Non-invasive placentation in the marsupials *Macropus eugenii* (Macropodidae) and *Trichosurus vulpecula* (Phalangeridae) involves redistribution of uterine Desmoglein-2

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In mammalian pregnancy, the uterus is remodeled to become receptive to embryonic implantation. Since non-invasive placentation in marsupials is likely derived from invasive placentation, and is underpinned by intra-uterine conflict between mother and embryo, species with non-invasive placentation may employ a variety of molecular mechanisms to maintain an intact uterine epithelium and to prevent embryonic invasion. Identifying such modifications to the uterine epithelium of marsupial species with non-invasive placentation is key to understanding how conflict is mediated during pregnancy in different mammalian groups. Desmoglein-2, involved in maintaining lateral cell–cell adhesion of the uterine epithelium, is redistributed before implantation to facilitate embryo invasion in mammals with invasive placentation. We identified localization patterns of this cell adhesion molecule throughout pregnancy in two marsupial species with non-invasive placentation, the tammar wallaby (*Macropus eugenii*; Macropodidae), and the brushtail possum (*Trichosurus vulpecula*; Phalangeridae). Interestingly, Desmoglein-2 redistribution also occurs in both *M. eugenii* and *T. vulpecula*, suggesting that cell adhesion, and thus integrity of the uterine epithelium, is reduced during implantation regardless of placental type, and may be an important component of uterine remodeling. Desmoglein-2 also localizes to the mesenchymal stromal cells of *M. eugenii* and to epithelial cell nuclei in *T. vulpecula*, suggesting its involvement in cellular processes that are independent of adhesion and may compensate for reduced lateral adhesion in the uterine epithelium. We conclude that non-invasive placentation in marsupials involves diverse and complementary strategies to maintain an intact epithelial barrier.

**KEYWORDS**
Desmoglein-2, epitheliochorial, implantation, pregnancy, uterus

1 | INTRODUCTION

The mammalian uterus is remodeled during pregnancy to become receptive to the implanting embryo (Murphy, 2004; Orchard & Murphy, 2002; Zhang et al., 2013). Receptivity involves morphological and biochemical alterations to the uterine epithelium, termed the plasma membrane transformation, that occur irrespective of placental type (Murphy, 2004).

Phylogenetic distribution of placentation types in mammals suggests that placentation in the common ancestor of living eutherian
mammals was invasive—either haemochorial or endotheliochorial (Figure 1a) (Carter & Mess, 2007; Elliot & Crespi, 2009; Enders & Carter, 2004; Martin, 2008). Recent molecular evidence and phylogenetic reconstructions also suggest that invasive placentalation is ancestral for living marsupials (Figure 1b) (Bininda-Emonds et al., 2007; Mess & Ferner, 2010). Therefore, non-invasive placentalation in both eutherian mammals (Carter & Enders, 2013; Elliot & Crespi, 2009) and marsupials (Ferner & Mess, 2011; Mess & Ferner, 2010) is likely secondarily derived (Carter & Mess, 2007; Capellini, 2012; Elliot & Crespi, 2009; Martin, 2008; Mess, 2014; Vogel, 2005).

A potential driver of this placental transition in mammals is intra-uterine conflict arising from genetic differences between mothers and offspring (Crespi & Semeniuk, 2004; Isles & Holland, 2005; Moore & Haig, 1991; Zeh & Zeh, 2000). For example, invasive placentalation—particularly haemochorial placentalation—can incur negative maternal fitness consequences (Crespi & Semeniuk, 2004; Haig, 1993), including destruction of uterine tissue (Roberts, Green, & Schulz, 2016), and can increase the potential for embryonic manipulation of maternal physiology to maximize resource allocation to the embryo, potentially beyond that which mothers are selected to provide (Crespi & Semeniuk, 2004; Fowden & Moore, 2012; Haig, 1993; Moore, 2012; Moore & Haig, 1991). Conflicts of interest result in an evolutionary “arms race” between mother and embryo to control placental function (Moore, 2012), resulting in the rapid evolution of diverse alterations to both sides of the maternal-embryonic interface in utero (Martin, 2008; Mess & Carter, 2007; Vogel, 2005; Zeh & Zeh, 2000). Uterine strategies that favor maternal control over resource allocation may thus underpin the transition from invasive to non-invasive placentalation in mammals (Carter & Enders, 2013; Crespi & Semeniuk, 2004). Identifying such uterine adaptations is critical to understanding how conflict is mediated during pregnancy among different mammalian groups.

