San Diego, California USA, www.graphpad.com.

2.3 Results

Epithelialization is the process by which epithelial cells migrate, proliferate and repair a denuded surface. This term can be broadened to include growth of epithelial tissues under various physiological conditions such as the growth that occurs following prior resorption in the estrous cycle, or growth to form endometrial glands, termed adenogenesis. In this study we investigated the contribution of MDM-derived cells (i.e., endometrial stromal cells) to epithelialization in mouse uteri during different physiological and hormonal circumstances.

MDM-derived epithelial cells fluctuate across the estrous cycle in postpartum and nulliparous uteri.

Previous studies using lineage tracing mouse models identified MDM-derived epithelial cells following postpartum and menses-like endometrial

regeneration but did not observe them in nulliparous mice. It was therefore hypothesized that MDM cells (i.e., endometrial stromal cells) underwent MET as a mechanism to regenerate the epithelium following endometrial damage. MDM-derived cells were shown to be present in the epithelium for at least two months postpartum, but additional evaluation of these cells was not conducted. Here, to further characterize MDM-derived epithelial cells, uteri were collected from *Amhr2-Cre; Rosa-EYFP* females at 96 hr, 2 wks, 3 wks, 2 mo and 3 mo postpartum. By flow cytometry, no significant differences in the percentage of EpCAM⁺EYFP⁺ cells (i.e. MDM-derived epithelial cells) were found by time postpartum or by number of implantation sites (**Fig. 3A and C**). This was due to the high variability of samples. For example, with five implantation sites, the range in EpCAM⁺EYFP⁺ cells was 0.03% to 61.4%. There was no difference in locational expression of YFP⁺ epithelial cells in implantation and inter-implantation sites (**Fig. 3B**). Subsequently samples were categorized by stage of the estrous cycle based on vaginal cytologies, and there was a significant decline in the percentage of EpCAM⁺EYFP⁺ cells from proestrus (47.35 ± 7.48) to diestrus (0.81 ± 0.09), with no difference between diestrus and control uteri (**Fig. 3D**). Because of the high variability in MDM-derived epithelial cells observed across the estrous cycle in postpartum uteri, a re-evaluation of nulliparous uteri (i.e. no endometrial damage and repair) was performed based on stage of the cycle. Similar to postpartum uteri, there was a high percentage of EpCAM⁺EYFP⁺ cells identified in nulliparous uteri during proestrus that significantly declined to indistinguishable from control uteri in diestrus *Amhr2-*

Figure 3. MDM-derived epithelial cells fluctuate across the estrous cycle in postpartum uteri. (A) Percent of MDM-derived epithelial (EpCAM⁺YFP⁺) cells is variable across number of implantation sites with no difference by direct fluorescence of YFP expression in epithelial cells (B) within the implantation site (white arrow) and inter-implantation sites (yellow arrow). (C) Variability in percent of EpCAM⁺YFP⁺ cells was also found at collection times following parturition. (D) When mice were characterized by estrous cycle stage, statistical significant ($p < 0.05$) was found in proestrus and estrus when compared to control mice (No Cre Ctrl). Diestrus uteri were not statistically different from control uteri ($p > 0.9999$).

Cre; Rosa-EYFP mice (**Fig. 4A-H and Fig. 5.A**). When compared by cycle stage, EpCAM⁺EYFP⁺ cells in postpartum and nulliparous uteri were statistically similar (**Fig. 5B**). These data suggest that MDM-derived epithelial cell populations fluctuate across the estrous cycle regardless of prior endometrial damage and repair events.

MDM-derived epithelial cells exhibit dynamic temporal expression patterns during postnatal uterine maturation and epithelialization.

