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Research Article

Uterine molecular changes for non-invasive embryonic attachment in the marsupials *Macropus eugenii* (Macropodidae) and *Trichosurus vulpecula* (Phalangeridae)[1]

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Short title: Talin in *M. eugenii* and *T. vulpecula*

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Abstract
Pregnancy in mammals requires remodelling of the uterus to become receptive to the implanting embryo. Remarkably similar morphological changes to the uterine epithelium occur in both eutherian and marsupial mammals, irrespective of placental type. Nevertheless, molecular differences in uterine remodelling indicate that the marsupial uterus employs maternal defences, including molecular reinforcement of the uterine epithelium, to regulate embryonic invasion. Non-invasive (epitheliochorial) embryonic attachment in marsupials likely evolved secondarily from invasive attachment, so uterine defences in these species may prevent embryonic invasion. We tested this hypothesis by identifying localization patterns of Talin, a key basal anchoring molecule, in the uterine epithelium during pregnancy in the tammar wallaby (*Macropus eugenii*; Macropodidae) and the brush tail possum (*Trichosurus vulpecula*; Phalangeridae). Embryonic attachment is non-invasive in both species, yet Talin undergoes a clear distributional change during pregnancy in *M. eugenii*, including recruitment to the base of the uterine epithelium just before attachment, which closely resembles that of invasive implantation in the marsupial species *Sminthopsis crassicaudata*. Basal localization occurs throughout pregnancy in *T. vulpecula*, although, as for *M. eugenii*, this pattern is most specific prior to attachment. Such molecular reinforcement of the uterine epithelium for non-invasive embryonic attachment in marsupials supports the hypothesis that less-invasive and non-invasive embryonic attachment in marsupials may have evolved via accrual of maternal defences. Recruitment of basal molecules, including Talin, to the uterine epithelium may have played a key role in this transition.

Keywords: focal adhesion, Talin, implantation, uterus, pregnancy

Introduction

Successful pregnancy in mammals requires intimate contact between the embryo and the epithelial cell lining of the uterus (Schlafke and Enders, 1975; Wu et al, 2011). Contact occurs as the embryo implants and forms a placenta, which provides the embryo's nutrient, gas exchange, and waste removal requirements throughout its growth and development in utero (Wildman et al, 2006; Ramirez-Pinilla et al, 2012). Under normal conditions, implantation is prevented by unsuitable uterine conditions, particularly by cell morphology of the uterine epithelium, which forms the first physical barrier to an implanting embryo. Implantation can only occur when the uterine epithelium is remodelled to become receptive to the embryo (Orchard and Murphy, 2002; Murphy, 2004; Zhang et al, 2013). Remodelling occurs in all plasma membrane regions, and the resulting alterations are collectively termed the plasma membrane transformation (Murphy, 2004).

Alterations to the basal plasma membrane are particularly important as they affect how strongly the uterine epithelium attaches to the underlying tissue (Kaneko et al, 2008; 2013), and thus how easily the epithelium can be breached by an invading embryo. Focal adhesions are important basal anchors of uterine epithelial cells (Kaneko et al, 2008; 2009; 2011). In laboratory rodents, focal adhesions disassemble before implantation of the embryo to facilitate both sloughing of the uterine epithelium and highly invasive implantation (Enders and Schlafke, 1967; Schlafke and Enders, 1975). Disassembly involves loss of key focal adhesion proteins, including Talin, from the basal plasma membrane early in pregnancy (Kaneko et al, 2008; 2013). In contrast, focal adhesions remain intact in a marsupial species with moderately invasive placentation, *Sminthopsis crassicaudata* (Laird et al, 2015; 2017a), and Talin is recruited to reinforce the base of the uterine

epithelium, likely in response to the implanting embryo. This pattern of recruitment indicates that invasive implantation is not facilitated in *S. crassicaudata* in the same manner as in rodent pregnancy, and that basal reinforcement may be a maternal strategy to regulate embryonic invasion in *S. crassicaudata* (Fowden and Moore, 2012; Laird et al, 2017a).

Embryonic invasion can involve significant maternal tissue destruction, so strategic uterine regulation of this process is critical (Moffett and Loke, 2006). For example, contact with maternal blood maximizes the potential for embryonic manipulation of maternal physiology (Wooding and Flint, 1994; Vogel, 2005), and may enable the embryo to access additional nutrients from the blood that the mother may not otherwise provide (Crespi and Semeniuk, 2004; Vogel, 2005; Moore, 2012).

Marsupial pregnancy is characterized by less invasive embryonic attachment than that of eutherian mammals (Freyer et al, 2003). Since embryonic implantation in the common ancestor of living marsupials was probably invasive, non-invasive placentation in marsupials is most likely secondarily derived (Freyer et al, 2003). Thus, the persistence of maternal defences against the marsupial embryo in pregnancy suggest that less-invasive and non-invasive modes of embryonic attachment may have evolved by accumulating maternal defences against the invading embryo (Crespi and Semeniuk, 2004; Vogel, 2005; Carter and Mess, 2007; Laird et al, 2015; 2017a). We tested this hypothesis by conducting the first study of focal adhesion dynamics during pregnancy in marsupial species with non-invasive embryonic attachment – the tammar wallaby, *Macropus eugenii,* and the brush tail possum, *Trichosurus vulpecula*. Non-invasive attachment in phalangerid and macropodid marsupials likely evolved independently (Freyer et al, 2003; Binida-Edmonds et al. 2007), so comparison of these species can identify if maternal

defences are required for successful embryonic attachment in marsupials, and how varied such maternal strategies are across lineages.

