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Immunology of the gray short-tailed opossum during pregnancy and prenatal development

Victoria L. Hansen
University of New Mexico

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Victoria L. Hansen

Candidate

Biology

Department

This dissertation is approved, and it is acceptable in quality and form for publication:

Approved by the Dissertation Committee:

Robert Miller , Chairperson

Paul Samollow

Stephen Stricker

Christina Vesbach

**IMMUNOLOGY OF THE GRAY SHORT-TAILED
OPOSSUM DURING PREGNANCY AND
PRENATAL DEVELOPMENT**

BY

VICTORIA L. HANSEN

B.A., Biology, University of Puget Sound, 2007

DISSERTATION

Submitted in Partial Fulfillment of the
Requirements for the Degree of

**Doctor of Philosophy
Biology**

The University of New Mexico
Albuquerque, New Mexico

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DEDICATION

This dissertation is dedicated to my parents, Jeanne and Walt, who are unconditionally supportive and loving. Thank you, Mommy and Daddy.

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**IMMUNOLOGY OF THE GRAY SHORT-TAILED OPOSSUM *MONODELPHIS*
DOMESTICA DURING PREGNANCY AND PRENATAL DEVELOPMENT**

by

Victoria L. Hansen

B.S., Biology, University of Puget Sound, 2007

PhD, Biology, University of New Mexico, 2017

ABSTRACT

Vertebrate species that bear live young have overcome the evolutionary challenge of maintaining both a functional adaptive immune system and viviparous life cycle. In normal pregnancy, viviparous mothers are able to sustain and nourish a fetus that is genetically half non-self without mounting an immunological attack. In 1953 Sir Peter Medawar brought attention to the “enigma of the fetal graft” and immunologists have been puzzling out the intricacies of fetal tolerance ever since. Although viviparity has evolved in all jawed vertebrate lineages aside from Aves, the vast majority of reproductive immunology research has been limited to eutherian mammals. There are insights to be gained by examining the reproductive immunology of other viviparous lineages. Marsupials, which share a common viviparous ancestor with eutherians, could be key in highlighting the most ancestral of mammalian characteristics.

The gray short-tailed opossum, *Monodelphis domestica*, is a model marsupial species with a sequenced and well-annotated genome. Transcriptomes of pregnant and non-pregnant *M. domestica* uterine tissues were assessed by Next Generation RNA-Seq techniques. Global gene transcription profiles were generated for opossum uterine tissue from pregnant and control animals. Using multiple differential expression algorithms thousands of genes were determined to be differentially transcribed. Notable was the contribution of immune related genes to those most differentially abundant among the transcripts. Of particular interest were genes coding for pro-inflammatory cytokines and complement components, both known to be regulated in eutherian pregnancy. To extend the RNA-Seq results further, quantitative real-time PCR was used to assess transcript abundance of a subset of cytokines and complement components. The results revealed that the spike of increased inflammation is associated with parturition in the opossum consistent with its ancient role in birth in mammals. In addition, complement components were suppressed in the data set throughout pregnancy, consistent with regulation of this immune mechanism being critical for sustaining normal pregnancy in marsupials as it is in eutherians. Overall, the results presented support active regulation of some immune mechanisms during marsupial pregnancy and provide a basis for understanding the evolution of parturition mechanisms.

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CHAPTER 1

INTRODUCTION

By Victoria L. Hansen

Center for Evolutionary & Theoretical Immunology, Department of Biology, University
of New Mexico, Albuquerque, New Mexico, 87131, USA

INTRODUCTION TO THIS DISSERTATION

The vertebrate immune system evolved to be able to detect and respond with exquisite specificity to foreign invading pathogens as well as abnormal self-tissues and cells (*e.g.* tumors). With the advent of organ transplantation in the 20th century came the realization that our immune system could detect allogeneic differences even among humans and reject the grafted tissue (Moore et al., 1960). Organ transplantation however is an artifact of modern medicine raising the question as to whether our immune system evolved to cope with allogeneic transplants. That is, transplants between non-identical members of the same species. This question has intrigued immunologists for decades and drove research on allo-recognition in species as diverse as humans, colonial tunicates, and cnidarians for insights into the origin of the vertebrate immune system (Scofield et al., 1982; Fuchs et al., 2002).

Although the natural fusion of adult tissues that occurs in some marine invertebrates does not have equivalence in vertebrates, there are examples where the possibility of allo-recognition and the need for tolerance exist in species as distant as sharks and humans. The evolution of viviparity, the transition from egg-laying to live-birth, in vertebrates is an example of the immune system having to co-evolve with changing reproductive strategies. The potential exposure to allogeneic antigens in the form of an invasive placenta is an evolutionary challenge (Medawar, 1953).

This dissertation describes research exploring this co-evolution in species that are humans' most distant viviparous mammalian relatives, the marsupials. Chapters herein describe immune ontogeny in developing marsupials, changes in gene expression during pregnancy, conserved immune regulation associated with mammalian pregnancy, and

expose the role the immune system played in the evolution of parturition. This introductory chapter is meant to provide background and perspective to the chapters that follow.

THE EVOLUTION OF LIVE BIRTH IN JAWED VERTEBRATES

The transition from egg-laying to live-bearing has evolved independently many times in the vertebrate lineages (Blackburn, 2015). By the mid-twentieth century this adaptation to viviparity was a question perplexing to immunologists. All jawed vertebrates have an adaptive immune system that is able to distinguish between self and non-self (or altered-self) cells with extreme precision (Litman et al., 2010). Yet, when viviparous animals mate and recombine the genes of two parents, the mother typically tolerates that genetically non-self tissue until parturition. In a now-famous lecture by the Nobelaureate and immunologist, Sir Peter Medawar brought attention to this apparent enigma in a lecture to the Society for Experimental Biology by asking, “how does the pregnant mother contrive to nourish...a foetus that is an antigenically foreign body?” (Medawar, 1953; Billington, 2003). In the past sixty years significant progress has been made with regard to understanding how the adaptive immune system and viviparity coexist, primarily in mammals (Erlebacher, 2012; Erlebacher, 2013; Racicot et al., 2014). Such studies have revealed that many mechanisms can help to prevent rejection of the fetus during normal pregnancy. What is not well understood is which particular mechanisms are the most important and most conserved.

MAMMALIAN RELATIONSHIPS

Extant mammals fall into three distinct lineages: the prototherians, metatherians, and eutherians (Figure 1). The egg-laying prototherians, or monotremes, last shared a common ancestor with viviparous mammals 186 million years ago (MYA) and include only echidna species and the duck-billed platypus (Bininda-Emonds et al., 2007). Monotremes are true mammals and use lactation to nourish their newborn young (Griffiths, 1968; Hopson, 1973). Together the metatherians and eutherians are known as the Theria and share a common ancestor approximately 166 MYA that was the origin of viviparity in mammals (Baker et al., 2004; Bininda-Emonds et al., 2007; Killian et al., 2001). Eutherians, sometimes called “placental” mammals, bear live offspring and include the most well-studied vertebrate species in biology: humans and mice. Metatherians, or marsupials, are also viviparous, but tend to have shorter gestation periods and more altricial offspring compared to eutherians (Tyndale-Biscoe & Renfree, 1987). Marsupials invest less in offspring during gestation than they do during their complex lactation program, which spans the time from birth and being permanently affixed to a teat to intermittent suckling when offspring are outside of the pouch or nest (Tyndale-Biscoe & Renfree, 1987). While there has been speculation that marsupials simply never evolved the ability to gestate their young long-term as eutherians do, there are trade-offs for investing less in prenatal than postnatal development.

EVOLUTION OF VIVIPARITY IN MAMMALS

The evolution of live birth offered therian mammals both advantages and disadvantages. Viviparity allows for extended prenatal development sheltered from

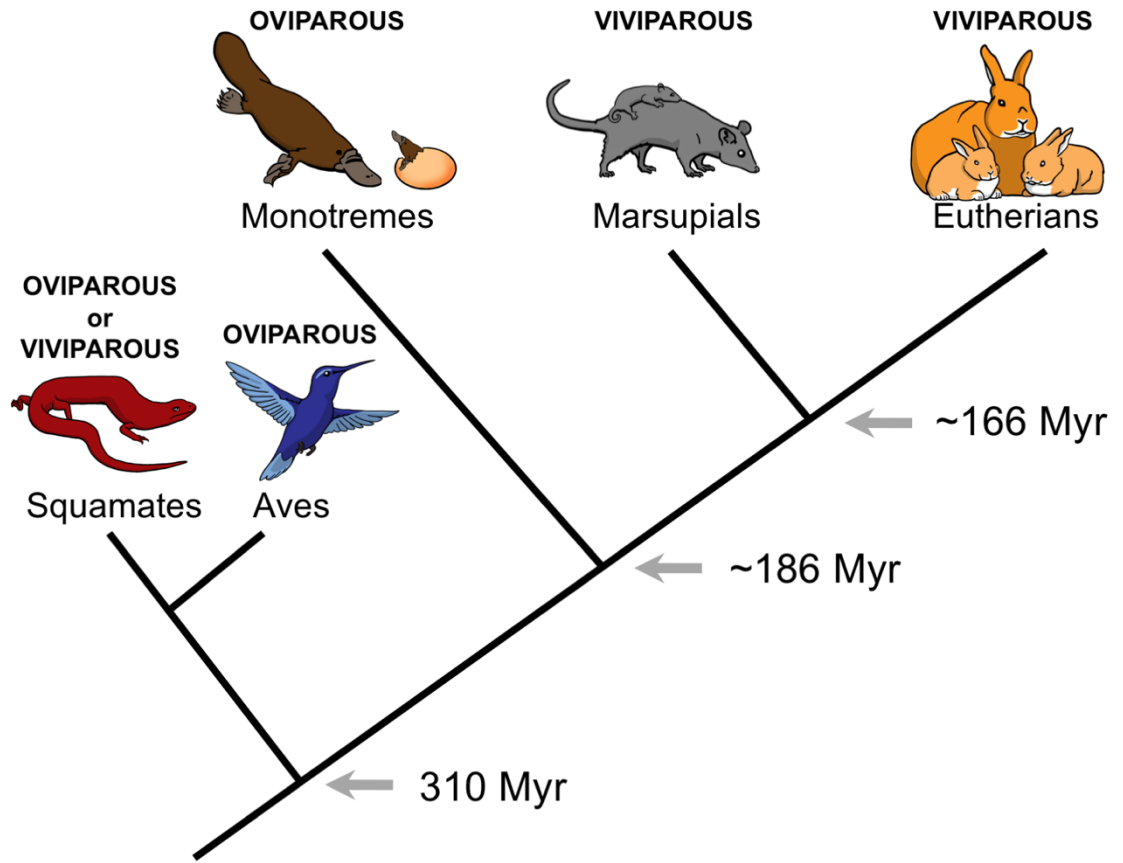


Figure 1. Diagram describing phylogeny of oviparous and viviparous mammals.

predators and other outside environmental hazards. Some eutherians give birth to extremely precocial offspring. Giraffe neonates are able to walk within hours of being born. Humans give birth to neonates that are physically altricial but nutrients and prenatal development are instead invested in a large and complex brain (Dunsworth et al., 2012). However there are costs to extended gestation. In eutherians with well-developed large neonates the mother may be burdened with additional weight and that causes her to be more prone to predation herself.

Marsupials mitigate some of the disadvantages of viviparity by investing less in prenatal development. Marsupial neonates are born in a more fetal state compared to eutherians, and thus a substantial portion of early development occurs outside the womb (Russell, 1982; Tyndale-Biscoe & Renfree, 1987). A newborn marsupial has well-developed forelimbs and climbs to a teat, often within a pouch, without the mother's aid (Russell, 1982; Sharman, 1973). After reaching a teat, the neonate suckles continuously for an extended period. Some marsupial species have morphological specializations aside from a pouch, particularly in the teats and neonate mouth, to prevent early detachment (Merchant & Sharman, 1966; Russell, 1982). Much of early postnatal development in marsupials would occur in mid- or late prenatal gestation in eutherians (Tyndale-Biscoe & Renfree, 1987).

PLACENTATION IN MAMMALS

All therian mammals have a placenta to facilitate prenatal nourishment and protection of embryos. Mammalian placentation morphology is highly variable across lineages and even species (Mossman, 1987). Eutherian placental types are generally

classified by level of invasiveness into maternal tissues. Humans and mice have hemochorial placentas, the most invasive type, where fetal placental membranes not only invade into maternal tissues, but also penetrate maternal circulation such that fetal trophoblast is in direct contact with maternal blood (Moffett & Loke, 2006). Carnivores, such as cats and dogs, are an example of a lineage that has endotheliochorial placentas where the trophoblast invades into maternal membranes but does not contact circulation. Ungulates, such as cows, tend to have the least invasive epitheliochorial placentas where fetal and maternal membranes are merely apposed next to each other.

There are four extra-embryonic membranes that make up a mammalian conceptus along with the embryo itself (Telugu & Green, 2007). The amnion, or amniotic sac, is the innermost membrane that cushions the embryo in amniotic fluid. The yolk sac functions as nutrient storage for the embryo, and the allantois functions in waste storage and gas exchange. The chorion is the outermost fetal membrane that surrounds the whole conceptus and contacts maternal tissues. In eutherians most of nutrient, gas, and waste exchange between embryo and mother occurs through a chorioallantoic placenta. That is, a placenta that is composed of fused mesoderm layers from both the chorion and allantois.

Most eutherians use chorioallantoic placentation through the majority of gestation (Mossman, 1987). Eutherians also may or may not form a transient choriovitelline, or yolk sac, placenta in addition to the chorioallantoic one (Mossman, 1987; Telugu & Green, 2007). Marsupials, on the other hand, have a definitive choriovitelline placenta that persists throughout pregnancy without the allantois contacting the chorion (Freyer et al., 2003; Tyndale-Biscoe & Renfree, 1987; Zeller & Freyer, 2001). A few species of marsupials, such as bandicoots, do form a chorioallantoic placenta as well in the last 24 hours of

pregnancy (Hill, 1898; Padykula & Taylor, 1976). Marsupials have placentas that are either epitheliochorial-like, such as in the tammar wallaby, or endotheliochorial-like, such as in the gray short-tailed opossum (Freyer et al., 2007; Zeller & Freyer, 2001). Even in less invasive placenta types the fetal membranes are apposed to maternal membranes and therefore at risk of being recognized and rejected by the maternal immune system.

REPRODUCTIVE IMMUNOLOGY OF EUTHERIANS

Since the 1950's there has been extensive research into fetal tolerance mechanisms, but the vast majority of this research has been conducted in eutherian mammals. This has revealed a surprisingly high degree of diversity of placentation strategies and fetal protection mechanisms across eutherian groups (Bainbridge, 2000; Moffett & Loke, 2006). The immune system components described below are not the only ones known in eutherian reproductive immunology, but they are the specific ones that will be explored further in this dissertation.

Inflammation, which plays an important role in eutherian implantation and parturition, can lead to pre-term labor and fetal injury during other stages of pregnancy (Mor et al., 2011). Cytokines are essential cell signaling molecules in the immune system, many of which enhance inflammation. Pro-inflammatory cytokines attract and activate immune cells at the fetomaternal interface (Romero et al., 2007). Pro-inflammatory cytokines such as interleukin-1 (IL-1), interleukin-6 (IL-6), interleukin-8 (IL-8), and tumor necrosis factor α (TNF α) are highly expressed in placental and uterine tissues in normal term labor (Bowen et al., 2002; Keelan et al, 2003). These cytokines also have effects on non-immune cells at the fetomaternal interface leading to effects like rupture of fetal

membranes and cervical ripening in normal term labor (Christiaens et al., 2008; Sennstrom et al, 1997). Outside of implantation or parturition, inflammation at the fetomaternal interface is associated with complications like preeclampsia, preterm labor, and recurrent spontaneous abortions (Lockwood et al., 2008; Romero et al., 2006).

Another example of critical immune regulation during pregnancy is that of the complement system. Complement is an ancient component of the immune system with homologues found in species as distant from mammals as starfish and other echinoderms (Al-Sharif et al., 1998; Gross et al., 1999; Smith et al., 1998; Smith et al., 1999). Complement mediates a wide range of immune effects including cellular lysis via a membrane attack complex (MAC), enhanced phagocytosis, and stimulation of inflammation and cell chemotaxis (Sarma & Ward, 2011). Controlling the complement system at the feto-maternal interface appears to be critical to maintaining a successful pregnancy. Several complement regulators are expressed on human trophoblast cells (Holmes et al., 1992) and mouse embryos lacking the murine complement regulator Crry do not survive to parturition (Xu et al., 2000). However, since Crry^{-/-} embryo lethality does not appear to be dependent on the MAC, it has been hypothesized that it is inflammation caused by complement cleavage products that ultimately leads to embryonic death (Mao et al., 2003).

REPRODUCTIVE IMMUNOLOGY OF MARSUPIALS

Comparatively little attention has been paid to the fetal allograft problem in marsupials (van Oorschot & Cooper, 1988). Indeed, in 1974 Philip J. Moors speculated that perhaps marsupials never evolved fetal tolerance mechanisms and this is correlated

with the short duration of pre-natal development common to this mammalian lineage (Moors, 1974).

Successful marsupial pregnancy, despite intimate contact between maternal and fetal membranes, indicates a means of fetal tolerance in marsupials contrary to Moors' hypothesis. However, studies of marsupial immune systems thus far have revealed characteristics in conflict with Moors' hypothesis *per se*. Immunization of female tammar wallabies, *Macropus eugenii*, with male alloantigens did not reduce their fecundity (Rodger et al., 1985), nor could anti-paternal antibodies be detected in blood of wallabies who had a successful pregnancy (van Oorschot and Cooper, 1988). This is in contrast to normal human pregnancy where such antibodies are commonly found (Payne & Rolfs, 1958; van Rood et al., 1958). This may suggest that the marsupial maternal immune system is not "aware" or exposed to fetal alloantigens, or perhaps they are not sufficiently allogenic to induce a detectable response. Unfortunately these two studies represented the only attempts to experimentally determine if the marsupial immune system is "aware" of the fetal allograft.

Recent results from genomic analyses are consistent with marsupials lacking several known fetal protection mechanisms found in eutherians. Non-eutherian mammals, for example, lack the transcription enhancer CNS1 at the *Foxp3* gene, FoxP3 is a transcription factor essential to the development of regulatory T cells that inhibit immune responses. The CNS1 enhancer allows for peripheral regulatory T-cells (pTregs) to be generated during pregnancy in mice (Samstein et al., 2012). Depletion of pTregs in pregnant mice has been shown to increase fetal reabsorption rates (Samstein et al., 2012) and decreased levels of Tregs in human maternal circulation are associated with pre-

eclampsia (Santner-Nanan et al., 2009; Sasaki et al., 2007). Marsupials also lack a promoter to enable expression of decidual prolactin (dPRL), an important suppressor of inflammation during pregnancy in some eutherian mammals (Bao et al., 2007; Emera & Wagner, 2012). Though marsupials lack these evolutionary adaptations for viviparity, it is not evidence that they lack fetal any protection mechanisms, or the need for them.

A PRIMER ON MAMMALIAN COMPLEMENT

The complement (C') cascade is a system of proteins present in vertebrate serum that, left unchecked, will primarily puncture holes in cellular membranes and lead to apoptosis of both bacterial and animal cells (Sarma & Ward, 2011). C' proteins are activated and cleaved leading to downstream C' components to do the same. Some cleavage products act in the downstream cascade while others act as proinflammatory chemoattractants or activators of immune cells. Certain C' components can bind to and opsonize antigens as well. That is, complement can enhance the phagocytosis of antigens by immune cells. The end result of the complement cascade is the formation of a tunnel-like membrane attack complex (MAC) that inserts itself into a cellular membrane.

