Research Article

Non-invasive placentation in the marsupials *Macropus eugenii* (Macropodidae) and *Trichosurus vulpecula* (Phalangeridae) involves redistribution of uterine Desmoglein-2[1]

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Abstract

In mammalian pregnancy, the uterus is remodelled 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: uterus, Desmoglein-2, pregnancy, implantation, epitheliochorial

1 Introduction

The mammalian uterus is remodeled during pregnancy to become receptive to the implanting embryo (Orchard and Murphy, 2002; Murphy, 2004; 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) (Enders and Carter, 2004; Carter and Mess, 2007; Martin, 2008; Elliot and Crespi, 2009). Recent molecular evidence and phylogenetic reconstructions also suggest that invasive placentation is ancestral for living marsupials (Figure 1b) (Bininda-Emonds et al, 2007; 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 secondarily derived (Vogel, 2005; Carter and Mess, 2007; Martin, 2008; Elliot and Crespi, 2009; Capellini, 2012; Mess, 2014).

A potential driver of this placental transition in mammals is intra-uterine conflict arising from genetic differences between mothers and offspring (Moore and Haig, 1991; Zeh and Zeh, 2000; Crespi and Semeniuk, 2004;

Isles and Holland, 2005). For example, invasive placentation –particularly haemochorial placentation– can incur negative maternal fitness consequences (Haig, 1993; Crespi and Semeniuk, 2004), including destruction of uterine tissue (Roberts et al. 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 (Moore and Haig, 1991; Haig, 1993; Crespi and Semeniuk, 2004; Moore, 2012; Fowden and Moore, 2012). Conflicts of interest result in an evolutionary 'arms race' between mother and embryo to control placental function (Moore, 2012), resulting in rapid evolution of diverse alterations to both sides of the maternal-embryonic interface in utero (Zeh and Zeh, 2000; Vogel, 2005; Mess and Carter, 2007; Martin, 2008). Uterine strategies that favor maternal control over resource allocation may thus underpin the transition from invasive to non-invasive placentation in mammals (Crespi and Semeniuk, 2004; Carter and Enders, 2013). 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 and Loke, 2006), the process of cellular transformation of stromal cells into decidual cells (Wagner et al, 2014). Decidual cells are a uniquely eutherian cell type that develop primarily in species with invasive placentation (Wagner et al. 2014), and regulate embryonic invasion of the uterine stroma following breaching of the uterine epithelium (Moffett and Loke, 2006). Marsupials, in contrast, do not undergo

decidualization (Wagner et al. 2014; Kin et al. 2014). Therefore, different uterine strategies are likely involved in mitigating intra-uterine conflict in marsupials compared with eutherian mammals.

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 and Moore, 2012; Laird et al, 2015; Laird et al, 2017a,b). 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 (Murphy, 2000; Kaneko et al, 2008; 2013).

Lateral adhesion between adjacent epithelial cells is also critical for maintaining integrity of the uterine epithelium (Preston et al, 2004; 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 and Denker, 1990; Sarani et al, 1999; Illingworth et al, 2000; Preston et al, 2004; 2006). 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 (Preston et al, 2006; Dudley et al, 2015), 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, *Sminthopsis crassicaudata* (Dudley et al, 2015) in which implantation is invasive endotheliochorial (Roberts and 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 (Orchard et al, 1999; Dudley et al, 2017). 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) (Pilton and Sharman, 1962; Freyer et al, 2003). Both species have non-invasive placentation and undergo basal reinforcement of the uterine epithelium leading to implantation (Laird et al, 2017b). As non-invasive placentation likely evolved independently in macropodids and phalangerids (Mess and 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.

Macropus eugenii has a predictable annual breeding cycle (Tyndale-Biscoe and Renfree, 1987; Renfree and Shaw, 2014; see Laird et al (2016) and Laird et al (2017b) for summary timelines). Mating occurs during a post-partum oestrus (Tyndale-Biscoe and Renfree, 1987; Renfree, 1993; Rudd, 1994), 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 and Tyndale-Biscoe, 1982; Renfree and Shaw, 2000). After the winter solstice, diapause shifts to photoperiodic control and the embryo is held in arrest until the summer solstice (Tyndale-Biscoe and Renfree, 1987; Renfree, 1993; Renfree and Shaw, 2000; Renfree and Shaw, 2014). After reactivation, non-invasive placentation occurs between Days 17-18 post-conception following rupture of the shell coat (Denker and Tyndale-Biscoe, 1986; Menzies et al, 2011). Birth occurs on Day 26.5 (Renfree et al, 1989).