Involvement of MDM-derived epithelial cells in postnatal uterine maturation has not been previously explored. To investigate the temporal origin and possible contribution of MET during epithelialization events postnatally, uteri were collected from *Amhr2-Cre; Rosa26-tTa; H2B-GFP* females at postnatal day (P) 0.5, P 3, P 8, P 14, and P 21 to encompass epithelial differentiation and adenogenesis. Flow cytometry data showed that MDM-derived epithelial cells, EpCAM⁺GFP⁺ cells, arose rapidly after birth (P 0.5) as seen by a high variability in samples ranging from 0.21% to 81.84%, before a significant decline at P 8, during initiation of adenogenesis (**Fig. 6A**). EpCAM⁺GFP⁺ and EpCAM⁺GFP⁻ cells also segregated into two distinct populations by side scatter (**Fig. 6C**), an indicator of cellular complexity in all samples at P 0.5 and in one sample at P 21. P 3 similarly showed high variability between samples as seen at P 0.5 (**Fig. 6A, D and E**). The variability in the data may indicate dynamic turnover of the two epithelial cell populations leading up to adenogenesis, which is predominated by EpCAM⁺GFP⁻ (non-MDM-derived) cells. At P 14, during the shift from adenogenesis to glandular morphogenesis, the percentage of EpCAM⁺GFP⁺

Figure 4. MDM-derived epithelial populations differ across the estrous cycle. Direct fluorescence of YFP⁺ epithelial cells during (A) proestrus, (C) estrus, (E) metestrus, and (G) diestrus, with corresponding EpCAM⁺YFP⁻ and EpCAM⁺YFP⁺ epithelial populations by flow cytometry (B-proestrus, D-estrus, F-metestrus, H-diestrus).

Figure 5. MDM-derived epithelial cells fluctuate across the estrous cycle in nulliparous and postpartum uteri. (A) EpCAM⁺YFP⁺ populations during proestrus, estrus, and metestrus were statistically different ($p < 0.0001$, 0.001 , and 0.05 , respectively) from diestrus and control uteri. Diestrus and control uteri were not statistically different ($p > 0.9999$). **(B)** Based on estrous cycle stage, nulliparous and postpartum uteri did not differ during the same stage.

Figure 6. MDM-derived epithelial populations demonstrate dynamic temporal patterns. (A) MDM-derived epithelial (EpCAM⁺GFP⁺) populations fluxuate dynamically during postnatal uterine maturation. High variability is seen in P 0.5 and P 3 before the population averages less than 2% during adenogenesis initiation at P 8. During glandular morphogenesis (P 14) through the peripubertal stage (P 21), MDM-derived epithelial cells increase and are maintained. (B) No difference is seen between *Amhr2-Cre; Rosa-EYFP* (AYFP) and *Amhr2-Cre; Rosa26-tTa; H2B-GFP* (ATG) females at P 21. (C) EpCAM⁺GFP⁺ and EpCAM⁺GFP⁻ populations segregate based on sidescatter (SSC; cellular complexity), with EpCAM⁺GFP⁺ cells showing greater complexity (population 2). (D and E) Representative images by direct fluorescence of variability in *Amhr2-Cre; Rosa26-tTa; H2B-GFP* uteri at P 3.

cells increased slightly and was maintained through P 21 (**Fig. 6A**). Variation at P 21 Some variability was noted at P 21 with results from *Amhr2-Cre; Rosa-EYFP* females supporting the same findings at P 21 (**Fig. 6B**). Together, these data indicate that epithelial cells originate from the MDM very early after birth, presumably through MET, and are quickly replaced by non-MDM-derived epithelial cells during adenogenesis. However, they return during gland morphogenesis and differentiation. Analyses of blood serum estradiol (E2) and progesterone (P4) concentrations at P 21 did not show any correlation with MDM-derived epithelial cell populations in *Amhr2-Cre; Rosa-EYFP* and *Amhr2-Cre; Rosa26-tTa; H2B-GFP* females. These results suggest that the mechanism, MET, is independent of ovarian steroids postnatally through P 21.

MDM-derived epithelial cells persist in the adult and are characteristic of functional epithelial cells.