The complement cascade can be kicked off from three starting points. In the Classical Pathway, the first complement pathway to be discovered, the complement system is activated by immunoglobulins bound to an antigen (Figure 2). This pathway utilizes all the original complement components from C1 through C9. The Classical Pathway is the only one that is antibody-dependent and therefore has an adaptive immunity role. In the Lectin Pathway an innate immune defense molecule such as mannose binding lectin (MBL) binds to a bacterial cell surface. Then the activated MBL

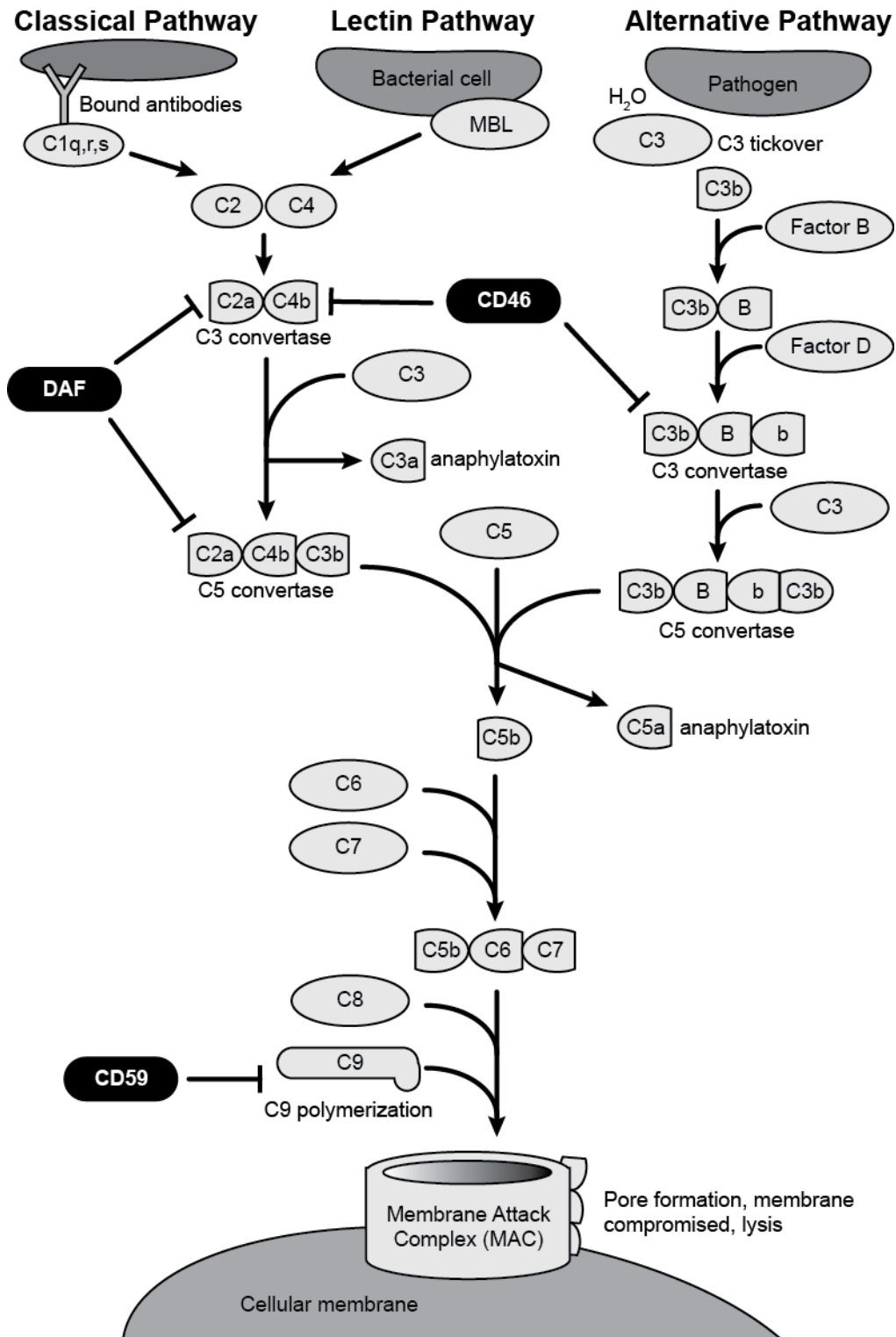


Figure 2. Diagram of the Classical, Lectin, and Alternative Pathways of the complement cascade. Major components of the complement system are light gray and complement regulators are black with white text.

complex cleaves C4 and the cascade proceeds from there in the same fashion as the Classical Pathway. The Alternative Pathway relies on the inherent instability of the C3 molecule. Simply in the presence of bacterial and other pathogen surfaces C3 has a tendency to spontaneously cleave and start the complement cascade with the help of a few additional serum proteins, Factor B and Factor D (Figure 2).

***MONODELPHIS DOMESTICA*: A MODEL MARSUPIAL**

The gray short-tailed opossum, *Monodelphis domestica*, is an excellent model organism in which to study marsupial pregnancy for several reasons. *M. domestica* is not a seasonal breeder; rather estrus is induced by exposure to a male (Fadem, 1985). Timing of gestation (Mate et al., 1994) and *M. domestica* placentation are well documented (Freyer et al., 2002; Freyer et al., 2007; Freyer & Zeller, 2001). The genome of *M. domestica* has been sequenced, assembled, and annotated as well (Mikkelsen et al., 2007), making it a good candidate for studies for next generation sequencing-based gene expression analyses.

The gestation of *M. domestica* is short – only 14.5 days in duration from copulation to parturition (Mate et al., 1994). See Figure 3 for a timeline of *M. domestica* gestation. After copulation, ovulation occurs and eggs are fertilized within 24 hours. As a fertilized oocyte is transported through the oviduct it acquires an uneven mucoid coat derived from oviduct epithelial cell secretions (Baggott & Moore, 1990; Zeller & Freyer, 2001). The zygote's proteinaceous shell coat is also maternally-derived and originates from uterine secretions (Zeller & Freyer, 2001). The *M. domestica* embryo has a long preimplantation period in the uterus where the shell coat remains intact for 80% of the gestation period.

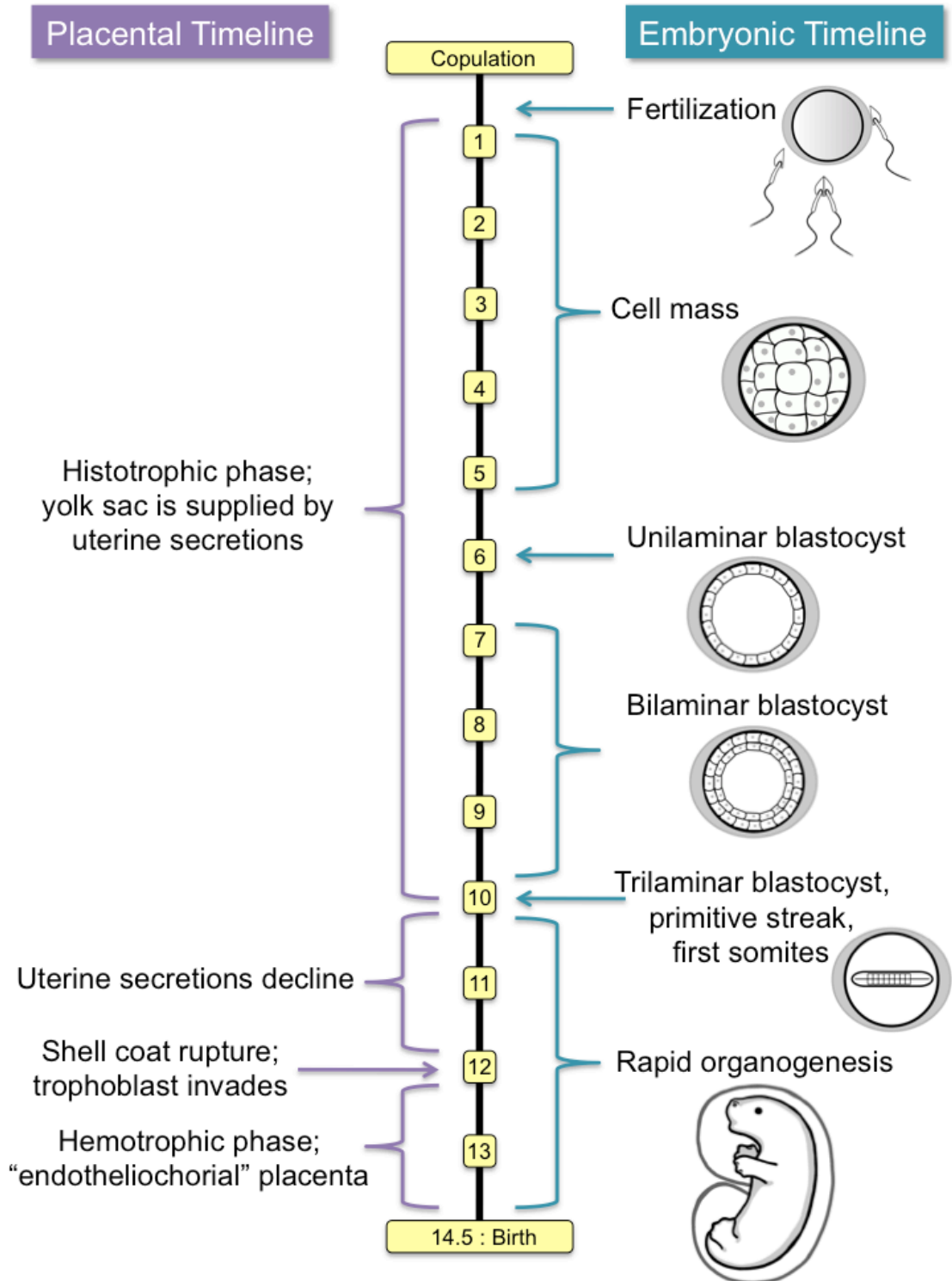


Figure 3. Timeline of placentation and organogenesis stages in *Monodelphis domestica*.

The maternally derived shell coat may mask the conceptus's alloantigens, thus shielding it from maternal immune detection. During this time the embryo is nourished by the yolk sac, which is supplied by uterine glandular secretions from the endometrium (Freyer et al., 2006). Uterine secretions decline by two-thirds between the couple days leading up to shell coat rupture on day 12 of gestation. Once the shell coat is no longer a barrier, the fetal membranes and uterine epithelium are truly in apposition to one another. The trophoblast of the vascular portion of the yolk sac infiltrates the endometrium with a bias toward uterine epithelium near maternal capillaries (Freyer et al., 2006). The trophoblast does not take over the maternal circulation; instead the maternal capillaries secrete hemotrophe into the stroma and the trophoblast takes up the nutrients until parturition (Freyer et al., 2006). After neonates are born they latch onto the mother's teats and remain permanently affixed for approximately two weeks and then nurse intermittently until weaning at 8 weeks old (VandeBerg, 1990).

The combination of convenient reproductive characteristics, and documentation of placentation, and genetic resources available for *M. domestica* make it an ideal marsupial in which to study reproductive immunology.

THE AIMS OF THIS DISSERTATION

The aims of this dissertation were centered on investigating potential immune mechanisms at work during marsupial pregnancy. First I characterized and compared the whole uterine transcriptomes of pregnant and non-pregnant opossums by next generation RNA sequencing. The differentially transcribed genes included immune system genes, particularly pro-inflammatory cytokines that were significantly more transcribed in

pregnant samples. However, as the samples were taken from animals less than 24 hours from giving birth, it was unclear as to whether higher transcription was a general condition during pregnancy or a reflection of impending parturition. Therefore, with a limited set of target genes, more time points in pregnancy were examined for differential gene transcription using quantitative polymerase chain reaction (qPCR).

Chapter 2:

Hansen, V. L., & Miller, R. D. (2017) On the prenatal initiation of T cell development in the opossum *Monodelphis domestica*. *Journal of Anatomy*. ***in press***.

This chapter has been accepted for publication in *Journal of Anatomy*. It is currently available in online early view from December 2016 and will likely be printed in the April 2017 issue. This manuscript presents evidence of prenatal initiation of T cell development in *M. domestica* embryos. All other marsupials examined thus far do not initiate T cell development until after birth. This was a serendipitous discovery while examining histological sections from pregnant uteruses for T cell localization.

Chapter 3:

Hansen, V. L., Schilkey, F., and Miller, R. D. 2016. Transcriptomic changes associated with pregnancy in a marsupial, the gray short-tailed opossum *Monodelphis domestica*. *PLoS ONE* 11(9): e0161608.

This chapter has been published. The manuscript presents global analyses of *M. domestica* uterine transcriptomes from both pregnant and non-pregnant individuals.

Chapter 4:

Hansen, V. L., Faber, L., Salehpoor, A., Miller, R. D. Expression of cytokines during marsupial pregnancy and the evolution of parturition.

This chapter is a manuscript in preparation and presents analyses of cytokine gene expression at the fetomaternal interface for additional pregnancy time points beyond the ones explored in Chapter 3. Here we show that high cytokine expression at parturition, similar to that seen in eutherians, is present in *M. domestica*. However, inflammation during implantation seems to be reduced or absent in *M. domestica*.

Chapter 5:

Hansen, V. L., Salehpoor, A., Faber, L., Miller, R. D. Regulation of complement during pregnancy in the opossum *Monodelphis domestica*.

This chapter is a manuscript in preparation and presents analyses of gene expression for complement components and their regulators at the fetomaternal interface during *M. domestica* pregnancy. Here we show that transcription levels of most complement component genes are reduced during all stages of pregnancy compared to non-pregnant controls. We also observed a different pattern of complement regulator gene transcription in opossum than in human pregnancy.

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CHAPTER 2

ON THE PRENATAL INITIATION OF T CELL DEVELOPMENT IN THE *OPOSSUM MONODELPHIS DOMESTICA*

By Victoria L. Hansen¹ and Robert D. Miller^{1,2}

¹Center for Evolutionary and Theoretical Immunology, Department of Biology,
University of New Mexico, Albuquerque, NM, 87131, USA

²National Science Foundation, 4200 Wilson Blvd, Arlington VA 22230, USA

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ABSTRACT

Thymus dependent lymphocytes (T cells) are a critical cell lineage in the adaptive immune system of all jawed vertebrates. In eutherian mammals the initiation of T cell development takes place prenatally and the offspring of many species are born relatively immuno-competent. Marsupials, in contrast, are born in a comparatively altricial state and with a less well developed immune system. As such, marsupials are valuable models for studying the peri- and postnatal initiation of immune system development in mammals. Previous results supported a lack of prenatal T cell development in a variety of marsupial species. In the gray short-tailed opossum, *Monodelphis domestica*, however, there was evidence that $\alpha\beta$ T cells were present on postnatal day 1 and likely initiated development prenatally. Demonstrated here is the presence of CD3 ϵ^+ lymphocytes in late stage embryos at a site in the upper thoracic cavity, the site of an early developing thymus. CD3 ϵ^+ cells were evident as early as 48 hours prior to parturition. In day 14 embryos, where there is clear organogenesis, CD3 ϵ^+ cells were only found at the site of the early thymus, consistent with no extra-thymic sites of T cell development in the opossum. These observations are the first evidence of prenatal T cell lineage commitment in any marsupial.

Key words: marsupial; thymus; embryonic development; T cell.

INTRODUCTION

Marsupials are noted for their relatively short gestation times and being born in a highly altricial state. Much of marsupial postnatal development resembles that of fetal eutherian stages. This extended postnatal development has made marsupials useful models of organogenesis and systems ontogeny (reviewed in Borthwick et al. 2014).

Hubbard and colleagues (1991) demonstrated the presence of cell clusters resembling an early stage thymus in 1 day postnatal *M. domestica* neonates. Clear cortical and medullary regions were not evident, however, until the end of the first postnatal week. Previous analyses of T cell receptor transcription in the gray short-tailed opossum, *Monodelphis domestica*, clarified many aspects of the postnatal progression of T cell development (Parra et al. 2009). Parra and colleagues (2009) reported that transcripts that could encode fully functional T cell receptor (TCR) α and β chains were present in the first 24 postnatal hours. These studies were performed using RNA extracted from the upper thoracic cavity, as a macroscopic thymus was not yet visible at this early stage. Unlike eutherian mammals, opossum $\alpha\beta$ T cell development precedes that of $\gamma\delta$ T cells (Parra et al. 2009). The presence of functional α and β TCR transcripts at birth in *M. domestica* neonates is consistent with progression of T cell development prenatally. To reach this stage by birth, T cells would have progressed from the pre-T cell stage through to having fully rearranged their TCR β and TCR α chain genes. What was not determined was the site of this T cell development in prenatal opossums, although the likely candidate is the early immature thymus. Here we demonstrate the presence of CD3 ϵ ⁺ cells in *M. domestica* as early as at least 48 hours prior to birth and during the last 24 hours clearly located in the early developing thymus.

METHODS

Tissue collection and fixation

All opossums originated from a captive-bred research colony housed at the University of New Mexico, Albuquerque, New Mexico. Animals were bred and used under protocols approved by the Institutional Animal Care and Use Committee, protocol numbers 13-100920-MCC and 15-200334-B-MC. Embryos, neonates, and spleen tissue were collected from adult pregnant or nursing female opossums euthanized by isoflurane overdose. Pregnant uteruses were excised, opened laterally, and embryos were removed. All tissues were fixed in buffered formalin solution (10%, Sigma-Aldrich) overnight and then stored at 4°C in 70% ethanol solution until paraffin-embedding. Prior to embedding, tissues were dehydrated in a series of ethanol washes of increasing concentration and then submerged in chloroform overnight. Then tissues were embedded in paraffin wax blocks after being submerged in wax and incubated at 65°C under vacuum. Paraffin-embedded tissues were sliced 6µm thick and mounted on charged glass slides.

Immunohistochemistry

Slides with mounted formalin-fixed paraffin-embedded (FFPE) tissues were deparaffinized and rehydrated in xylene and ethanol solution washes of decreasing concentration (100%-70%) and then put into 1x PBS. Antigen retrieval was performed by microwaving slides submerged in 10mM sodium citrate solution, pH 6 for 20 min. Tissue sections were incubated with 80µL 1% hydrogen peroxide solution in 1:1 methanol and 1x PBS solution in a humidified chamber for 2 hours. For immunohistochemistry (IHC) all antibodies were diluted in 0.1% fish gelatin (Sigma-Aldrich) 1x PBS solution and slides

were rinsed 3x in 1x PBS between incubations. For IHC visualization of T cells, 80 μ L of 1:100 dilution of polyclonal rabbit anti-human CD3 ϵ (Dako A0452) was incubated on slides in a humidified chamber overnight at 4°C. The next day 80 μ L of 1:100 dilution of biotin-conjugated goat anti-rabbit IgG (Dako E0432) was added and incubated in the humidified chamber for 2 hours at room temperature (RT). For negative controls no primary antibody was added to slides. Antibody staining was visualized using Vectastain ABC HRP Kit (Vector Laboratories) and DAB Substrate Kit (Abcam) according to manufacturer's recommendations. Slides were counter-stained in hematoxylin solution, Harris Modified (Sigma-Aldrich), dehydrated in ethanol and xylene washes, and mounted with glass coverslips and DPX mountant for histology (Sigma-Aldrich).

