

Uterine epithelial remodelling during pregnancy in the marsupial *Monodelphis domestica* (Didelphidae): Implications for mammalian placental evolution

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Abstract

Mammalian pregnancy involves remodelling of the uterine epithelium to enable placentation. In marsupials, such remodelling has probably played a key role in the transition from ancestral invasive placentation to non-invasive placentation. Identifying uterine alterations that are unique to marsupials with non-invasive placentation can thus elucidate mechanisms of marsupial placental evolution. We identified apical alterations to uterine epithelial cells prior to implantation in *Monodelphis domestica*, a member of the least derived living marsupial clade (Didelphidae) with invasive (endotheliochorial) placentation. We then compared these traits with those of *Macropus eugenii* (Macropodidae) and *Trichosurus vulpecula* (Phalangeridae), both with non-invasive placentation, to identify which alterations to the uterine epithelium are ancestral and which facilitate secondarily evolved non-invasive placentation. In *M. domestica*, remodelling of the uterine epithelium involves reduced cellular heterogeneity and development of uterodome-like cells, suggesting that similar alterations may also have occurred in the marsupial common ancestor. These alterations also overlap with those of both *T. vulpecula* and *Ma. eugenii*, suggesting that the placental shift from invasive to non-invasive placentation in marsupials involves essential, conserved characteristics, irrespective of placental mode. However, unique apical alterations of both *T. vulpecula* and *Ma. eugenii*, relative to *M. domestica*, imply that lineage-specific alterations underpin the evolutionary shift to non-invasive placentation in marsupials.

KEYWORDS

marsupial, morphology, placentation, plasma membrane transformation

1 | INTRODUCTION

Amniote viviparity often involves formation of a placenta during pregnancy to meet the physiological needs of a developing embryo in utero (Murphy et al., 2000; Thompson et al., 2002). In mammals, viviparity has evolved once in the common ancestor of eutherian mammals and marsupials (Lillegraven, 1975; Zeller and Freyer, 2001). Placental morphology in mammals is highly diverse

(Mossman, 1987; Murphy, 1998) and invasion of the uterus by the embryo at implantation occurs along a penetrative spectrum (Wildman, 2016), from non-invasive (epitheliochorial) placentation (Ferner and Mess, 2011), involving relatively superficial maternal-embryonic contact, through to highly invasive (haemochorial) placentation with direct transfer between maternal and embryonic blood streams (Mess, 2014). Remodelling of the uterine epithelium, termed the plasma membrane transformation, is required

for uterine receptivity to the embryo, irrespective of placental type (Murphy, 2004). In eutherian mammals, many aspects of remodelling facilitate embryonic invasion at implantation, including reduction of baso-lateral adhesion of uterine epithelial cells, such that uterine cells are more readily displaced by invasive embryonic cells (Enders and Schlafke, 1967; Schlafke and Enders, 1975; Preston et al., 2004; Kaneko et al., 2008). By contrast, remodelling in marsupial pregnancy involves maternal defences against the implanting embryo, including reinforcement of the basal connections of the uterine epithelium that potentially reduce or prevent invasion (Laird et al., 2017a, 2018). Lack of similar defences in eutherian placentae, which are typically more invasive than those of marsupials (Amoroso, 1952; Renfree et al., 2013), may reflect decidualisation of fibroblasts in the uterine stroma during pregnancy. This uniquely eutherian phenomenon serves to regulate stromal invasion by the embryo after the uterine epithelium has been breached (Kin et al., 2014; Wagner et al., 2014). Since marsupials lack decidualisation, and thus a stromal mechanism of regulating embryonic invasion, the uterine epithelium itself may thus play a greater role in regulating marsupial implantation than it does in eutherian mammals, resulting in maternal reinforcement of the existing epithelial barrier (Laird, 2017).

Phylogenetic distribution of placental types in mammals suggests that the eutherian common ancestor had invasive placentation that was either haemochorial or endotheliochorial (Carter and Mess, 2007; Elliot and Crespi, 2009). Recent molecular evidence suggests that invasive placentation is also ancestral for marsupials (Mess and Ferner, 2010); therefore, non-invasive placentation in both eutherian mammals (Elliot and Crespi, 2009; Carter and Enders, 2013) and marsupials (Mess and Ferner, 2010; Ferner and Mess, 2011) is likely to be secondarily derived (Carter and Mess, 2007). Accumulation of maternal defences to the embryo may be a mechanism by which non-invasive placentation has evolved secondarily in marsupials (Crespi and Semeniuk, 2004; Vogel, 2005; Carter and Mess, 2007; Laird et al., 2017a). Hence, unique aspects of remodelling involved in non-invasive marsupial placentation can identify how the uterus has been modified to support this placental type.

Non-invasive placentation occurs in both phalangerid and macropodid marsupials (Figure 1; Pilton and Sharman, 1962; Tyndale-Biscoe and Renfree, 1987; Freyer et al., 2003; Mess and Ferner, 2010; Laird et al., 2018), and has probably evolved independently in these two families. The uterine epithelium of both *Macropus eugenii* (tammar wallaby; Macropodidae) and *Trichosurus vulpecula* (brushtail possum; Phalangeridae) undergoes similar morphological and molecular modifications during pregnancy (Freyer et al., 2002; Laird et al., 2017b, 2018), yet apical remodelling of the uterine epithelium also involves species-specific differences. For example, cellular diversity of the uterine epithelium increases before embryonic attachment in *T. vulpecula* (Laird et al., 2017b), yet decreases in *Ma. eugenii* as the uterine epithelium becomes more uniform (Freyer et al., 2002). In addition, cells of *T. vulpecula* develop extremely domed and elongated apices during the

implantation period (Laird et al., 2017b), but these cells do not develop in *Ma. eugenii* (Freyer et al., 2002).