The annual breeding cycle of *M. eugenii* is highly predictable and involves both lactational and seasonal diapause (Tyndale-Biscoe and Renfree, 1987; Renfree and Shaw, 2014). Ovulation alternates between ovaries, and only one of the two separate uteri carries an embryo at a time (monovular) (Renfree, 2000; Tyndale-Biscoe, 2005). Mating occurs immediately after birth during a post-partum oestrus (Tyndale-Biscoe and Renfree, 1987; Renfree, 1993; Rudd, 1994). Ovulation occurs the day after birth, and the embryo enters the uterus 1 day later. The embryo develops to the unilaminar blastocyst stage by Day 7-8 of gestation before it enters diapause, which is initiated by the sucking stimulus of the pouch young. Diapause is controlled by lactational inhibition of growth of the corpus luteum resulting from this suckling stimulus between January and May (Renfree and Shaw, 2000; Hinds and Tyndale-Biscoe, 2013), and after the winter solstice is under photoperiodic control until the summer solstice, when the diapausing blastocyst reactivates (Tyndale-Biscoe and Renfree, 1987; Renfree, 1993; Renfree and Shaw, 2000; 2014). The reactivated, unilaminar blastocyst then continues development. Embryonic attachment occurs around Day 18 after conception, when the shell coat ruptures (Denker and Tyndale-Biscoe, 1986; Menzies et al, 2011), with birth on Day 26.5 (Renfree et al*,* 1989).

Trichosurus vulpecula has an oestrous cycle of 28 days (Tyndale-Biscoe, 2005), a 17.5-day gestation period (Pilton and Sharman, 1962; Sizemore et al, 2004), and does not undergo developmental arrest. Like *M. eugenii*, *T. vulpecula* is monovular, and ovulation of a single egg occurs 1-2 days after oestrus. Non-invasive attachment of the embryo occurs approximately 14 days after conception, with birth

3-4 days later (Tyndale-Biscoe, 2005). Lactation suppresses ovulation, and the female enters oestrus again after the young is weaned, at approximately 110 days post-oestrus.

We combined immunofluorescence microscopy and Western blotting to identify patterns of Talin localization in the uterus throughout pregnancy in these two marsupials. As the embryo does not breach the uterine epithelium in *M. eugenii* (Freyer et al, 2003) or *T. vulpecula* (Pilton and Sharman, 1962), specific recruitment of Talin to the uterine lining before implantation, as occurs in *S. crassicaudata*, would strongly suggest that uterine defences prevent invasion of the embryo and are ubiquitous in marsupial pregnancy.

Results

Light microscopy

Uterine epithelial cells of *M. eugenii* were pseudostratified columnar and arranged irregularly after reactivation of the embryo (Stage 1) (Figure 1a). Underlying stromal cells were densely packed, and glandular epithelial cells resembled those of the uterine epithelium. By Stage 2 (pre-implantation), uterine epithelial cells were less elongated and more regularly arranged than in Stage 1 (Figure 1b). Most cells were no longer pseudostratified and had rounded nuclei. Glandular epithelial cells were similar to uterine epithelial cells, but more elongated. Stromal cells underlying the uterine epithelium were sparse relative to Stage 1. Uterine epithelial cells had developed domed apices by Stage 3 (implantation), and possessed basal, rounded nuclei (Figure 1c). Intercellular spaces between adjacent epithelial cells spaces occurred along the uterine epithelium. Glandular epithelial

cells lacked these spaces, and did not develop rounded apices. Stromal cells remained relatively sparse at this stage. By Stage 4 (post-implantation), uterine epithelial cells were low and cuboidal with large, rounded nuclei and often possessed domed apices (Figure 1d). No intercellular spaces occurred at this stage. Stromal cells were more abundant at Stage 4 than for Stages 2 and 3. Glandular epithelial cells resembled those of Stage 3.

Uterine epithelial cells of *T. vulpecula* at Stage 1 of pregnancy were columnar with uniform basal nuclei (Figure 2a). The uterine epithelium was also highly folded, and the underlying stromal region was densely packed with cells as well as uterine glands. Uterine epithelial cells at Stage-2 (pre-implantation) were pseudostratified columnar with large elongated nuclei (Figure 2b). By Stage 3 (implantation), uterine epithelial cells were irregularly arranged and elongated. Some small intercellular spaces were observed, and the underlying stromal cells were sparse relative to Stages 1 and 2 (Figure 2c). No uterine glands were observed in Stage 3. By Stage 4 (post-implantation), the uterine epithelium was highly folded, with irregular uterine epithelial cells (Figure 2d). These cells were low and cuboidal, and large spaces occurred between cells. Stromal cells at Stage 4 were more sparsely distributed than for Stage 3. No glands were observed in the uterus at this stage.

Immunofluorescence microscopy

Talin localization was punctate and diffuse in the stromal cells underlying the uterine epithelium after embryonic reactivation in *M. eugenii* (Stage 1) (Figure 3a). Talin was not specifically localized to the basal plasma membrane of uterine epithelial cells, although some cytoplasmic staining occurred in these cells. By Stage 2 (pre-implantation), Talin was present as a diffuse, prominent band at the base of

the uterine epithelium (Figure 3b). Stromal staining of Talin appeared to be less diffuse and more punctate by this stage, particularly concentrated around stromal cell nuclei. Cytoplasmic staining still occurred in the uterine epithelium. By Stage 3, Talin remained as a prominent band that was tightly localized to the basal plasma membranes of uterine epithelial cells; stromal staining was faint and diffuse (Figure 3c). Localization of Talin at Stage 4 (post-implantation) resembled that of Stage 1 as talin was present as a diffuse, basal band of staining in stromal cells that was no longer tightly localized to the base of the uterine epithelium (Figure 3d). A prominent basal band of Talin also occurred at the base of embryonic cells. Folds of the uterine epithelium interdigitated with those of the placental membranes (Figure 3e), resulting in extremely close apposition of uterine and embryonic tissue.