Imaging and quantification

All IHC images were taken on an Eclipse Ti-S (Nikon) inverted microscope. Whole embryo and neonate sagittal section images were composed by NIS-Elements software (Nikon) stitching together overlapping images. Images were edited for clarity by enhanced color contrast in Photoshop (Adobe). For CD3 ϵ ⁺ cell counting in thymic areas of tissue sections, only stained sections with >150 total visible cells in the thymus were used. Five tissue sections from each 14 days postcoital embryo (n = 2) and 1 day postnatal neonate (n = 2) were assessed for CD3 ϵ ⁺ cell counts by a single researcher. Statistical analysis of cell count data was performed in Prism 7 (GraphPad Software).

RESULTS

While performing IHC using an anti-CD3 ϵ polyclonal antibody to detect maternal T cells in *M. domestica* uterine tissues before, during, and after pregnancy, positive staining cells in 13 days postcoital embryos in the uterine lumen were observed (Fig. 1). CD3 ϵ is a component of the TCR complex and is useful as a pan-T cell marker (Clevers et al. 1988) and the anti-CD3 ϵ polyclonal antibody used has been shown to cross-react with marsupial epitopes (Jones et al. 1993; Kreiss et al. 2009). No similar staining was observed in 12 days postcoital embryos (not shown).

To investigate this staining further, isolated 14 days postcoital embryos were examined. Day 14 embryos are less than 24 hours from birth and organogenesis is more progressed (Mate et al. 1994). A patch of CD3 ϵ ⁺ staining cells is clearly visible at a site in the upper thoracic cavity along the esophagus (Fig. 2). The morphology of this patch resembles the early developing thymus in 1 day postnatal *M. domestica* neonates described previously (Hubbard et al. 1991). To reproduce the histology described by Hubbard and colleagues, day 1 neonates were also investigated. Not surprisingly CD3 ϵ ⁺ cells were present at this age as well (Fig. 3). CD3 ϵ ⁻ cells with the morphology of lymphocytes are also evident at both pre- and postnatal stages. Based on cell counts from day 14 embryos (n = 2) and postnatal day 1 neonates (n = 2), 48 to 57% CD3 ϵ ⁺ cells were observed in the embryonic thymuses and 55 to 59% in the postnatal day 1 thymuses (Fig. 2C, 3B). There was no statistically significant difference in the number or proportion of CD3 ϵ ⁺ cells between the two ages (Student's t-test, Two-way ANOVA, data not shown). Distinct cortical and medullary regions were not yet evident. CD3 ϵ staining was found

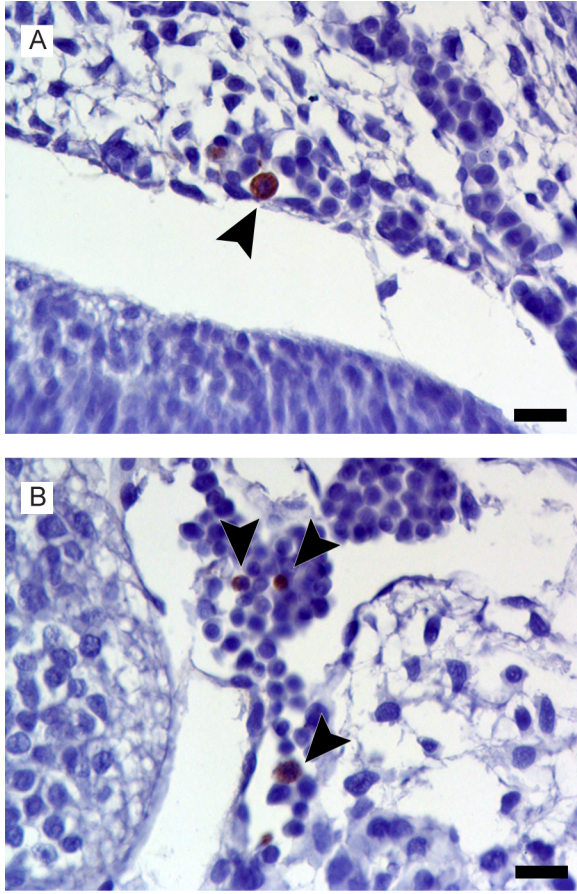


Fig. 1 CD3 ϵ ⁺ cells in 13 days postcoital *M. domestica* embryos. (A-B) IHC staining of CD3 ϵ reveals sparse developing T cells as indicated by arrows. Scale bars: 20 μ m.

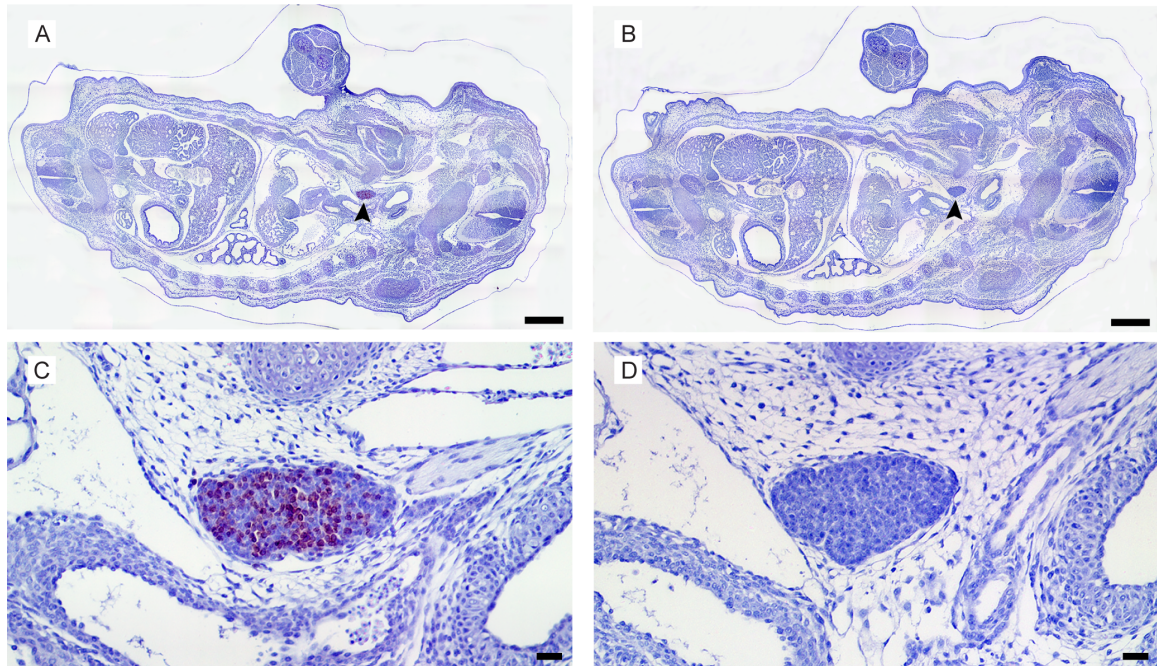


Fig. 2 CD3 ϵ^+ cells in 14 days postcoital *M. domestica* embryos. (A-B) Sagittal sections of whole embryos. Left is posterior, right is anterior, ventral is at the top, dorsal is at the bottom. Scale bars: 500 μ m. (A) CD3 ϵ^+ staining in the developing thymus area as indicated by arrow. (B) No staining in developing thymus in negative control using no primary antibody. (C-D) Developing thymuses as seen in A and B at 10x magnification. Scale bars: 20 μ m. (C) Developing thymus with both CD3 ϵ^+ and CD3 ϵ^- cells. 48 to 57% of thymic cells in 14 days postcoital embryo sections examined were CD3 ϵ^+ and pictured section is representative. (D) Similar morphology to C in thymic cells but no staining in developing thymus in negative control using no primary antibody.

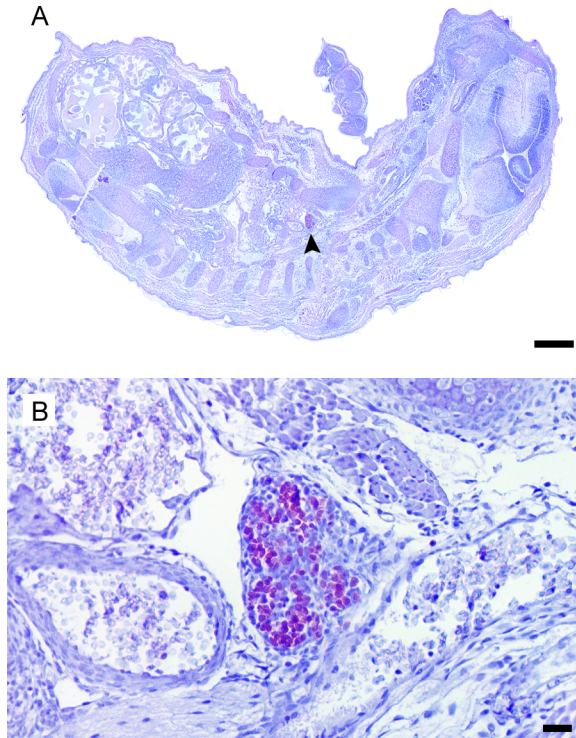


Fig. 3 CD3 ϵ^+ cells in 1 day postnatal *M. domestica* neonates. (A) Sagittal section of whole neonate with CD3 ϵ^+ staining in the developing thymus area as indicated by arrow. Left is posterior, right is anterior, ventral is at the top, dorsal is at the bottom. Scale bar: 500 μ m. (B) Developing thymus as seen in A at 10x magnification with both CD3 ϵ^+ and CD3 ϵ^- cells. 55 to 59% of thymic cells in 1 day postnatal neonate sections were CD3 ϵ^+ and pictured section is representative. Scale bar: 20 μ m.

nowhere else in the day 14 embryo (Fig. 2 and data not shown), consistent with T cell development being restricted to the thymus.

DISCUSSION

Marsupials are a distinct mammalian lineage noteworthy for giving birth to highly altricial young (Tyndale-Biscoe & Renfree, 1987). Comparatively, much of postnatal development in marsupials occurs during prenatal development in most eutherian species. This is particularly true for the adaptive immune system (Baker et al. 1999; Old & Deane, 2003a,b). Analyses of lymphocyte ontogeny using reverse transcriptase PCR of B and T cell specific transcripts as proxies for developmental stages were consistent with the appearance of early committed cell lineages around the time of birth. Parra and colleagues (2009) demonstrated the presence of complete, functional TCR α and β transcripts on postnatal day 1, consistent with the initiation of T cell development in prenatal opossums.

Thymus ontogeny and T cell development has been examined in a variety of American and Australasian marsupials. The American marsupials, like eutherians, all appear to have a single bi-lobed thoracic thymus (Yadav, 1973; Haynes, 2001). The prenatal appearance of CD3 ϵ ⁺ T cells described here, however, is restricted to *M. domestica*. In the white-eared opossum, *Didelphis albiventris*, CD3 ϵ ⁺ cells were absent from newborn (10mm crown-rump length) opossums but were detectable at later postnatal stages (≥ 14 mm) (Coutinho et al. 1995). Although not analyzed for CD3 ϵ expression, newborn North American opossums (*Didelphis virginiana*) did have a distinct thymus composed primarily of epithelial cells and also some large lymphocytes (Block, 1963; Krause, 1998). The cortical and medullary regions, however, were not apparent until the third postnatal week, consistent with a delayed appearance of maturing T cells (Klug et al. 1998). In contrast, differentiation of the cortical and medullary regions was evident by the end of the first postnatal week in *M. domestica* (Hubbard et al. 1991).

Some Australasian marsupial species, such as the macropods (kangaroos and wallabies) have two thymuses, a thoracic thymus and a cervical thymus (Yadav, 1973). In these species the cervical thymus matures earlier in postnatal development than the thoracic thymus, and the two thymuses appear to be functionally redundant (Stanley et al. 1972; Ashman et al. 1977; Basden et al. 1996; Wong et al. 2011; Duncan et al. 2012). Previous attempts to detect the initiation of T cell development in perinatal Australasian marsupials have not been successful. CD3 ϵ ⁺ cells were not detectable in either thoracic or cervical *Macropus eugenii* thymus until after postnatal day 5 (Old & Deane, 2003a). CD3 ϵ ⁺ cells were not evident in *Trichosurus vulpecula* neonates until postnatal day 2 (Baker et al. 1999). In the stripe-faced dunnart, *Sminthopsis macroura*, CD3 ϵ ⁺ cells were not observed in 12 day old neonates, and though 1 day neonates were not tested for CD3 expression their thoracic thymus appeared to be composed mainly of stromal cells at birth (Old et al. 2004a,b; Borthwick et al. 2014).

In *M. domestica* fertilization takes place within 24 hours following copulation. Organogenesis first becomes apparent around 10 days postcoitus with the formation of the primitive streak and first somites (Mate et al. 1994). On postnatal day 12 the maternal shell coat ruptures and the embryo implants into the endometrium. At this point organogenesis proceeds swiftly and birth occurs at 14.5 days postcoitus. CD3 ϵ ⁺ thymocytes were not apparent in 12 days postcoital embryos but were present in day 13 embryos or, in other words, soon after implantation. By comparison, *Mus musculus* fertilization occurs approximately 1 day postcoitus and embryos implant 4 days postcoitus while the embryo is still a blastocyst (Theiler, 1989). The primitive streak and first somites form 7-8 days postcoitus and organogenesis stages similar to those seen in 12-14.5 days post coital *M.*

domestica embryos occur 9-12 days postcoitus in *M. musculus*. CD3⁺ cells appear 14 days postcoitus in mouse embryos (Bluestone et al. 1987; Clevers et al. 1988; Wilson et al. 1995). In summary the progression of development, from the initiation of organogenesis to milestones such as the first appearance of clear T cell progenitors is compressed in opossum relative to mouse.

There are several possibilities for why *M. domestica* may initiate T cell development earlier than other marsupial species. One is the relatively swift development of *M. domestica* offspring with a gestation period of 14.5 days and a weaning age of 56 days, which is one of the most rapid among marsupials (Fadem et al. 1982; VandeBerg, 1983; Tyndale-Biscoe & Renfree, 1987). Australian marsupials like *M. eugenii* and *T. vulpecula*, by comparison, have longer times to weaning at 270 and 230 days, respectively (Murphy & Smith, 1970; Renfree & Tyndale-Biscoe, 1973; Lyne et al. 1959; Hughes & Hall, 1984). *D. albiventris*, which is more closely related to *M. domestica*, has an even shorter gestation of 13 days but also has a longer lactation period of 90-100 days (Talice & Lagomarsino, 1959; Streilein, 1982; Tyndale-Biscoe & Renfree, 1987; Cabello, 2006; Perez Carusi et al. 2009). Perhaps *M. domestica* adapted to having an accelerated T cell development to accommodate a rapid birth to weaning period. Alternatively, *M. domestica* is a marsupial that lacks the protective environment of a pouch. This may create a vulnerability that has been compensated for by a more rapid development of T cells in the newborn.

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AUTHOR CONTRIBUTIONS

Concept and design: VLH, RDM; acquisition of data: VLH; data analysis and interpretation: VLH, RDM; drafting of manuscript: VLH, RDM; critical revision of the manuscript: VLH, RDM.

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CHAPTER 3

TRANSCRIPTOMIC CHANGES ASSOCIATED WITH PREGNANCY IN A MARSUPIAL, THE GRAY SHORT-TAILED OPOSSUM *MONODELPHIS* *DOMESTICA*

By Victoria L. Hansen^{1*}, Faye D. Schilkey², Robert D. Miller^{1,#a}

¹Center for Evolutionary and Theoretical Immunology, Department of Biology,
University of New Mexico, Albuquerque, New Mexico, United States of America

²National Center for Genome Resources/New Mexico IDeA Networks of Biomedical
Research Excellence, Santa Fe, New Mexico, United States of America

^{#a}Current Address: Division of Integrative Organismal Systems, National Science
Foundation, 4201 Wilson Blvd, Arlington, Virginia, United States of America

*Corresponding author

Email: vhansen@unm.edu (VLH)

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ABSTRACT

Live birth has emerged as a reproductive strategy many times across vertebrate evolution, however mammals account for the majority of viviparous vertebrates. Marsupials are a mammalian lineage that last shared a common ancestor with eutherians (placental mammals) over 148 million years ago. Marsupials are noted for giving birth to highly altricial young after a short gestation, and represent humans' most distant viviparous mammalian relatives. Here we ask what insight can be gained into the evolution of viviparity in mammals specifically and vertebrates in general by analyzing the global uterine transcriptome in a marsupial. Transcriptome analyses were performed using NextGen sequencing of uterine RNA samples from the gray short-tailed opossum, *Monodelphis domestica*. Samples were collected from late stage pregnant, virgin, and non-pregnant experienced breeders. Three different algorithms were used to determine differential expression, and results were confirmed by quantitative PCR. Over 900 opossum gene transcripts were found to be significantly more abundant in the pregnant uterus than non-pregnant, and over 1400 less so. Most with increased abundance were genes related to metabolism, immune systems processes, and transport. This is the first study to characterize the transcriptomic differences between pregnant, non-pregnant breeders, and virgin marsupial uteruses and helps to establish a set of pregnancy-associated genes in the opossum. These observations allowed for comparative analyses of the differentially transcribed genes with other mammalian and non-mammalian viviparous species, revealing similarities in pregnancy related gene expression over 300 million years of amniote evolution.

INTRODUCTION

The transition from oviparity to viviparity has occurred independently multiple times in vertebrate evolution. Indeed, most vertebrates including cartilaginous fishes, bony fishes, amphibians, squamate reptiles, and mammals have lineages that transitioned to viviparity from oviparous ancestors [1]. In many viviparous vertebrates the transition away from oviparity involved the evolution of the placenta, which serves as an interface between the maternal system and the conceptus. The placenta is a specialized organ that facilitates the transfer of gases, nutrients, and waste as well as providing a physical barrier to protect the fetus from pathogens [2].

Based on phylogenetic relationships between metatherian (marsupial) and eutherian ("placental") mammals, viviparity appears to have evolved once early in the therian lineage [3-5]. There are three general categories of placental invasiveness in therians. The least invasive is epitheliochorial placentation, which is a superficial apposition of fetal and maternal membranes, whereas the most is the hemochorial placenta where fetal trophoblast invades the uterine endometrium and remodels maternal capillaries [6, 7]. The third major type, an intermediary endotheliochorial placenta, also invades into the endometrium but does not directly contact maternal circulation [7]. Eutherians vary from species to species in placental invasiveness [6]. Primates and rodents typically have hemochorial placentation, whereas ruminants like cows and sheep use epitheliochorial placentas [8, 9]. Most branches of eutherians also have species that use endotheliochorial placentation, such as canines and bats [6, 9].

Prior to the widespread use of molecular phylogenetics, most theories of placental evolution were based on assuming a progression from less invasive to more invasive [10].

The current view is that the ancestral eutherian placenta was either hemochorial or endotheliochorial [9, 11-15]. The less invasive epitheliochorial placenta in eutherian species is likely an evolutionary innovation to give the mother greater control over nutrient and gas exchange [14, 15]. There is a correlation between forming an epitheliochorial placenta and giving birth to more precocial offspring in eutherians [14]. Garratt and colleagues [15] hypothesized that this may be due to maternal and fetal interests deviating relatively quickly after birth in many eutherian species with epitheliochorial placentation.

The marsupials are the sister group to eutherians that give birth to relatively altricial young [16]. In contrast to the eutherian placentation trend, marsupials tend to form epitheliochorial-like placentas despite their altricial offspring. Moreover most marsupials use the yolk sac, as opposed to the allantois, to contact the chorion and form the trophoblast that is apposed to maternal membranes [16, 17]. A notable exception is within the family Peramelidae (bandicoots and echymiperas) where an invasive chorioallantoic placenta forms in the last few days before parturition [16, 18].