Members of Didelphidae, the least derived clade of living marsupials (Nilsson et al., 2010), represent ancestral marsupial reproductive traits (Harder et al., 1993; Freyer et al., 2003; Freyer and Renfree, 2009; Kin et al., 2014; Hansen et al., 2016, 2017; Griffith et al., 2017) and are therefore ideal for testing evolutionary theories of how and when marsupial placental modes arose (Freyer et al., 2002; Hansen et al., 2016). The didelphid *Monodelphis domestica* (grey short-tailed opossum), in which embryonic implantation is invasive (Harder et al., 1993; Kin et al., 2014), can identify the ancestral states of remodelling characteristics, and thus the mechanisms involved in the evolutionary transition from ancestrally invasive to non-invasive embryonic attachment.

In the present paper, we report the first detailed investigation of the uterine surface alterations required for receptivity to the embryo in *M. domestica*, using scanning electron microscopy. We compare these alterations with those of *T. vulpecula* (Laird et al., 2017b) and *Ma. eugenii* (Freyer et al., 2002) to identify which apical alterations are ancestral and which have developed secondarily for non-invasive embryonic attachment in marsupials.

2 | METHODS

2.1 | Study species

Monodelphis domestica has a gestation period of 14.5–15 days (Harder et al., 1993; Mate et al., 1994; Zeller and Freyer, 2001). Unlike other marsupial species, females of *M. domestica* do not undergo a true (spontaneous) oestrous cycle as oestrus is induced by male pheromones (Fadem, 1985; Hinds et al., 1992; Harder et al., 1993). Ovulation occurs approximately 20–24 hr post-copulation (Baggott and Moore, 1990; Mate et al., 1994; Zeller and Freyer, 2001). The shell coat ruptures 12 days post-copulation, followed by invasive (endotheliochorial) implantation of the trophoblastic syncytium of the trilaminar yolk sac (Zeller and Freyer, 2001), which persists until birth.

2.2 | Animal husbandry and tissue collection

This study was approved under protocol numbers 13-100920-MCC and 15-200334-B-MC from the University of New Mexico Institutional Animal Care and Use Committee. Animals used in this study were from a captive-bred research colony housed at the Department of Biology Animal Research Facility at the University of New Mexico and housed as per Hansen et al. (2016). Adult males and females were housed separately when not breeding. Oestrus was induced by introducing the female into a cage with a male. Pregnancies were timed once mating was observed. Pregnant animals were killed by inhaled isoflurane overdose (>5%) until breathing had ceased for >1 min. Uterine

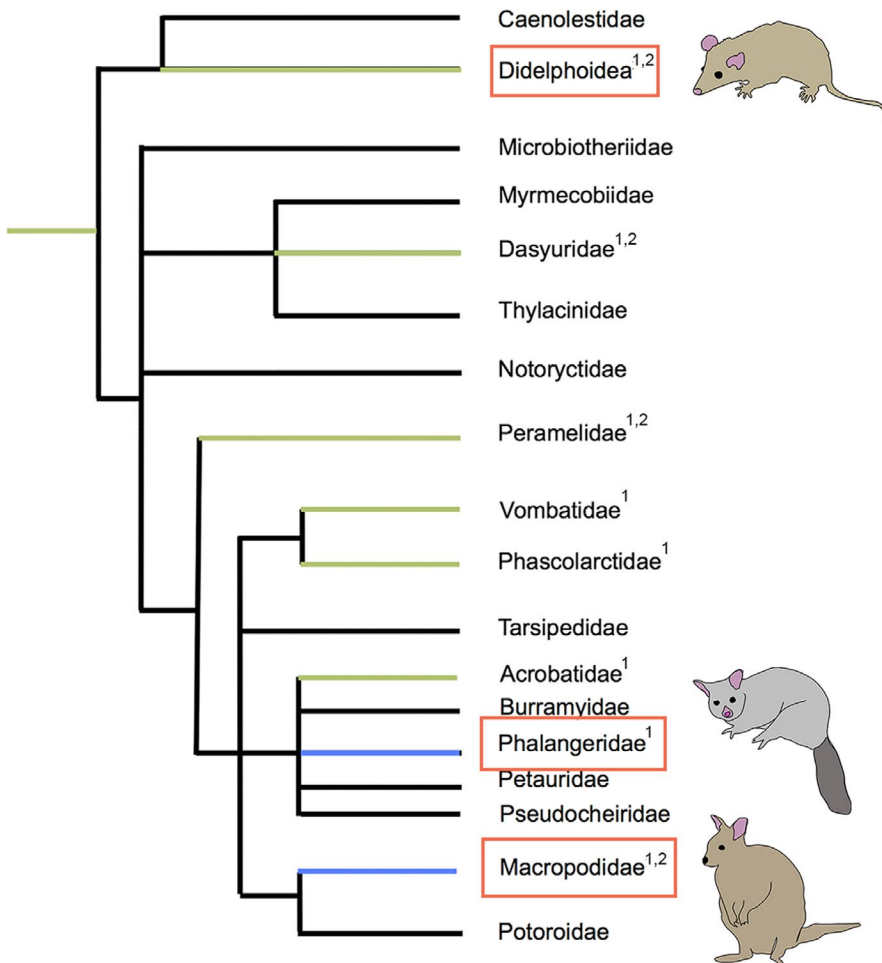


FIGURE 1 Phylogenetic distribution of placental types in Metatheria (redrawn from Freyer et al. (2003) and Laird et al. (2018)). Endotheliochorial placentation (green); epitheliochorial placentation (blue); unknown placentation (black). Clades compared in this studied are indicated by red boxes. ¹Mess and Ferner (2010); ²Freyer et al. (2003)

tissues were collected from 15 pregnant females between 7 days 0 hr and 14 days 0 hr post-conception. Females were grouped into three reproductive stages over the period of implantation (Mate et al., 1994): Stage 1 (pre-implantation, between 7 days 0 hr and 10 days 12 hr post-copulation; $n = 6$); Stage 2 (implantation, between 11 days 1 hr–12 days 2 hr post-copulation; $n = 5$); Stage 3 (post-implantation; between 13 days 0 hr and 14 days 0 hr post-copulation; $n = 4$). The morphology of the non-pregnant (cycling) uterine epithelium is described in Wick and Kress (2002).