In eutherian mammals, embryonic invasion, and thus intra-uterine conflict, is mediated via decidualization (Moffett & Loke, 2006), the process of cellular transformation of stromal cells into decidual cells (Wagner, Kin, Muglia, & Pavličev, 2014). Decidual cells are a uniquely eutherian cell type that develop primarily in species with invasive placentalation (Wagner et al., 2014), and regulate embryonic invasion of the uterine stroma following breach of the uterine epithelium (Moffett & Loke, 2006). Marsupials, in contrast, do not undergo decidualization (Kin, Maziarz, & Wagner, 2014; Wagner et al., 2014). Therefore, different uterine strategies are likely involved in mitigating intra-uterine conflict in marsupials compared to eutherian mammals.

**FIGURE 1** Phylogenetic distribution of placental types in Eutheria and Metatheria. Redrawn from Mess (2014) for Eutheria (a) and Freyer et al. (2003) for Metatheria (b). Occurrence of haemochorial placentalation is indicated in orange; endotheliochorial placentalation in green; epitheliochorial placentalation in blue; and unknown placentalation type in black. Reprinted, with permission, from: 1Martin (2008); 2Enders and Carter (2004); 3Mess and Ferner (2010); 4Freyer et al. (2003)
Since marsupials appear to lack mechanisms of regulating embryonic invasion in the uterine stroma, the uterine epithelium likely plays a more important role in regulating implantation in marsupials than in eutherian mammals. This tenet is supported by molecular reinforcement of focal adhesions—basal connections between the uterine epithelium and the underlying stromal cells—prior to implantation in marsupials, irrespective of placentation type, thus strengthening the uterine epithelium as a barrier to embryonic invasion (Fowden & Moore, 2012; Laird, Turancona, McAllan, Murphy, & Thompson, 2015; Laird, Dargan, et al., 2017; Laird, Turancona, McAllan, Murphy, & Thompson, 2017). In contrast, basal adhesion of the uterine epithelium is lost during this same period in eutherian mammals as focal adhesions disassemble, thus facilitating embryonic invasion (Kaneko, Lindsay, & Murphy, 2008; Kaneko, Day, & Murphy, 2013; Murphy, 2000).

Lateral adhesion between adjacent epithelial cells is also critical for maintaining integrity of the uterine epithelium (Preston, Lindsay, & Murphy, 2004; Preston, Lindsay, & Murphy, 2006). In eutherian mammals, lateral adhesion is reduced during uterine receptivity as desmosomes, lateral adhesion points, become fewer and concentrate at the apical region of the lateral plasma membrane (Classen-Linke & Denker, 1990; Illingworth et al., 2000; Preston et al., 2004, 2006; Sarani, Ghaffari Novin, Warren, Dockery, & Cooke, 1999). Since desmosomes confer cell–cell adhesion in the uterine epithelium, alterations to desmosome abundance and distribution can alter the permeability of the uterine epithelium and thus the ease with which it can be breached by an invading embryo (Preston et al., 2004, 2006).

Desmoglein-2, an important component and marker of desmosomes, also apically redistributes during the period (Dudley, Murphy, Thompson, & McAllan, 2015; Preston et al., 2006), indicating that cell-cell contact in the uterine epithelium is weakened in preparation for invasive implantation (Preston et al., 2004, 2006). Interestingly, this redistribution of Desmoglein-2 also occurs in the marsupial, Smynthopsis crassicaudata (Dudley et al., 2015) in which implantation is invasive endotheliochorial (Roberts & Breed, 1994), and is accompanied by loss of the lateral molecule, E-cadherin, suggesting further loss of lateral cell adhesion through modification of the adherens junction (Dudley, Murphy, Thompson, & McAllan, 2017; Orchard, Shaw, & Murphy, 1999). Investigating cell adhesion dynamics in the uterine epithelium during marsupial pregnancy is thus critical to understanding the extent to which the uterine epithelium regulates embryonic invasion.