The morphological changes of the placenta throughout pregnancy have been well characterized in several marsupial species such as *Sminthopsis crassicaudata*, *Macropus eugenii*, and *M. domestica* [19-23]. *M. domestica* forms an invasive endotheliochorial yolk sac placenta in the last 60 hours prior to birth and, characteristic for marsupials, a permeable shell coat separates the fetal and maternal membranes for the majority of gestation [21]. *M. domestica* is a member of the Didelphidae family, considered to be among the least derived marsupial lineage and likely represents the ancestral reproductive characteristics of marsupials [17, 21]. Therefore *M. domestica* is potentially an important model for discovering other basal characteristics of reproduction in marsupials and perhaps

even therians in general.

More recent analyses of gene expression in reproductive tissues during opossum pregnancy have made important contributions to our understanding of the evolution of the placenta in eutherian mammals [24, 25]. Kin and colleagues described the distribution of important eutherian transcription factors (TFs) in *M. domestica* endometrium, determining the ancestral cell type of eutherian endometrial stromal fibroblasts (ESFs) that undergo decidualization [24]. Lynch and colleagues recently revealed that endometrially-expressed transposable elements (TEs), ancient in the eutherian lineage, have played an important role in the evolution of decidual tissues; a tissue type lacking in marsupials [25]. This has demonstrated key adaptations in endometrial tissue that may have enabled the successful extended gestation periods seen in eutherians.

Needed for a greater understanding of the mammalian adaptation to viviparity are analyses of those genes differentially transcribed between pregnant and non-pregnant tissues within a non-eutherian mammal. This may also facilitate the discovery of genes crucial to marsupial pregnancy that may or may not be shared with eutherians or, indeed, other viviparous vertebrates. Presented here is just such a comparison with an analysis of the pregnant and non-pregnant uterine transcriptomes of *M. domestica*.

RESULTS

To investigate changes in gene expression patterns associated with opossum pregnancy, transcriptome analyses were performed on uterine tissues from late stage pregnant (P), virgin (V), and non-pregnant (N) experienced breeders. Three biological replicates were used for each condition. Paired-end reads were generated on the Illumina HiSeq 2000 platform and aligned to the *M. domestica* genome. The number of mapped reads per sample ranged from 17.1 to 25.9 million at the high end, with an average of 20.4 million (S1 Table). The numbers of aligned reads per sample were more than sufficient for differential expression analysis with treatment groups containing three biological replicates [26].

Comparing the global transcriptomes individually, the nine samples segregated into pregnant (P) and non-pregnant groups (N and V) based on Jensen-Shannon (JS) distances (Fig 1). The P transcriptomes grouped together and were more similar to each other than they were to any other samples. Of the non-pregnant individuals, all three N grouped together and two of the three V individuals grouped together. Virgin opossum V1 did not group with the other virgin animals, but her uterine transcriptome was more similar to the other non-pregnant animals than to the pregnant animals (Fig 1). For this reason analyses both including and excluding V1 were performed when testing the P group against the V and N groups. No other replicate in any group was dissimilar enough to warrant being separated for further analyses. Three replicates per treatment group appeared to be adequate since they grouped together well overall indicating consistency between biological replicate transcriptomes (Fig 1). The P group was tested for differentially transcribed genes compared to N, all three V, and V2 and V3 alone (V2 & V3) (Fig 2).

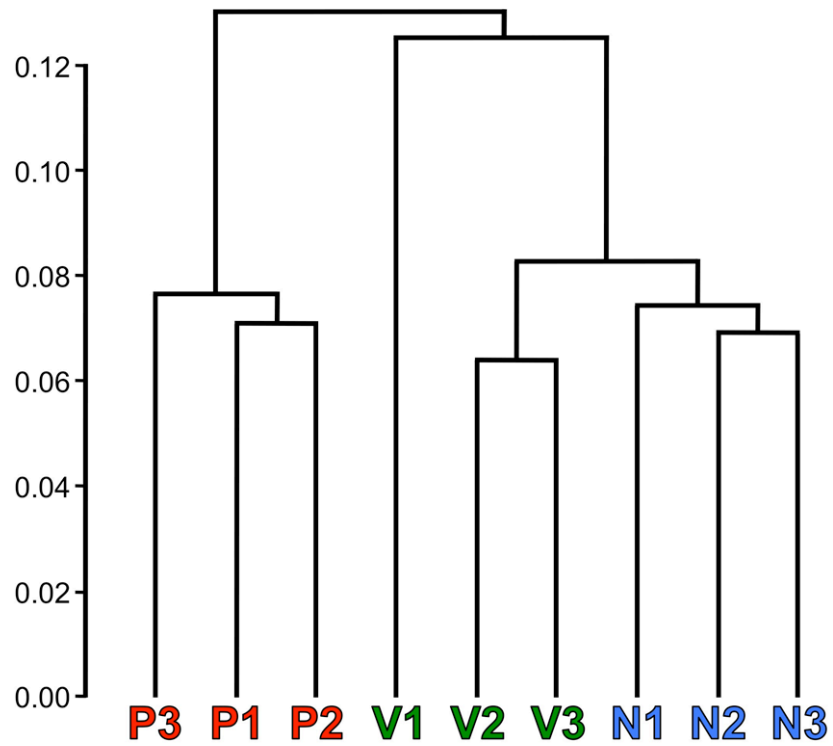


Fig 1. Global transcriptomic similarities between samples. Dendrogram of Jensen-Shannon (JS) distances between transcriptomes of replicates with the pregnant (P), virgin (V), and non-pregnant past breeder (N) transcriptomes.

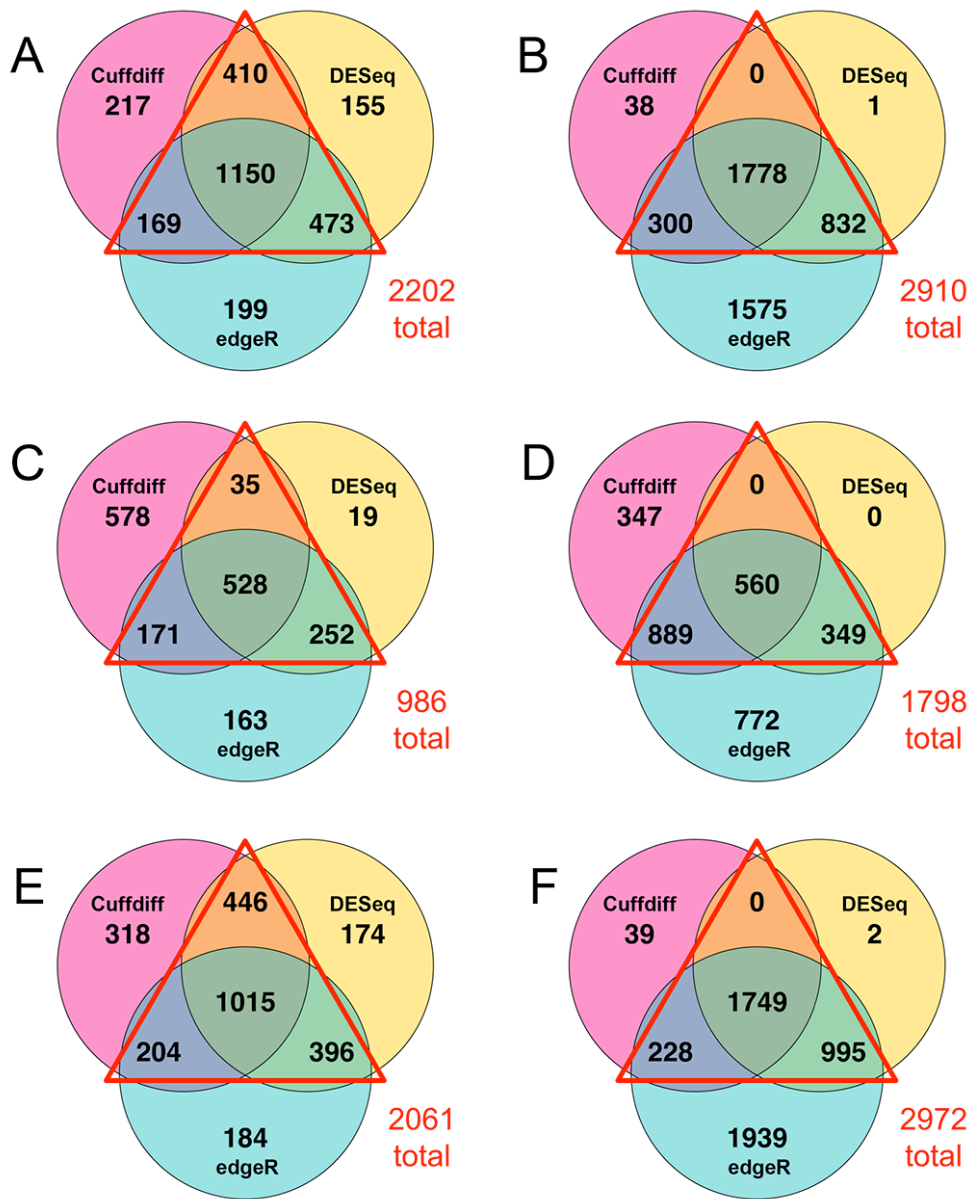


Fig 2. Overlap of genes called as significant in differential expression programs. Venn diagrams of the numbers of gene transcripts found to be significantly more abundant (A, C, and E) or less abundant (B, D, and F) when comparing the dataset from the P group to N (A and B), V (C and D) and V2 & V3 only (E and F). Genes were tested for differential expression according to Cuffdiff (pink), DESeq (yellow), and edgeR (blue). Red triangles denote the cases where at least two out of three programs were in agreement.

The three sample groups, V, N, and P, were compared in a pair-wise manner and independently assessed for differential transcription using Cuffdiff, DESeq, and edgeR. Since these algorithms assess differential expression using different parameters they identified partially, but not fully, overlapping sets of genes (Fig 2). Genes that were identified as being differentially transcribed by at least two of the three programs were used for this study (red triangles in Fig 2).

Transcripts of 2202 genes were significantly more abundant in the P uterus compared to N (Fig 2A), whereas 2910 genes were significantly less (Fig 2B). When the P samples were compared to all three V only 986 gene transcripts increased in abundance (Fig 2C). However when V1 was excluded this number was 2061 (Fig 2E), more similar to the comparison with N (Fig 2A). There were 1798 and 2972 decreased gene transcripts in the same comparisons, respectively (Figs 2D and 2F). Therefore, including the outlier V1 suppressed the number of differentially genes. Its exclusion made the V group more similar to N in numbers of differentially transcribed genes.

Validation of differential gene transcript abundance

To validate the Illumina data, we developed quantitative PCR (qPCR) assays for a subset of genes that were differentially represented in the different groups. These were *membrane cofactor protein (CD46)*, *MAC-inhibitory protein (CD59)*, *interleukin-6 (IL6)*, *lysosomal-associated membrane protein 1 (LAMP1)*, and *lumican (LUM)*. The direction of log₂-fold change of gene expression in P compared to N samples in the Illumina datasets was recapitulated by the qPCR (Fig 3). The same was found in a P vs V comparison. These genes were not significantly differentially expressed in V compared to the N group in either

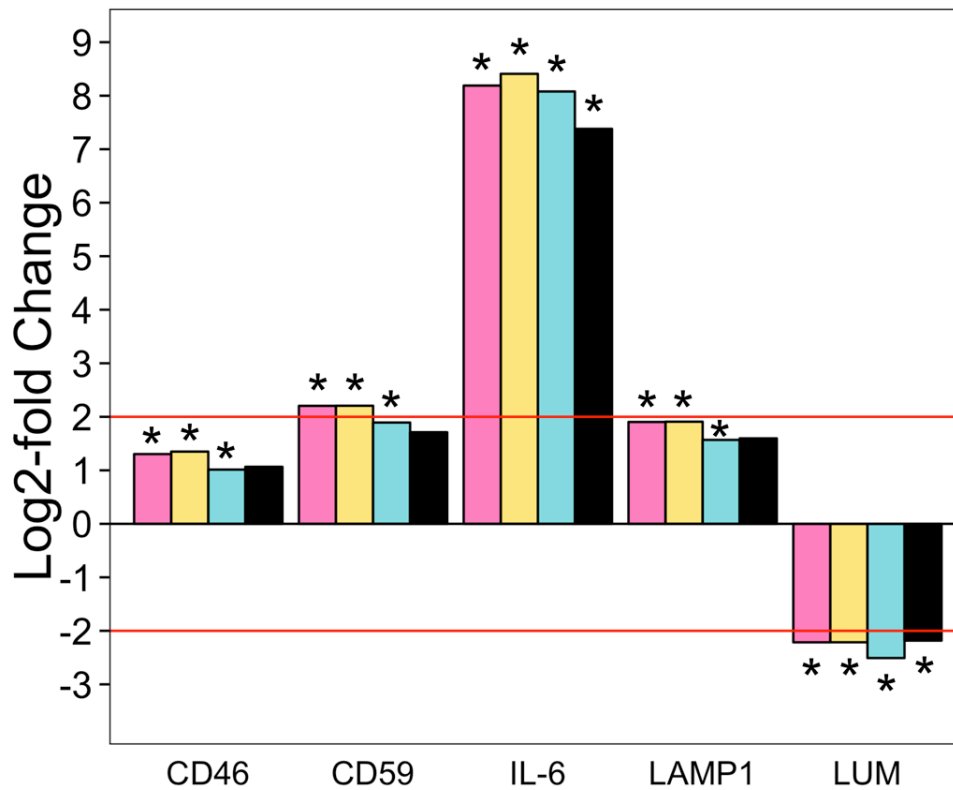


Fig 3. Gene transcription levels in P vs N according to differential expression programs compared to qPCR data. Log₂-fold changes in the transcription of membrane cofactor protein (CD46), MAC-inhibitory protein (CD59), interleukin 6 (IL-6), lysosomal-associated membrane protein 1 (LAMP1), and lumican (LUM) in the pregnant group compared to the non-pregnant past breeder group. Log₂-fold changes were according to Cuffdiff (pink bars), DESeq (yellow bars), edgeR (blue bars), and qPCR using the Vandesompele method (black bars). Red line indicates the threshold of log₂-fold change needed for significance according to the Vandesompele method of relative quantification of qPCR data. * Adjusted p < 0.05 for differential expression programs or >2 log₂-fold change for qPCR.

Illumina or by qPCR (S1 Fig).

Pregnancy-associated genes in the opossum

To determine gene sets that were explicitly differentially transcribed during pregnancy, the intersections of differentially transcribed genes in the P set compared to both non-pregnant sets (N and V) were identified. Such genes should represent a minimal, high confidence “pregnancy-associated” set for the opossum. In the intersections of all three comparisons 932 genes were significantly more abundant and 1482 genes were less abundant in the P group (Fig 4). These increased and decreased differentially transcribed pregnancy-associated genes are listed in S2 and S3 Tables. Gene Ontology (GO) analyses of the pregnancy defining genes revealed 18 GO terms were overrepresented and 6 GO terms were underrepresented (Table 1). In the pregnancy-associated genes with decreased transcription during pregnancy, 25 GO terms were overrepresented and two were underrepresented (Table 2). The most overrepresented GO term in the increased in pregnant gene set was ion transport ($p = 9.86E-06$) (Table 1); the most overrepresented in the decreased in pregnant gene set was ectoderm development ($p = 2.23E-09$) (Table 2).

Differential transcript abundance between pre- vs. post-estrus

There is evidence that marsupial uterine differentiation is not completed until the first estrus occurs [16, 27]. The data sets generated for this study allow for investigation of transcriptomic differences between the N and V groups that might be linked to this

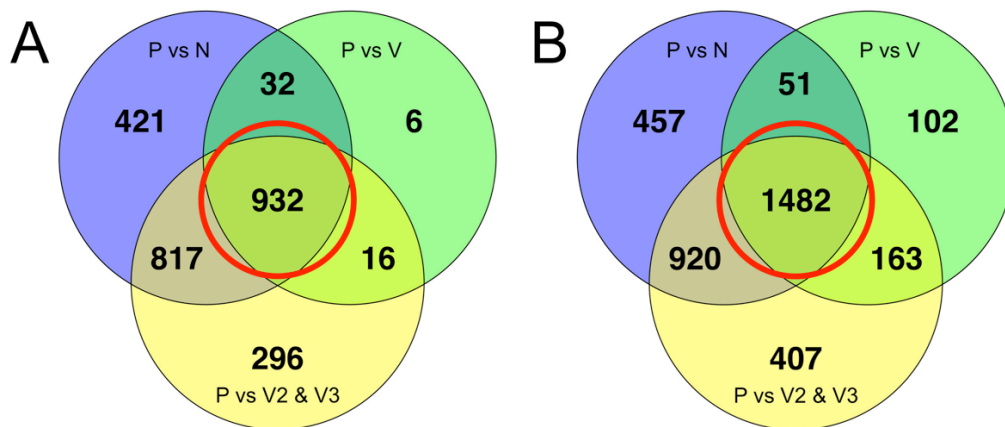


Fig 4. Pregnancy-associated gene sets. Venn diagrams describing the intersection of genes that were considered differentially transcribed in comparisons of the P group to the N, V, and V2 & V3 control groups. The genes that were differentially transcribed in the P group compared to all three control groups (red circles) were considered to be differentially transcribed at a high level of confidence for this study.

Table 1. Overrepresented and underrepresented Biological Process GO terms in the increased transcription during pregnancy gene set.

Overrepresented GO-Slim Biological Process	P-value
ion transport	9.86E-06
immune system process	9.15E-05
proteolysis	1.08E-04
cellular amino acid metabolic process	2.12E-04
amino acid transport	3.04E-04
cation transport	4.99E-04
lipid metabolic process	7.04E-04
cell-matrix adhesion	1.08E-03
blood coagulation	1.12E-03
extracellular transport	1.40E-03
cell-cell adhesion	3.53E-03
angiogenesis	4.64E-03
response to external stimulus	7.30E-03
transport	1.14E-02
response to stimulus	1.52E-02
system development	1.61E-02
localization	2.15E-02
blood circulation	2.42E-02
Underrepresented GO-Slim Biological Process	P-value
nucleobase-containing compound metabolic process	1.98E-06
RNA metabolic process	4.21E-05
cell cycle	2.90E-03
translation	4.79E-03
DNA metabolic process	1.34E-02
mRNA processing	1.42E-02

Table 2. Overrepresented and underrepresented Biological Process GO terms in the decreased transcription during pregnancy gene set.

Overrepresented GO-Slim Biological Process	P-value
cellular process	2.92E-14
ectoderm development	2.23E-09
nervous system development	5.17E-09
multicellular organismal process	9.91E-09
single-multicellular organism process	9.91E-09
chromosome segregation	3.10E-08
cell-cell adhesion	9.10E-08
developmental process	2.02E-07
system process	2.76E-07
cell adhesion	4.81E-07
biological adhesion	4.81E-07
system development	5.78E-07
cell cycle	2.31E-06
neurological system process	4.95E-06
mitosis	4.59E-05
cellular component organization	1.68E-04
cell communication	4.66E-04
cellular component organization or biogenesis	6.41E-04
muscle organ development	1.35E-03
cell-cell signaling	1.73E-03
cellular component morphogenesis	8.77E-03
mesoderm development	1.18E-02
cellular component movement	1.81E-02
cytokinesis	2.99E-02
muscle contraction	3.23E-02
Underrepresented GO-Slim Biological Process	P-value
primary metabolic process	2.47E-03
metabolic process	8.20E-03

differentiation. The V and N samples were transcriptomically similar to each other when compared to the P group (Fig 1). In order to determine the genes that might be linked to uterine differentiation, the pre-estrus V dataset was compared to the post-estrus N dataset. The intersections of N vs V and N vs V2 & V3 were examined and 8 genes were significantly more abundant and 4 were less abundant in V relative to N, respectively (Table 3).