2.3 | Tissue processing for scanning electron microscopy

Uterine horns were excised and opened to expose the internal surface, and embryos and amnions were removed. Tissue samples were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (PB) for 1 hr, followed by rinsing in 0.1 M PB. Samples were then post-fixed in 4% osmium tetroxide for 1 hr, followed by further rinsing in 0.1 M PB and gradual dehydration to 100% ethanol. These samples were then dried with a Leica EM CPD300 Critical Point Dryer (Leica) using carbon dioxide as the drying agent. Dried tissue was mounted onto

aluminium stubs and coated with gold (15 nm). Images were captured on a JEOL NeoScope JCM-600 Tabletop scanning electron microscope and a Zeiss Sigma HD VP STEM (Zeiss).

2.4 | Tissue processing for transmission electron microscopy

For transmission electron microscopy, a small portion of excised uterus from each animal was fixed in glutaraldehyde as per the scanning electron microscopy samples, then post-fixation was carried out for 1 hr in 4% osmium tetroxide with 0.8% potassium ferrocyanide [$K_4Fe(CN)_6$] to enhance membrane contrast (Hulstaert et al., 1983). Samples were rinsed in 0.1 M PB, then 50%, 70% and 100% ethanol. Larger pieces of tissue were cut to $\sim 0.5 \text{ mm}^3$. Ethanol was replaced by Spurr's resin (Agar Scientific) in increments of 25%, and tissue pieces were transferred to individual BEEM capsules and polymerized at 62°C overnight. Semi-thin (200- μm) and ultra-thin (70- μm) sections were cut using a Leica Ultrastat 7 cryostat (Leica) and ultra-thin sections were transferred to 200- μm mesh copper grids (ProSci Tech). Grids were post-stained by floating each on a drop of 2% uranyl acetate for 10 min, rinsing in warm water, then floating on a drop of Reynolds lead citrate surrounded by sodium hydroxide (NaOH)

pellets for 10 min (Hayat, 1986). Grids were rinsed and air dried before imaging. Similar to the process for scanning electron microscopy, images were captured using a Zeiss Sigma HD VP STEM (Zeiss).

2.5 | Morphometrics

Cellular heterogeneity of the uterine epithelium of *M. domestica* was determined by comparing the relative abundance of distinct cell types over the three pregnancy stages (Laird et al., 2017b). The entire uterine surface was examined using scanning electron microscopy to confirm uniformity. At least five high magnification images were randomly selected for morphometric analysis per animal (500 \times and 3,000 \times ; \sim 100–400 cells per image), with most at \sim 1,400–1,700 \times (200–300 cells per image). Cells were counted systematically using an overlaid grid. Each cell in an image was allocated to one of five cell types: bare surface; sparse microvilli; dense microvilli; budding cells and ciliated cells (as per Laird et al., 2017b; see Results for examples of cell types). Damaged cells, or cells that could not be clearly identified, were excluded from the count. Proportions were obtained by dividing the total number of each cell type by the total number of cells in the image. Proportions for each animal were arcsine-transformed (Dytham, 2011). Cells were counted and the analysis was applied until SEM < 0.05 for each cell type for each animal (Aherne and Dunhill, 1982). Arcsine-transformed proportions were log-transformed to satisfy assumptions of normality. These values were analysed using a generalized linear model with respect to cell type

by ANOVA in STATISTICA v9.0 (Statsoft), with pregnancy stage as a covariate.

3 | RESULTS

3.1 | Scanning electron microscopy

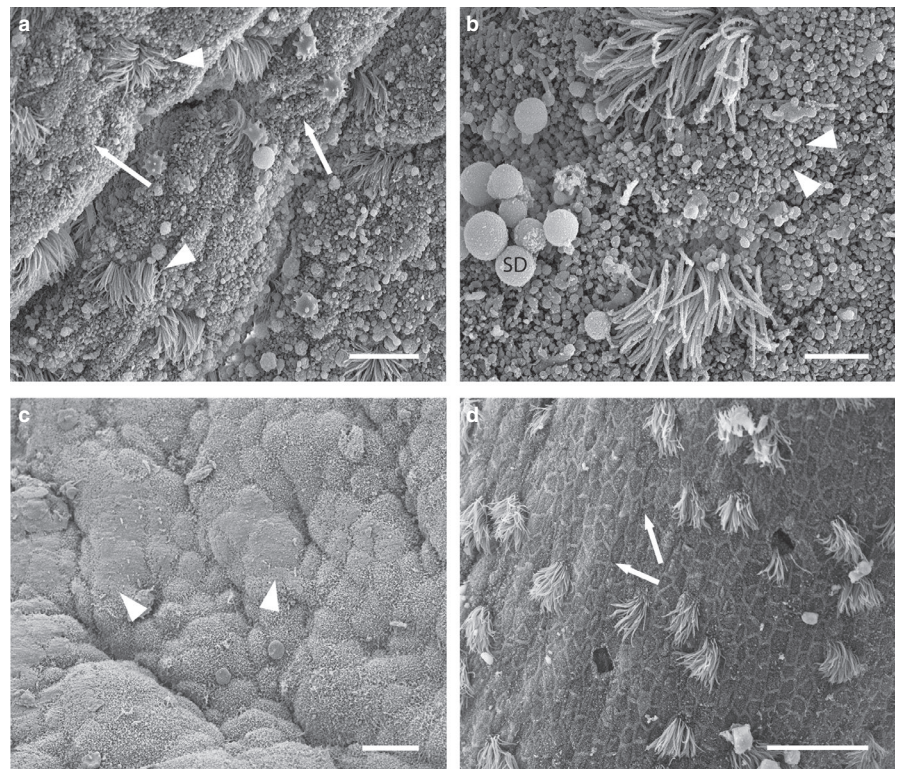
3.1.1 | Stage 1: Pre-implantation (7 days 0 hr–10 days 12 hr post-copulation)