Molecular changes in the lateral plasma membrane that are involved in non-invasive placentation in marsupials are unknown. We addressed this by identifying Desmoglein-2 localization in the uterus throughout pregnancy in the marsupial species Macropus eugenii (Macropodidae) and Trichosurus vulpecula (Phalangeridae) (Figure 1b) (Freyer, Zeller, & Renfree, 2003; Pilton & Sharman, 1962). Both species have non-invasive placentation and undergo basal reinforcement of the uterine epithelium leading to implantation (Laird, Dargan, et al., 2017). As non-invasive placentation likely evolved independently in macropods and phalangerids (Mess & Ferner, 2010), comparison of the placental features of M. eugenii and T. vulpecula can identify the shared, essential mechanisms of non-invasive placentation in marsupials. Since redistribution of Desmoglein-2 is associated with invasive implantation (Preston et al., 2006), we predict that marsupial species with non-invasive implantation undergo different lateral alterations to maintain an intact uterine epithelium.

M. eugenii has a predictable annual breeding cycle (Renfree & Shaw, 2014; Tyndale-Biscoe & Renfree, 1987; see Laird, Hearn, Shaw, & Renfree [2016]; and Laird, Dargan, et al. [2017] for summary timelines). Mating occurs during a post-partum oestrous (Renfree, 1993; Rudd, 1994; Tyndale-Biscoe & Renfree, 1987), and ovulation of a single egg occurs the following day, with ovulation alternating between ovaries (monovular). The embryo develops to the unilaminar blastocyst stage (approximately Day 7–8 of gestation), and then enters embryonic diapause. Between January and May, the suckling stimulus of the pouch young holds the embryo in arrest by inhibiting growth of the corpus luteum (Hinds & Tyndale-Biscoe, 2013; Renfree & Shaw, 2000). After the winter solstice, diapause shifts to photoperiodic control and the embryo is held in arrest until the summer solstice (Renfree, 1993; Renfree & Shaw, 2000, 2014; Tyndale-Biscoe & Renfree, 1987). After reactivation, non-invasive placentation occurs between Days 17–18 post-conception following rupture of the shell coat (Denker & Tyndale-Biscoe, 1986; Menzies, Pask, & Renfree, 2011). Birth occurs on Day 26.5 (Renfree et al., 1989).

The brushtail possum T. vulpecula has a 28-day oestrous cycle (Pilton & Sharman, 1962; Tyndale-Biscoe, 2005) and a 17.5-day gestation period (Pilton & Sharman, 1962; Sizemore, Hurst, & McLeod, 2004). Like M. eugenii, T. vulpecula is monovular, with ovulation occurring 1–2 days after oestrus (see Laird, Dargan, et al., 2017; Laird, McShea, McAllan, Murphy, & Thompson, 2017 for summary timelines); however, embryos of T. vulpecula do not undergo developmental arrest. The embryo attaches non-invasively approximately 14 days after conception, with birth 3–4 day later (Pilton & Sharman, 1962; Tyndale-Biscoe, 1955). Lactation suppresses ovulation, and the female enters oestrus again after weaning approximately 110 days post-oestrus.

2 | RESULTS

2.1 | Immunofluorescence of uterine tissue of M. eugenii

After embryonic reactivation (stage 1), Desmoglein-2 is localized throughout the cytoplasm of uterine epithelial cells, and is not present at the lateral plasma membrane (Figure 2a). Prominent localization of Desmoglein-2 was observed around clusters of mesenchymal stromal cells underlying the uterine epithelium, and laterally in glandular epithelial cells, particularly at the apical region of the lateral plasma membrane. Glandular epithelial cells were elongated with basal nuclei (Figure 2b).