Conservation of genes regulated during pregnancy

Transcriptomic changes in *M. domestica* uterus during pregnancy were compared to previously published uterine transcriptome studies in pig (*Sus scrofa*), skink (*Chalcides ocellatus*), and seahorse (*Hippocampus abdominalis*) [28-30]. For this meta-analysis, “top-100” gene sets were identified. The top-100 significantly increased genes in opossum pregnancy are the 100 genes with the greatest transcript abundance among the 932 increased in P (Fig 4A). These were then compared to the published analyzed differentially expressed gene sets for the pig, skink, and seahorse [28-30]. Fifty-five of the top 100 gene transcripts most abundant during pregnancy for the opossum were identified as also increased in pregnant skink (Table 4). Only 11 and 1 of the selected top 100 opossum genes were identified as increased in pregnant pig and seahorse, respectively (Table 4). Of the 1482 genes identified as being decreased in pregnant (Fig 4B), the top 100 is that subset that had the greatest abundance of transcripts in the opossum N samples. Forty-three of these genes were also identified as significantly decreased in skink pregnancy (Table 5). Only 8 of these were identified in the pig pregnancy and none in the seahorse analyses (Table 5).

Table 3. Significantly differentially transcribed genes in the virgin group as compared to the non-pregnant past breeder group.

Expression in V Group	Gene Symbol	Ensembl Gene ID	Gene Description
Increased	SLC6A15	ENSMODG00000020492	Solute carrier family 6 (neutral amino acid transporter), member 15
Increased	TFAP2C	ENSMODG00000016445	Transcription factor AP-2 gamma (activating enhancer binding protein 2 gamma)
Increased	GPR115	ENSMODG00000018861	G protein-coupled receptor 115
Increased	ARG1	ENSMODG00000017584	Arginase 1
Increased	ZAR1	ENSMODG00000020634	Zygote arrest 1
Increased	ACTC1	ENSMODG00000001384	Actin, alpha, cardiac muscle 1
Increased	KRT13	ENSMODG00000014608	Keratin 13
Increased	RAB3B	ENSMODG00000000791	RAB3B, member RAS oncogene family
Decreased	NTSR1	ENSMODG00000016798	Neurotensin receptor 1 (high affinity)
Decreased	IGK ^a	ENSMODG00000009815	Ig kappa chain constant domain
Decreased	IGH ^a	ENSMODG00000029124	IgG heavy chain constant domain
Decreased	IGJ	ENSMODG00000019083	Immunoglobulin J polypeptide, linker protein for immunoglobulin alpha and mu polypeptides

^a Not annotated for *M. domestica* in Ensembl; BLASTN of gene sequence

Table 4. Comparison of “top 100” genes with significantly higher transcription during pregnancy than a non-pregnant state for opossum (*M. domestica*), skink (*C. ocellatus*), pig (*S. scrofa*), and seahorse (*H. abdominalis*).

Gene Symbol	Gene Description	Average Opossum Count in Pregnant	Average Opossum Log2-fold Change	Skink Log2-fold Change ^a	Pig Log2-fold Change ^b	Seahorse Log2-fold Change ^c
RBP4	retinol binding protein 4 plasma	144035	8.14	9.04	0.8	N/A
SOD3	superoxide dismutase 3 extracellular	136902	7.53	1.64	N/A	N/A
LAMC1	laminin gamma 1	90448	3.47	0.93	N/A	N/A
MFGE8	milk fat globule-EGF factor 8 protein	68679	2.34	0.96	NA	N/A
VEGFA	vascular endothelial growth factor A	57448	5.16	1.68	N/A	N/A
FN1	fibronectin 1	56309	2.86	2.91	0.9	N/A
OAT	ornithine aminotransferase	55577	3.91	1.25	N/A	N/A
TC2N	tandem C2 domains nuclear	46083	4.62	4.22	N/A	N/A
FUCA2	fucosidase alpha-L- 2 plasma	45619	5.74	8.13	N/A	N/A
HPGD	hydroxyprostaglandin dehydrogenase 15-(NAD)	44649	3.00	4.46	N/A	N/A
APOA4	apolipoprotein A-IV	35713	11.53	2.33	N/A	N/A
FBLN1	fibulin 1	35636	5.01	0.53	N/A	N/A
APOA1	apolipoprotein A-I	34757	10.18	6.19	N/A	6.7
CD9	Tetraspanin	33256	2.12	1.13	N/A	N/A
GLUL	glutamate-ammonia ligase	31378	2.42	4.47	N/A	N/A
BSG	basigin	30580	2.41	2	N/A	N/A
CTGF	connective tissue growth factor	27365	4.11	2.09	-1.5	N/A
S100A6	S100 calcium binding protein A6	25414	9.61	1.43	N/A	N/A
UGP2 ^d	UDP-glucose pyrophosphorylase 2	23790	4.07	2.77	0.8	N/A
CA15 ^d	carbonic anhydrase 15-like)	22857	7.98	4	N/A	N/A
SGK1	serum/glucocorticoid regulated kinase 1	21241	4.43	3.79	0.9	N/A
CLU	clusterin	18230	1.88	1.88	N/A	N/A

Table 4. (cont.)

LAMP1	lysosomal-associated membrane protein 1	17351	1.96	0.6	N/A	N/A
SLC39A9	solute carrier family 39 member 9	16924	2.04	1.57	N/A	N/A
KRT18	keratin 18	16287	2.98	2.6	-1.6	N/A
SLC41A2	solute carrier family 41 member 2	16039	4.65	0.71	N/A	N/A
CTSL1-like ^d	cathepsin L1-like	15665	3.12	9.8	N/A	N/A
SLC16A5	solute carrier family 16 member 5	15270	5.02	1.86	N/A	N/A
LGMN	legumain	14692	1.69	7.8	N/A	N/A
SPINT2	serine peptidase inhibitor Kunitz type 2	14629	2.29	2.39	N/A	N/A
ABCA2	ATP-binding cassette sub-family A member 2	14471	1.37	0.45	N/A	N/A
HEXB	hexosaminidase B	13839	2.82	7.09	0.7	N/A
PCK1	phosphoenolpyruvate carboxykinase 1	13158	4.54	7.76	7.7	N/A
GLRX	glutaredoxin	12803	2.58	2.46	1.5	N/A
HIF1A	hypoxia inducible factor 1 alpha subunit	12033	1.43	1.13	N/A	N/A
EDEM3	ER degradation enhancer mannosidase alpha-like 3	11608	3.14	1.18	N/A	N/A
CTSA	cathepsin A	11252	2.72	3.49	N/A	N/A
EZR ^d	ezrin	11085	2.00	1.67	N/A	N/A
ATP6V0A4	ATPase H ⁺ transporting lysosomal V0 subunit a4	10973	5.30	3.53	N/A	N/A
TACSTD2	tumor-associated calcium signal transducer 2	10907	2.53	3.21	N/A	N/A
F3	coagulation factor III	10317	3.88	0.56	N/A	N/A
FERMT1	fermitin family member 1	9972	3.47	1.2	N/A	N/A
GPX3	glutathione peroxidase 3	9890	2.81	7.01	1.9	N/A

Table 4. (cont.)

FGA	fibrinogen alpha chain	9829	9.29	4.25	N/A	N/A
MDM2	MDM2 oncogene E3 ubiquitin protein ligase	9771	2.56	0.49	N/A	N/A
DPP4	dipeptidyl-peptidase 4	9723	2.94	5.17	N/A	N/A
RHOU	ras homolog family member U	9413	2.59	2.55	N/A	N/A
CD164	CD164 molecule sialomucin	9376	1.72	0.97	N/A	N/A
PRCP	prolylcarboxypeptidase (angiotensinase C)	9109	4.63	4.81	1.8	N/A
CCNG2	cyclin G2	9076	2.77	0.51	N/A	N/A
CDO1	cysteine dioxygenase type 1	8886	5.03	2.98	-1.9	N/A
ENTPD5	ectonucleoside triphosphate diphosphohydrolase 5	8853	2.53	2.33	N/A	N/A
HEBP1	heme binding protein 1	8800	3.28	1.71	N/A	N/A
LIMA1	LIM domain and actin binding 1	8781	1.39	1.72	N/A	N/A
DAG1	dystroglycan 1	8257	1.71	0.96	N/A	-1.1
AURKAI P1 ^d	aurora kinase A interacting protein 1	175222	10.98	Not DE	N/A	N/A
SLC16A1	solute carrier family 16 member 1	90219	5.11	Not DE	3.5	N/A
GJA1	gap junction protein alpha 1	28936	2.98	Not DE	N/A	N/A
CLSTN1	calsyntenin 1	25541	3.10	Not DE	N/A	N/A
SEC62	SEC62 homolog	22111	1.58	Not DE	N/A	N/A
SAT1	spermidine/spermine N1-acetyltransferase 1	20683	3.02	Not DE	N/A	N/A
SDC4	syndecan 4	17065	2.21	Not DE	N/A	N/A
CCNG1	cyclin G1	15842	1.56	Not DE	N/A	N/A
GNAI1	G protein alpha inhibiting activity polypeptide 1	10469	2.27	Not DE	N/A	N/A
ERBB3	Erb-B2 receptor tyrosine kinase 3	8182	2.20	Not DE	1.2	N/A
TIMP3	TIMP metalloproteinase inhibitor 3	423559	5.04	-2.51	N/A	N/A

Table 4. (cont.)

IGFBP3	insulin-like growth factor binding protein 3	365182	5.62	-1.4	N/A	N/A
PLAT	plasminogen activator tissue	58851	3.70	-0.61	N/A	N/A
SLCO2A1	solute carrier organic anion transporter family member 2A1	53304	6.80	-0.56	N/A	-1.6
PTGS2	prostaglandin-endoperoxide synthase 2	52902	6.44	-4.64	-1.7	N/A
SRD5A2	steroid-5-alpha-reductase alpha polypeptide 2	40909	7.54	-4.03	N/A	N/A
UCK2	uridine-cytidine kinase 2	34080	4.08	-0.72	N/A	N/A
TSC22D1	TSC22 domain family member 1	21251	2.85	-1.46	N/A	N/A
WFDC2	WAP four-disulfide core domain 2	18812	1.88	-0.91	N/A	N/A
SLC39A8	solute carrier family 39 member 8	18386	3.51	-1.83	1.2	N/A
PROM1	prominin 1	18127	4.68	-1.7	N/A	N/A
RNASEL	ribonuclease L	14498	2.67	-2.12	N/A	N/A
MET	met proto-oncogene	12403	3.11	-0.7	N/A	N/A
APOB ^d	apolipoprotein B	8530	9.77	-3.32	N/A	N/A
ULK2	unc-51 like autophagy activating kinase 2	8521	1.81	-0.63	N/A	N/A
SERPINA1	serpin peptidase inhibitor clade A member 1	124987	8.30	N/A	N/A	N/A
STC1	stanniocalcin 1	93090	9.48	N/A	N/A	N/A
HBE1-like ^d	hemoglobin subunit epsilon-like	43294	7.45	N/A	N/A	N/A
AFP	alpha-fetoprotein	42044	10.50	N/A	N/A	N/A
GABRP	gamma-aminobutyric acid A receptor pi	35781	2.29	N/A	N/A	N/A
PIGR	polymeric immunoglobulin receptor	28437	4.90	N/A	N/A	N/A
CSTL1-like ^d	cystatin-like	26624	3.46	N/A	N/A	N/A

Table 4. (cont.)

SLC34A2-like ^d	sodium-dependent phosphate transport protein 2B-like	13004	5.03	N/A	N/A	N/A
ESM1	endothelial cell-specific molecule 1	12730	6.66	N/A	N/A	N/A
MDFIC	MyoD family inhibitor domain containing	12031	2.08	N/A	N/A	N/A
RBBP9	retinoblastoma binding protein 9	10542	5.81	N/A	N/A	N/A
IL6	interleukin 6	10525	8.59	N/A	N/A	N/A
FXYD3 ^d	FXYD domain containing ion transport regulator 3	10495	3.86	N/A	N/A	N/A
SLC2A1	solute carrier family 2 member 1	10334	3.24	N/A	3.3	N/A
SUCO	SUN domain containing ossification factor	10163	2.08	N/A	N/A	N/A
OXTR	oxytocin receptor	9637	4.46	N/A	N/A	N/A
PC	pyruvate carboxylase	9626	2.40	N/A	N/A	N/A
IL1R2	interleukin 1 receptor type II	9248	4.28	N/A	4.5	N/A
CLIC6	chloride intracellular channel 6	9202	4.03	N/A	N/A	N/A
FAM213A	family with sequence similarity 213 member A	8574	4.33	N/A	N/A	N/A

^a Data pulled from Brandley et al. 2012

^b Data pulled from Samborski et al. 2013

^c Data pulled from Whittington et al. 2015

^d Not annotated for *M. domestica* in Ensembl; BLASTN of gene sequence

Table 5. Comparison of “top 100” genes with significantly lower transcription during pregnancy than a non-pregnant state for opossum (*M. domestica*), skink (*C. ocellatus*), pig (*S. scrofa*), and seahorse (*H. abdominalis*).

Gene Symbol	Gene Description	Average Opossum Count in Non-pregnant	Average Opossum Log2-fold Change	Skink Log2-fold Change ^a	Pig Log2-fold Change ^b	Seahorse Log2-fold Change ^c
MYH11	myosin heavy chain 11 smooth muscle	146637	-2.06	-2.3	N/A	N/A
RPL3	ribosomal protein L3	60395	-1.79	-0.11	N/A	N/A
MYLK	myosin light chain kinase	39309	-1.73	-2.52	N/A	N/A
RPS24	ribosomal protein S24	30918	-1.35	-1.38	N/A	N/A
DPT	dermatopontin	23739	-2.58	-4.91	N/A	N/A
RPL23	ribosomal protein L23	23312	-1.29	-0.97	N/A	N/A
SMOC2	SPARC related modular calcium binding 2	22716	-3.88	-2.52	N/A	N/A
IGFBP5	insulin-like growth factor binding protein 5	21266	-3.18	-4.11	N/A	N/A
RPS8	ribosomal protein S8	20773	-1.33	-1.67	N/A	N/A
EEF1B2	eukaryotic translation elongation factor 1 beta 2	20740	-1.70	-1.02	N/A	N/A
RPL35	ribosomal protein L35	20346	-1.80	-0.48	N/A	N/A
RPS20	ribosomal protein S20	20268	-1.82	-0.53	N/A	N/A
RPL22	ribosomal protein L22	20209	-1.72	-1.02	N/A	N/A
RGS5	regulator of G-protein signaling 5	19385	-2.50	-3.33	N/A	N/A
VCAN	versican	18616	-2.19	-2	N/A	N/A
RPS27	ribosomal protein S27	18449	-1.23	-0.43	N/A	N/A
DST	dystonin	17957	-2.22	-2.43	N/A	N/A
PTRF	polymerase I and transcript release factor	17692	-1.70	-0.99	N/A	N/A
FGFR2	fibroblast growth factor receptor 2	13337	-2.12	-1.33	N/A	N/A
PGR	progesterone receptor	12650	-2.77	-3.05	N/A	N/A

Table 5. (cont.)

RPL30	ribosomal protein L30	11864	-1.28	-1.94	N/A	N/A
DDX50	DEAD (Asp-Glu-Ala-Asp) box polypeptide 50 protein	11747	-1.92	-1.53	N/A	N/A
PPP1R12 B	phosphatase 1 regulatory subunit 12B	11508	-2.37	-1.93	N/A	N/A
CD55	decay accelerating factor for complement	10820	-4.27	-1.23	N/A	N/A
LMNB2	lamin B2	9510	-1.41	-1.04	N/A	N/A
HNRNPA1 ^d	heterogeneous nuclear ribonucleoprotein A1	8817	-2.49	-1.53	N/A	N/A
PDGFRA	platelet-derived growth factor receptor alpha polypeptide	8580	-1.94	-1.15	N/A	N/A
MYH10	myosin heavy chain 10 non-muscle	7912	-2.43	-1.01	N/A	N/A
ALCAM	activated leukocyte cell adhesion molecule	7551	-1.88	-1.18	0.8	N/A
LAMA4	laminin alpha 4	7452	-2.36	-2.6	N/A	N/A
CCDC135	coiled-coil domain containing 135	7242	-3.56	-1.63	N/A	N/A
NR2F2	nuclear receptor subfamily 2 group F member 2	6722	-1.68	-2.12	N/A	N/A
EMILIN1	elastin microfibril interfacier 1	6590	-1.77	-1.62	-0.7	N/A
PPP1R12 A	protein phosphatase 1 regulatory subunit 12A	6479	-1.72	-1.27	N/A	1.7
TPPP3	tubulin polymerization-promoting protein family member 3	6362	-2.21	-0.66	N/A	N/A
SYNM	synemin intermediate filament protein	6298	-1.86	-1.7	N/A	N/A
EDNRA	endothelin receptor type A	6105	-3.05	-1.96	N/A	N/A

Table 5. (cont.)

LTBP1	latent transforming growth factor beta binding protein 1	6017	-1.61	-1.6	N/A	N/A
EFEMP1	EGF containing fibulin-like extracellular matrix protein 1	6003	-1.89	-2.77	-0.8	N/A
SEMA3C	semaphorin 3C	5929	-2.06	-0.74	-1.3	N/A
MDN1	midasin homolog (yeast)	5918	-1.22	-2.92	N/A	N/A
HMGB2	high mobility group box 2	5717	-1.81	-1.28	1	N/A
LBR	lamin B receptor	5421	-1.94	-2.78	N/A	N/A
COL15A1	collagen type XV alpha 1	29091	-4.40	Not DE	N/A	N/A
SERPINE2	serpin peptidase inhibitor clade E member 2	21379	-2.17	Not DE	1.6	1.6
SPOCK3	testican 3	12675	-6.37	Not DE	N/A	N/A
WDR52	WD repeat domain 52	12099	-3.03	Not DE	N/A	N/A
CCDC40	coiled-coil domain containing 40	10227	-3.37	Not DE	N/A	N/A
OLFM4	olfactomedin 4	10012	-2.97	Not DE	N/A	N/A
SYNE1	spectrin repeat containing nuclear envelope 1	9834	-2.62	Not DE	N/A	N/A
ZAN	zonadhesin	8676	-5.96	Not DE	N/A	N/A
SULF1	sulfatase 1	7471	-2.01	Not DE	N/A	N/A
WDR78	WD repeat domain 78	7202	-3.58	Not DE	N/A	N/A
PDLIM3	PDZ and LIM domain 3	7000	-2.16	Not DE	-3	N/A
GPAM	glycerol-3-phosphate acyltransferase mitochondrial	6496	-1.60	Not DE	N/A	N/A
ADAMTS2	ADAM metallopeptidase with thrombospondin type 1 motif 2	6288	-2.07	Not DE	N/A	N/A
DNAH9	dynein axonemal heavy chain 9	6100	-2.72	Not DE	N/A	N/A
HTRA3	HtrA serine peptidase 3	5795	-3.19	Not DE	N/A	N/A
CCDC39	coiled-coil domain containing 39	5639	-3.13	Not DE	N/A	N/A
TEKT2	tektin 2	5538	-4.33	Not DE	N/A	N/A

Table 5. (cont.)