The brushtail possum *T. vulpecula* has a 28-day oestrous cycle (Pilton and Sharman, 1962; Tyndale-Biscoe, 2005) and a 17.5-day gestation period (Pilton and Sharman, 1962; Sizemore et al, 2004). Like *M. eugenii*, *T. vulpecula* is monovular, with ovulation occurring 1-2 days after oestrus (see Laird et al. 2017b-c 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 (Tyndale-Biscoe, 1955; Pilton and Sharman, 1962). 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 occurs 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 also occurs (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 occurred in trophoblastic cells.

No Desmoglein-2 localization occurred 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 occurs along the lateral plasma membrane of uterine epithelial cells (Figure 3a). Faint localization also occurs along the basal plasma membrane and in the cytoplasm. Similar staining

occurred 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 also occurred 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 occurred 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 localization occurred 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 also occurred in the uterine and glandular epithelium.

No Desmoglein-2 staining was observed in the negative control tissue of *T. vulpecula* (primary antibody replaced 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).

3 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 also occur in both *M. eugenii* and *T. vulpecula* during pregnancy. Desmoglein-2 is localized to epithelial cell nuclei during implantation and post-implantation in *T. vulpecula*. In *M. eugenii*, Desmoglein-2 is 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 and Denker, 1990; Sarani et al, 1999; Illingworth et al, 2000; Murphy, 2000; Preston et al, 2004; 2006) 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, as it suggests that cell to cell adhesion also reduces during pregnancy in these species and 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 and 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 (Biazik et al, 2010). Since the ancestral placental type for viviparous lizards is non-invasive, in contrast to mammals, apical redistribution of Desmoglein-2 is unlikely to be a mechanism to reduce embryonic invasion. Instead, it may facilitate non-invasive placentation and uterine remodeling by creating a more-plastic uterine epithelium (Biazik 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, this 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 et al, 2017b). 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, thus enabling successful placentation (Poon et al, 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 et al, 2014]). Molecular compensation may also explain account for the apparent loss of Desmoglein-2 protein at Stage 4 of pregnancy in *T. vulpecula* (Hartlieb et al, 2014). In addition, other junctional regions of the lateral plasma membrane may also be involved in compensation. In rodents, as well as *S. crassicaudata*, the adherens junction becomes displaced by extension of the tight junction down the lateral plasma membrane (Murphy, 2000; Laird et al, 2014), which further reduces cell-cell adhesion. In addition, cadherins associated with the adherens junction, including E-cadherin, are also down-regulated before implantation (Murphy, 2000) in rabbits (Denker, 1994) and humans (Getsios 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, suggests that Desmoglein-2 plays additional cellular roles that are independent of desmosomes during pregnancy in *M. eugenii* and *T. vulpecula* (Nava et al, 2007; Hartlieb et al, 2014; Ebert et al, 2016) that differ between marsupial lineages of non-invasive placentation (Bininda-Emonds et al, 2007; Mess and Ferner, 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 et al, 2017c), 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 also 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 et al, 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

desmogleins can trigger intracellular signaling pathways (Schlegel et al, 2010) that may relate to maintenance of epithelial cell polarity (Madawala et al, 2014). Apical localization of Desmoglein-2 can also stabilize epithelial cells against apoptosis (Nava et al, 2007; Singh and Aplin, 2015), as demonstrated in intestinal epithelia following inflammation by consequent loss of cell-to-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 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 et al, 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; Meredith et al, 2009a, b; Kin et al, 2014; Westerman et al, 2016; Hansen 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 on of 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 et al. (2017b, c). 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 et al, 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 et al (2017b): 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 et al, 2017b, c), and females were allocated to a reproductive stage using ovarian and uterine morphology (Laird et al. 2017c): 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 N_2. 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). 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, USA), with 100 µl of reagent from the Micro BCA™ Protein Assay Kit (Thermo Scientific, Rockford, IL). 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 h 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 h in 5% skim milk in Tris-buffered saline with 0.05% Tween20 (TBS-t) (Sigma), and then 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, Melbourne, VIC, Australia). Membranes were rinsed in TBS-t, and then incubated for 1.5 h with horseradish peroxidase-conjugated sheep anti-rabbit IgG in TBS-t containing 1% skim milk (1:2000 dilution of GEHENA931) (GE Healthcare, Buckinghamshire, UK). Proteins on the rinsed membrane were visualized using a Chemidoc MP Imaging System (Biorad), with the ECL Plus Western Blotting Detection System (Amersham, GE Healthcare, Buckingham-shire, UK). The membranes were then incubated for 45 min at 60°C in stripping buffer containing β-mercaptoethanol and reprobed for actin as above, substituting the primary antibody with monoclonal anti-β-actin antibody (1:2000 dilution of A1978) (Sigma-Aldrich, Castle Hill, Sydney).