By pre-implantation (stage 2), Desmoglein-2 localizes to the apical region of the lateral plasma membrane of uterine epithelial cells (Figure 2c). Staining of mesenchymal stromal cells and glandular epithelial cells was similar to that of stage 1.
During the implantation period (stage 3), Desmoglein-2 remained tightly localized to the apical region of the lateral plasma membrane of uterine epithelial cells. Prominent staining of mesenchymal stromal cells was also observed (Figure 2d; representative of two females), whereas its distribution in glandular epithelial cells remains the same as in stage 1.

Post-implantation (stage 4), Desmoglein-2 localization resembled that of stage 1, although staining of mesenchymal stromal cells was less prominent and more diffuse (Figure 2e). Glandular epithelial cell staining was also similar to stage 1. Folds of the uterine epithelium closely interdigitated with folds of placental membranes.

Both lateral and basal localization of Desmoglein-2 was present in trophoblastic cells.

No Desmoglein-2 staining was observed in negative-control tissue of *M. eugenii* (primary antibody replaced with IgG antibody) (Figure 2f).

### 2.2 Immunofluorescence of uterine tissue of *T. vulpecula*

At stage 1 of pregnancy, Desmoglein-2 was present along the lateral plasma membrane of uterine epithelial cells, as well as faint
localization along the basal plasma membrane and in the cytoplasm (Figure 3a). Similar staining was observed for glandular epithelial cells. Diffuse staining occurred in stromal cells.

At pre-implantation (stage 2), Desmoglein-2 localization in the uterine epithelium was similar to that of stage 1, with more prominent staining in the apical region of the lateral plasma membrane (Figure 3b; representative of two females). Punctate localization of Desmoglein-2 was also present along the apical plasma membrane. Glandular epithelial cell staining was similar to that of uterine epithelial cells.

During implantation (stage 3), Desmoglein-2 was tightly localized to the apical region of the lateral plasma membrane of uterine epithelial cells (Figure 3c). Cytoplasmic staining was reduced, although some punctate staining was observed along the apical plasma membrane. Desmoglein-2 also localized to cell nuclei in some regions of the uterine and glandular epithelium (Figure 3d).

Post-implantation (stage 4), Desmoglein-2 localized in the apical region of the lateral plasma membrane, as well as along the apical plasma membrane (Figure 3e; representative of a single female). As for stage 3, nuclear staining was also present in the uterine and glandular epithelium.

No Desmoglein-2 staining was observed in the negative control tissue of *T. vulpecula* (primary antibody substituted with IgG antibody) (Figure 3f).

### 2.3 Western blot

Desmoglein-2 was detected in *M. eugenii* uteri (Figure 4a) at ~150 kDa, at all stages of pregnancy. A possible cleaved fragment was also detected.
at ∼55 kDa. In *T. vulpecula* (Figure 4b), Desmoglein-2 was detected at ∼150 kDa at stage 3 of pregnancy, with a cleavage fragment of ∼72 kDa at stages 1–3; no bands were detected at stage 4. Both the uncleaved (150 kDa) and cleaved-fragment bands (55 kDa) were detected in positive-control tissue (rat uterus at Day 1 of pregnancy) (Figure 4c).

### DISCUSSION

Changes in distribution of Desmoglein-2 occur during pregnancy in both *M. eugenii* and *T. vulpecula*, demonstrating that lateral alterations of the uterine epithelium occur in preparation for non-invasive placentation in marsupials. Specifically, in both species, Desmoglein-2 redistributed to the apical region of the lateral plasma membrane of uterine epithelial cells before implantation. Additional patterns of Desmoglein-2 localization were observed in both *M. eugenii* and *T. vulpecula* during pregnancy. Desmoglein-2 localized to epithelial cell nuclei during implantation and post-implantation in *T. vulpecula*. In *M. eugenii*, Desmoglein-2 localized to the mesenchymal stromal cells underlying the uterine epithelium throughout pregnancy.