In *T. vulpecula*, Talin was present as a prominent band at the base of the uterine epithelium immediately post-oestrus (Stage 1), with some faint cytoplasmic staining (Figure 4a). Talin was also tightly localized to the base of glandular epithelial cells at this stage, while punctate Talin localization occurred at the peripheries of stromal cells. Talin remained localized to the base of the uterine epithelium at Stage 2 (pre-implantation), although the basal band was more prominent than at Stage 1 (Figure 4b). Basal localization of Talin still occurred for glandular epithelial cells, while that of stromal cells was less punctate than the previous stage. By Stage 3 (implantation period), Talin was specifically localized to the basal plasma membrane of uterine epithelial cells, although cytoplasmic was also present in these cells (Figure 4c). Talin was less tightly localized to the base of the uterine epithelium at Stage 4 (post-implantation) than for Stages 2 and 3, yet still occurred as a basal band (Figure 4d). Diffuse cytoplasmic staining also occurred in the uterine epithelium at Stage 4, with diffuse and punctate stromal staining.

Controls

No Talin localization occurred in negative control samples (primary antibody replaced with IgG antibody) (Figure 5a-b). In contrast, prominent basal localization of Talin occurred in positive control tissue (rat uterus on Day 1 of pregnancy) (Figure 5c).

Western blotting

Talin was detected as a doublet at approximately 225 kDa at all stages of pregnancy for *M. eugenii* (Figure 6a) and *T. vulpecula* (Figure 6b). Talin was also detected as a doublet at approximately 225 kDa in isolated rat epithelial cells at Day 1 of pregnancy (Figure 6c). The loading control (monoclonal β-actin antibody) showed no difference for the amount of protein loaded for each sample (not shown).

Discussion

Changes in basal plasma membrane dynamics occur in, and are likely to be important for, preparation for non-invasive embryonic attachment in *M. eugenii* and *T. vulpecula.* Talin was redistributed in both species prior to uterine receptivity and implantation, and specifically localized to the basal plasma membrane of uterine epithelial cells during the attachment period.

Talin localization in the uterus at all stages of pregnancy indicates that focal adhesions do not disassemble in *M. eugenii* and *T. vulpecula.* In contrast, focal adhesion disassembly in rodents involves loss of Talin from the base of the uterine epithelium, reducing adhesion between the uterine epithelium and the underlying stromal tissue and facilitating highly invasive implantation (Kaneko et al, 2008;

2013). Maintenance of focal adhesions throughout pregnancy in *M. eugenii* and *T. vulpecula*, with non-invasive embryonic attachment, was expected since focal adhesions also remain intact in eutherian mammal species with non-invasive implantation (Moffett and Loke, 2006; Kaneko et al, 2013). Additional apical focal adhesions form to create a cellular connection between the uterine epithelium and the embryo in both pigs and sheep (Johnson et al, 2001; Garlow et al, 2002), although no apical recruitment of Talin occurs in *M. eugenii* and *T. vulpecula*.

Talin became tightly localized to the base of the uterine epithelium during the period of uterine receptivity in both *M. eugenii* and *T. vulpecula*. This pattern likely corresponds with the period of strongest adhesion between the uterine epithelium and underlying cells (Kaneko et al, 2008). Similar localization of Talin occurs in preparation for invasive implantation in *S. crassicaudata,* in which Talin is recruited to reinforce the uterine epithelium against the invading embryo (Laird et al, 2015; 2017a). Such molecular reinforcement of the uterine epithelium may be a maternal strategy to regulate embryonic invasion in *S. crassicaudata* (Laird et al, 2015; 2017a), and likely evolved to mitigate conflict between mother and embryo (Fowden and Moore, 2012).

Although under-restriction of embryonic invasion enables greater access to maternal resources, it can lead to adverse maternal consequences, including tissue damage and manipulation of maternal physiology (Crespi and Semeniuk, 2004; Vogel, 2005; Fowden and Moore, 2012; Moore, 2012). In contrast, mechanisms that restrict embryonic invasion also potentially limit embryonic access to maternal resources. Placentation is thus a trade-off between facilitation and restriction of embryonic invasion that ensures both maternal protection and successful embryonic development (Moffett and Loke, 2006). Indeed, the specific, basal recruitment of

Talin in *M. eugenii*, *T. vulpecula*, and *S. crassicaudata* suggests that this pattern may also be a uterine defence mechanism that prevents removal of the uterine epithelium, irrespective of placentation mode in marsupials. This hypothesis is supported by cellular alterations of both *M. eugenii* and *T. vulpecula* that likely compensate for restricted resource access by facilitating haemotrophic nutrient transfer from maternal blood across the uterine epithelium, including apical migration of maternal blood vessels and folding of the base of uterine epithelial cells to increase surface area (Freyer et al, 2002; Laird et al, 2017b). Thus, maintenance of the uterine epithelium in *M. eugenii* and *T. vulpecula* likely affords maternal protection without compromising embryonic development, and thus plays a critical role in balancing maternal and embryonic requirements in marsupial pregnancy, irrespective of placentation mode.