KIF9	kinesin family member 9	5504	-3.09	Not DE	N/A	N/A
MAK	male germ cell-associated kinase	5409	-2.23	Not DE	N/A	N/A
GSTA4	glutathione S-transferase alpha 4	5073	-6.82	Not DE	N/A	N/A
GSN	gelsolin	27062	-1.64	2.53	N/A	N/A
ZWILCH	zwilch kinetochore protein	18376	-1.73	0.86	N/A	N/A
EIF3D	eukaryotic translation initiation factor 3 subunit D	16024	-1.24	0.24	N/A	N/A
MSLN	mesothelin	14443	-4.24	6.88	-2.9	N/A
ANXA6	annexin A6	10093	-1.57	1.35	N/A	N/A
ENPP2	ectonucleotide pyrophosphatase/phosphodiesterase 2	8322	-2.93	8.52	N/A	N/A
PCOLCE	procollagen C-endopeptidase enhancer	8165	-2.65	3.07	N/A	N/A
TOP2A	topoisomerase II alpha	8162	-4.78	5.36	N/A	N/A
TNFRSF19	tumor necrosis factor receptor superfamily member 19	6330	-3.23	4.17	N/A	N/A
CALCOCO2	calcium binding and coiled-coil domain 2	6299	-1.65	1.16	N/A	N/A
LDHB	L-lactate dehydrogenase B chain	6010	-3.21	0.7	N/A	N/A
DNAH2	dynein axonemal heavy chain 2	5401	-4.42	1.62	N/A	N/A
DMBT1	deleted in malignant brain tumors 1	5398	-3.91	9.5	N/A	N/A
MFSD4	major facilitator superfamily domain containing 4	5167	-5.69	5.17	-1.4	N/A
KRT7	keratin 7	5166	-1.73	1.86	N/A	N/A
EIF5A2	eukaryotic translation initiation factor 5A2	20527	-1.89	N/A	N/A	N/A
CCDC78	coiled-coil domain containing 78	18291	-3.31	N/A	N/A	N/A

Table 5. (cont.)

NPTX2	neuronal pentraxin II	14803	-7.71	N/A	N/A	N/A
COL14A1	collagen type XIV alpha 1	13659	-4.09	N/A	N/A	N/A
CCDC108	coiled-coil domain containing 108	9196	-4.18	N/A	N/A	N/A
CCDC141	coiled-coil domain containing 141	9031	-2.23	N/A	N/A	N/A
FILIP1L	filamin A interacting protein 1-like	8940	-2.14	N/A	N/A	N/A
COL11A2	collagen type XI alpha 2	8803	-2.17	N/A	N/A	N/A
CIRBP	cold inducible RNA binding protein	8169	-1.30	N/A	N/A	N/A
ARMC3	armadillo repeat containing 3	7039	-4.05	N/A	N/A	N/A
VTCN1	V-set domain containing T cell activation inhibitor 1	6889	-3.22	N/A	-3	N/A
F10	coagulation factor X	6794	-2.32	N/A	N/A	N/A
C15orf26	chromosome 15 open reading frame 26	6624	-4.31	N/A	N/A	N/A
C1orf87	chromosome 1 open reading frame 87	6467	-3.59	N/A	N/A	N/A
unknown	Uncharacterized protein ENSMODG0000023422	6271	-2.56	N/A	N/A	N/A
CA3	carbonic anhydrase III	6219	-3.87	N/A	4	N/A
CKAP5	cytoskeleton associated protein 5	6212	-1.21	N/A	N/A	N/A
MBL2	mannose-binding lectin 2	6109	-6.70	N/A	N/A	N/A
MXRA5	matrix-remodelling associated 5	5872	-3.67	N/A	N/A	N/A
TWSG1	twisted gastrulation BMP signaling modulator 1	5738	-2.08	N/A	N/A	N/A
HDAC2	histone deacetylase 2	5627	-1.24	N/A	N/A	N/A
DPYSL3	dihydropyrimidina se-like 3	5169	-2.07	N/A	-0.9	N/A

Table 5. (cont.)

^a Data pulled from Brandley et al. 2012

^b Data pulled from Samborski et al. 2013

^c Data pulled from Whittington et al. 2015

^d Not annotated for *M. domestica* in Ensembl; BLASTN of gene sequence

DISCUSSION

Pregnancy involves substantial tissue remodeling and physiological changes. This study was aimed at investigating the changes that occur at the level of uterine gene transcription in a model marsupial species. Marsupials are a distinct lineage of mammals noteworthy for their short gestation times, relative immaturity at birth, and minimal placentation [16]. Viviparity likely has a single common origin in marsupials and eutherians, which last shared a common ancestor at least 148 million years (MY) ago, making marsupials an important comparative contrast to pregnancy in eutherians [3-5].

The comparison between pregnant and non-pregnant samples was chosen to identify the most differentially transcribed uterine genes during pregnancy. All the P and N samples used in this study came from opossums that had successfully given birth to at least one litter prior to this study. Based on what genes were being transcribed and the transcript number, the P transcriptomes were distinctly different from those of non-pregnant animals, whether N or V (Fig 1).

Sets of pregnancy-associated genes were identified as those at the intersection of differentially transcribed in the P versus all non-pregnant animals (N and V). Identifying the overlap in differentially transcribed genes in P vs N, P vs V, and P vs V2 & V3 generated gene sets with of high confidence for being significantly increased or decreased during opossum pregnancy. It is noteworthy that there were more genes with decreased transcription in the opossum uterus than increased transcription during pregnancy (n = 550; the difference between Figs 4A and 4B).

The top 100 most differentially transcribed opossum genes in the P and N groups were compared to results from pregnant uterine transcriptome studies in the pig, skink, and

seahorse [28-30]. The pattern of differential expression during pregnancy in skink had the most in common with the results in *M. domestica*. This similarity is unlikely due to shared structural similarities between the opossum and skink since the skink (*C. ocellatus*) has an epitheliochorial chorioallantoic placenta [28, 31, 32]. Rather the similarity in transcriptomes is likely due to the skink study focusing on a pregnancy time point more similar to that used here in the opossum. The opossum P samples were from uteruses preceding parturition by only 12-24 hours and there was likely to be late-pregnancy and parturition-specific gene transcription represented in the datasets.

In contrast there was less overlap with the transcriptome in the pig. This may be due to the pig study focusing primarily on early pregnancy stages and also the pig's chorioallantoic epitheliochorial placenta type and the source of embryonic nutrition, which is primarily histotrophic rather than hemotrophic [29, 33, 34]. Likewise, there was very little overlap with the seahorse, which may be due to evolutionary distance or, again, the pregnancy time points examined.

Since there was substantial overlap in genes differentially transcribed in both the opossum and the skink, we focused on this comparison further. *Keratin 18 (KRT18)* has been previously shown to be essential to maintaining pregnancy in mice [35] and *KRT18* is increased during pregnancy in both opossum and skink (Table 4). *KRT18* is the only keratin gene that is increased in pregnant opossum (S2 Table). This is in contrast to eutherian pregnancy, such as in pigs and humans, where in addition to *KRT18*, *KRT8* and *KRT19* are also up-regulated [29, 36]. Other notable genes transcribed encoding structural molecules that are more abundant consistently in opossum and skink pregnancy are *fibronectin (FNI)* and *fibulin (FBLN1)* (Table 4). Both of these have been shown to be

important to maintaining pregnancy in eutherian species such as mice [37, 38] and this conservation across a long evolutionary history is consistent with such proteins being critical to placental formation. In contrast *serpin peptidase inhibitor clade member 2* (*SERPINE2*), an up-regulated tissue remodeling gene in human, mouse, pig, and even seahorse pregnancy [29, 30, 39, 40], was among the most decreased gene transcripts during opossum pregnancy (Table 5). *SERPINE2* is also not differentially regulated in skinks [28]. Therefore, although *SERPINE2* transcription in pregnancy is shared between a teleost and eutherians, this does not appear to be a critically conserved gene.

Not surprisingly, an area of broad conservation was hormonal regulation. For example, *progesterone receptor* (*PGR*) had fewer transcripts during pregnancy in both opossum and skink (Table 5), and down-regulation of *PGR* in endometrial cells is observed in eutherian species at implantation [41-44]. Kin and colleagues used immunohistochemistry (IHC) to demonstrate that term pregnant *M. domestica* uterine epithelium and uterine glands express PGR weakly in comparison to non-pregnant uterine tissues [24]. The transcriptome results described here are consistent with that finding (S4 Table). *Oxytocin receptor* (*OXTR*) is more abundant in opossum at terminal pregnancy and in human pregnancy *OXTR* expression increases over time peaking prior to parturition and playing a role in inducing contractions [45-48]. Interestingly, Yamashita and Kitano [49] hypothesized that the evolution of *OXTR* associated with labor contractions in eutherians occurred after the marsupial-eutherian split. However, *OXTR* was a top 100 most abundant gene in pregnant opossum raising the possibility of its role in parturition being more ancient than the marsupial-eutherian split.

Prostaglandins have been shown to be important to normal birth mechanisms in

other marsupials such as the tammar wallaby [50, 51]. Injection of exogenous prostaglandin F2 (PGF2) have also been shown to induce birthing in marsupials [50, 52]. The pregnant samples used in this study were taken from opossums that were within 24 hours of giving birth and it is probable that gene transcript values in these pregnant transcriptomes were influenced by birth mechanisms at work. Genes involved in prostaglandin synthesis and detection such as *prostaglandin E synthase (PTGES)*, *prostaglandin-endoperoxide synthase 2 (PTGS2)*, and *prostaglandin E receptor 4 subtype EP4 (PTGER4)* were all included in the increased in pregnancy-associated set (S2 Table).

Eight solute carrier proteins (*SLC39A9*, *SLC41A2*, *SLC16A5*, *SLC16A1*, *SLCO2A1*, *SLC39A8*, *SLC34A2L*, *SLC2A1*) are included in the top 100 most abundant genes in opossum during pregnancy (Table 4). This is consistent with ion transport being the most highly overrepresented GO term in the datasets. Two of these, *SLC39A9* and *SLC41A2* are zinc and magnesium ion transporters that are also up-regulated during skink pregnancy [28, 53, 54]. Transporters of cholesterol and chylomicrons like *ATP-binding cassette sub-family A member 2 (ABCA2)* and apolipoprotein genes *APOA1*, *APOA4*, and *APOB* are also in the top 100 most abundant gene transcripts in opossum pregnancy (Table 4) which is consistent with what is known about cholesterol transport across eutherian placentas [55].

Two different non-pregnant control groups were used in this study due to concern over previous observations on sexual maturation in *M. domestica* [27]. Briefly, *M. domestica* females do not appear to reach reproductive maturity until they enter estrus and mate for the first time, a process dependent on male pheromones. Therefore, both virgin (V) females (pre-first estrus) as well as non-pregnant (N) experienced past breeders (post-first estrus) were used. In addition to providing controls for the pregnant state, this also

allowed for a direct comparison between presumably immature pre-estrus and more mature post-estrus uterine tissues. In the analyses comparing the V group to P and N, only 12 genes were uniquely differentially transcribed in common (Table 3). Interestingly three of the four genes less abundant (*Ig J chain*, *Ig kappa chain*, *IgG heavy chain constant domain*) in virgin uterine tissue were genes associated with antibody expression (Table 3). This may indicate a need for humoral immunity in uterine tissues only after copulation and could point to a mechanism for controlling sexually transmitted infections in opossums. The morphological changes described by Stonerook and Harder [27], which is primarily organ weight, do not appear to be substantially reflected in changes in gene regulation in the uterus. *Actin alpha cardiac muscle 1 (ACTC1)* and *keratin 13 (KRT13)* are two of eight significantly more abundant genes in the virgin samples but no other major structural genes are differentially transcribed between the non-pregnant groups (Table 3).

The V group had the most variation between biological replicates than any other group as illustrated by V1 being an outlier (Fig 1). Nonetheless, V1 was transcriptomically more similar to the non-pregnant samples than to the pregnant ones. V1's outlier status may have been due to her being 4.8 months old at the time of collection, whereas the other two virgin animals (V2 and V3) were 10 months old. *M. domestica* individuals are generally considered to be sexually mature at 5-6 months old [56], but V1's uterus was likely in a different stage of development from the older virgin animals. Aside from V1, all biological replicates were more similar to each other than to samples from other treatment groups (Fig 1). Non-pregnant individuals (groups N and V) were more similar to each other than they were to the pregnant individuals (Fig 1). This was an expected result and provided justification for retaining V1 in the analyses of differential transcription.

There were more differentially transcribed genes shared between the P vs N and P vs V2 & V3 comparisons than between the P vs V and P vs V2 & V3 comparisons (Fig 4). This confirmed that V1 was skewing the differential transcription results in the P vs V comparison.

Kin and colleagues identified TFs spatially in *M. domestica* endometrium tissue sections in non-pregnant, pre-implantation pregnancy, and term pregnancy using IHC [24]. They concluded that the stromal mesenchymal cells seen in *M. domestica* endometrium are homologous to eutherian ESFs based on the expression of TFs and certain cytoskeletal proteins, and no marsupial has definitive decidualization. Two of the transcription factors (TFs) examined by Kin and colleagues were found in our pregnancy-associated gene sets. These were PGR, which was less abundant in pregnant samples, and CEBPB which was more abundant in pregnant samples (S4 Table). All samples examined in this study did have gene transcripts from the TFs evaluated in the Kin et al study (S4 Table). This is not surprising since Kin and colleagues observed transcription factors in a spatial manner by comparing IHC-stained endometrium of pregnant and non-pregnant *M. domestica*, whereas our study used whole sections of tissue without separating by cell type. Indeed, a limitation of the data sets used in this study is that they contain multiple cell and tissue types and therefore a specific transcript cannot be ascribed to a particular cell or tissue. Therefore in future studies on the genes identified as pregnancy-associated here, a spatial analysis of gene expression would be informative.

In conclusion, the analysis of the opossum late pregnancy uterine transcriptome enabled the discovery of a set of genes that can be described as being pregnancy-associated in the opossum. Curiously, morphological changes associated with sexual maturation in

the opossum uterus reported previously are not obviously reflected in the transcriptome of this species. These analyses also revealed significant conservation of gene regulation among distantly related species. Reptiles and mammals are separated by over 300 MY of evolution and the opossum and skink do not share a common viviparous ancestor [57, 58]. While the similar nature of the gene regulation is likely indicative of convergence on common mechanisms in the evolution of live birth, the transcriptomic similarities in these studies could be explained by the similar pregnancy stages examined here and in the skink.

MATERIALS AND METHODS

Animals and tissue collection

This study was approved under protocol numbers 13-100920-MCC and 15-200334-B-MC from the University of New Mexico Institutional Animal Care and Use Committee. Animals were from a captive-bred research colony housed at the University of New Mexico Department of Biology Animal Research Facility. Founders were obtained from *M. domestica* Populations 1 and 2 at the Southwest Foundation for Biomedical Research, San Antonio, TX [59]. All animals were fed *ad libitum* a diet of Labdiet Short Tailed Opossum #2 pellets (90%, Lab Supply TX) and dried mealworms (10%, Lab Supply TX). They were given drinking water *ad libitum* in sterile glass sipper bottles. All animals were housed individually in standard rat caging and bedding. When not breeding adult females and males were caged separately.

Uterine tissues were collected from isoflurane-euthanized virgin (V), pregnant (P), and non-pregnant (N) experienced breeders. Non-pregnant past breeders had at least one successful pregnancy prior to harvesting. Uterine horns were excised and separated from surrounding tissues. In the case of pregnant uteri, the uterine horns were opened laterally and the intact embryos and amnions removed. Endometrium was separated from myometrium by teasing apart the tissue layers in shallow petri dishes filled with RNALater buffer (Ambion). In pregnant samples invasive fetal chorion and yolk sac membranes could not be fully separated from maternal endometrium. RNA extractions were either performed immediately or tissues were stored in RNALater overnight at 4°C. For longer-term storage, excess RNALater was removed and tissues were stored at -80°C until use.

RNA extraction

Total RNA was isolated by homogenizing tissue in TRIzol (Ambion) using a sterile glass homogenizer followed by chloroform extraction and centrifugation at 4°C for 15 min. Protein and DNA contamination was removed using the PureLink RNA Mini (Ambion) and On-Column DNase Treatment (Invitrogen) kits following manufacturers' recommended protocols. RNA was stored in RNase-free water at -80°C. RNA quality was assessed using a 2100 Bioanalyzer (Agilent) and concentration was determined using a Qubit 2.0 Fluorometer (Life Technologies).

cDNA library synthesis and Illumina sequencing

All cDNA library synthesis for high-throughput RNA-seq and Illumina sequencing procedures were performed at the National Center for Genome Resources (NCGR) in Santa Fe, New Mexico, USA. A Sciclone G3 Automated Liquid Handling Workstation (Caliper Life Sciences) with a Multi TEC Control for heating and cooling steps (INHECO) was used for the majority of liquid handling. Poly-A RNA was isolated using magnetic RNA Purification Beads (Dynabeads: Invitrogen). RNA was fragmented by high temperature (95°C for 8 min). SuperScript II enzyme (Invitrogen), First Strand Master Mix (Illumina), and random hexamer primers (Illumina) were added to RNA to conduct first strand cDNA synthesis by reverse transcription. Second Strand Master Mix (Illumina) was used to conduct second strand cDNA synthesis, resulting in double-stranded cDNA. Double-strand cDNA was isolated using Agencourt AMPure XP beads (Beckman Coulter). Blunt end cDNA was prepared by removing 3' overhangs and filling in 5' overhangs using End Repair Mix (Illumina). 3' ends were then adenylylated using A-Tailing Mix (Illumina).

Universal and barcoded TruSeq Adapters (Illumina) were ligated to cDNA ends. Polymerase Chain Reaction (PCR) was conducted with KAPA HiFi HotStart ReadyMix (KAPA Biosystems) and PCR Primer Cocktail (Illumina) to selectively amplify the cDNA fragments that had TruSeq adapters ligated to both ends. PCR products were purified using Agencourt AMPure XP beads. PCR product quality was assessed using a 2100 Bioanalyzer and cDNA quantity was measured by Nanodrop ND-1000 (Thermo Scientific). Samples were normalized to 10nM equimolar concentrations and pooled prior to flow cell injection. All Illumina sequencing was performed on an Illumina HiSeq 2000 instrument (Illumina) to generate 50bp paired-end reads. Read data was de-multiplexed (segregated by library index) using Illumina CASAVA v1.8.2 to produce FASTQ files. Thereafter, NCGR's custom contaminant filtering pipeline removed anomalous sequences (i.e. Illumina PhiX control, library adapters, primer dimmers, and library indexes not part of the experiment). Quality assessment of read data was performed using FASTQC.

Data access

All high-throughput sequence data sets generated for this study have been deposited at NCBI Sequence Read Archive (SRA) under the accession numbers SRR2969483, SRR2969536, SRR2970443, SRR2972728, SRR2972729, SRR2972792, SRR2972837, SRR2972840, and SRR2972848.

Illumina read quality control, alignment, and quantification

All alignment and differential expression bioinformatics programs were run through the online Lumenogix platform (api.lumenogix.com, [60]). Reads were aligned to the annotated *M. domestica* genome (assembly: monDom5, annotation: Ensembl release 76) using Tophat2 [61, 62]. For a summary of Illumina read counts for this study see S1 Table. Read-count based differential expression was performed by Cufflinks, which normalizes by transcript length and apportions multi-mapped reads, and HTSeq which counts at the gene level and counts only uniquely mapped reads. Differential expression analysis was performed in three different algorithms: Cuffdiff, DESeq, and edgeR. Filtering was performed using log₂-fold changes and adjusted p-value ≤ 0.05 .

