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PARACELLULAR AND TRANSCELLULAR TRANSPORT ACROSS THE SQUAMATE UTERINE EPITHELIUM

JOANNA M. BIAZIK[1,2,3], MICHAEL B. THOMPSON[2], CHRISTOPHER R. MURPHY[1]

[1]*School of Medical Sciences (Anatomy and Histology) and Bosch Institute. The University of Sydney, New South Wales 2006, Australia*
[2]*School of Biological Sciences, The University of Sydney, New South Wales 2006, Australia.*
[3]*e-mail: jbiazik@anatomy.usyd.edu.au*

***Abstract*.—We have previously investigated the paracellular pathway by confirming the presence of the integral tight junctional proteins occludin and claudin-5. In this present study we used enzyme histochemistry of alkaline phosphatase (AP) activity to understand the mechanisms associated with active transcellular movement of ions and solutes across the plasma membrane. Alkaline phosphatase activity was identified in the apical region of the plasma membrane of uterine epithelial cells in the skinks, and we propose that this activity in the omphaloplacenta may move molecules across the plasma membrane of the skink uterine epithelium as a component of histotrophic transfer.**

Key Words.—paracellular pathways, Squamata, transcellular pathways, uterine epithelium

INTRODUCTION

Viviparity (live birth), occurs in many animal groups and is common in the Order Squamata (lizards and snakes) and is particularly common in the Scincidae family (Blackburn 2006). Skinks are an ideal animal model for studying the evolution of viviparity because some species exhibit oviparity (egg laying), while others are viviparous with placentae ranging from simple chorioallantoic placenta (Weekes 1935) to complex placentae (Thompson et al. 2002). The evolution of viviparity involves the maternal transfer of water, oxygen and in some cases nutrients (Guillette 1993) across six distinct layers that make up the epitheliochorial placenta present in all squamate reptiles (Mossman 1974; Luckett 1977). Understanding the role of the uterine epithelium is crucial because in the case of epitheliochorial placentation, the epithelium takes on a very important role allowing active placental transport to occur. This placental transport transforms the uterus to a nourishing chamber in which the developing embryo relies on matrotrophy (Blackburn 1993; Stewart and Thompson 1993). In squamate reptiles with complex placentae, the chorioallantoic membranes become regionalized and membranes of the chorioallantoic region show evidence of gas exchange (Weekes 1935; Luckett 1977). In contrast, membranes associated with the abembryonic omphaloplacenta exhibit features of nutrient transfer via histotrophy (Corso et al. 2000; Flemming and Branch 2001). Recent studies have also isolated the mechanisms associated with the paracellular pathway in the uterine epithelium of skinks (Biazik et al. 2007; Biazik et al. 2008) and new work on enzyme histochemistry using AP activity is increasing our understanding of the transcellular pathway and how molecules, glucose, and lipids traverse the uterine epithelium.

Molecules must pass through cells (transcellular pathway) or go between cells (paracellular pathway; Citi and Cordenonsi 1998; Anderson 2001) when crossing epithelia. The tight junction (TJ), the most apical part of the junctional complex, provides a barrier that is an essential feature of epithelial and endothelial cells for the regulation of passive diffusion of water and solutes (Anderson and Van Itallie 1995; Nusrat et al. 2000; Van Itallie and Anderson 2004). Occludin (~ 60 kDa) was the first integral protein to be isolated in chicken liver (Furuse et al. 1993) and is exclusively associated with tight junctional strands (Saitou et al. 1998). For this reason, occludin has been widely used in studies to determine permeability of the paracellular pathway by correlating occludin expression with the number of tight junctional strands present (Furuse et al. 1993). Work carried out in recent studies on the uterine epithelium in skinks found occludin in one of the lineages studied, the *Eugongylus* group of skinks and occludin expression increased with increasing gestation (Biazik et al. 2007).

Both the number of strands and molecular composition of strands determine the level of permeability of the TJ barrier (Saitou et al. 1998; Kojima et al. 2002). In addition to occludin, claudins are 22 kDa TJ proteins that are cell- and tissue-specific and are associated with the formation of ion selective pores (Furuse et al. 1998; Tsukita and Furuse 2000; Heiskala et al. 2001). Claudin-5 is of interest as it is a key protein associated with endothelial tight junctions and plays and important role in the blood brain barrier (Morita et al. 1999). Claudin-5 was detected in the uterine epithelium of all skinks studied and there was a redistribution of claudin-5 from the cytosol and basolateral membranes in the non-pregnant/gravid uterus, to the tight junction located on the apical

region of the lateral plasma membrane in late stage pregnant/gravid skinks (Biazik et al. 2008).