We have shown in addition to endometrial regeneration, MDM-derived epithelial cells contribute to epithelialization postnatally and in nulliparous mice. Due to the high turnover or replacement of MDM-derived epithelial cells by non-MDM-derived epithelial cells during postnatal uterine maturation and the estrous cycle, we investigated their epithelial characteristics to determine if they are capable of functioning as bone fide endometrial epithelial cells. MDM-derived epithelial cells expressed the epithelial marker, EpCAM, at all postnatal and adult time points. Importantly, EpCAM was specific to LE and GE, indicating mesenchymal cells were not inadvertently analyzed by flow cytometry (**Figs. S2 and S3**). FOXA2, a marker of GE, was first observed at P 8 (adenogenesis

initiation), in which few cells showed nuclear expression, and was also found cytoplasmically in some stromal cells (**Fig. 7A-C**). Because very few MDM-derived epithelial cells (GFP⁺) were found at this time, subsequently no co-expression of GFP and FOXA2 was observed. From P 14 through adulthood, expression of FOXA2 was restricted to nuclei of GE cells and was expressed by both MDM-derived and non-MDM-derived epithelial cells (**Fig. 7D-L and Fig. S6A-I**).

During postnatal maturation, initially there was high proliferation in both epithelial and stromal compartments (P 0.5) in which both GFP⁺ (MDM-derived) and GFP⁻ (non-MDM-derived) epithelial cells expressed Ki67 (**Fig. 8A-C**). After a decline in proliferation at P 3 (**Fig. 8D-F**), there was another increase in proliferation in the stroma at P 8 (**Fig. S7A-C**) and in the epithelia at P 14. Few GFP⁺ epithelial cells expressed Ki67 at P 14, with majority of expression found in GFP⁻ epithelial cells, specifically GE cells, indicating that few MDM-derived epithelial cells contribute to proliferating epithelium, thus maintaining GFP⁺ populations from P 14 to P 21 (**Fig. 8G-L**). Additionally, both ESR1 and PGR were present in both non-MDM-derived and MDM-derived epithelia, and stroma, at P 0.5 but were absent by P 3 (**Figs. S8A-F and S9A-F**). The abundance of ESR1 returned by P 8, specifically within the stroma, and began to be highly expressed within glands at P 14 and was maintained at P 21 (**Fig. S8G-O**). Conversely, PGR was highly expressed within the epithelia at P 8 and increased through P 21 (**Fig. S9G-O**). At all postnatal stages in this study, there were no qualitative differences in marker expression between MDM-derived and non-

Figure 7. MDM-derived epithelial cells co-express FOXA2 by immunofluorescence in postnatal and adult tissue. MDM-derived and non-MDM-derived epithelia showed mosaicism in the LE and GE. Few MDM-derived epithelial cells were found by direct fluorescence at P 8 during adenogenesis initiation, supporting flow cytometry data. **(A-C)** FOXA2 expression was found either cytoplasmically in subsets of epithelial and stromal cells (white arrow-stromal cell expressing FOXA2 cytoplasmically), with few epithelial cells showing nuclear expression. **(D-F)** Robust nuclear expression of FOXA2 was found specific to the GE at P 14, with a proportion of these cells being GFP⁺ (white arrow). **(G-I)** Like P 14, FOXA2 expression at P 21 was found in GE cells with a proportion of these cells being GFP⁺ (white arrow). **(J-K)** Maintained from the peripubertal uterus, FOXA2 expression was nuclear in GE cells, and a subset of these cells were YFP⁺ (white arrow).

Figure 8. MDM-derived epithelial cells proliferate in a dynamic temporal pattern postnatally. (A-C) Both stromal and epithelial layers showed Ki67 expression, indicating stromal and epithelial cells proliferate at P 0.5. No difference in Ki67 expression was found between GFP⁺ and GFP⁻ epithelial cells, suggesting MDM-derived epithelial cells proliferate and contribute to the epithelial layer at this time (white arrow). (D-F) Proliferation decreased at P 3, with greater proliferation found in the epithelium compared to the stroma. Few GFP⁺ epithelial cells were found to express Ki67 during this time (white arrow) in comparison to GFP⁻ epithelial cells. (G-I) At P 14, both stroma and epithelia were found proliferating, with more epithelial cells expressing Ki67 than stromal cells. Some GFP⁺ epithelial cells also expressed Ki67 (white arrow), but majority of epithelial cells expressing Ki67 were GFP⁻. (J-L) Proliferation decreased from P 14 in epithelial and stromal compartments. With little-to-no Ki67 expression in epithelia at P21, GFP⁺ and GFP⁻ epithelial cells expression was similar.