Declaration of interest

The authors have no conflict of interest to declare.

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Author contributions

GS and MBR collected the tissue of *M. eugenii* used in this study. MKL 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 BMM 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.

References

Adams AP, Antczak DF. 2001. Ectopic transplantation of equine invasive trophoblast. Biology of Reproduction 64: 753– 763.

Biazik JM, Thompson MB, Murphy CR. 2010. Desmosomes in the uterine epithelium of noninvasive skink placentae. Anat Rec 293:502-512.

Bininda-Emonds ORP, Cardillo M, Jones KE, MacPhee RDE, Beck RMD, Grener R. 2007. The delayed rise of present-day mammals. Nature 446:507–512.

Bonné S, van Hengel J, Nollet F, Kools P, van Roy F. 1999. Plakophilin-3, a novel Armadillo-like protein present in nuclei and desmosomes of epithelial cells. J Cell Sci 112:2265-2276.

Capellini I. 2012. The evolutionary significance of placental interdigitation in mammalian reproduction: contributions from comparative studies. Placenta 33:763-768.

Carter AM, Mess A. 2007. Evolution of the placenta in eutherian mammals. Placenta 28:259–262.

Carter AM, Enders AC. 2013. The evolution of epitheliochorial placentation. Ann Rev Ani BioSci 1:443-467.

Classen-Linke I, Denker HW. 1990. Preparation of rabbit uterine epithelium for trophoblast attachment: histochemical changes in the apical and lateral membrane component. In: Denker HW, Aplin JD, editors,. *Trophoblast invasion and endometrial receptivity. Novel aspects of the cell biology of embryo implantation*. New York: Plenum Press. p307-322.

Crespi B, Semeniuk C. 2004. Parent-offspring conflict in the evolution of the vertebrate reproductive mode. Amer Nat 163(5):635-653.

Denker H-W. 1994. Endometrial receptivity: cell biological aspects of an unusual epithelium. A review. Ann Anat 176:53-60.

Denker HW, Tyndale-Biscoe CH. 1986. Embryo implantation and proteinase activities in a marsupial (Macropus eugenii). Histochemical patterns of proteinases in various gestational stages. Cell Tissue Res. 246(2):279-91.

Dudley JS, Murphy CR, Thompson MB, McAllan BM. 2015. Desmoglein- during pregnancy and its role in the evolution of viviparity in a marsupial (*Sminthopsis crassicaudata*; Dasyuridae). J Morphol 276:261-272.

Dudley JS, Murphy, CR, Thompson MB, McAllan BM. 2017. Epithelial cadherin disassociates from the lateral plasma membrane of uterine epithelial cells throughout pregnancy in a marsupial. J Anat doi:10.1111/joa.12648.

Ebert LM, Tan LY, Johan MZ, Min KKM, Cockshell MP, Parham KA et al. 2016. A non-canonical role for desmoglein-2 in endothelial cells: implications for neoangiogenesis. Angiogenesis 19:463-486.

Elliot MG, Crespi BJ. 2009. Phylogenetic evidence for early haemochorial placentation in Eutheria. Placenta 30:949-967.

Enders AC, Carter AM. 2004. What can comparative studies of placental structure tell us? A review. Placenta 25: Supp A, Troph Res 18:S3-S9.

Ferner K, Mess A. 2011. Evolution and development of fetal membranes and placentation in amniote vertebrates. Respir Physiol Neurobiol 178:39-50.