The uterine surface is folded at \sim 7 days post-conception (Figure 2a). Cell apices are slightly domed and are densely covered by short blunt microvilli, and ciliated cells are interspersed with microvillous cells (Figure 2a,b). Small secretory droplets occur in the uterine lumen. By \sim 10 days post-conception, microvilli are spiky, rather than blunt, and plaques of glycocalyx occur on the uterine surface (Figure 2c). Additionally, throughout Stage 1, cells in some uterine regions are flattened with raised borders, and are uniformly arranged in a 'honeycomb' pattern (Figure 2d). Ciliated cells are evenly distributed in these regions (Figure 2d).

3.1.2 | Stage 2: Implantation (11 days 1 hr–12 days 2 hr post-copulation)

Uterine epithelial cells at Stage 2 possess spiky microvilli, and cell apices are more domed than for Stage 1 (Figure 3a). Many microvillous cells have a 'dimple' on the apical surface (Figure 3b), as well as clumps

FIGURE 2 Scanning electron micrographs of the uterine surface of *Monodelphis domestica* at Stage 1 of pregnancy (7 days 0 hr–10 days 12 hr post-copulation). (a) Ciliated cells (arrowheads) are interspersed with densely microvillous cells (arrows). (b) Microvillous cells are densely covered in short blunt microvilli (arrowheads). Small secretory droplets (SD) also occur in the uterus. (c) Plaques of glycocalyx (arrowheads) occur on the uterine surface. (d) In some uterine regions, uterine epithelial cells are flattened, with raised borders, and arranged in a 'honeycomb' pattern (arrows). Scale bars = 10 μ m (a), 4 μ m (b), 15 μ m (c), 20 μ m (d)



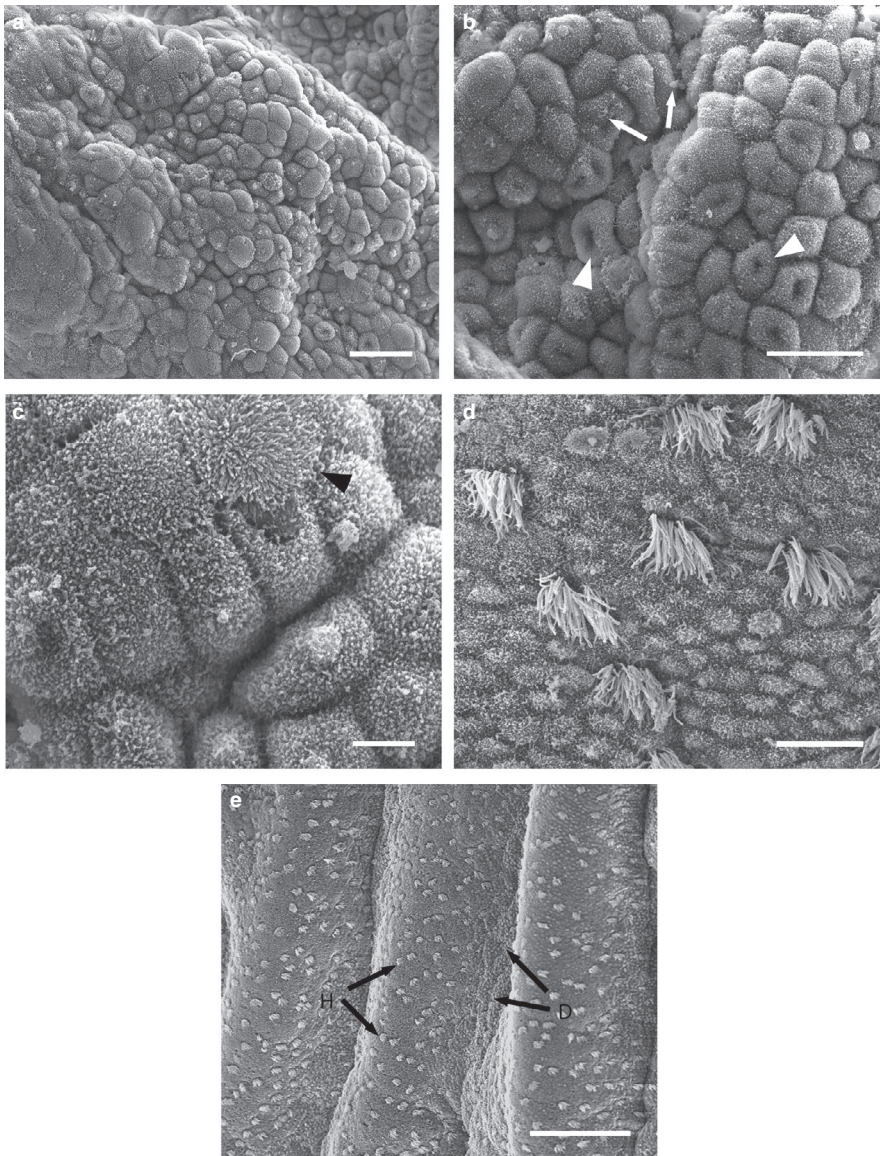


FIGURE 3 Scanning electron micrographs of the uterine surface of *Monodelphis domestica* at Stage 2 of pregnancy (11 days 1 hr–12 days 2 hr post-copulation). (a) Uterine region consisting of cells with domed apices. (b) Higher magnification of (a): depicts apical 'dimples' (arrowheads) and clumps of glycocalyx (arrows). (c) Cells with elongated microvilli (arrowhead) occur. (d) Uterine region consisting of uniform 'honeycomb' cells. (e) Domed uterine epithelial cells (d, arrows) are restricted to the bases of uterine folds, while flattened 'honeycomb' regions (h, arrows) occur on the crests. Scale bars = 25 μm (a), 20 μm (b), 5 μm (c), 10 μm (d), 100 μm (e)

of glycocalyx. Cells with longer microvilli occur at this stage (Figure 3c), alongside the ciliated cells, which have become fewer by ~12 days 2 hr post-conception. Similarly to Stage 1, regions of the uterus retain a uniform 'honeycomb' appearance that is morphologically distinct throughout Stage 2 (Figure 3d). These 'honeycomb' regions are restricted to the crests of uterine folds (Figure 3e), whereas regions of uterine epithelium with domed cells are restricted to the bases of these folds (Figure 3e).

