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Unlocking Amniote Live Birth: the 'Other' Mammalian Model

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Abstract

Amniotes (birds, reptiles and mammals) exhibit a remarkable range of reproductive strategies. The transition from oviparity (egg-laying) to viviparity (live birth) has occurred independently multiple times in squamate reptiles (snakes and lizards) and once in therian mammals (placental mammals and marsupials) and requires many changes to the uterus to allow the embryo to develop inside the mother. An important step in this transition is the evolution of a placenta. Formation of a placenta in early pregnancy requires substantial remodelling of the surface of the uterus, termed the plasma membrane transformation. Similar cellular changes occur in both placental mammals and live-bearing squamate reptiles which suggests this phenomenon plays an important role in the evolution of amniote viviparity.

Marsupials are ideally placed to test theories of the generality and importance of the plasma membrane transformation of the uterus. Similar morphological changes also occur in a marsupial species (*Sminthopsis crassicaudata*; Dasyuridae), suggesting these changes are ubiquitous in amniote pregnancy, but remodelling appears to be underpinned by different molecular changes in each group. This study demonstrates that not all uterine changes are common across vertebrate lineages. Thus, the transition from egg-laying to live birth may involve flexible molecular recruitment as common molecules do not play the same roles in pregnancy in different live bearing groups. This study highlights the necessity of including marsupials as a separate mammalian group in comparative studies, and the valuable and novel contribution marsupials can make to evolutionary theories.

Introduction

The transition from egg-laying (oviparity) to live birth (viviparity) has occurred many times within the vertebrates and produced a remarkable diversity of live-bearing species (Blackburn and Flemming, 2009): from mammals, to live-bearing skinks and snakes, and even to male pregnancy in seahorses (Whittington et al., in review). This life history transition involves many complex steps, including a reduction of the eggshell, and development of mechanisms to fulfill the embryo's gas exchange and waste removal requirements during development in the uterus. Repeated evolution of live birth within the vertebrate clade Amniota (birds, reptiles and mammals; Figure 1) has resulted in major differences in gestation length, and the amount of nutrients provided to the embryo during pregnancy. Despite these differences, viviparous members of this clade overcome the novel problems of viviparity in common ways.

The best example of a common strategy is the co-option of the characteristic extra-embryonic membranes of this group, which in egg-laying taxa meet an embryo's needs within an eggshell, to form a placenta in early pregnancy. The placenta is a complex organ that forms from intimate contact between the embryo and the cells of the uterus as the embryo implants, and enables exchange of gases, wastes and, to varying extents, nutrients, to occur between the mother and the embryo within the uterus (Ramirez-Pinilla et al., 2012).

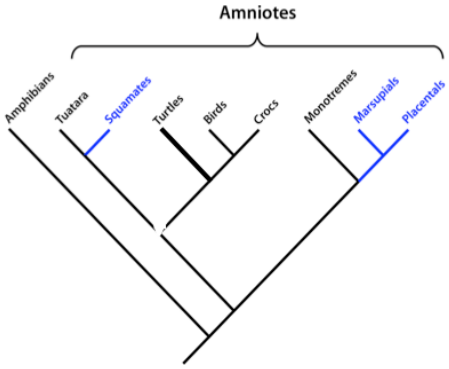

Figure 1: Phylogeny of the amniotes. Blue branches indicate lineages which contain viviparous members.

Placentation requires significant remodelling of the epithelial cells lining the uterus in early pregnancy to enable intimate contact between the uterus and the embryo. Without remodelling, the uterus will not become receptive to implantation by the blastocyst, and pregnancy will fail (Kaneko et al., 2008; Murphy, 2004; Murphy et al., 2000; Orchard and Murphy, 2002; Zhang et al., 2013). Hence, the cell changes that occur during this period, termed the plasma membrane transformation (Figure 2), are critical to determining the success of the pregnancy (Murphy, 2000; 2004; Murphy et al., 2000).