Fowden AL, Moore T. 2012. Maternal-fetal resource allocation: Co-operation and conflict. Placenta 33:e11-e15.

Freyer C, Zeller U, Renfree MB. 2003. The marsupial placenta: A phylogenetic analysis. J Exp Zool 299A:59–77.

Getsios S, Chen GTC, Stephenson MD, Leclerc P, Blaschuk OW, MacCalman CD. 1998. Regulated expression of cadherin-6 and cadherin-11 in the glandular epithelial and stromal cells of the human endometrium. Develop Dynam 211:238-147.

Haig D. 1993. Genetic conflicts in human pregnancy. Quart Rev Biol 68:495-532.

Hansen VL, Schilkey FD, Miller RD. 2016. Transcriptomic changes associated with pregnancy in a marsupial, the gray short-tailed opossum *Monodelphis domestica*. PLOS One doi:10.1371/journal.pome.0161608.

Hartlieb E, Rötzer V, Radeva M, Spindler V, Waschke J. 2014. Desmoglein 2 compensates for desmoglein 3 but does not control cell adhesion via regulation of p38 mitogen-activated protein kinase in keratinocytes. J Biol Chem 289:17043-17053.

Hinds LA, Tyndale-Biscoe CH. 2013. Daily prolactin pulse inhibits the corpus luteum during lactational quiescence in the marsupial, *Macropus eugenii*. Reprod Fertil Dev 25:456-461.

Illingworth IM, Kiszka I, Bagley S, Ireland GW, Garrod DR, Kimber SJ. 2000. Desmosomes are reduced in the mouse uterine luminal epithelium during the preimplantation period of pregnancy: a mechanism for facilitation of implantation. Biol Reprod 63:1764-1773.

Isles AR, Holland AJ. 2005. Imprinted genes and mother-offspring interactions. Early Hum Develop 81:73-77.

Kaneko Y, Lindsay LA, Murphy CR. 2008. Focal adhesions disassemble during early pregnancy in rat uterine epithelial cells. Reprod Fertil Dev 20:892–899.

Kaneko Y, Day ML, Murphy CR. 2013. Uterine epithelial cells: serving two masters. Int J Biochem and Cell Physiol 45:359-363.

Kin K, Maziarz, Wagner GP. 2014. Immunohistological study of the endometrial stromal fibroblasts in the opossum, *Monodelphis domestica*: evidence for homology with eutherian stromal fibroblasts. Biol Reprod 90(5):1-12.

Laird MK, Thompson MB, Murphy CR, McAllan BM. 2014. Uterine epithelial cell changes during pregnancy in a marsupial (*Sminthopsis crassicaudata*; Dasyuridae). J Morphol 275:1081-1092.

Laird MK, Turancova M, McAllan BM, Murphy CR, Thompson MB. 2015. Unlocking amniote live birth: the ‘other’ mammalian model. J Proc Roy Soc NSW 148:52-59.

Laird MK, Hearn CM, Shaw G, Renfree MB. 2016. Uterine morphology during diapause and early pregnancy in the tammar wallaby (*Macropus eugenii*). J Anat 229:459-472.

Laird MK, Turancova M, McAllan BM, Murphy CR, Thompson MB. 2017a. Uterine focal adhesion dynamics during pregnancy in a marsupial (*Sminthopsis crassicaudata*; Dasyuridae). Anat Rec 300:1150-1159.

Laird MK, Dargan J, Paterson L, Murphy CR, McAllan BM, Shaw G, Renfree MB, Thompson MB. 2017b. Uterine molecular changes for non-invasive embryonic attachment in the marsupials, *Macropus eugenii* (Macropodidae) and *Trichosurus vulpecula* (Phalangeridae) (*in press, Mol Reprod Dev*).

Laird MK, McShea H, McAllan BM, Murphy CR, Thompson MB. 2017c. Uterine remodeling during pregnancy and pseudopregnancy in the brushtail possum (*Trichosurus vulpecula*; Phalangeridae). J Anat 231:84-94.

Madawala RJ, Dowland S, Poon CE, Lindsay LA, Murphy CR. 2014. Caveolins redistribute in uterine epithelial cells during early pregnancy in the rat: an epithelial polarisation strategy? Histochem Cell Biol 142:555-567.