MDM-derived epithelial cells, suggesting MDM-derived epithelial cells acquired non-MDM-derived epithelial cell characteristics throughout postnatal maturation.

During the estrous cycle, Ki67, ESR1 and PGR expression vary dynamically from stage to stage (Wood et al, 2007, Lessey et al, 1988). Regarding each stage, ESR1 and PGR expression did not differ between MDM-derived and non-MDM-derived epithelial cells (**Figs. S10A-L and S11A-L**). Interestingly, Ki67 expression did not differ between YFP⁺ (MDM-derived) and YFP⁻ (non-MDM-derived) epithelial cells in proestrus (**Fig. 9A-C**) but began to become restricted to YFP⁻ epithelial cells through subsequent stages of the estrous cycle (**Fig. 9D-L**). These data support MDM-derived epithelial cells retain epithelial characteristics through the estrous cycle, but do not continue to proliferate. Total epithelial proliferation decreased during progesterone-dominant stages, metestrus and diestrus, with few proliferating cells being non-MDM-derived epithelial cells.

Discussion

Prior research using the same lineage tracing models showed that MDM-derived epithelial cells populated the epithelium following postpartum or menses-like repair (Huang et al, 2012, Patterson et al, 2013, Cousins et al, 2014). These cells were not observed in pre-pubertal or nulliparous mice. It was thus hypothesized that stromal cells (e.g. MDM cells) underwent MET as a mechanism of endometrial epithelialization following physiological damage. Contrary, in a recent report, MDM-derived epithelial cells were identified in nulliparous mice using the same lineage tracing model and it was concluded that

Figure 9. Proliferation of MDM-derived epithelial cells decreases during the estrous cycle. (A-C) Epithelial proliferation was greatest during proestrus. No difference was seen in Ki67 expression of MDM-derived and non-MDM-derived epithelia (white arrow). (D-F) Epithelial proliferation decreased from proestrus to estrus, and few MDM-derived epithelial cells expressed Ki67 during estrus, suggesting they do not continue to contribute greatly to the epithelia at this time. (G-I) Glandular proliferation increased during metestrus, while few luminal epithelial cells showed Ki67 expression. Proliferating glandular epithelial cells were found to be both YFP⁺ and YFP⁻ (white arrow). (J-L) Proliferation by Ki67 expression was minimal in diestrus, though some stromal cells and few YFP⁻ epithelial cells expressed this marker. YFP⁺ epithelial cells were rare and did not contribute to epithelial proliferation during diestrus.

these cells were embryonically derived rather than by MET in endometrial repair. Evidence suggesting embryonic origin is lacking though. *Amhr2* promotor activity has been investigated previously embryonically, postnatally, and in adults: at no point during Müllerian duct development, postnatal uterine maturation or in cyclic adult uteri have epithelia demonstrated *Amhr2* promotor activity, supporting epithelial cells found in lineage tracing models are MDM-derived epithelial cells (Arango et al, 2008, Patterson et al, 2013). In the current study, using more sensitive techniques, MDM-derived epithelial cells were also identified in nulliparous mice and during postnatal uterine maturation.

At birth, fetuses experience a sudden change in their environment hormonally. The gestational environment is dominated by progesterone produced by the mother, with progesterone (P4) withdrawal occurring when the fetuses are removed from the uterine environment at birth. In humans, some newborn females experience uterine bleeding similar to that seen during menstruation in the adult and has been suggested to be due to P4 withdrawal (Ober and Bernstein, 1955, Puttemans et al, 2018). Due to the extreme variation of MDM-derived epithelial cells from negligible amounts to over 80% at P 0.5, we speculate that these cells arise rapidly after birth, instead of embryonically, influenced by P4 withdrawal. In humans and rats, circulating estrogen (E2) is high at birth and may further aid MDM-derived epithelial cell contribution in neonates (Bell, 2018). However, since mice do not show detectable levels of E2 at birth, the future use of rats may better recapitulate neonatal events in humans. Although E2 is known to not be required for postnatal epithelialization