Common Uterine Changes

Recent studies of pregnancy in live-bearing lizards have identified changes that are remarkably similar to those changes involved in preparation for mammalian pregnancy (Biazik et al., 2007; Murphy et al., 2000). In both lizards and placental mammals, uterine cells flatten, and lose microvilli from their apical surfaces (Figure 2), creating a smooth, flat surface to which the embryo can adhere (Murphy, 2004; Murphy et al., 2000; Orchard and Murphy, 2002).

Junctions in the lateral membrane undergo distinct structural changes (Murphy, 2000; Murphy et al., 2000; Orchard and Murphy, 2002). Tight junctions, which regulate fluid transport across the uterine lining, are modified to block unregulated solute movement into the uterus (Biazik et al., 2007; Orchard and Murphy, 2002). This modification enables precise control of the fluid environment surrounding the embryo during implantation and development (Murphy, 2000; Murphy et al., 1982).

The number of desmosomes, or attachment points between epithelial cells, decreases at implantation as the remodelled cells become more labile (Biazik et al., 2010). Thus, the apical and lateral morphological changes shared by squamate reptiles and placental mammals influence both the structural and chemical environment to which the embryo is exposed in the uterus.

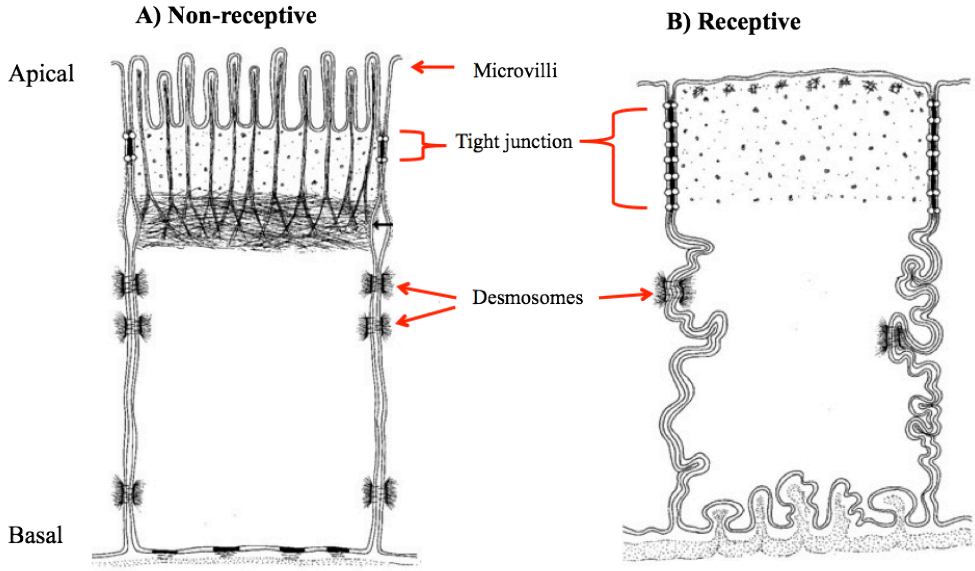

Figure 2: Remodelling of the uterine lining during pregnancy. A) Non-receptive cells have long apical microvilli, short lateral tight junctions and numerous lateral desmosomes. B) Receptive cells have undergone a 'plasma membrane transformation' (PMT) and are remodelled. Apical microvilli are replaced by a smooth flat surface. Laterally, tight junctions extend further down the membrane and the number of desmosomes is reduced. Receptive cells are ready to receive the embryo at implantation (adapted from Murphy, 2000).

Molecular Changes

A common beginning to pregnancy in diverse live-bearing groups suggests that this suite of morphological changes is essential for receptivity and successful pregnancy in live-bearing species. Remarkably, these apical and lateral changes occur whether the embryo breaches the uterine lining and invades maternal tissue, even maternal blood vessels, as it implants (haemochorial placentation), or whether the embryo simply adheres to the uterine lining and does not invade (epitheliochorial placentation; Biazik et al., 2010; Wooding and Flint, 1994). Repeated evolution of morphological changes in diverse groups suggests that the plasma membrane transformation is a fundamental characteristic of live birth in the amniotes (Murphy et al., 2000; Thompson et al., 2002).