Marsupial species likely achieve non-invasive embryonic attachment by utilizing a variety of uterine strategies to exclude the embryo. Additional strategies may involve redistribution of other focal adhesion molecules, including integrins and focal adhesion kinases, which play important structural and signalling roles in rodents, humans, and pigs (Garlow et al, 2002; Kaneko et al, 2012; Burkin et al, 2013). Alterations to lateral adhesion between adjacent epithelial cells may also be involved. In *S. crassicaudata*, for example, lateral adhesion is reduced before implantation as adhesion points (desmosomes) decrease in abundance and redistribute (Dudley et al, 2015). Lateral changes may also occur in *M. eugenii* and *T. vulpecula* to reinforce the uterine epithelium and prevent invasion.

Basal reinforcement of the uterine epithelium in *M. eugenii, T. vulpecula*, and *S. crassicaudata,* irrespective of the mode of embryonic attachment, suggests that maternal defences are ubiquitous in marsupial pregnancy. As reinforcement occurs

even in species with non-invasive attachment, this study supports the hypothesis that maternal uterine strategies may regulate, and even prevent, embryonic invasion, and that accumulation of such defences could provide a mechanism by which less invasive and non-invasive modes of attachment have evolved secondarily from invasive implantation in mammals (Vogel, 2005; Carter and Mess, 2007; Martin, 2008; Capellini, 2012; Fowden and Moore, 2012; Mess, 2014). Indeed, non-invasive implantation is a derived trait of several eutherian groups, including ungulates (Mess and Carter, 2007; Ferner and Mess, 2011). Embryos of pigs (Samuel and Perry, 1972) and horses (Adams and Antczak, 2001) removed from the uterus undergo highly invasive implantation at an ectopic site, demonstrating that the uterus actively prevents invasion in these species under the conditions of normal pregnancy. Similar studies of marsupial embryos will help determine how the marsupial uterus affects embryonic invasion, and identify the role of maternal defences in the evolution of mammalian pregnancy.

Materials and methods

Tissue harvest and processing

All collection of samples of *M. eugenii* 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 samples of *T. vulpecula* was a secondary use from a cull approved by the Animal Ethics Committee of Landcare Research, New Zealand.

Tissue from wild *M. eugenii* was collected on Kangaroo Island, South Australia, from wild animals at all stages of pregnancy. Uterine tissue from animals

with a new pouch young before developmental arrest of the embryo (approximately Day 8 of gestation) was not included in this study as uterine changes during this period are associated with preparation for initiation of diapause, not embryonic attachment (Laird et al, 2016). We divided post-diapause, active pregnancy of *M. eugenii* pregnancy into four time periods relating to major reproductive events (Figure 7): Stage 1 (after embryonic reactivation; Days 9-12 of gestation; n = 2), Stage 2 (pre-implantation; Days 13-17; n = 3), Stage 3 (shell coat rupture and early implantation; Days 18-20; n = 2), and Stage 4 (post-implantation; Days 21-26; n = 6).

Uterine tissue of wild *T. vulpecula* was obtained opportunistically from a cull undertaken in the Orongorongo Valley near Wellington, New Zealand. Brush-tail possums in New Zealand breed from February to April (Crawley, 1973; Tyndale-Biscoe, 1955), so a complete set of reproductive-stage tissues from 13 females was collected over two seasons (April 2014 and March 2015). We divided normal gestation in *T. vulpecula* into four time periods (approximate number of days post-oestrus) relating to reproductive events and based on uterine and ovarian morphology following Laird et al. (2017b) (Figure 7): Stage 1 (0-6d post-oestrus; n= 6), Stage 2 (7-11d post-oestrus; n= 2), Stage 3 (12-14d post-oestrus; n= 4), Stage 4 (15-17.5d post-oestrus; n= 1).

Uterine tissue of both *M. eugenii* and *T. vulpecula* was processed for light microscopy, immunofluorescence microscopy, and Western blotting.

Light microscopy

Uterine tissue from *M. eugenii* was fixed in 4% paraformaldehyde for 24 hours under gentle rotation, washed in three changes of phosphate buffered saline (PBS), and stored in 100% methanol. This tissue was then embedded in paraffin, serially

sectioned (8 μm) using a Tissue-Tek Accu-Cut™ microtome (Sakura, Tokyo, Japan), and mounted on gelatin-coated slides. Sections on slides were dried overnight at 40˚C, and then dehydrated through a graded series of ethanol before staining with haemotoxylin and eosin (Drury and Wallington, 1980).

Uterine tissue of *T. vulpecula* was coated with Tissue-Tek OCT cryoprotectant (Sakura, Tokyo, Japan), and frozen by brief immersion in super-cooled isopentane. Frozen tissue was then sectioned (8 μm) at -25˚C using a Leica CM3050 S cryostat (Leica, Heerbrugg, Switzerland). Sections were collected on gelatin-coated slides, and stained as for *M. eugenii*, omitting the dehydration step.

Stained sections for both *M. eugenii* and *T. vulpecula* were examined using an Olympus DX-53 digital microscope (Olympus, Tokyo, Japan), with CellSens imaging software.