Quantitative PCR

All RNA used for qPCR originated from the same RNA samples used for Illumina sequencing. Prior to cDNA synthesis, all RNA used in cDNA synthesis was treated with DNase using the TURBO DNA-free Kit (Ambion) according to manufacturers' recommended protocols. 500ng of total RNA was used to make cDNA libraries by reverse transcriptase PCR (RT-PCR) using the SuperScript III First Strand Synthesis kit (Invitrogen) according to the manufacturer's instructions. All cDNA libraries were made

in triple replicates and then pooled to reduce bias generated during reverse transcription. Transcription levels of specific genes were assessed by qPCR using ABsolute Blue QPCR SYBR Green ROX Mix (Thermo Scientific) according to manufacturer's instructions. Primers used in qPCR were designed for the *M. domestica* genome and according to manufacturer's recommendations for qPCR primer properties. Primer sequences and properties are in S2 Table. All qPCR reactions were performed in triplicate repeats on an ABI Prism 7000 Sequence Detection System (Applied Biosystems) using the default cycling parameters with a dissociation step.

qPCR data were assessed by the Vandesompele method [63, 64] using *tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta polypeptide (YWHAZ)* and *TATA box-binding protein (TBP)* as reference genes. These reference genes were chosen based on relatively consistent transcription across Illumina data samples (S5 Table), as well as literature recommending them as reference genes for placental gene expression studies [65, 66]. All pregnant and virgin qPCR gene transcription levels described here were relative to the non-pregnant past breeder group, and greater than 2-fold change was considered significant. Genes were chosen for qPCR confirmation based on being differentially transcribed in the Illumina data set, having >20 reads per sample in the Illumina data set, and novel qPCR primers passing efficiency testing. All primers were generated using the online tool Primer 3 (<http://primer3.ut.ee/>, [67]) with *M. domestica* gene sequences from the MonDom5 genome assembly. Primer sets used for qPCR and their associated properties are listed in S5 Table.

Differential transcription analysis

Three separate differential expression analysis tools, Cuffdiff version 2.2.1, [68, 69], DESeq version 1.2.1 [70], and edgeR version 2.0.5 [71], were used to assess relative gene transcription between the P, N and V groups. All Venn diagrams were created using the R package VennDiagram [72]. The R package cummeRbund [73] was used to produce a dendrogram describing Jensen-Shannon (JS) distances between sample transcriptomes. The dendrogram was modified for readability in Microsoft PowerPoint. Since DESeq and edgeR use read counts and Cuffdiff uses Fragments Per Kilobase of transcript per Million mapped reads (FPKM) to calculate differential expression, the log₂-fold change and associated adjusted p-value of genes were reported in cases where differential expression analysis results of specific genes were compared. The adjusted p-value threshold was ≤ 0.05 for all used differential expression programs.

Gene Ontology analysis

Gene Ontology (GO) analysis was performed using online PANTHER tools (<http://www.pantherdb.org/> [74, 75]. Specific gene sets were tested for statistical overrepresentation or underrepresentation of PANTHER GO-Slim Biological Process terms. Gene sets were compared to the whole uterine transcriptome gene set (all genes with expression of ≥ 1 FPKM in at least one uterine sample transcriptome) and GO terms with $p < 0.05$ (Bonferroni corrected for multiple testing) were considered significant. The “Unclassified” GO category was ignored in all analyses.

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SUPPLEMENTAL TABLES AND FIGURES

<http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0161608#sec017>

CHAPTER 4

A PRONOUNCED UTERINE PRO-INFLAMMATORY RESPONSE AT PARTURITION IN A MARSUPIAL

By Victoria L. Hansen¹, Lauren S. Faber¹, Ali A. Salehpoor¹, and Robert D. Miller^{1,2}

¹Center for Evolutionary and Theoretical Immunology, Department of Biology,
University of New Mexico, Albuquerque, NM 87131 USA

²National Science Foundation, 4201 Wilson Blvd, Arlington, VA 22230 USA

ABSTRACT

Marsupials are a lineage of viviparous mammals that last shared a common ancestor with eutherians (placental mammals) over 148 million years ago. Marsupials are distinct from eutherians in that they give birth to highly altricial young. Whether or not there is regulation of maternal immune responses during marsupial pregnancy has been a matter of debate. Indeed, the evolution of short gestation times has been hypothesized to be due to a lack of immune regulation during marsupial pregnancy. Alternatively, the maternal immune system may be entirely unaware of the presence of the fetal allograft in marsupials due to lack of a highly invasive placenta. In an earlier report, we used NextGen transcriptome sequencing and found that transcripts encoding proinflammatory cytokines were differentially overrepresented in terminal pregnancy in the gray short-tailed opossum, *Monodelphis domestica*. To investigate this observation further, immune gene transcripts were quantified by real-time PCR at multiple gestational timepoints in the opossum. Analyses revealed transcripts encoding pro-inflammatory cytokines remained relatively low during pregnancy, even at implantation, but increased dramatically in abundance immediately preceding birth, peaking within twelve hours prior to parturition. These results contrast the two spikes of inflammation seen in eutherian pregnancy, first at implantation and second at parturition. Our results are consistent with the marsupial immune system being "aware" of the allogeneic fetus but under regulation and that inflammation may be an ancient contributor to parturition in mammals.

INTRODUCTION

Viviparity has evolved independently multiple times among the vertebrate lineages. The transition from oviparity to viviparity has occurred in cartilaginous fishes, teleosts, amphibians, reptiles, as well as the mammals (Blackburn, 2015). In squamates alone viviparity appears to have evolved independently over one hundred times (Blackburn, 1999a,b). With so many lineages having made the transition from egg-laying to live birth it is clear that the evolutionary challenges are readily surmountable. By looking for common mechanisms among different lineages, insights may be gained into the evolutionary solutions needed to transition from oviparity to viviparity.

Viviparity in mammals appears to have evolved once at least 166 million years ago (MYA) in a common ancestor of therian mammals (Baker et al., 2004; Bininda-Emonds et al., 2007). The sister group to the therians is the oviparous monotremes, the platypus and the echidnas, which last shared a common ancestor with therians 186 MYA (Bininda-Emonds et al., 2007). The therians include the eutherians, sometimes referred to as “placental” mammals, and the marsupials. Reproductive modes of eutherians and marsupials differ notably in the levels of pre-natal versus post-natal maternal investment. Eutherians invest more during gestation and thus give birth to more highly developed young. Marsupials, in contrast, invest comparatively less during gestation, giving birth to highly altricial young. Marsupials then utilize a complex lactation scheme to nourish the externally developing neonates (Tyndale-Biscoe & Renfree, 1987).

Among the mammals, the eutherians have been the focus of reproductive biology due to its importance to human health and the economics of livestock husbandry. Marsupials therefore provide an important comparative outgroup for understanding the

transition to viviparity in early mammals. The therian ancestor likely exhibited an intermediate of eutherian and marsupial reproductive characteristics in terms of altriciality and prenatal investment time (Werneburg et al., 2016). The reproductive characteristics shared between extant marsupials and eutherians are likely to be among those most essential, or ancestral, to viviparous mammals.

The transition from oviparity to viviparity in vertebrates creates a number of evolutionary challenges. One is the co-evolution with the immune system (Medawar, 1953). All jawed vertebrates possess an adaptive immune system capable of specific recognition of non-self antigens. Consequently, organ transplants even among closely-related (but non-identical) individuals may be immunologically rejected (Moore et al., 1960). The fetus represents an allogeneic tissue that the adaptive immune system should recognize as foreign and, if not regulated, reject (Medawar, 1953). In eutherians the fetally-derived placenta is in close contact with maternal tissues, and in some cases such as humans, in direct contact with maternal blood circulation (Moffett & Loke, 2006). Analyses of pregnancy in eutherians, particularly humans and mice, has revealed numerous mechanisms for fetal protection (reviewed in Erlebacher, 2012; Erlebacher, 2013; Racicot et al., 2014). Examples include regulation of inflammation and the complement system during pregnancy (Denny et al., 2013; Romero et al., 2007). Pregnancy complications associated with the maternal immune system have been well studied but remain poorly understood (Matthiesen et al., 2012; Roberts & Bell, 2013; Romero et al., 2006). By comparing reproductive immunology of humans (and other eutherians) to the other viviparous mammalian lineage, marsupials, we may gain a better understanding of the importance of shared characteristics.

Studies of marsupial reproductive immunology have been limited. Attempts to immunize female tammar wallabies, *Macropus eugenii*, with male alloantigens failed to reduce fecundity (Rodger et al., 1985). In addition, antibodies against paternal antigens were undetectable in maternal wallaby serum after successful pregnancies (van Oorschot and Cooper, 1988). In contrast, maternal anti-paternal antibodies are normal during pregnancy in humans and other eutherian species (Mowbray et al., 1985; Cauchi et al., 1991). So far the results are consistent with the maternal immune system either being "unaware" of the conceptus or being capable of regulating its immune response. Marsupial embryos have a maternally-derived mucoid shell coat that potentially masks paternal alloantigens prior to implantation into maternal endometrium (Freyer et al., 2003; Zeller & Freyer, 2001). This shell coat is intact for at least the first two thirds of pregnancy in marsupial species thereby leaving little time for an allogeneic response.

Previously we reported that gene transcripts associated with the immune system were overrepresented among those with increased abundance at terminal pregnancy in the opossum, *Monodelphis domestica* (Hansen et al., 2016). However, it was unclear whether the high transcription levels in these genes is only at terminal pregnancy and possibly due to imminent parturition, or if there is a lack of regulation of inflammation throughout pregnancy. In order to examine the expression of these and other cytokines known to have effects on pregnancy outcome, transcription levels were examined at multiple time points in opossum pregnancy, concentrating particularly on the stages ranging just prior to embryo implantation on day 12 to within 24 hours of parturition.

MATERIALS AND METHODS

Opossum husbandry and tissue collection

This study was approved under protocol numbers 13-100920-MCC and 15-200334-B-MC from the University of New Mexico Institutional Animal Care and Use Committee. All opossums used in this study were from a captive-bred research colony housed at the University of New Mexico Department of Biology Animal Research Facility. Founders were obtained from *M. domestica* Populations 1 and 2 at the Southwest Foundation for Biomedical Research, San Antonio, TX (VandeBerg & Robinson, 1997). All animals were fed *ad libitum* on a diet of Labdiet Short Tailed Opossum #2 pellets (90%, Lab Supply TX) and dried mealworms (10%, Lab Supply TX).

Uterine tissues were collected from opossums euthanized by isoflurane overdose followed by decapitation. For both pregnant and non-pregnant animals, uterine horns were removed, separated from ovaries and lateral vaginal canals, and dissected in shallow petri dishes filled with RNALater buffer (Ambion). For histology samples, one intact uterine horn was put into buffered formalin solution and incubated for 48 hours at 4°C before being transferred to 70% ethanol solution for storage at 4°C until paraffin-embedding. For RNA samples, uterine horns were opened laterally, any visible embryos were removed, and endometrial tissue was excised in 100-200mg samples. Tissues were collected from time points on embryonic days 3 (e3), 9 (e9), 10 (e10), 11 (e11), 12 (e12), 13 (e13), 14 (e14), and postnatal day 1 (p1). In addition, non-pregnant (np) control uterine tissue was collected from females who had given birth three to six months prior to collection. There were three biological replicates collected for each pregnancy time point. In pregnant samples from time points e12, e13, and e14 the invasive fetal placenta membranes invaded maternal

endometrium and could not be separated out and therefore these samples contained fetal membranes. After a 24-hour incubation at 4°C in RNALater, the tissue samples were removed and stored at -80°C until RNA extraction.

RNA extraction and cDNA synthesis

All RNA extractions were performed by first freezing tissues in liquid nitrogen and then homogenizing them using a sterile liquid-nitrogen cooled mortar and pestle. Subsequently, 1mL TRIzol (Ambion) for every 100mg of tissue was added to the mortar and homogenized with the powdered tissue until the mixture warmed enough to become liquid again. The homogenized tissue was then phase-separated by adding 0.2mL chloroform for every 1mL TRIzol used and shaken vigorously for 15 seconds. The samples were incubated at room temperature for three minutes and then centrifuged at 12,000g for 15 minutes at 4°C. The clear aqueous upper phase was transferred to a sterile tube and an equal amount of RNase-free 70% ethanol was added to the tube. Then the RNA was isolated using the PureLink RNA Mini Kit (Ambion) according to manufacturer's instructions for purifying RNA from animal tissues. The resulting total RNA samples were purified of DNA contamination using the TURBO DNA-free Kit (Ambion) according to manufacturers' recommended protocols.

All cDNA libraries were generated by reverse transcriptase PCR (RT-PCR) using the SuperScript III First Strand Synthesis Kit (Invitrogen) according to manufacturer's instructions for generating cDNA from poly(A) RNA. A total of 500ng of RNA was used for each RT-PCR reaction and reactions were performed in triplicate for each sample. The RT-PCR reactions were pooled by individual tissue sample and 87µL of PCR-grade water

was added to bring the total volume of cDNA to 150 μ L. cDNA samples were stored at -20°C until use in qPCR reactions.

Quantitative PCR

All qPCR reactions were performed in triplicate using 18 μ L SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) and 2 μ L cDNA for each sample. All reactions were performed on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad) under the following conditions: 95°C for 3 minutes, then 40 cycles of 95°C for 10 seconds followed by 60 seconds at the appropriate annealing temperature during which data was collected. All primer sequences may be found in Table 1. qPCR data was analyzed in the CFX Manager Software (Bio-Rad) using tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta polypeptide (*YWHAZ*) and TATA box-binding protein (*TBP*) as reference genes for normalization. These reference genes were chosen based on their relatively uniform transcription across pregnant and non-pregnant opossum uterine tissues (Hansen et al., 2016). Transcription levels were tested for significant change in a time series (e3 through p1) using one-way ANOVA with Sidak test of multiple comparisons. Pregnant and post-natal samples were also tested for significantly different transcription against the non-pregnant samples using one-way ANOVA with Dunnett's multiple comparisons test. The threshold for significance in all statistical tests was $p < 0.005$. All statistical tests and graphs were generated using Prism 7 (GraphPad) and edited for clarity in Illustrator (Adobe).

Table 1. qPCR primer sequences.

Gene	Primer Direction	Primer Sequence
YWHAZ*	Forward	AAAGACGGAAGGTGCTGAG
	Reverse	CCTCAGCCAAGTAACGGTAG
TBP*	Forward	GTGCCCGAAATGCTGAATAC
	Reverse	TTTCCTGGCTGCTAATCTGG
IL-1 α	Forward	TGACAATGACAGCCTTCCAG
	Reverse	TGCTGCCCTGTATTGGAAC
IL-1 β	Forward	TTTGACCAACGCCAGTGAAC
	Reverse	CTTCCCATCACGCTTCACAC
IL-6	Forward	ATCACAGAGCGAGATGGATG
	Reverse	GTGCTCAGCTTTAGGGTTTG
IL-8	Forward	CCAAACCTTTCCATCCCAAG
	Reverse	CCCAATTTGCATGAGGATCC
IL-10	Forward	CCAACATGCTCCGAGATCTTC
	Reverse	CCCTCCTCATTCTCTGCCTG
IL-17A	Forward	CCCGATTACAAGAACCGCTC
	Reverse	ATTGATGCAGCCTGAGTAGC
TNF α	Forward	CCTGTGGCTCATGTTGTAGC
	Reverse	GGAGCTGAGAGTAGACCAGG

* Reference gene for normalization

RESULTS

In a previous study of opossum uterine transcriptomes we found that some cytokine gene transcripts were substantially more abundant at terminal pregnancy relative to non-pregnant samples (Hansen et al. 2016). These included pro-inflammatory cytokines such as *IL-1 α* , *IL-6*, *IL-10*, *IL-17A*, and *TNF α* . Due to the fact that the pregnancy time point assessed in that study was during the last 24 hours of gestation, the apparent inflammation could not be definitively attributed to pregnancy in general versus implantation and/or parturition specifically. Here we have assessed the transcription of these pro-inflammatory cytokine genes in additional pregnancy time points, focusing in particular on time points around implantation and parturition. Instead of performing RNAseq, we used qPCR to quantify cytokine transcript levels. We normalized transcript levels relative to two reference genes, *YWHAZ* and *TBP*.

In a study of uterine transcriptomes, the *IL-1 α* gene had significantly more transcripts in terminal pregnant than non-pregnant samples (Hansen et al. 2016). After examination of transcription by qPCR, *IL-1 α* gene did not appear to be transcribed at the fetomaternal interface at the earliest time point, embryonic day 3 (Figure 1A). However, *IL-1 α* was transcribed on embryonic days 9, 10, and 11 (Figure 1A). The transcript level decreased on e12, the day the embryo implants into the maternal endometrium, and remained low on e13 as well (Figure 1A). *IL-1 α* transcript levels significantly increased on e14, the last day of pregnancy (Figure 1A). Transcription decreased significantly on post-natal day 1 (Figure 1A). The only time point that *IL-1 α* transcription significantly differed from non-pregnant levels was e14 ($p < 0.005$).

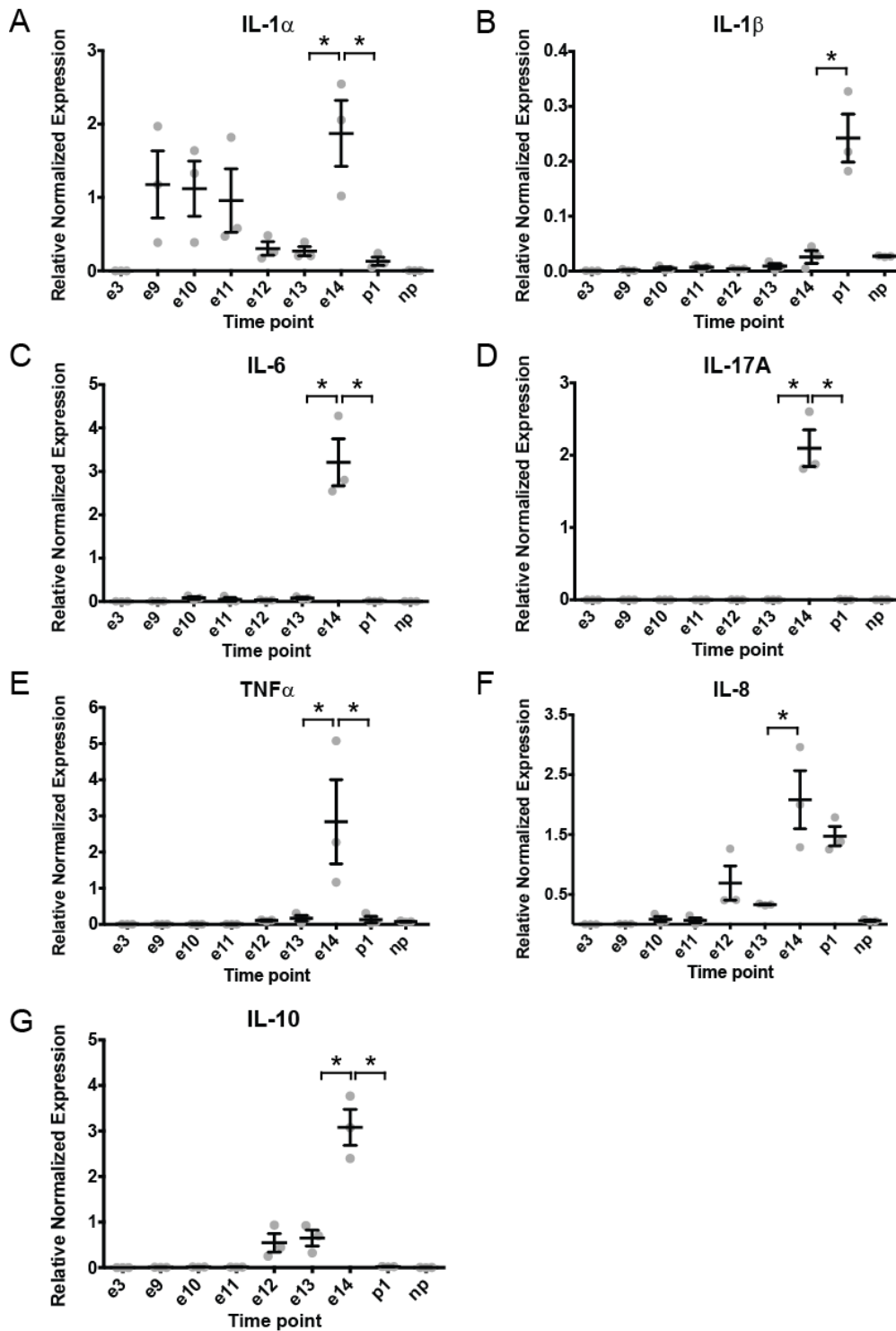


Figure 1. Relative normalized cytokine transcript abundance as measured by qPCR. Lines and bars represent mean and upper and lower SEM. Gray dots represent expression levels of individual samples. Transcription level difference was tested between consecutive time points by one-way ANOVA with Sidak's multiple comparisons test, * $p < 0.005$.