3.1.3 | Stage 3: Post-implantation (13 days 0 hr–14 days 0 hr post-copulation)

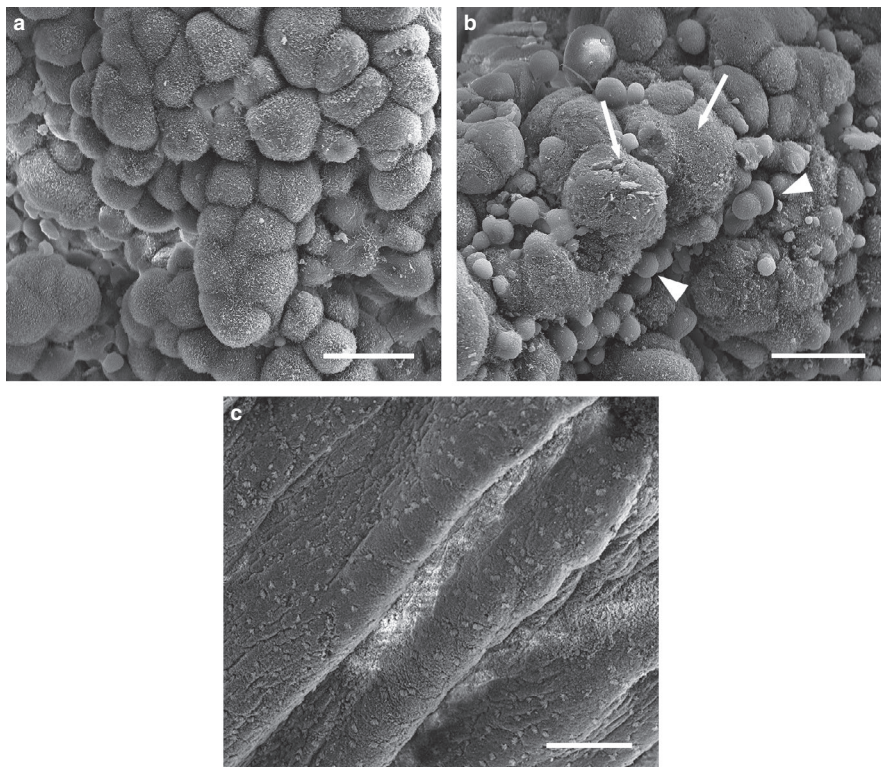
Uterine epithelial cells are domed and irregular by Stage 3, with sparse spiky apical microvilli (Figure 4a). In some regions, cells are extremely domed (budding cells; Figure 4b) and lack apical projections. Glycocalyx plaques cover large regions of uterine epithelium (Figure 4b). Ciliated

cells are few, except in regions of regularly arranged 'honeycomb' cells, which still occur at the crests of uterine folds (Figure 4c).

3.2 | Transmission electron microscopy

At Stage 1 (pre-implantation), both uterine epithelial cells and trophoblastic cells are densely covered in microvilli (Figure 5a–c). Uterine epithelial cells are elongated and form a single columnar layer, underlain by large blood vessels close to their basal membranes (Figure 5b). Trophoblastic cells are large and domed and underlain by a thin layer of mesenchyme (Figure 5c). By Stage 2 (implantation), uterine epithelial cells are less uniform and have apices that project into the uterine lumen (Figure 5d). These projections are more pronounced by Stage 3 (post-implantation) when the uterine surface is highly folded, and rounded budding cells extend into the lumen (Figure 5e).

FIGURE 4 Scanning electron micrographs of the uterine surface of *Monodelphis domestica* at Stage 3 of pregnancy (13 days 0 hr–14 days 0 hr post-copulation). The uterine epithelium consists of domed and irregular cells (a), and budding cells (b, arrowheads) with large glycocalyx plaques (b, arrows). Regions of honeycomb cells also occur (c). Scale bars = 20 μm (a, b), 100 μm (c)



3.3 | Morphometrics

Densely microvillous cells dominate the uterine surface across all three stages (Figure 6). Budding cells are significantly more abundant at Stage 3 ($F = 11.6649$, $df = 2$; $p < .001$) than at other stages, while bare surface cells appear to be more abundant at Stage 1. A reduction in abundance of ciliated cells occurs between Stages 1 and 3. Overall cellular diversity reduces after Stage 1 and the uterine epithelium is least diverse at Stage 2, after which diversity increases (Figure 6).