Immunofluorescence microscopy

Tissue for immunofluorescence was coated with Tissue-Tek OCT cryoprotectant (Sakura, Tokyo, Japan), briefly immersed in super-cooled isopentane, and stored in liquid nitrogen. Tissues were sectioned (8 μm) at -25˚C using a Leica CM3050 S cryostat (Leica, Heerbrugg, Switzerland), and mounted on gelatin-coated slides. Tissue sections were fixed for 10 min at room temperature in 4% paraformaldehyde, blocked for 30 min with 1% bovine serum albumin (BSA) in PBS, and incubated for 2 h with mouse monoclonal anti-Talin antibody (Sigma-Aldrich, Castle Hill, Sydney) (1:2000 dilution of T3287 in 1% BSA in PBS) Slides were then rinsed in PBS, and incubated for 1 h with goat anti-mouse fluorescein isothiocynate-conjugated IgG antibody (Jackson Immunoresearch Laboratories, West Grove, PA) (1:5000 dilution of 115-095-116 in 1% BSA in PBS). After further rinsing, the tissue

was allowed to air dry before mounting with Vectashield mounting medium with DAPI (Vector Laboratories, Burlingame, CA). Images were captured using a Zeiss deconvolution microscope (Carl Zeiss, Australasia) fitted with a Zeiss AxioCam HR monochrome CCD camera, using Zen imaging software (Version 7.1). Negative controls were prepared as above by substituting the primary antibody with 1 mg/mL mouse IgG purified immunoglobulin (item I5381) (Sigma-Aldrich). Positive control slides were of rat uterine tissue at Day 1 of pregnancy (Kaneko et al, 2008).

Western blot analysis

Uterine tissue, including positive control tissue (isolated rat uterine epithelial cells on Day 1 of pregnancy), was snap frozen immediately after excision. Samples were prepared by homogenization using short bursts of vigorous shaking with homogenizing beads in lysis buffer with protease inhibitor cocktail (1:100 dilution) (Sigma-Aldrich). Extracted protein samples (5 µl) were diluted 1:100, 1:200, and 1:400 with distilled water for determining protein concentration using the Micro BCA™ Protein Assay Kit (Thermo Scientific, Rockford, IL), and read on a CLARIOstar Microplate reader (BMG LabTech, Durham, NC).

Total protein samples (20 µg) were denatured at 90˚C for 5 min in Laemmli sample buffer (Kaneko et al, 2008), separated on a pre-cast 10% SDS-PAGE gel for 1.5 h at 100 V, and then transferred to a polyvinylidene fluoride membrane (Millipore Corporation, Bedford, MA). The membrane was blocked for 1 h in 5% skim milk in TBS-t (Tris-buffered saline with 0.1% Tween-20), and then incubated overnight at 4˚C with mouse monoclonal anti-Talin antibody (1:2000 dilution of T3287 in 1% skim milk in TBS-t) (Sigma-Aldrich). After rinsing in TBS-t, the membrane was incubated for 2 h with sheep anti-mouse IgG conjugated to horseradish peroxidase (GE

Healthcare, Buckinghamshire, UK) (1:2000 dilution of GEHENA931 in 1% skim milk in TBS-t). Following further rinsing, the membrane was imaged using a Chemidoc MP Imaging System (Bio Rad Laboratories, Hercules, CA) using the ECL Plus Western Blotting Detection System (Amersham, GE Healthcare, Buckinghamshire, UK). Proteins were then stripped from the membrane by incubation in stripping buffer containing β-mercaptoethanol for 45 min at 60˚C. The membrane was then re-probed for actin following the same procedure as above, using a monoclonal primary antibody against β-actin (Sigma-Aldrich)(1:2000 dilution of A1978 in 1% skim milk in TBS-t).

Declaration of interest

The authors have no conflict of interest to declare.

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Figure legends

Figure 1: Light micrographs of uterine structure during pregnancy in *M. eugenii*. (a) Stage 1; (b) Stage 2; (c) Stage 3; (d) Stage 4. Sections were stained with haemotoxylin and eosin. Scale bars, 40 μm. Arrows indicate domed apices; arrowheads indicate intercellular spaces. GEC, glandular epithelial cells; GL, glandular lumen; L, lumen; S, stroma (S); UEC, uterine epithelial cells.

Figure 2: Light micrographs of uterine structure during pregnancy in *T. vulpecula*. (a) Stage 1; (b) Stage 2; (c) Stage 3; (d) Stage 4. Sections were stained with haemotoxylin and eosin. Scale bars, 40 μm. Arrowheads indicate intercellular spaces. L, lumen; S, stroma; UEC, uterine epithelial cells.

Figure 3: Immunofluorescence micrographs of Talin localization in uterine epithelial cells during pregnancy in *M. eugenii*. (a) Stage 1; (b) Stage 2; (c) Stage 3; (d-e) Stage 4. Talin localization (arrow) is green, nuclei are labeled with DAPI (blue). Arrowhead indicates trophoblastic cells. Scale bars, 20 μm (a-d), 100 μm (e). EC, trophoblastic cells; GEC, glandular epithelial cells; L, lumen; S, stroma; UEC, uterine epithelial cells.

Figure 4: Immunofluorescence micrographs of Talin localization in uterine epithelial cells during pregnancy in *T. vulpecula*. (a) Stage 1; (b) Stage 2; (c) Stage 3; (d) Stage 4. Talin localization (arrow) is green, nuclei are labeled with DAPI (blue). Scale bars, 20 µm. GL, glandular lumen; L, lumen (L); S, stroma; UEC, uterine epithelial cells.

Figure 5: Immunofluorescence micrographs of control tissue. No specific staining for Talin occurred in negative control tissue (primary antibody substituted with IgG antibody) for *M. eugenii* (a) or *T. vulpecula* (b). (c) Positive control tissue (rat uterus on Day 1 of pregnancy) showed a distinct band of basal Talin localization (arrow). Scale bars, 20 µm. GL, glandular lumen; L, lumen; S, stroma; UEC, uterine epithelial cells.