The occludin and claudin-5 studies indicate evolutionary similarities between reptiles and some mammalian species which also exhibit an increased paracellular barrier at the time of implantation (Orchard and Murphy 2002). Because the paracellular pathway is slowly being understood, more clarification is needed in association with the transcellular pathway. For this study, histochemistry using AP activity was used to assess whether active transfer of molecules was occurring transcellularly (Danielli 1954).

Alkaline phosphatase is a member of a growing family of membrane-bound proteins anchored to the outer leaflet of the lipid bilayer via a glycosyl-phosphatidylinositol moiety (Low 1989). Alkaline phosphatase activity has been widely implicated in transport phenomena including cellular secretory and absorptive functions across cell membranes in a wide variety of tissues including small intestines and kidney (Harris 1989) where active transport occurs (Fernley 1971). Close correlation between AP activity and secretion of molecules such as glucose (Danielli 1954), fatty acids and triglycerides (Mahmood et al. 1994) has also been found. The purpose of this study is to test the hypothesis that active transcellular transport occurs across the uterine epithelium in squamate reptiles by evaluating the distribution of AP activity in different species and at different stages of the reproductive cycle.

MATERIALS AND METHODS

Species and embryonic stages.—We chose oviparous and viviparous species of Australian skinks from two lineages, *Eugongylus* and *Sphenomorphus* groups (Greer 1989) for this investigation to avoid confounding effects attributable to evolutionary history rather than the evolution of viviparity. we included a viviparous skink with a simple reptilian placenta from the *Sphenomorphus* lineage *Eulamprus tympanum* as well as the bimodally reproductive *Saiphos equalis* (Smith and Shine 1997). Within the *Eugongylus* lineage, we chose *Pseudemoia entrecasteauxii* and *P. spenceri* because they have the most complex reptilian placentae to be described in Australia (Weekes 1935; Stewart and Thompson 1998). *Lampropholis guichenoti* was chosen for this investigation because it is oviparous, and *Lerista bougainvillii* has been included as it is bimodally reproductive (contains both oviparous and viviparous populations) which allows for changes in the transition from egg laying to live bearing to be documented within one species. We allocated five individuals from each species to different reproductive stages using the Dufaure and Hubert (1961) staging method. In oviparous skinks, we chose two reproductive stages (mid–embryonic stages 25–27 and late-stage 30, which coincides with oviposition). In viviparous species, we recognized three reproductive stages (early-embryonic stages 20–25, mid–embryonic stages ranged from stage 30–37 and late–stages 38–40). We also examined five vitellogenic and post-parturient (2 weeks) skinks from each species.

Uterus excision and enzyme histochemistry.—At the appropriate reproductive stage, we euthanized skinks and a central incision was made to expose the uterus. We isolated incubation chambers of the uterus containing the embryos and uterine tissue was peeled away. We coated tissue for enzyme histochemistry in Tissue–Tek OCT cryoprotectant (Merck, Victoria, Australia), immersed in super-cooled isopentane (Unilab, New South Wales, Australia), and we stored it in liquid nitrogen. We collected 16 sections (7 μm thick) on gelatin coated slides, four replica slides per animal. Incubation substrate for alkaline phosphatase localisation was carried out using a modified Gomori (1952) method consisting of 2.97% sodium β-glycerophosphate, 5 ml; 0.284% lead nitrate, 20 ml; 0.05 M barbitone buffer, 20 ml. We filtered this solution and allowed it to stand at 35°C for 15 min prior to incubation of tissue. We incubated slides in substrate at 35°C for 1 hr. We washed slides in 0.05 M barbitone buffer and transferred them into 2% cobalt chloride for 5 min; 4% formaldehyde for 5 min; and then transferred them to 1% ammonium sulphide for 2 min to form a brown/black precipitate visible with the light microscope. Control slides were carried out by omitting the incubation substrate. Each successive step was alternated by a wash in 0.05 M barbitone buffer.

RESULTS

Results from previous studies are incorporated to show occludin (Biazik et al. 2007) and claudin-5 (Biazik et al. 2008) expression. Occludin was most abundant in skinks at mid-stage and late-stage pregnancy/gravidity in the uterus of species from the *Eugongylus* lineage which included *P. entrecasteauxii* (Fig. 1A), *P. spenceri* (Fig. 1B) and *L. guichenoti*. Claudin-5 expression was greatly reduced and migrated to the apical region of the lateral plasma membrane, corresponding to the ultrastructural location of the TJ. Uterus of *Pseudemoia spenceri* (Fig. 1C) and *L. guichenoti* (Fig. 1D) showing apical claudin-5 expression is also included.