4 | DISCUSSION

The uterine epithelium of *M. domestica* undergoes substantial apical remodelling during the implantation period that is consistent with a plasma membrane transformation (Murphy et al., 2000; Murphy, 2004). Short, blunt microvilli are replaced by spiky microvilli prior to embryonic implantation in *M. domestica*, corresponding to a peak in plasma progesterone ~9–10 days post-copulation (Fadem and Rayve, 1985; Hinds et al., 1992; Bradshaw and Bradshaw, 2011), as also occurs in rats and mice (Ljungkvist, 1972; Murphy, 2000). This change in microvilli also corresponds to a shortening of microvilli on the trophoctodermal surface, enabling subsequent interdigitation of trophoblastic and endometrial microvilli and reducing the barrier between fetal and maternal blood (Zeller and Freyer, 2001). Ciliated cells are reduced in the uterine epithelium by Stage 3 in *M. domestica*, similar to post-oestrus deciliation in the cycling non-pregnant uterus in response to oestrogen reduction (Wick

and Kress, 2002). Loss of ciliated cells is typical of remodelling in a range of eutherian mammals (Barberini et al., 1978; Stroband et al., 1986; Murphy, 2004) and marsupials (Enders and Enders, 1969; Ward and Renfree, 1988; Rose et al., 1999; Laird et al., 2014). Since *M. domestica* most closely resembles the reproductive traits of the most recent marsupial common ancestor (Harder et al., 1993; Freyer et al., 2002; Kin et al., 2014; Hansen et al., 2016; Griffith et al., 2017), similarities in apical cell remodelling during pregnancy in *M. domestica* and eutherian mammals suggest that these alterations are ubiquitous in mammalian pregnancy and that a plasma membrane transformation may also have occurred in the therian common ancestor.

Interestingly, the uterine epithelium of *M. domestica* is not uniformly remodelled. Regions of flattened 'honeycomb' cells with raised borders, interspersed with ciliated cells, are retained throughout pregnancy and occur at the crests of uterine folds. In contrast, remodelled cells occur only at the bases of these folds. This morphological difference between remodelled and non-remodelled regions could relate to the high functional differentiation of embryos of *M. domestica*, which have an invasive trilaminar yolk sac and a non-invasive bilaminar yolk sac (Freyer et al., 2002), resulting in a mixed epitheliochorial-endotheliochorial placenta (Zeller and Freyer, 2001; Carter and Enders, 2016). Implantation of the trilaminar yolk sac side forms a trophoctodermic syncytium (Freyer et al., 2002) and placental cells closely interdigitate with those of the uterine epithelium, enabling the syncytium to erode the uterine epithelium at regular intervals (Zeller and Freyer, 2001). In contrast, microvillous cells of the bilaminar side interact non-invasively with microvilli of uterine epithelial cells at implantation (Zeller and Freyer, 2001). Hence, in

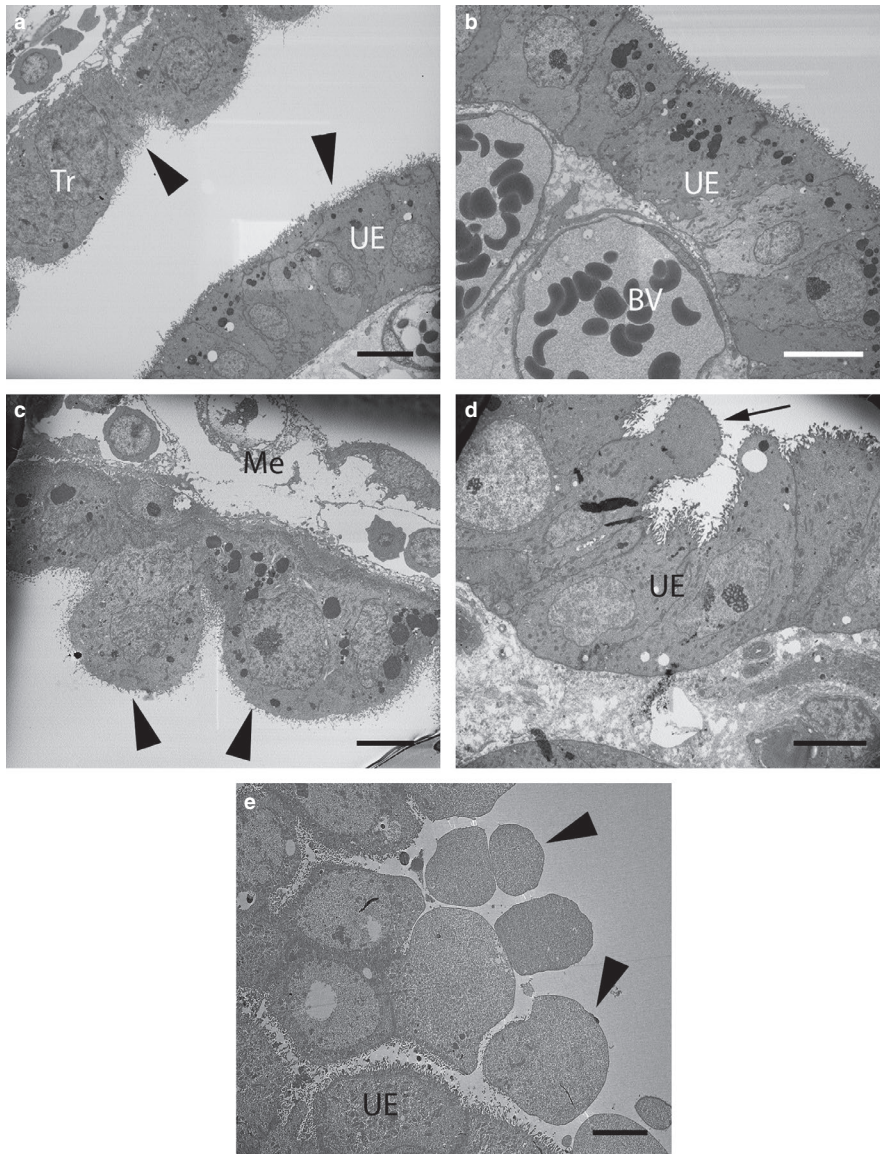


FIGURE 5 Transmission electron micrographs of the uterus of *Monodelphis domestica*. (a–c) Stage 1 (Pre-implantation), (d) Stage 2 (Implantation) and (e) Stage 3 (Post-implantation). (a) Both uterine epithelial cells (UE) and trophoblastic cells (Tr) have elongated microvilli (arrowheads). (b) Higher magnification of (a), depicting single layer of columnar UE underlain by maternal blood vessels (BV). (c) Higher magnification of (a), depicting large trophoblastic cells (arrowheads) underlain by mesenchyme (Me). (d) UE cells at implantation are less regular and project into the uterine lumen (arrow). (e) Budding cells (arrowheads) are prominent post-implantation. Scale bars = 10 μm (a–c), 6 μm (d), 5 μm (e)