(adenogenesis and differentiation) (Ogasawara et al, 1983, Bigsby and Cunha, 1985, Branham et al, 1995), it is crucial for adult epithelial turnover (Galand et al, 1971, Cooke et al, 1986). We show that MDM-derived epithelial cells may arise by MET in the adult due to increasing E2 as the highest percentages were observed in proestrus and estrus. This is somewhat substantiated by a report of MET *in vitro*. Cultured adult uterine mesenchymal cells were shown to transition to epithelial cells after 10 days of E2 treatment (Yin et al, 2019), but the mechanistic process of this has not been further explored. Because postnatal uterine maturation is ovarian steroid independent until approximately P 25 (Ogasawara et al, 1983), we hypothesize that MET following birth is correlated to sudden withdrawal of P4 whereas in the adult, it may be both P4 withdrawal and E2 stimulation. At P 21, our results showed no correlation between MDM-derived epithelial populations and serum E2 or P4 concentrations, supporting the uterus is still E2/P4 independent at this time. Because of this, MET in the postnatal uterus is likely mechanistically different from MET occurring in adult uteri until at least P 21. Further exploration during peripubertal time points is necessary to determine the temporal change of the MET initiation mechanism in the uterus.

In addition to this *in vivo* model, *in vitro* analyses can continue to elucidate how E2 and P4 affect MET in postnatal cells. Cultured adult uterine mesenchymal cells have previously been shown to transition to epithelial cells after 10 days of estradiol treatment (Yin et al, 2019), but the mechanistic process of this transition postnatally has not been explored. Because postnatal uterine

maturation is ovarian steroid independent, we hypothesize that increased MET following birth is correlated to sudden withdrawal of P4 instead of increased E2 and will be addressed in future research.

Previous research suggested MET occurred following natural and artificial decidualization, indicating epithelial damage was necessary. Uteri were also found with MDM-derived epithelial cells persisting for 2 months postpartum (Hu et al, 2012). This study identified MDM-derived epithelial cells during homeostatic conditions of the estrous cycle without prior damage/repair. Peak percentages were observed during the E2-dominant stages, proestrus and estrus. The MDM-derived epithelial populations during diestrus did not differ from control uteri, but interestingly increased significantly to comprising approximately 50% of the epithelia in proestrus. Since the estrous cycle in mice is 4-5 days long, these data indicates that there is a rapid contribution from the stroma to epithelial expansion in no more than one day's time. Yet these MDM-derived epithelial cells are cleared or replaced in an exceptionally short amount of time, as well. With the current findings, it is likely that the uteri with cells identified to persist for 2 months postpartum were collected during E2-dominant stages of the estrous cycle. Furthermore, MDM-derived epithelial cells did not continue to proliferate beginning in estrus and are rarely found during diestrus, suggesting that they do not continue to populate and contribute to the epithelium during these stages. Previous studies have identified putative endometrial epithelial stem/progenitor cells in mice using label-retaining cell (LRC) and single-cell lineage tracing (Chan and Gargett, 2006, Kaitu'u-Lino et al, 2010,

Patterson and Pru, 2013, Jin, 2019). Together with current results, it is speculated that both putative epithelial and stromal stem cell populations contribute to epithelialization in the uterus postnatally and in the adult. Since MDM-derived cells arose rapidly, appear to be cleared or replaced quickly, and do not continue to proliferate through the estrous cycle, it is likely that they are replaced via this putative epithelial stem cell population. Because MDM-derived epithelial cells demonstrate rapid turnover during homeostasis, further analyses are needed during initial hours following natural or artificial decidualization to determine the direct contribution of these cells to epithelial regeneration. Additionally, endometrial organoids may be used to better recapitulate the uterine environment in an *in vitro* model since epithelial-stromal interactions are crucial to respond correctly to hormones. Future 3D culture systems with human endometrial cells can then provide further understanding of the role of MET within women.