Redistribution of Desmoglein-2 to the apical-lateral region of uterine epithelial cells occurs in preparation for invasive implantation in both eutherian (Classen-Linke & Denker, 1990; Illingworth et al., 2000; Murphy, 2000; Preston et al., 2004, 2006; Sarani et al., 1999) and marsupial mammals (*S. crassicaudata*; Dudley et al., 2015), reducing lateral cell–cell adhesion in the uterine epithelium. This redistribution pattern in *M. eugenii* and *T. vulpecula*, which both have non-invasive placentation, is thus unexpected, and suggests that cell-cell adhesion also reduces during pregnancy in these species, which could compromise the function of the uterine epithelium as a barrier to the embryo.

Since non-invasive placentation has likely evolved secondarily in marsupials as a derived character (Mess & Ferner, 2010), we predicted that a different pattern of Desmoglein-2 redistribution occurs in *M. eugenii* and *T. vulpecula* to that of species with invasive placentation to maintain an intact uterine epithelium and prevent embryonic invasion. Yet, apical redistribution of Desmoglein-2 in both *M. eugenii* and *T. vulpecula* suggests that this molecule may play an important role in facilitating implantation and placentation, rather than restricting invasion. For example, non-invasive placentation in the viviparous skinks *Pseudemoia entrecasteauxii* and *P. spenceri* involves apical redistribution of both Desmoglein-2 and morphological desmosomes (Blazik, Thompson, & Murphy, 2010). Since the ancestral placentation type for viviparous lizards is non-invasive, in contrast to mammals, apical redistribution of Desmoglein-2 is unlikely to be a mechanism to

![FIGURE 4](image)

**FIGURE 4** Immunoblot of whole uterine tissue lysate, incubated with rabbit anti-Desmoglein-2 antibody (20 µg of protein per well). Numbers above the lanes indicate the respective stage of (a) *M. eugenii* uteri or (b) *T. vulpecula* uteri. (c) Positive-control lysate of rat uterine tissue from Day 1 of pregnancy. The loading control expressed equal amounts of β-actin at 42 kDa (not shown).
reduce embryonic invasion. Instead, it may facilitate non-invasive placentation and uterine remodeling by creating a more-plastic uterine epithelium (Blazik et al., 2010). This hypothesis is supported by the fact that lateral localization of Desmoglein-2 in intestinal epithelia maintains epithelial integrity (Schlegel et al., 2010), and desmosomes in gut epithelia remain evenly distributed along the lateral plasma membrane, even in response to pathogen invasion (Takeuchi, 1967). Indeed, redistribution of Desmoglein-2 in the marsupial S. crassicaudata may facilitate apposition of embryonic cells to the uterine epithelium before invasion (Dudley et al., 2015, 2017). Redistribution also occurs pre-implantation (stage 2) in both M. eugenii and T. vulpecula, and persists throughout the implantation period. Hence, this redistribution pattern may be an important mechanism for uterine remodeling and early attachment of the embryo to the uterine epithelium in marsupials.

Maternal-embryonic interactions involve a precise balance of strategies that facilitate implantation, with those that mediate conflict to prevent uncontrolled embryonic invasion. The marsupial endometrium does not undergo decidualization, so the uterine epithelium is critical for maintaining this balance during marsupial pregnancy relative to that of eutherian mammals. Given that uterine Desmoglein-2 redistribution is relatively conserved across mammalian groups, and occurs irrespective of placental type, the specific pattern of localization may be an important uterine strategy involved in facilitating placentation, rather than as a response to increase maternal control over resource allocation, and may play a role in initial embryonic implantation. In marsupials, including M. eugenii and T. vulpecula, reduced cell-cell adhesion resulting from Desmoglein-2 redistribution may be compensated for by molecular reinforcement of the basal plasma membrane of the uterine epithelium prior to implantation (Laird, Dargan, et al., 2017). Hence, the molecular patterns of Desmoglein-2 in the lateral plasma membrane and Talin in the basal plasma membrane may play complementary roles during pregnancy in M. eugenii and T. vulpecula, resulting in facilitation and restriction of embryonic invasion, respectively, and thus enabling successful placentation (Poon, Madawala, Dowland, & Murphy, 2016).