The most intense AP activity was detected in the uterine epithelium of the two viviparous species, *P. entrecasteauxii* and *P spenceri,* in late stage gestation. The typical dark precipitate was found along the apical plasma membrane and was most prominent in the omphaloplacental region (Fig. 1E). No AP activity was detected in any other region of the uterus. In non-pregnant/gravid skinks and oviparous species, the AP activity was restricted to glandular epithelium. Control sections where AP incubating substrate was

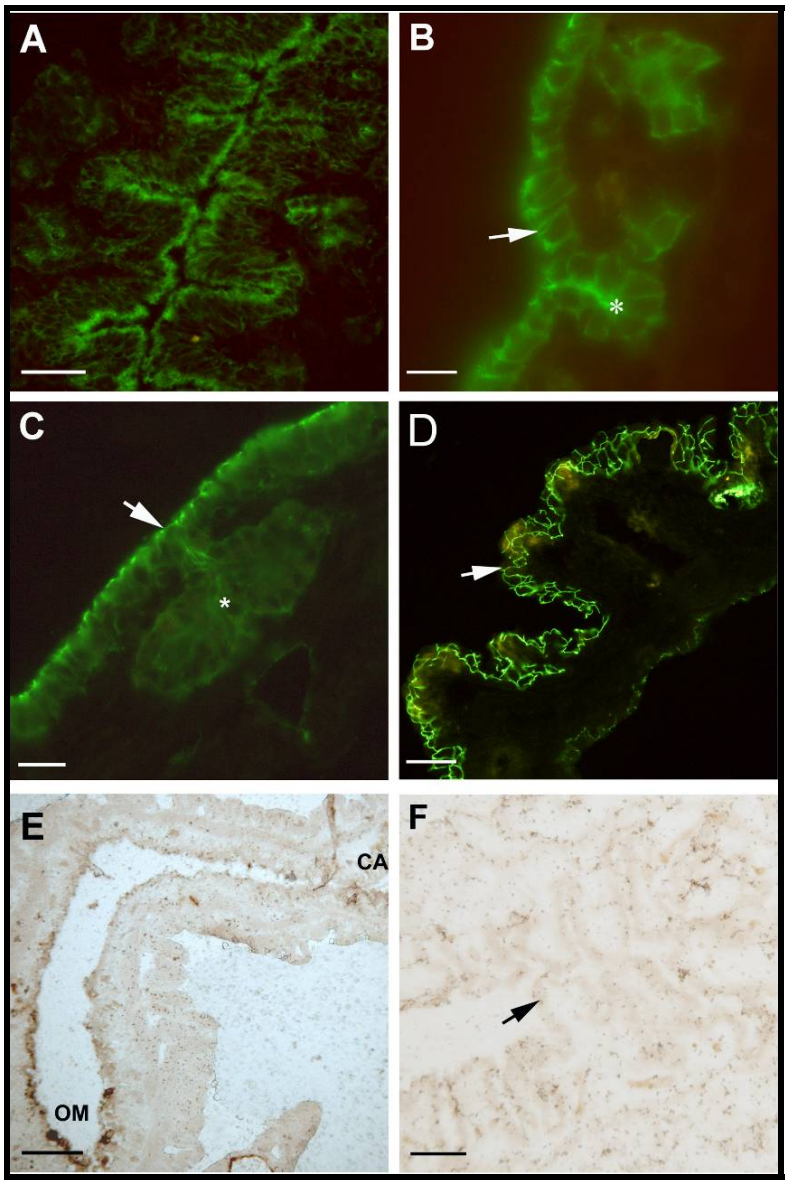

FIGURE 1. A) Low power occludin immunofluorescence micrograph of *Pseudemoia entrecasteauxii* uterus in mid stage pregnancy showing tissue convolution after embryo removal. Occludin staining present predominantly along the apical region of the lateral plasma membrane. Scale bar = 30µm. B) High power occludin immunofluorescence micrograph of *P. spenceri* with occludin located in the apical region of the lateral plasma membrane (arrow) and presence of glands (⋆). Scale bar = 10µm. C) High power claudin-5 immunofluorescence micrograph of *P. spenceri* in late stage pregnancy showing apical claudin-5 distribution along the lateral plasma membrane (arrow) and presence of glands (⋆). Scale bar = 10µm. D) Low power claudin-5 immunofluorescence micrograph of *Lampropholis guichenoti* with fully calcified egg showing claudin-5 distribution along the apical region of the lateral plasma membrane (arrow). Scale bar = 10µm. E) Low power enzyme histochemistry micrograph of late stage pregnant *P. entrecasteauxii*, showing AP activity in the omphaloplacental (OM) region of the uterus. Scale bar = 30µm. F) High power micrograph for AP control showing no AP activity along the apical region of uterine epithelial cells (arrow) in *P. entrecasteauxii*. Scale bar = 20µm.

omitted showed no alkaline phosphatase activity (Fig. 1F).