This research has demonstrated MET occurs during homeostatic conditions using lineage tracing models, contrary to previous belief. MDM-derived epithelial cells were found during E2-dominant stages of the estrous cycle, as well as during postnatal maturation, suggesting MET has greater contribution to epithelialization events than solely being a mechanism for endometrial repair. In both postnatal maturation and during the estrous cycle, MDM-derived epithelial cells exhibited rapid turnover, likely contributing to the lack of evidence that MET occurs during homeostasis in the uterus. Despite rapid turnover, MDM-derived epithelial cells expressed similar markers to non-

MDM-derived epithelial cells, supporting that these cells have gained epithelial characteristics during MET. Our data suggests E2 may have a role in MET in the adult, but P4 withdrawal may contribute during ovarian steroid independent stages: the mechanism inducing MET is still unknown and requires further study. Future research will continue to characterize MDM-epithelial cells with lineage tracing models, organoids, and begin to address the mechanism behind MET.

CONCLUSION

Previous research has indicated MET occurs as an endometrial repair mechanism following damage, but the current research has indicated MET occurs during endometrial epithelialization events as a mechanism of homeostatic epithelial turnover in the adult and during epithelialization events postnatally. Adult *Amhr2-Cre; Rosa26-EYFP* females were used to assess MDM-derived epithelial populations by flow cytometry during estrous cycle stages in nulliparous and postpartum mice. Estrogen-dominant stages, proestrus and estrus, showed the greatest MDM-derived epithelial populations, with up to 80% of the uterine epithelia being EpCAM⁺YFP⁺ in proestrus. Additionally, progesterone-dominant stages, metestrus and diestrus, had little to no MDM-derived epithelial cells. Together, these results suggest this population may be responsive to E2 in the adult.

Since *Amhr2* promotor activity is restricted to mesenchyme embryonically, postnatally, and in the adult, our postnatal data using *Amhr2-Cre; Rosa-flox-stop-tTA; TRE-H2B-GFP* female mice indicate a rapid contribution of MDM-derived epithelial cells at birth (P 0.5), contrary to recent report. Through postnatal uterine maturation, MDM-derived epithelial populations demonstrated dynamic temporal expression patterns: by P 8 during adenogenesis initiation, MDM-derived epithelial populations were less than 2% before increasing to a mean of approximately 11% at P 14 during glandular morphogenesis. This population was maintained from P 14 to P 21. Because there was some variation at P 14 and P 21, E2 and P4 serum concentrations were evaluated, but showed no

correlation to MDM-derived epithelial populations in *Amhr2-Cre; Rosa26-EYFP* or *Amhr2-Cre; Rosa-flox-stop-tTA; TRE-H2B-GFP* females, indicating this population is not dependent on ovarian steroids through P 21. The mechanism inducing MET is still unknown and requires further study in both postnatal uteri and adults.

To address embryonic contribution in mice, future studies aim to collect embryonic uterine horns during late gestation. Furthermore, *Amhr2-Cre; Rosa-flox-stop-tTA; TRE-H2B-GFP* female mice can be used to identify the time of contribution: this inducible model can block labeling during embryogenesis with maternal administration of doxycycline, and upon removal of doxycycline treatment, labeling will begin. This model can better identify the temporal origin of MDM-derived epithelial cells during adenogenesis, as well. Additionally, despite rapid turnover, MDM-derived epithelial cells expressed similar markers to non-MDM-derived epithelial cells, supporting that these cells have gained epithelial characteristics during MET. Future research will continue to characterize MDM-epithelial cells with lineage tracing models, organoids, and begin to address the mechanism behind MET.

SUPPLEMENTAL

Figure S1. Flow Cytometry Gating. Flow cytometry gating for EpCAM⁺YFP⁺/EpCAM⁺YFP⁻ and EpCAM⁺GFP⁺/EpCAM⁺GFP⁻ (MDM-derived/non-MDM-derived) epithelial cells on FlowSight machine.

Figure S2. EpCAM is specific to epithelial cells postnatally.
 Immunofluorescence showed EpcAM was specific to epithelial cells, further supporting mesenchymal cells were not inadvertently analyzed during flow cytometry.

Figure S3. EpCAM is specific to epithelial cells during the estrous cycle. Immunofluorescence showed EpCAM was specific to epithelial cells during cyclic adult mice. These data support mesenchymal cells were not inadvertently analyzed during flow cytometry.