Compensation for reduced cell-cell contact may involve other lateral molecules, including claudins and desmosomal cadherins, many of which are able to compensate for the functional loss of other molecules (e.g., Desmoglein-3 in keratinocytes; [Hartlieb, Rötzer, Radeva, Spindler, & Waschke, 2014]). Molecular compensation may also account for the apparent loss of Desmoglein-2 protein at stage 4 of pregnancy in T. vulpecula (Hartlieb et al., 2014). Other junctional regions of the lateral plasma membrane may also be involved in compensation. In rodents, as well as in S. crassicaudata, the adherens junction becomes displaced by extension of the tight junction down the lateral plasma membrane (Laird, Thompson, Murphy, & McAllan, 2014; Murphy, 2000), which further reduces cell-cell adhesion. In addition, cadherins associated with the adherens junction, including E-cadherin, are down-regulated in rabbits (Denker, 1994) and humans (Getios et al., 1998) before implantation (Murphy, 2000), as well as in the marsupial S. crassicaudata (Dudley et al., 2017). In rats, loss of the adherens junction is partially compensated for by recruitment of Nectin-3, a molecule associated with the basal plasma membrane, to the lateral junctional complex (Poon et al., 2016). Different morphological and molecular alterations to the adherens junction, including patterns of Nectin-3 localization, to those of species with invasive placentation may help to maintain lateral cell adhesion during non-invasive placentation in M. eugenii and T. vulpecula.

The unusual localization patterns of Desmoglein-2, in addition to the apical redistribution in the uterine epithelium, suggest that Desmoglein-2 plays additional cellular roles that are independent of desmosomes during pregnancy in M. eugenii and T. vulpecula (Ebert et al., 2016; Hartlieb et al., 2014; Nava et al., 2007) and differ between marsupial lineages of non-invasive placentation (Bininda-Emonds et al., 2007; Mess & Fener, 2010). Hence, different additional patterns of Desmoglein-2 localization in M. eugenii and T. vulpecula, as well as differences in uterine cell morphology following remodeling (Laird, McShea, et al., 2017), suggest that the evolutionary transition from invasive to non-invasive placentation likely involved lineage-specific selective pressures (Martin, 2008), resulting in diverse molecular patterns at the maternal-embryonic interface.

These additional localization patterns may compensate for reduced cell-cell adhesion. In T. vulpecula, Desmoglein-2 is localized in nuclei of uterine epithelial cells during and after implantation (stages 3 and 4), similar to that of Plakophilin-3, which is also associated with desmosomes (Bonné, van Hengel, Rollet, Koos, & van Roy, 1999). The additional nuclear and lateral localization suggests a dual role for Desmoglein-2 in both cell signaling and cell adhesion during and after implantation in T. vulpecula (Bonné et al., 1999; Schlegel et al., 2010). This conclusion is supported by localization of Desmoglein-2 to the apical plasma membrane during pregnancy in T. vulpecula, as apical binding of desmosoleins can trigger intracellular signaling pathways (Schlegel et al., 2010) that may relate to maintenance of epithelial cell polarity (Madawala, Dowland, Poon, Lindsay, & Murphy, 2014). Apical localization of Desmoglein-2 can also stabilize epithelial cells against apoptosis (Nava et al., 2007; Singh & Aplin, 2015), as demonstrated in intestinal epithelia following inflammation by consequent loss of cell-cell adhesion (Nava et al., 2007). Since inflammation also occurs during mammalian pregnancy (Kin et al., 2014), Desmoglein-2 may be involved in prevention of apoptosis and maintenance of the uterine epithelium following pregnancy-induced inflammation.