Martin RD. 2008. Evolution of placentation in primates: implications of mammalian phylogeny. Evol Biol 35:125.

Menzies BR, Pask AJ, Renfree MB. 2011. Placental expression of pituitary hormones is an ancestral feature of therian mammals. EvoDevo 2:16.

Meredith RW, Krajewski C, Westerman M, Springer MS. 2009a. Relationships and divergence among orders and families of Marsupialia. In: Albright BL, editor. *Papers in geology, paleontology, and paleostratigraphy in honour of Michael O. Woodburne*, Vol. 65. Flagstaff, Arizona: Museum of Northern Arizona Bulletin.

Meredith RW, Westerman M, Springer MS. 2009b. A phylogeny of Diprotodontia (Marsupialia) based on sequences for five nuclear genes. Mol Phylogen Evol 51:554-571.

Mess A. 2014. Placental evolution within the supraordinal clades of Eutheria with the perspective of alternative animal models for human placentation. Advances in Biology 1-21.

Mess A, Carter AM. 2007. Evolution of the placenta during the early radiation of placental mammals. Comparative Biochemistry and Physiology A 148:769–779.

Mess AM, Ferner KJ. 2010. Evolution and development of gas exchange structures in Mammalia: the placenta and the lung. Respir Physiol Neurobiol 173S:S74-S82.

Moffett A, Loke C. 2006. Immunology of placentation in eutherian mammals. Nat Rev Immunol 6:584-594.

Moore T. 2012. Parent-offspring conflict and the control of placental function. Placenta 33: Supp: S33-S36.

Moore T, Haig D. 1991. Genomic imprinting in mammalian development: a parental tug-of-war. Trends Genet. 7(2): 45-49.

Murphy CR. 2000. Junctional barrier complexes undergo major alterations during the plasma membrane transformation of uterine epithelial cells. Hum Reprod 15:182-188.

Murphy CR. 2004. Uterine receptivity and the plasma membrane transformation. Cell Res 14:259–267.

National Health and Medical Research Council. 2013. Australian code of practice for the care and use of animals for scientific purposes. 8th ed. Canberra: National Health and Medical Research Council.

Nava P, Laukoetter MG, Hopkins AM, Laur O, Gerner-Smidt K, Green KJ, Parkos CA, Nusrat A. 2007. Desmoglein-2: a novel regulator of apoptosis in the intestinal epithelium. Mol Biol Cell 18:4565-4578.

Orchard MD, Shaw TJ, Murphy CR. 1999. Junctional plaque proteins shift to the apical surface of uterine epithelial cells during early pregnancy in the rat. Acta Histochem 101:147–156.

Orchard M, Murphy CR. 2002. Alterations in tight junction molecules of uterine epithelial cells during early pregnancy in the rat. Acta Histochem 104:149–155.

Pilton PE, Sharman GB. 1962. Reproduction in the marsupial *Trichosurus vulpecula*. J Endocrin 25:119-136.

Poole WE, Simms NG, Wood JT, Lubulwa M. 1991. *Tables for age determination of the Kangaroo Island wallaby (tammar),* Macropus eugenii*, from body measurements*. CSIRO Division of Wildlife and Ecology, Canberra, Technical Memorandum No. 31.

Poon CE, Madawala RJ, Dowland SN, Murphy CR. 2016. Nectin-3 is increased in the cell junctions of the uterine epithelium at implantation. Reprod Sci 23:1580-1592.

Preston AM, Lindsay LA, Murphy CR. 2004. Progesterone treatment and the progress of early pregnancy reduce desmoglein 1&2 staining along the lateral plasma membrane in rat uterine epithelial cells. Acta Histochem 106:345-351.

Preston AM, Lindsay LA, Murphy CR. 2006. Desmosomes in uterine epithelial cells decrease at the time of implantation: an ultrastructural and morphometric study. J Morphol 267(1):103-108.

Renfree MB. 1993. Diapause, pregnancy and parturition in Australian marsupials. J Exp Zool 266:450-462.

Renfree MB, Fletcher TP, Blanden DR, Lewis PR, Shaw G, Gordon K, Short RV, Parer-Cook E, Parer D. 1989. Physiological and behavioural events around the time of birth in macropodid marsupials. In: Grigg G, Jarman P, Hume ID, editors. Kangaroos, Wallabies and Rat Kangaroos. Sydney: Surrey Beatty & Sons Pty. Ltd. p323-337.