While morphological changes are common between viviparous groups, the molecular changes that underpin them are much more variable. For example, modification of tight junctions involves the molecule claudin-5 in both placental mammals and squamate reptiles (Biazik et al., 2008). Occludin, another key tight junction molecule, is also involved in this process in placental mammals, but not all viviparous skink lineages (Biazik et al., 2007). Differences in patterns of key molecules involved in pregnancy suggest that the roles of these molecules differ between lineages (Biazik et al., 2007), and that the common morphological changes shared by different viviparous groups are underpinned by

flexible, or variable, molecular mechanisms (Brandley et al., 2012).

The 'Other' Mammals

Marsupials, while part of the same viviparous lineage as placental mammals, are a highly unusual mammalian group which has been distinct for at least 125 million years (Graves, 1996). The unique features of pregnancy in this group mean that marsupials provide an important and novel perspective to studies of amniote viviparity (Graves and Westerman, 2002; Shaw and Renfree, 2006). A short gestation period (as short as 12 days in *Smithopsis. crassicaudata*), followed by an extended period of lactation in the pouch (Carter, 2008; McAllan, 2011), during which most organ growth and differentiation of the young occurs (Freyer and Renfree, 2009; McAllan, 2011; Renfree, 2010; Shaw and Renfree, 2006). The marsupial embryo is surrounded by a shell for most of pregnancy and 'hatches' in the uterus several days before birth. As a result, implantation and formation of a choriovitelline placenta do not occur until late in pregnancy, and in some cases, the placenta functions for only a few days (Roberts and Breed, 1994).

Despite the unique features of marsupial pregnancy, a plasma membrane transformation occurs in the marsupial species *Sminthopsis crassicaudata* (the fat-tailed dunnart; Laird et al., 2014), demonstrating that uterine changes are a ubiquitous and essential requirement of amniote pregnancy. The next step is to identify the molecular changes that underpin the plasma membrane transformation in marsupial pregnancy, and to compare the molecular mechanisms with those of other live-bearing mammals. In this way, marsupials provide an ideal model system to test the generality of uterine changes in mammalian pregnancy and to identify their importance in the evolution of live birth in this group.

Basal membrane changes in *Sminthopsis crassicaudata*

We conducted a study of the molecules involved in changes in the basal plasma membrane of uterine cells during pregnancy in a marsupial (*Sminthopsis crassicaudata*; Dasyuridae). Changes in the basal plasma membrane, particularly molecular changes, are potentially the most interesting and informative as they are directly involved in the cellular dynamics of implantation. In the rat, implantation involves sloughing of regions of the uterine lining to facilitate invasion of the embryo into the underlying maternal blood vessels (Enders and Schlafke 1967). Sloughing requires disassembly of protein complexes (focal adhesions) that anchor the uterine lining to the underlying uterine tissue. This process involves loss of focal adhesion molecules, including talin and paxillin, from the basal region of cells before the embryo can implant (Kaneko et al., 2008; 2009).

Highly invasive implantation and sloughing are rare among viviparous species. Most eutherian embryos breach only the uterine lining, and not the maternal blood vessels, while in most live-bearing squamate reptiles, the embryo adheres to the uterine lining and does not invade (Wu et al., 2011). It is therefore unlikely that preparation for implantation would involve the same basal changes as the rat in all viviparous groups. Instead, a specific set of basal changes may be required for different modes of implantation. As implantation in *S. crassicaudata* is less invasive than that of the rat and no sloughing occurs (Roberts and Breed, 1994), this species is ideal to test the

relationship between uterine changes and implantation mode.

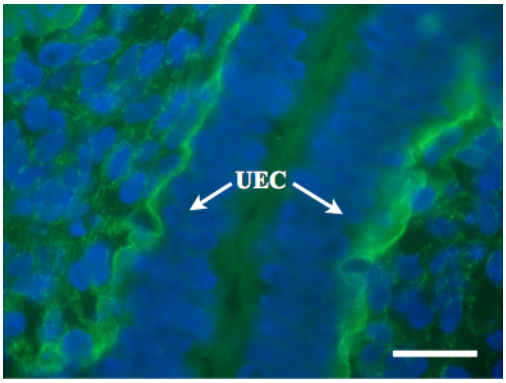

Figure 3: Immunofluorescence micrograph of talin localisation in uterine epithelial cells (UEC) during pregnancy in *Sminthopsis crassicaudata*. A prominent line of talin localisation (green) occurs at the base of both opposing rows of uterine epithelium pre-implantation.
Blue staining=cell nuclei; scale bar = 10μm.