In addition to apical redistribution in M. eugenii, Desmoglein-2 is tightly localized to the mesenchymal stromal cell population underlying the uterine epithelium throughout pregnancy, which suggests that this population may possess a fibroblast-like function. Mesenchymal stromal cells of the marsupial Monodelphis domestica express a range of cytoskeletal proteins during pregnancy, and are therefore considered homologous to the endometrial fibroblasts of eutherian mammals (e.g., fibroblast-like) (Kin et al., 2014; Wagner et al., 2014) that are the precursors to eutherian decidual cells. Desmoglein-2 indirectly interacts with the cell cytoskeleton (Yashiro, Nishioka, & Hirakawa, 2006); therefore, specific localization of Desmoglein-2 to the mesenchymal stromal cells of M. eugenii provides some evidence that these cells are also fibroblast-like, although this requires
verification using cytoskeletal proteins and transcription factors as fibroblast markers (Kin et al., 2014). Fibroblast-like cells in the uteri of both M. eugenii and M. domestica, species from two of the most phylogenetically divergent living marsupial clades, respectively (Freyer et al., 2003; Hansen, Schilkey, & Miller, 2016; Kin et al., 2014; Meredith, Krajewski, Westerman, & Springer, 2009; Meredith, Westerman, & Springer, 2009; Westerman et al., 2016), but not in the uterus of T. vulpecula, would imply that endometrial stromal fibroblasts play an important and interesting role during marsupial pregnancy, deserving of further investigation.

We conclude that apical redistribution of Desmoglein-2 is an important and conserved uterine strategy that occurs in both eutherian mammals and marsupials, irrespective of placental type. This pattern is likely involved in uterine remodeling and placentation, rather than mitigation of intra-uterine conflict by restricting embryonic invasion. The species-specific patterns of Desmoglein-2 localization suggest that Desmoglein-2 is also involved in important cellular processes during pregnancy, independent of cell adhesion, including potentially preventing apoptosis in the uterine epithelium. Thus non-invasive placentation in marsupials, and maintenance of the uterine epithelium throughout pregnancy, is likely underpinned by diverse and complementary molecular mechanisms.

4 | MATERIALS AND METHODS

4.1 | Tissue collection and reproductive staging

Collection of tammar wallaby samples was approved by the University of Melbourne Institutional Animal Ethics Committees, and conformed to the Australian National Health and Medical Research Council (2013) guidelines. Collection of possum samples was a secondary use from a cull approved by the animal ethics committees of Landcare Research, New Zealand.

Uterine tissues were collected from both M. eugenii and T. vulpecula, as described by Laird, Dargan, et al. (2017) and by Laird, McShea, et al. (2017). Uterine tissue of M. eugenii was collected from animals with new pouch young from wild colonies on Kangaroo Island, South Australia. Development staging was based on the age of pouch young using published growth curves (Poole, Simms, Wood, & Lubulwa, 1991) or from a known time after a detected birth or mating. The day of birth was designated as Day 0 post-partum. Tissue was collected throughout pregnancy following reactivation of the embryo on Day 8 post-partum. Uterine tissue prior to reactivation was not included in this study since uterine changes are associated with initiation of diapause, not embryonic attachment (Laird et al., 2016). Reproductive stages were determined following Laird, Dargan, et al. (2017): stage 1 (after embryonic reactivation; Days 9–12 of gestation; n = 4); stage 2 (pre-implantation; Days 13–16; n = 5); stage 3 (implantation, post-rupture of shell coat; Days 17–18; n = 2); and stage 4 (post-implantation; Days 19–26; n = 4).

Uterine tissue of Trichosurus vulpecula was collected from wild females in the Orongorongo Valley near Wellington, New Zealand, over two breeding seasons (April 2014 and May 2015) (Laird, Dargan, et al., 2017; Laird, McShea, et al., 2017), and females were allocated to a reproductive stage using ovarian and uterine morphology (Laird, McShea, et al., 2017): stage 1 (0–6 days post-oestrus; n = 6), stage 2 (pre-implantation; 7–11 days post-oestrus; n = 2), stage 3 (implantation; 11–14 days post-oestrus; n = 4), and stage 4 (post-implantation; 15–17.5 days post-oestrus; n = 1).