M. domestica, remodelled regions in the bases of uterine folds may facilitate invasive embryonic interactions (Enders and Schlafke, 1967) with the trilaminar side, while non-remodelled regions may facilitate non-invasive contact with the bilaminar yolk sac. The receptive uterus of *Sminthopsis crassicaudata* (fat-tailed dunnart) also has remodelled and non-remodelled sites (Laird et al., 2014), and embryos of this species show similar functional differentiation to those of *M. domestica* (Roberts and Breed, 1994). In contrast, the uterus is uniformly remodelled in both *T. vulpecula* (Laird et al., 2017b) and *Ma. eugenii* (Freyer et al., 2002), two species for which embryos are non-invasive. Hence, uniform remodelling of the entire uterine surface may be a modification to facilitate non-invasive embryonic attachment in marsupials.

An alternate explanation is that restriction of remodelled cells to certain regions may be a maternal innovation to reduce parent-offspring conflict at the embryonic–maternal interface. Invasive trophoblasts are probably ancestral for marsupials (Mess and Ferner, 2010). Since microvilli greatly increase the surface area for active

transport, the conflict hypothesis predicts that maternal microvillous regions are smaller than those of embryonic surfaces to restrict transfer of resources (Kazemian et al., 2019). Confinement of domed and densely microvillous cells to discrete regions in *M. domestica* and *S. crassicaudata* may thus regulate embryonic invasion by limiting physical contact between maternal and embryonic microvillous surfaces. This hypothesis also accounts for uniform remodelling in *T. vulpecula* and *Ma. eugenii*, as non-invasive placentation affords greater maternal control over resource allocation (Kazemian et al., 2019). Indeed, the plasma membrane transformation itself may also be a maternal mechanism to regulate the timing of placentation by restricting uterine receptivity to a brief window. Embryonic attachment in *M. domestica*, and potentially in the marsupial stem species, thus appears to be regulated both temporally, via a strict receptivity window, and spatially, by constricting remodelled epithelia to discrete regions.

Cellular diversity of the receptive uterine epithelium decreases prior to implantation in *M. domestica* (Figure 7a). A similar decrease

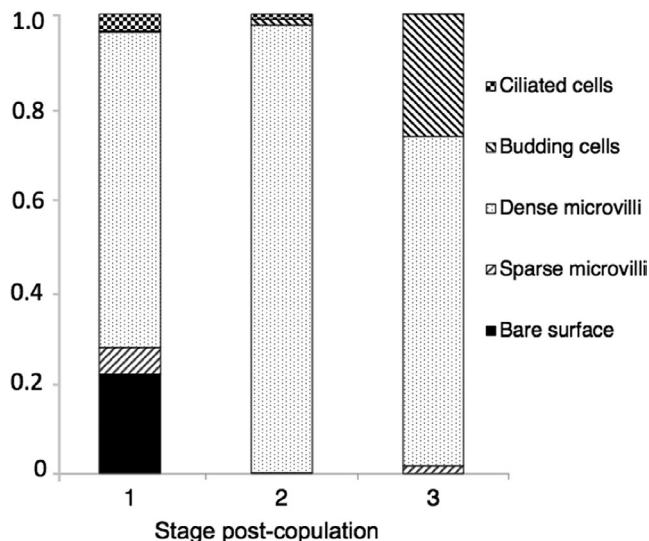


FIGURE 6 Cellular composition of the uterine epithelium of *Monodelphis domestica*. Graph depicts relative proportions of each of five distinct cell types on the uterine surface over the three pregnancy stages. Data are presented as means of arcsine-transformed proportions for each stage of pregnancy ($SEM < 0.05$)

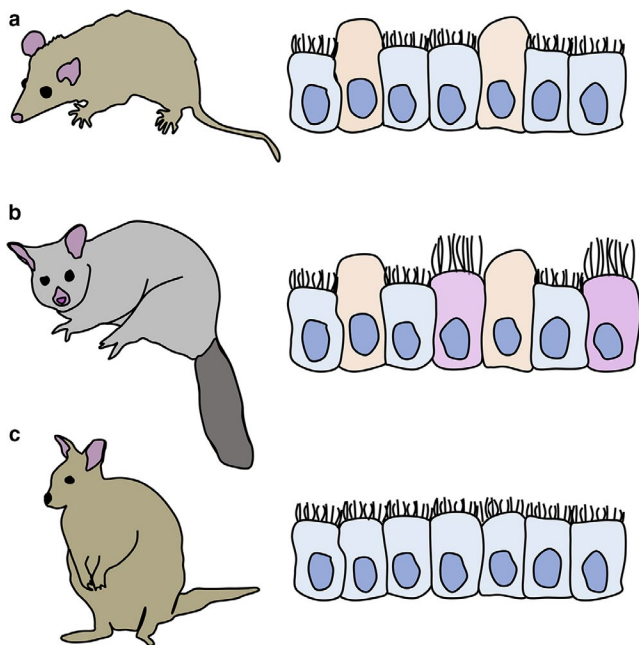


FIGURE 7 Diagrammatic representation of receptive uterine epithelial cell morphology in marsupials. (a) *Monodelphis domestica* (present study; invasive endotheliochorial placentation). (b) *Trichosurus vulpecula* [non-invasive epitheliochorial placentation (Laird et al., 2017b)]. (c) *Macropus eugenii* [non-invasive epitheliochorial placentation (Freyer et al., 2002)]. Blue cells = microvillous cells; orange cells = pinopods/uterodomes; pink/mauve cells = ciliated cells