We fluorescently labelled talin and paxillin and identified the patterns of localisation of these molecules in uterine epithelial cells throughout pregnancy in *S. crassicaudata*. We then compared these patterns to those that occur during rat pregnancy. Interestingly, we found that both of these key basal molecules are also involved in pregnancy in *S. crassicaudata*, but the patterns of localisation differ to those of the rat. While talin and paxillin are lost from the uterine lining in the rat before implantation, in preparation for sloughing, these molecules are most tightly localised to the base of the uterine lining during this period in *S. crassicaudata* (Figure 3). This localisation pattern indicates that connections between the uterine lining and underlying tissue are strongest just before the embryo implants.

Different patterns of localisation in uterine cells indicate that these molecules play different roles in marsupial pregnancy compared with rats. As these two species have different modes of implantation, different molecular patterns imply that not all uterine changes in early pregnancy are common, as basal changes differ with implantation mode. Importantly, different molecular patterns in these two species also highlight a fundamental difference in the response of the uterus to the embryo. While loss of the basal molecules talin and paxillin facilitates invasion in the rat (Kaneko et al., 2008, 2009), recruitment of these molecules to the basal plasma membrane of cells lining the uterus appears to strengthen the underlying connections of the uterine lining in *S. crassicaudata*.

Conflict *in utero*

Different uterine responses to the embryo in both the rat and *S. crassicaudata* may be explained in terms of conflict between the mother and the embryo. All vertebrate embryos can manipulate maternal reproductive physiology by releasing hormones and signalling molecules (Crespi and Semeniuk, 2004; Haig, 1993). As the plasma membrane transformation enables intimate contact between embryonic and maternal membranes, embryos can manipulate maternal physiology to a greater extent, and thereby maximise their share of resources (Crespi and Semeniuk, 2004). Manipulation is potentially greatest in species in which the embryo invades the maternal vasculature, including rats and humans, as contact is most intimate (Wooding and Flint, 1994).

Recent evidence suggests that less invasive types of implantation may evolve secondarily from highly invasive implantation in therian mammals through the accumulation of maternal defences to the embryo (Carter, 2008; Crespi and

Semeniuk, 2004). Conflict in utero creates an 'arms race' which results in the evolution of counter strategies on the part of both the mother and embryo to gain control over resource allocation.

The molecular changes in the uterus of *S. crassicaudata*, which has less invasive implantation, support this theory of placental evolution. Reinforcement of the uterine lining, the first barrier to embryonic attachment, just before implantation of the embryo is likely to be an example of a maternal strategy to regulate invasion by the embryo in this species. Hence, this study suggests that conflict occurs *in utero* during marsupial pregnancy, despite the relatively short gestation length in and brief direct contact between the uterus and the embryo.

Identifying additional maternal defences involved in pregnancy in *S. crassicaudata*, and other marsupial species, will allow us to determine the extent to which conflict occurs in the marsupial uterus. Such maternal defences may involve other key molecules which facilitate basal modification of the uterine lining, including members of the integrin molecular family (Kaneko et al., 2011).

In particular, comparison with marsupial species with non-invasive implantation, such as wallabies and kangaroos, will be most informative as maternal defences to the embryo are likely to play a greater role in pregnancy in these species.

Conclusion

While shared morphological changes to the surface of the uterus facilitate attachment of the embryo in both marsupials and placental mammals, this study demonstrates that the molecular responses of the uterus to embryonic invasion differ between mammalian species. The complex response of the marsupial uterus to invasion challenges the assumption that marsupial pregnancy is 'primitive' relative to the placental condition (e.g. Lillegraven, 1975), and highlights the need for these 'alternative' mammals (Renfree, 2010) to be included as a distinct group alongside placental mammals in comparative studies, as both are critical to understanding the evolution of mammalian live birth.

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