Renfree MB, Shaw G. 2000. Diapause. Annu Rev Physiol 62:353-375.

Renfree MB, Shaw G. 2014. Embryo-endometrial interactions during early development after embryonic diapause in the marsupial tammar wallaby. Int J Dev Biol 58:175-181.

Roberts CT, Breed WG. 1994. Embryonic-maternal cell interactions at implantation in the fat-tailed dunnart, a dasyurid marsupial. Anat Rec 240:59-76.

Roberts RM, Green JA, Schulz LC. 2016. The evolution of the placenta. Reprod doi:10.1530/REP-16-0325.

Rudd CD. 1994. Sexual behavior of male and female tammar wallabies (*Macropus eugenii*) at post partum oestrus. J Zool 232:151-162.

Sarani SA, Ghaffari Novin M, Warren MA, Dockery P, Cooke ID. 1999. Morphological evidence for the implantation window in human luminal endometrium Hum Reprod 14:3101-3106.

Schlegel N, Meir M, Heupel W-M, Holthöfer B, Leube RE, Waschke J. 2010. Desmoglein 2-mediated adhesion is required for intestinal epithelial barrier integrity. Am J Phyiol Gastrointest Liver Physiol 298:G774-G783.

Singh H, Aplin JD. 2015. Endometrial apical glycoproteomic analysis reveals roles for cadherin 6, desmoglein-2 and plexin b2 in epithelial integrity. Mol Hum Reprod 21:81-94.

Sizemore RJ, Hurst PR, McLeod BJ. 2004. Effect of steroid hormones on tissue remodelling and progesterone receptors in the uterus of seasonally anoestrous brushtail possums (*Trichosurus vulpecula*). Reproduction 127:255-264.

Takeuchi A. 1967. Electron microscope studies of experimental *Salmonella* infection. Am J Pathol 50:109-136.

Tyndale-Biscoe CH. 1955. Observations on the reproduction and ecology of the brush-tailed possum, *Trichosurus vulpecula* Kerr (Marsupialia) in New Zealand. Aust J Zool 3:162-184.

Tyndale-Biscoe CH. 2005. *Life of Marsupials*. CSIRO publishing, Victoria, AU.
Tyndale-Biscoe CH, Renfree MB. 1987. *Reproductive Physiology of Marsupials*. Cambridge: Cambridge University Press.

Vogel P. 2005. The current molecular phylogeny of eutherian mammals challenges previous interpretations of placental evolution. Placenta 26:591–596.

Wagner GP, Kin K, Muglia L, Pavličev. 2014. Evolution of mammalian pregnancy and the origin of the decidual stromal cell. Int J Dev Biol 58:117-126.

Westerman M, Krajewski C, Kear BP, Meehan L, Meredith RW, Emerling CA, Springer MS. 2016. Phylogenetic relationships of dasyuromorphian marsupials revisited. Zool J Linn Soc 176:686-701.

Yashiro M, Nishioka N, Hirakawa K. 2006. Decreased expression of the adhesion molecule desmoglein-2 is associated with diffuse-type gastric carcinoma. Euro J Cancer 42:2397-2403.

Zeh JA, Zeh DW. 2000. Reproductive mode and speciation: the viviparity-driven conflict hypothesis. BioEssays 22:938-946.

Zhang S, Kong S, Lu J, Wang Q, Chen Y, Wang W, Wang B, Wang H. 2013. Deciphering the molecular basis of uterine receptivity. Mol Reprod Dev 80:8–21.