4.2 | Immunofluorescence microscopy

Excised uterine tissue was coated with Tissue-Tek OCT cryoprotectant (Sakura, Tokyo, Japan) and briefly immersed in super-cooled isopentane, before storing in liquid N2. Samples were cut using a Leica CM3050 S cryostat (Leica, Heerbrugg, Switzerland) at −25°C to produce 8-µm sections, which were mounted on gelatin-coated slides. Sections on slides were fixed for 30 min at room temperature in acetone, and then blocked for 30 min with 1% bovine serum albumin (BSA) (0.1 g BSA/10 ml phosphate buffered saline [PBS]). Sections were then incubated for 1.5 h with rabbit anti-Desmoglein-2 antibody (1:250 dilution of ab150372 in 1% BSA) (Abcam, Melbourne, VIC, Australia), followed by rinsing in PBS and incubation for 45 min with goat anti-rabbit fluorescein isothiocyanate-conjugated IgG antibody (1:500 dilution of 111-095-144 in 1% BSA) (Jackson Immunoresearch Laboratories, West Grove, PA). Slides were rinsed again in PBS, and then mounted with Vectashield mounting medium containing 4′,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA). Images were captured using a Zeiss Deconvolution microscope (Carl Zeiss Pty. Australasia) fitted with a Zeiss AxioCam HR monochrome CCD camera, and using Zen imaging software, version 7.1.

Non-immune controls were prepared as above by substituting the primary antibody with 1 mg/ml rabbit IgG purified immunoglobulin (catalog number I5006) (Sigma–Aldrich, Castle Hill, Sydney). Positive-control slides of rat uterine tissue at Day 1 of pregnancy, in which Desmoglein-2 fluorescence has been confirmed (Preston et al., 2004), were also prepared as outlined above.

4.3 | Western blot

Uterine tissue was extracted by vigorous shaking in short bursts in a solution containing homogenizing beads, lysis buffer, and protease inhibitor cocktail (1:100 dilution) (Sigma–Aldrich). Protein content of samples was estimated by first diluting extracted protein samples 1:100, 1:200, or 1:400 with distilled water, and then measuring concentration against BSA standards in a 96-well plate (Thermo Scientific, Rockford, IL), with 100 μl of reagent from the Micro BCA™ Protein Assay Kit (Thermo Scientific). Protein content was estimated with a CLARIOStar Microplate reader (BMG LabTech, Durham, NC).

Samples (20 μg) were denatured at 90°C for 5 min in Laemmli sample buffer (Dudley et al., 2015). Proteins were separated for 1.5 hr at 100 V on a 10% denaturing polyacrylamide gel, and then transferred to polyvinylidene fluoride membranes (Millipore Corporation, Bedford, MA). These membranes were blocked for 1 hr in 5% skim milk in Tris-buffered saline with 0.05% Tween20 (TBS-t) (Sigma–Aldrich), and then

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probed overnight at 4°C with rabbit anti-Desmoglein-2 antibody in TBS-t containing 1% skim milk (1:10,000 dilution of ab150372 for M. eugenii samples; 1:5,000 dilution for T. vulpecula samples) (Abcam). Membranes were rinsed in TBS-t, and then incubated for 1.5 hr with horseradish peroxidase-conjugated sheep anti-rabbit IgG in TBS-t containing 1% skim milk (1:2,000 dilution of GEHENAg31) (GE Healthcare, Buckinghamshire, UK). Proteins on the rinsed membrane were visualized using a ChemiDoc MP Imaging System (Biorad, Gladesville, Australia), with the ECL Plus Western Blotting Detection System (Amersham, GE Healthcare, Buckinghamshire, UK). The membranes were then incubated for 45 min at 60°C in stripping buffer containing β-mercaptoethanol, and reprobed for β-actin as above, substituting the primary antibody with monoclonal anti-β-actin antibody (1:2,000 dilution of A1978) (Sigma-Aldrich).

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CONFLICTS OF INTEREST
The authors have no conflict of interest to declare.

AUTHORS’ CONTRIBUTIONS
GS and MBR collected the tissue of M. eugenii used in this study. M. K. Laird collected tissue of T. vulpecula, carried out the sample preparation and Western blot analysis, and wrote the manuscript. HM and MKL carried out the immunofluorescence microscopy. MBT, CRM, and MBR contributed to experimental design, technical advice, and image interpretation. MBT, CRM, BMM, GS, and MBR contributed to manuscript preparation and revision. We also thank two anonymous reviewers for their suggestions for improvement.

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