Figure 6: Immunoblot of whole-cell lysate from uterine tissue probed with a monoclonal mouse anti-Talin antibody (20 µl of total protein loaded per pregnancy stage). Talin was identified as a doublet at ~225 kDa at all pregnancy stages in *M. eugenii* (a) and *T. vulpecula* (b), as well as in rat uterine tissue on Day 1 of pregnancy (c).

Figure 7: Summary of reproductive staging for *M. eugenii* and *T. vulpecula*, based on major reproductive events. Stage 1 of *M. eugenii* pregnancy followed reactivation of the embryo after developmental arrest (Tyndale-Biscoe and Renfree, 1987; Renfree, 1993; Renfree and Shaw, 2000; 2014). Stage 1 of *T. vulpecula* pregnancy followed behavioural oestrus (Pilton and Sharman, 1962, Sizemore et al, 2004; Tyndale-Biscoe, 2005).

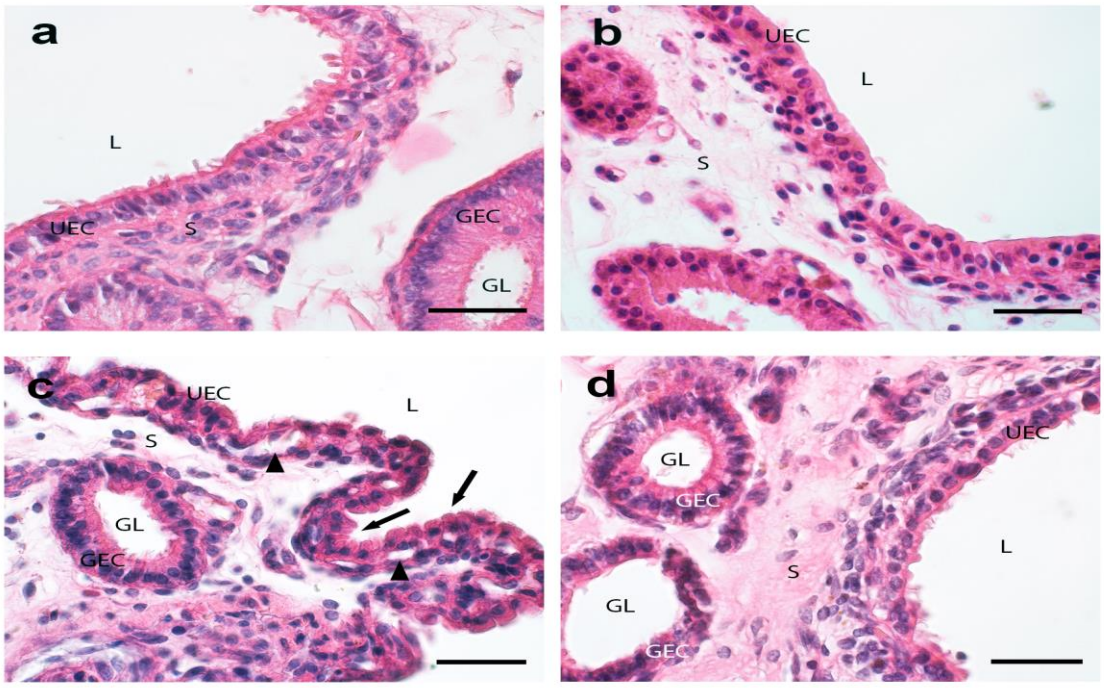

Figure 1

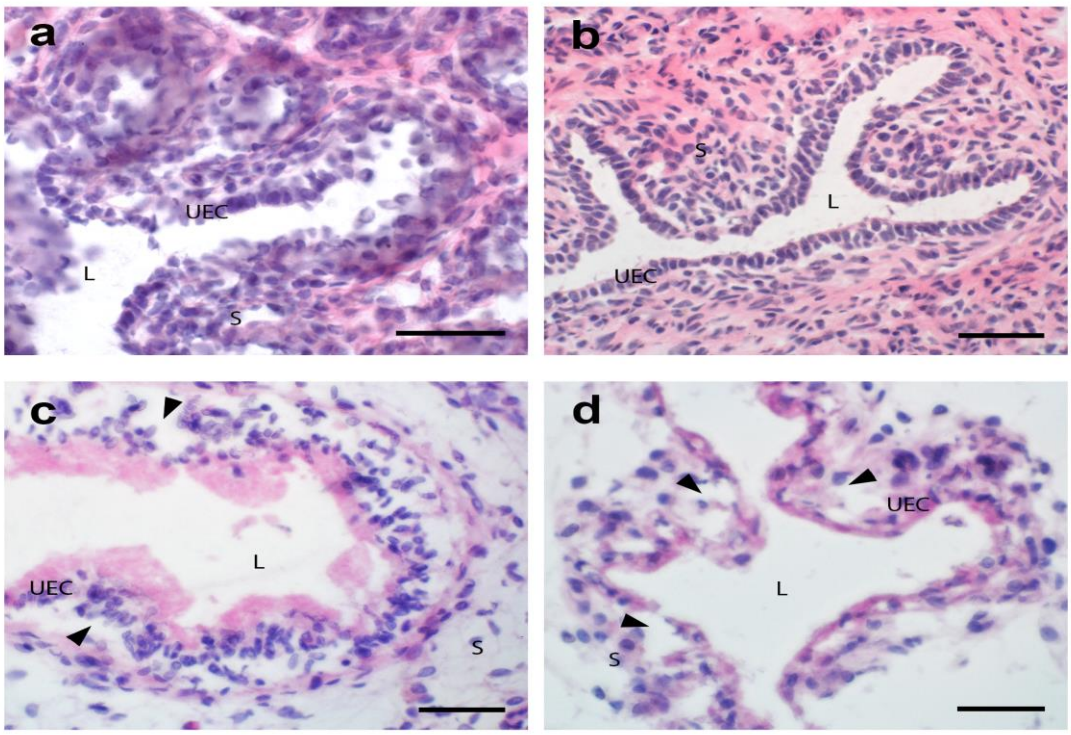

Figure 2

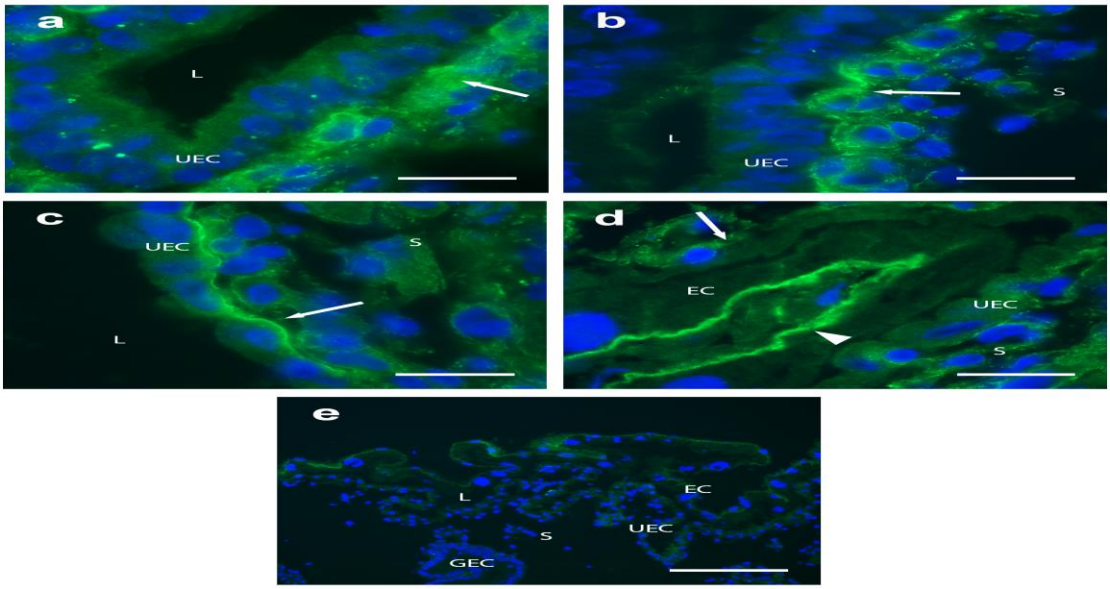

Figure 3

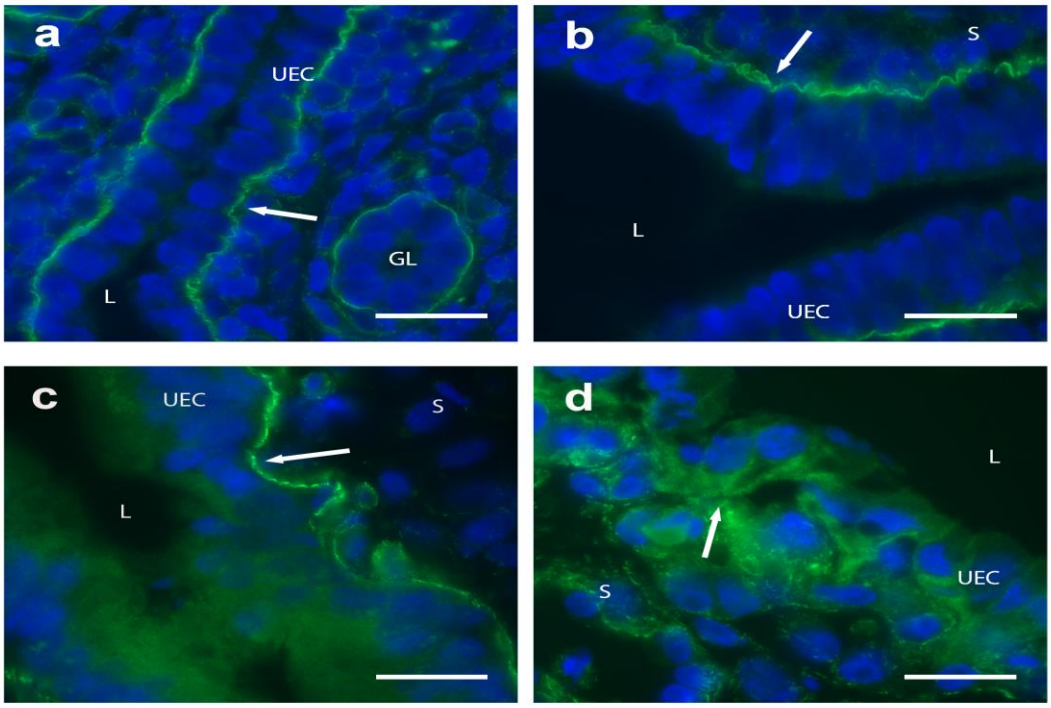

Figure 4

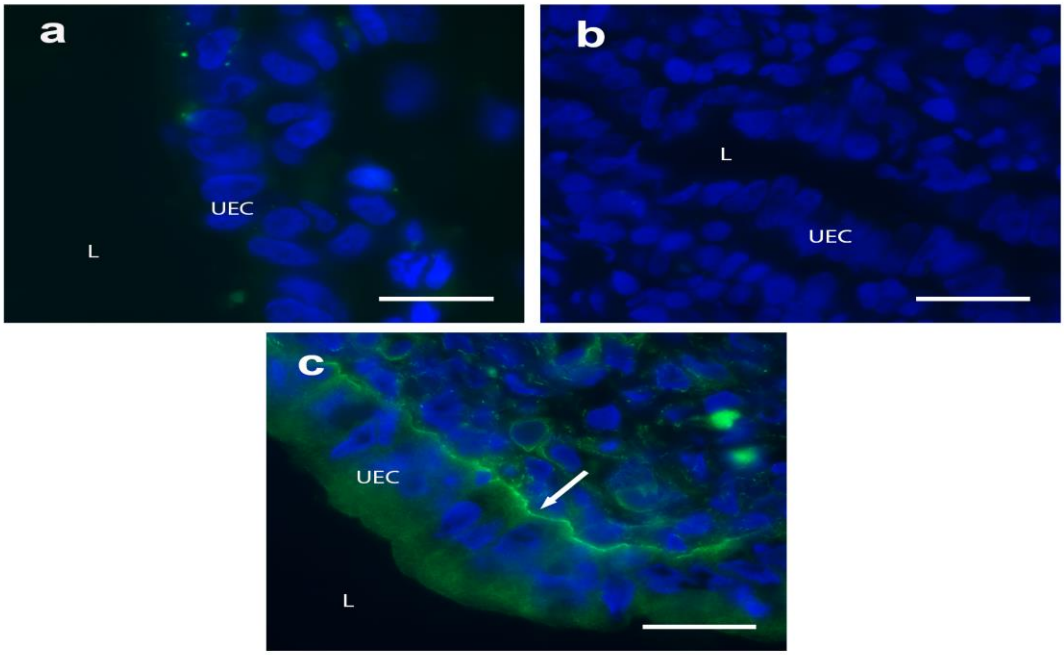

Figure 5

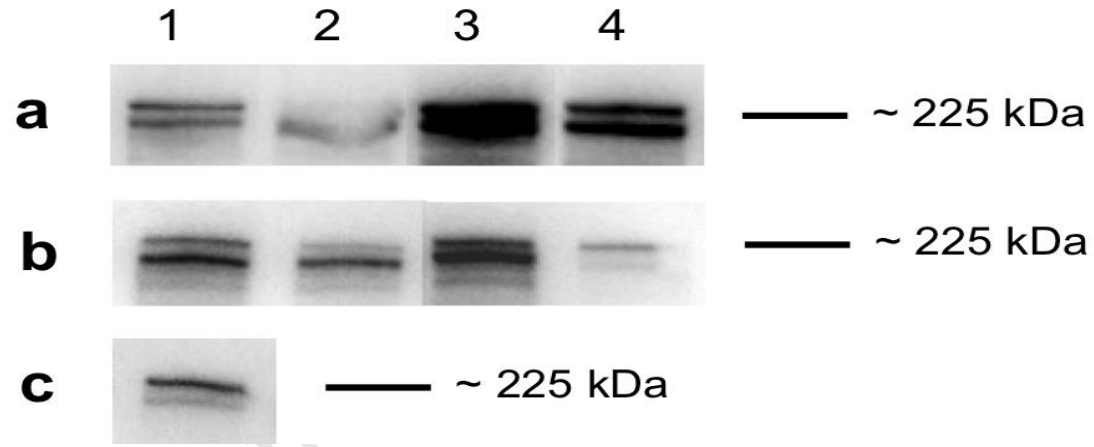

Figure 6

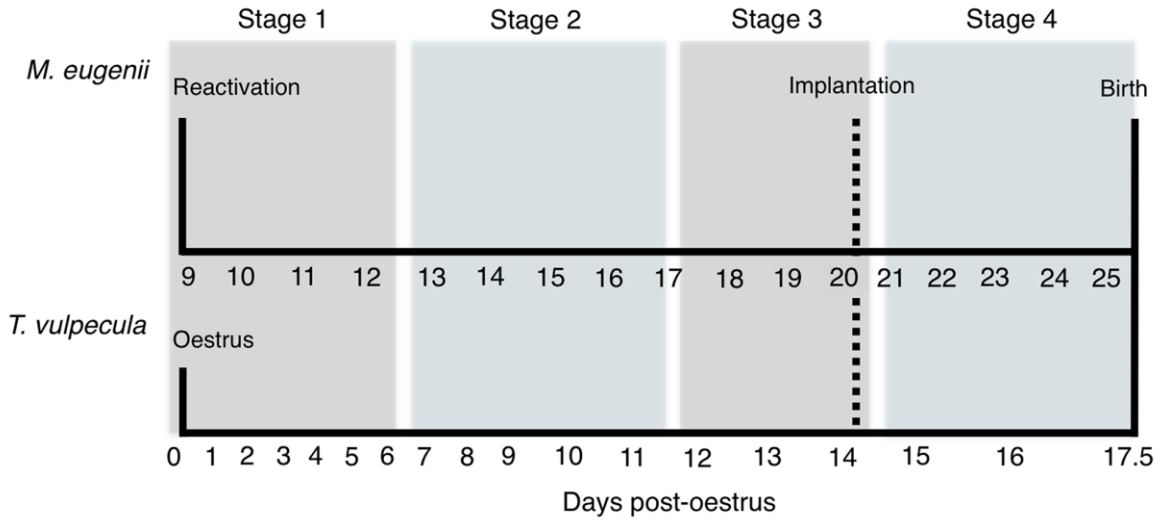

Figure 7