occurs in a range of eutherian mammal species (Potts and Racey, 1971; Winterhager and Denker, 1990; Murphy et al., 2000), as well as in *S. crassicaudata* (Laird et al., 2014) and potentially *Ma. eugenii* (Freyer et al., 2002). In these species, a uniform uterine epithelium

facilitates physical maternal-embryonic contact (Schlafke and Enders, 1975), as well as transfer of essential substances and signalling molecules between uterine and trophoblastic cells. In contrast, cell heterogeneity peaks at implantation for *T. vulpecula*, suggesting that a diverse uterine epithelium may be required for non-invasive embryonic attachment in this species (Laird et al., 2017b), but not *Ma. eugenii* (Figure 7b,c). Alternately, maternal recognition of pregnancy occurs in both *Ma. eugenii* (Renfree, 2000) and *M. domestica* (Griffith et al., 2019), but not *T. vulpecula* (Pilton and Sharman, 1962; Renfree, 2000; Laird et al., 2017b). Hence decreased cell diversity in *M. domestica* and *Ma. eugenii* may form part of an endometrial response to the presence of an embryo.

Elongated budding cells appear in the uterine epithelium of *M. domestica* ~12 days post-conception, and are abundant throughout Stage 3 (Figure 7a). These budding cells are consistent with previous observations of *M. domestica* (Griffith et al., 2017), and are similar in appearance to the domed uterine epithelial cells present at pro-oestrus (Wick and Kress, 2002). However, unlike budding cells of the pregnant uterus, domed cells at pro-oestrus are interspersed with solitary cilia, lack an apical 'dimple' and occur in the absence of uterine folds. Hence domed cells pro-oestrus and budding cells at implantation, while morphologically similar, probably serve different functions. Domed apices during pregnancy in *M. domestica* are also morphologically similar to pinopods in rats and mice (Nilsson, 1958; Murphy, 2000) and uterodomes in other mammals (Murphy, 2000; Murphy et al., 2000; Stavreus-Evers, 2005; Nikzad et al., 2010) and viviparous lizards (Hosie et al., 2003; Adams et al., 2005). In rodents, these cells are indicative of a uterus which is becoming receptive (Murphy, 2000; Stavreus-Evers, 2005). However, appearance of these cells is coincident with embryonic attachment in *M. domestica* (Zeller and Freyer, 2001; Freyer et al., 2002; Hansen et al., 2017), suggesting they play a role in later pregnancy in this species. Similar cells develop prior to implantation in *T. vulpecula* (Figure 7b; Laird et al., 2017b) and are thought to facilitate secretion of haematophages from the maternal blood circulation across the uterine epithelium for uptake by the embryo (Laird et al., 2017b). A similar role is likely for *M. domestica* as a shift in embryonic nutrition, from uterine secretions to haematophagy from the maternal circulation, occurs around the time of attachment (Zeller and Freyer, 2001; Freyer et al., 2007; Hansen et al., 2017), as also occurs for most marsupials. In contrast, uterodome-like cells do not develop during pregnancy in either *S. crassicaudata* (Laird et al., 2014) or *Ma. eugenii* (Figure 7c; Freyer et al., 2002), suggesting that these cells are not related to a particular placental form. Hence uterodome-like cells may have formed part of the receptive epithelium in the therian common ancestor, and have been subsequently lost in Dasyuridae and Macropodidae, or may have arisen independently in both *M. domestica* and *T. vulpecula*. Unusually, embryonic development post-implantation in *Ma. eugenii* is supported by both histotrophy and haematotrophy (Freyer et al., 2002, 2003). Hence, nutrient transfer via both mechanisms in *Ma. eugenii* may result in fewer or different uterine mechanisms to facilitate haematotrophic nutrient

transfer, potentially accounting for the lack of uterodome-like cells in the receptive uterus.

Uterine alterations in *M. domestica* prior to implantation, including decreased cellular heterogeneity of the uterine epithelium, reduction of ciliated cells and development of cells with spiky microvilli and uterodome-like cells, are likely to most closely represent those of the marsupial stem species. Conservation of these traits in more derived marsupial clades suggests that they are essential for marsupial pregnancy, irrespective of placental type. In contrast, *Ma. eugenii* and *T. vulpecula* undergo patterns of apical remodelling that differ both from those of each other and of *M. domestica*, including absence of uterodome-like cells in *Ma. eugenii* (Freyer et al., 2002) and increased cell diversity at implantation in *T. vulpecula* (Laird et al., 2017b). Molecular differences in the uterus also occur, including different localization patterns of the lateral plasma membrane protein, desmoglein-2, in *Ma. eugenii* and *T. vulpecula*, suggesting that desmoglein-2 plays different roles in these species (Laird et al., 2018) to eutherian mammals (Preston et al., 2004) and *S. crassicaudata* (Dudley et al., 2015). Hence, successful non-invasive embryonic attachment in marsupials requires lineage-specific alterations to the uterine epithelium, consistent with the independent evolution of non-invasive attachment in Macropodidae and Phalangeridae (Freyer et al., 2003; Laird et al., 2017b). Nevertheless, aspects of apical remodelling of both *Ma. eugenii* and *T. vulpecula* that overlap with those of *M. domestica* demonstrate that the transition from ancestrally invasive to non-invasive placentation in marsupials has not required innovation in all aspects of uterine remodelling, as some appear to be essential for placentation in therian mammals.

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CONFLICT OF INTEREST

None declared.

AUTHOR CONTRIBUTIONS

VH collected and fixed samples. MKL processed samples for microscopy, collected the data and wrote the manuscript. All authors contributed to experimental design, technical advice, image interpretation and manuscript preparation and revision.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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