DISCUSSION

The data indicates that AP activity is present in the glandular epithelium of non-pregnant/gravid skinks and oviparous skinks. The detection of AP activity in this region suggests that glands in the uterus of skinks are continually involved in active secretion or absorption irrespective of whether or not skinks are reproductive or non-reproductive. Alkaline phosphatase may participate in intracellular processes such as cellular differentiation and growth (Matsumoto et al. 1990), which might explain its presence in glands of non reproductive animals. In the uterus of

viviparous skinks, especially in late stage pregnant animals, AP activity occurs on the apical plasma membrane of uterine epithelial cells, however it seems to be regionalised to the omphaloplacenta. The omphaloplacenta is the abembryonic region of the skink uterus associated with nutrient transport via histotrophy (Blackburn 1993; Stewart and Thompson 1998; Thompson et al. 1999; Corso et al. 2000; Adams et al. 2005).

There is strong correlation between AP activity and sites of secretion, particularly with regard to molecules such as glucose (Danielli 1954) and active transport of lipid across the plasma membrane (DeSchryver et al. 1991). In the present study, a high concentration of AP activity in the omphaloplacenta suggests that comparable processes associated with lipid or glucose transport may be occurring across the apical plasma membrane in viviparous skinks.

In summary, this is the first study describing possible avenues of glucose and lipid transport via the transcellular pathway in the uterus of squamates. Recent studies on occludin and claudin-5 indicate that in early stages of pregnancy/gravidity, tight junctions are not tightly regulated so ions and solutes can pass freely through the paracellular space (Anderson and Van Itallie 1995; Balda and Matter 1998). With increasing length of gestation, however, the tight junction is established and forms a barrier impermeable to most ions and solutes. The redistribution of AP activity from the glandular epithelium to the apical plasma membrane, primarily in the omphaloplacental region, suggests active transcellular transport is a component of histotrophy or an independent mechanism associated with active transcellular transport of metabolites other than those transported via histotrophy. Histotrophy and AP activity are both increased in late stage gestation, during the same time as the paracellular pathway becomes more regulated and restricted. This suggests that different ions and metabolites traverse the uterine epithelium at different times and this sequential order may be important in the growth rate of the embryo.

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JOANNA BIAZIK completed her honors and Ph.D. degree in the Cell and Reproductive Biology Laboratory while collaborating with the School of Biological Sciences to investigate the evolution of viviparity (live-bearing) in Australian reptiles at The University of Sydney. Combining histology and molecular biology with different imaging techniques have always been a main focus of Joanna's research and resulted in the discovery of various components of placental structure and nutrient provision in Australian viviparous skinks. Currently Joanna is working at the Australian Centre for Microscopy and Microanalysis where she is involved in comparative hepatological studies of the lizard and rat liver. Using immunogold labeling and 3-D electron tomography, she is investigating the differences in the liver architecture as well as differences in protein distribution between the two species that results in variation in the rate of sinusoidal transport. (Photographed by Uli Eichhorn)

MICHAEL B. THOMPSON is Professor in Zoology at the University of Sydney. After completing his PhD on the nesting ecology and egg physiology of freshwater turtles at the University of Adelaide in the early 1980s, he moved to New Zealand to work on nesting and eggs of the tuatara. He then was awarded the Archie Carr Postdoctoral Fellowship at the University of Florida where he continued to work of eggs, particularly those of turtles and alligators. He took an academic position at the University of Sydney in 1989 where he continued to work of reptilian eggs, but also began to work on the evolution of viviparity in lizards. He is still at Sydney and is still working on reproduction in reptiles. (Photographed by Kelly Hare in the Amazon in 2008).

CHRIS MURPHY is Bosch Professor of Histology and Embryology and Professor of Female Reproductive Biology at The University of Sydney. After undergraduate study at The University of Adelaide in politics and zoology, Chris obtained his PhD from Flinders University in South Australia in cell biology and histology and more recently he was awarded a DSc by the University of Sydney. His research interests are in the biology of the uterus and in particular how its epithelial cells alter their structure and function to become receptive to the implanting blastocyst. A large part of this interest in uterine biology involves understanding how this organ evolved from a relatively passive egg holder into the active, nourishing, chamber seen in mammals and squamate lizards in particular.
Like most Australian academics, Chris is employed to teach as well as to research and earns his keep teaching histology, cell biology and some embryology to students of science, medical science, medicine and dentistry.