Figure S4. Verification of Epithelial EYFP and GFP Expression in Adult and Postnatal Tissue. Using a GFP antibody, YFP⁺ and GFP⁺ epithelial cells showed the same patterning as GFP antibody, validating their expression in postnatal and adult tissue.

Figure S5. There is no FOXA2 expression prior to adenogenesis initiation. No FOXA2 expression was found at (A-C) P 0.5 or (D-F) P 3.

Figure S6. MDM-derived epithelial cells co-express FOXA2 during the estrous cycle. Nuclear FOXA2 expression was maintained during all stages of the estrous cycle and was found in YFP⁺ and YFP⁻ epithelial cells during **(A-C)** estrus and **(D-F)** metestrus. **(G-I)** Nuclear FOXA2 expression was found in diestrus, but YFP⁺ epithelial cells were rarely found during this stage.

Figure S7. Ki67 expression at P 8. (A-C) Both stromal and epithelial cells were found expressing Ki67 during this time, but stromal proliferation was greater in comparison.

Figure S8. Postnatal ESR1 expression by immunofluorescence. (A-C) Moderate ESR1 expression was found throughout the stroma, with varying expression in the epithelium at P 0.5. GFP⁺ epithelial cells show similar ESR1 expression to GFP⁻ epithelial cells (white arrow). (D-F) ESR1 expression was not found at P 3 in GFP⁺ or GFP⁻ epithelial cells. (G-I) At P 8, stroma showed robust ESR1 expression with moderate expression in the epithelia. (J-L) ESR1 was found variably in epithelia at P 14 with maintained robust expression in the stroma. (M-O) ESR1 expression was found to be robust in GE but showed decreased expression in LE at P 21. GFP⁺ epithelial cells expressed ESR1 similar to GFP⁻ epithelial cells (white arrows).

Figure S9. Postnatal PGR expression by immunofluorescence. (A-C) Stromal cells showed little to no PGR at P 0.5, with epithelium showing greater expression. Both GFP⁺ and GFP⁻ epithelial cells were found to express PGR. (D-F) Little to no PGR expression was found in stromal or epithelial cells at P 3, indicating no difference between GFP⁺ and GFP⁻ epithelial cells. (G-I) At P 8, epithelia exhibited robust expression of PGR, while stroma showed mild to moderate expression. (J-L) Robust epithelial expression was maintained at P 14, while stromal cells showed moderate expression. (M-O) At P 21, PGR expression is high in both epithelia and stroma. GFP⁺ epithelial cells expressed ESR1 and PGR similar to GFP⁻ epithelial cells at P 14 and P 21 (white arrows).

Figure S10. MDM-derived epithelial cells show similar ESR1 expression as non-MDM-derived epithelial cells during the estrous cycle. (A-C) Stromal and epithelial cells showed robust ESR1 expression, with YFP⁺ epithelial cells maintaining robust expression during proestrus (white arrow). (D-F) ESR1 expression decreased in stromal cells during estrus but maintained robust expression in both YFP⁺ and YFP⁻ epithelial cells (white arrow). (G-I) Epithelial expression of ESR1 began to decrease during metestrus, though no difference was seen between MDM-derived and non-MDM-derived epithelial cells (white arrow), and expression in stroma was moderate to robust, similar to ESR1 stromal expression during estrus. (J-L) ESR1 expression was mild in both epithelium and stroma during diestrus.

Figure S11. MDM-derived epithelial cells show similar PGR expression as non-MDM-derived epithelial cells during the estrous cycle. (A-C) PGR expression was variable during proestrus but was found to be expressed similarly in YFP⁺ and YFP⁻ epithelial cells (white arrow). (D-F) Epithelial PGR expression decreased and became variable during estrus, though expression was similar between YFP⁺ and YFP⁻ epithelial cells (white arrow). (G-I) PGR expression began to increase in epithelial cells during metestrus, with both YFP⁺ and YFP⁻ epithelial cells showing moderate expression (white arrow). (J-L) PGR expression was robust in both compartments during diestrus.

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