Figure legends

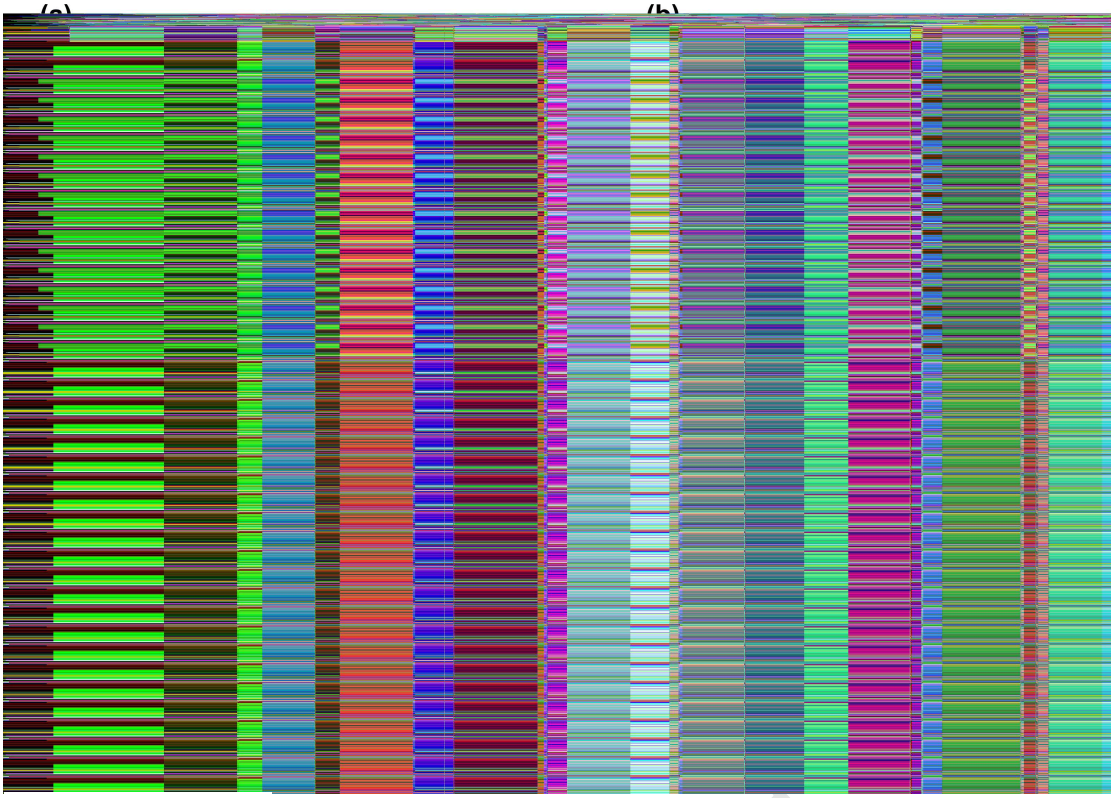
Figure 1: Phylogenetic distribution of placental types in Eutheria and Metatheria. Redrawn from Mess, 2014 for Eutheria (a) and Freyer et al, 2003 for Metatheria (b). Occurrence of haemochorial placentation is indicated in orange; endotheliochorial placentation in green; epitheliochorial placentation in blue; and unknown placentation type in black. Reprinted, with permission, from: [1]Martin, 2008; [2]Enders and Carter, 2004; [3]Mess and Ferner, 2010; [4]Freyer et al, 2003.

Figure 2: Immunofluorescence micrographs of Desmoglein-2 localization in the uterus of *M. eugenii* during pregnancy. (a-b) After embryonic activation / Stage 1 (n = 4); (c) Pre-implantation / Stage 2 (n = 5); (d) Implantation / Stage

3 (n = 2); (e) Post-implantation / Stage 4 (n = 4); (f) Negative-control staining of *M. eugenii* tissue (primary antibody substituted with IgG antibody). Scale bars, 20 µm. Desmoglein-2 staining is shown in green (arrow); nuclei are in blue. EC, embryonic cells; GEC, glandular epithelial cells; GL, glandular lumen; L, uterine lumen; MSC, mesenchymal stromal cells; UEC, uterine epithelial cells.

Figure 3: Immunofluorescence micrographs of Desmoglein-2 localization in uterine epithelial cells of *T. vulpecula* during pregnancy. (a) Stage 1 (n = 6); (b) Pre-implantation / Stage 2 (n = 2); (c-d) Implantation / Stage 3 (n = 4); (e) Post-implantation / Stage 4 (n = 1); (f) Negative-control staining of *T. vulpecula* tissue (primary antibody substituted with IgG antibody). Scale bars, 20 µm. Desmoglein-2 staining is shown in green (arrow); nuclei are in blue. GEC, glandular epithelial cells; GL, glandular lumen; L, uterine lumen; N, nuclei; S, stroma; UEC, uterine epithelial cells.

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).



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