Although *IL-1 β* was not a significantly differentially transcribed gene according to opossum uterine transcriptome results, its expression changes at the fetomaternal interface during pregnancy and labor in humans (Elliott et al., 2001; Hansen et al., 2016). According to qPCR results *IL-1 β* consistently had low transcript levels throughout pregnancy until after parturition in opossum uterine tissue (Figure 1B). Between terminal pregnancy and post-natal day 1 there was a significant increase in *IL-1 β* transcription (Figure 1B). Post-natal day 1 also differed significantly from the non-pregnant state of *IL-1 β* transcription ($p < 0.005$, Figure 1B).

IL-6, *-17A*, and *TNF α* were all cytokines with significantly more transcripts in pregnant versus non-pregnant tissue according to the opossum uterine transcription study as well (Hansen et al. 2016). All three cytokine genes had very similar transcription patterns wherein there was little to no transcription of these cytokines at any time point aside from e14 at impending parturition (Figures 1C,D,E). e14 was also the only time point where transcription of these cytokines was significantly different from non-pregnant transcription levels ($p < 0.001$).

Although *IL-8* was not one of the original cytokines identified as upregulated during opossum pregnancy, but it was examined here because it is associated with parturition in eutherian pregnancy (Hansen et al., 2016; Osmers et al., 1995). *IL-8* transcripts were negligible during pregnancy until the implantation time point e12 (Figure 1F). Transcript levels significantly increased on e14 at term pregnancy (Figure 1F). *IL-8* transcription did decrease, though not significantly, between e14 and p1 (Figure 1F). Non-pregnant transcription of *IL-8* was significantly lower compared to both e14 ($p < 0.001$) and p1 ($p < 0.005$).

IL-10 transcripts were identified as more abundant in pregnant than non-pregnant opossum tissue in the transcriptome study (Hansen et al., 2016). Moreover, *IL-10* is a cytokine that is thought to play an important part in regulating the maternal immune system during eutherian pregnancy (Thaxton & Sharma, 2010). In pregnant opossum uterine tissues *IL-10* transcripts were not detectable until e12 (Figure 1G). Transcription was evident on e12 and e13 (Figure 1G). Transcription of *IL-10* significantly increased at terminal pregnancy on e14 (Figure 1G). *IL-10* transcripts then fell to undetectable levels on post-natal day 1 (Figure 1G). Non-pregnant transcription levels were significantly lower only in comparison to e14 ($p < 0.0001$).

DISCUSSION

Inflammation at the fetomaternal interface is one of the major causes of pre-, peri-, and post-natal pregnancy complications (Romero et al., 2007). In humans, inflammation at the fetomaternal interface can lead to spontaneous abortion, pre-eclampsia, and is even associated with lifelong conditions affecting the child such as autism and schizophrenia (Meyer et al., 2009; Patterson, 2009). However, at two points during eutherian pregnancy inflammation appears to be beneficial and, perhaps, even necessary for successful gestation. The first is during the peri-implantation period when the embryo implants into the maternal endometrium (Mor et al., 2011). Studies have shown that human *in vitro* fertilization even benefits from a minor wound at the implantation site to induce an inflammation response (Dekel et al., 2010). The second point when inflammation is beneficial is at parturition. Furthermore, this inflammation appears to be dependent upon an allogeneic immune response. In cows, for example, if the mother and fetus are too genetically similar the allogeneic response is insufficiently strong to expel the fetal tissues from the womb (Davies et al., 2004). Since eutherians benefit from inflammation during both implantation and parturition, marsupials, which share a common viviparous ancestor with eutherians, would be expected to share this reproductive trait. Marsupial immunology at the fetomaternal interface has potential to reveal similarities and differences in eutherians, and shed light on previously overlooked characteristics.

Here we have shown that the transcription of cytokines during parturition at the fetomaternal interface in a model marsupial, *M. domestica*, is similar to the pattern of cytokine expression seen in eutherian parturition. On day 14 of opossum pregnancy, less than 24 hours before birth, there was significantly increased transcription of *IL-1 α* , *IL-6*,

IL-8, *IL-10*, *IL-17A*, and *TNF α* . However, there did not appear to be significant increases in cytokine transcription at implantation. These results possibly suggest a more ancient role of cytokines at parturition than during implantation for mammals.

IL-1 α was the only cytokine we assessed that exhibited moderate levels of transcription prior to implantation (Figure 1A). Embryonic day 12 is when the opossum embryo implants into the maternal endometrium and nourishment shifts from being provided by uterine secretions to hemotrophe from maternal circulation (Freyer et al., 2002; Zeller & Freyer, 2001). In mice *IL-1 α* is expressed by endometrial cells during the pre-implantation period (Noda et al., 2005; Takacs & Kauma, 1996). In murine pregnancy embryos implant around day 5 of gestation which is also when *IL-1 α* expression decreases (Noda et al. 2005). This is similar to the pattern we observed in *M. domestica* uterine tissues where *IL-1 α* transcription decreased on e12 (Figure 1A). The *IL-1 α* transcription levels did not significantly increase until e14 at parturition before dropping again on p1 (Figure 1A). *IL-1 α* is a cytokine that has increased expression at parturition in humans, though not as much as *IL-1 β* (Taniguchi et al., 1991).

Throughout opossum pregnancy there was very little transcription of *IL-1 β* and the only significant increase is on p1, after parturition has taken place (Figure 1B). Expression of *IL-1* is observed not only in mammalian term uterus, but also in those of squamate and cartilaginous fishes (Cateni et al, 2003; Paulesu et al., 1995; Paulesu et al., 2005a,b; Taniguchi et al., 1991). Therefore the *IL-1* system is likely an important component of parturition in even the earliest viviparous vertebrates that has been preserved in many extant lineages (Paulesu et al., 2005b). In humans, *IL-1 β* expression is upregulated in fetal membranes in the third trimester compared to the second and first (Elliott et al., 2001).

Since human blastocysts express IL-1, IL-1 receptors, and IL-1 antagonists, there is speculation that the IL-1 system is used as a means of communication and regulation between embryo and mother (van Mourik et al., 2009)

IL-6 transcription at the fetomaternal interface in the opossum was low prior to parturition on e14 and dropped after parturition as well (Figure 1C). In human labor IL-6 levels are also elevated at the fetomaternal interface (Dudley et al., 1996; Steinborn et al., 1998; Steinborn et al., 1999).

IL-17 is a highly pro-inflammatory cytokine that has been implicated as a factor in preeclampsia cases (Satner-Nanan et al., 2009). The transcription of *IL-17A* at the opossum fetomaternal interface was tightly restricted to only e14, with no detectable transcription in 40 cycles of qPCR for any other time point measured (Figure 1D). Pongchareon and colleagues showed that IL-17 is expressed in human terminal pregnancy explants as well (2007). Unfortunately, their examination did not include earlier pregnancy time points for comparison.

The pattern of transcription over the course of pregnancy for *TNF α* was virtually identical to that of *IL-6* and *IL-17A* in the opossum (Figure 1E). *TNF α* is a cytokine that has been shown to be elevated in human parturition (Dudley et al., 1996). However in other eutherians, such as cows, *TNF α* expression actually decreases at parturition (van Engelen et al., 2009), indicating that elevated *TNF α* levels at this time point is not a strongly conserved mammal characteristic.

Increased IL-8 expression is associated with parturition in eutherians (Osmers et al., 1995; Sennström et al., 1997; van Engelen et al., 2009). Therefore we expected to see *IL-8* transcription peak on e14 (Figure 1F). It is somewhat surprising that *IL-8* transcription

was not identified as significantly increased in our previous transcriptome study (Hansen et al., 2016). A possible explanation is that the differential transcription calling parameters used were too conservative to include *IL-8* in the list of significant genes.

There appeared to be minimal *IL-10* transcription prior to e12 in opossums (Figure 1G). On e12 *IL-10* transcription increased from e11 though not significantly. It was only on e14, at term parturition, when *IL-10* transcription significantly increased. By p1 *IL-10* transcription was again negligible. In human placenta *IL-10* expression has been reported as down-regulated at normal term labor compared to first and second trimester expression levels (Hanna et al., 2000). An earlier report in humans did not find a significant change in *IL-10* expression between labored and non-labored decidual cells (Jones et al., 1997). Regardless of which report is most representative of human cytokine production at labor, our observations of *IL-10* transcription in opossum placental tissues diverged from the human norm.

We discovered most of the high levels of cytokine transcription was restricted to the 24 hours pre-parturition time point on embryonic day 14 (e14). This is similar to the pre-parturition expression of cytokines seen in eutherians (Bowen et al., 2002). However we did not observe the cytokine expression during implantation that is present in pregnant eutherians (Mor et al., 2011). Therefore we propose that pro-inflammatory cytokine expression at the fetomaternal interface is an ancient characteristic of the therian lineage and is possibly a key factor in the evolution of mammalian parturitions.

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CHAPTER 5

TRANSCRIPTIONAL REGULATION OF COMPLEMENT COMPONENTS AND REGULATORS DURING PREGNANCY IS ANCIENT AND CONSERVED IN MAMMALS

By Victoria L. Hansen¹, Ali A. Salehpoor¹, Lauren S. Faber¹ and Robert D. Miller^{1,2}

¹Center for Evolutionary and Theoretical Immunology, Department of Biology,
University of New Mexico, Albuquerque, NM 87131 USA

²National Science Foundation, 4201 Wilson Blvd, Arlington, VA 22230 USA

ABSTRACT

Pregnancy in viviparous animals presents an evolutionary challenge of reconciling the presence of an adaptive immune system without inadvertently mounting an allogeneic attack on the fetus. One arm of the immune system that is regulated during pregnancy is the complement system. Complement can cause harm to fetal tissues by inducing inflammation and/or cell lysis. In eutherian mammals, such as humans and mice, the complement system is modulated at the fetomaternal interface largely by the complement regulators such as DAF, CD46, and CD59. Dysregulation of complement or complement regulators during pregnancy can lead to pregnancy complications at best or fetal loss in severe cases. By studying the role of complement during pregnancy in distantly related viviparous mammals, such as marsupials, we stand to gain better insight into the evolutionary solutions used to transition to viviparity. Here we demonstrate that regulation of the C' system is an ancient and conserved feature of mammalian pregnancy. In the opossum, *Monodelphis domestica*, transcript levels are reduced for complement components C3 and C4 throughout pregnancy. Known complement regulators CD46 and CD59 are present at the fetomaternal interface during *M. domestica* pregnancy as well. These results are consistent with controlling the complement system being ancient and conserved in mammals. These results also support regulating the complement system may have been critical in the transition from oviparity to viviparity in mammals over 165 million years ago.

INTRODUCTION

Mechanisms to protect the fetus and fetal membranes from immune injury are critical to successful pregnancy in viviparous vertebrates. This protection is mediated by regulating or inhibiting a variety of immune mechanisms (reviewed in Erlebacher, 2013). One of these mechanisms is the complement (C') system. C' is a collection of blood proteins that act in a cascade to mediate a variety of immune responses. C' is ancient in deuterostomes and highly conserved across vertebrates. The effects of C' are broad including puncturing holes in cellular membranes and stimulating apoptosis of both bacterial and animal cells, stimulating inflammation, and facilitating phagocytosis (Sarma & Ward, 2011).

The first evidence of the impact of C' at the fetomaternal interface in humans was produced by Faulk and colleagues (1980). C' at the fetomaternal interface is thought to contribute to pathology such as recurrent spontaneous abortions (RSA) and antiphospholipid antibody syndrome (APS) (Girardi et al., 2006). Mutations in C' genes is strongly correlated with women who suffer from pregnancy-associated atypical hemolytic uremic syndrome (Fakhouri et al., 2010). A deleterious mutation of a murine-specific C' regulator, Crry, results in lethal inflammation in mouse embryos (Xu et al., 2000; Mao et al., 2003). Most research into the effects of C' at the fetomaternal interface has been limited to eutherian mammals.

Evaluating the regulation of immune mechanisms such as C' during pregnancy in a wide variety of species should give insight into the role this regulation played in the evolution of viviparity. Marsupials are eutherians' nearest living viviparous relatives. Marsupials have been shown to lack some fetal protection mechanisms seen in eutherians

such as generating regulatory T cells at the fetomaternal interface (Samstein et al., 2012). Other mechanisms, such as the inhibition of inflammation appear to be conserved (Hansen et al., 2016). How "aware" the maternal immune system is of fetal tissues during marsupial pregnancy however, remains a debate. A study in tammar wallabies, *Macropus eugenii*, did not find evidence of anti-paternal antibodies generated by maternal immune systems (van Oorschot & Cooper, 1988). Anti-paternal antibodies are common in eutherian pregnancy, and even have been shown to be protective to the fetus in some species (Antczak et al., 1984; Chaouat et al., 1985; Orgad et al., 1999). Therefore it is possible that marsupials may have differences in C' regulation at the fetomaternal interface during pregnancy as well. So far C' activity has been shown for the Australian marsupial *Phascogale calura*, and American opossums *Didelphis virginiana* and *Monodelphis domestica* (Croix et al., 1989; Koppenheffer et al., 1998; Ong et al., 2015; Wirtz & Westfall, 1967). C' genes have been identified in marsupials with publicly available genomes (Ong et al., 2016).

Previously we have shown that a large number of genes involved in the immune response are differentially regulated during pregnancy in the opossum (Hansen et al., 2016). Here we follow up on these results focusing on the C' system and C' regulatory genes.

MATERIALS AND METHODS

This study was approved under protocol numbers 13-100920-MCC and 15-200334-B-MC from the University of New Mexico Institutional Animal Care and Use Committee. All uterine tissues collected were as in Chapter 4. Briefly, pregnant and post-natal *M. domestica* uterine horns were excised, dissected, treated with RNALater (Ambion), and stored at -80°C until use. Uterine samples were collected on embryonic days 3 (e3), 9 (e9), 10 (e10), 11 (e11), 12, (e12), 13 (e13), 14 (e14), post-natal day 1 (p1), and from non-pregnant past breeders (np) as a control.

All RNA and cDNA samples were as in Chapter 4. Briefly, RNA was phenol-extracted and isolated. cDNA libraries were generated from 500ng RNA using the SuperScript III First Strand Synthesis Kit (Invitrogen) according to manufacturer's instructions. All cDNA synthesis reactions were performed in triplicate to minimize bias in cDNA library generation.

qPCR methods performed were as described in Chapter 4. All reactions were performed on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad). Novel *M. domestica*-specific primers were generated according to specifications for SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) and sequences may be found in Table 1. Relative normalized expression levels were calculated in CFX Manager software (Bio-Rad) by the Livak method using tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta polypeptide (*YWHAZ*) and TATA box-binding protein (*TBP*) as reference genes. These reference genes were chosen due to their relatively uniform transcription levels in both pregnant and non-pregnant uterine transcriptome data (Hansen et al., 2016). Transcription levels were tested for significant change in a time series (e3

Table 1. qPCR primer sequences.

Gene	Primer Direction	Primer Sequence
YWHAZ*	Forward	AAAGACGGAAGGTGCTGAG
	Reverse	CCTCAGCCAAGTAACGGTAG
TBP*	Forward	GTGCCCCGAAATGCTGAATAC
	Reverse	TTTCCTGGCTGCTAATCTGG
C3	Forward	TTTGTCTGTGCCCTCAACGG
	Reverse	GACGCCCAGCCTTCAACATC
C4	Forward	GCTTCGTCTCTATGTGGCAG
	Reverse	GCCCTGTAGCAAGAAAGGG
C5	Forward	GAACATTGAACGGACCCTG
	Reverse	TGGGAGATGGCTTAGAGTAG
C6	Forward	AAGTGGAGAACAGGTGTACC
	Reverse	CAGCCACTCAGTAAACACAG
C7	Forward	GATGGTCTAGCAGTGGTTG
	Reverse	CCTTACTTTCTCCCTTCTG
C9	Forward	TTCTCCCTGCCATACTGATG
	Reverse	GCTTGCGGTAATAGGTTCC
CD46	Forward	TCCAGGTGTCAGGATTCGG
	Reverse	TGGTCTACTTGTAGCAGGTGG
DAF	Forward	CTTCAGAATGTGGTGTGGTC
	Reverse	GTCTTCTGCTCCCAATAACC

* Reference gene for normalization

through p1) using one-way ANOVA with Sidak's test of multiple comparisons. Transcription levels of pregnant and post-natal samples were tested against non-pregnant samples for differential transcription levels using one-way ANOVA with Dunnett's multiple comparisons test. The threshold for significance was $p < 0.05$ for all statistical tests. Statistical tests and graphs were generated using Prism 7 (GraphPad) and edited for clarity in Illustrator (Adobe).

RESULTS

Previous analyses of terminal pregnant uterine transcriptome in the opossum revealed immune related genes to be among the most represented among those with differential transcript abundance relative to non-pregnant (Hansen et al 2016). To extend these observations we focused primarily on transcripts encoding the C' system and its regulators and an increased number of timepoints in gestation.

The C' component C3 is among the most ancient and conserved of the components, and is central to all three pathways for C' activation (Sarma & Ward, 2011; Smith et al., 1996). Throughout pregnancy, C3 transcript levels were almost undetectable and significantly lower than non-pregnant uterine tissues. One day following birth, postnatal day 1 (p1) transcript levels appeared to be increasing, returning to non-pregnant levels (Figure 1A). The transcription of C3 was significantly lower in e3, e9, e10, e11, e12, e13, e14, and p1 than in non-pregnant samples. A similar pattern was found for transcripts encoding the C4 C' component (Figure 1B).

C5, C6, C7, C8, and C9 proteins each make up a portion of the terminal MAC that inserts into a cell membrane (Sarma & Ward, 2011). C5 transcript levels from e3 through e12 remained nearly undetectable. Beginning at e13 they began to rise in abundance (Figure 1C). Non-pregnant transcription levels of C5 were significantly lower in the samples from e3, e9, e10, e11, e12, e13, and p1. C6 had little to no transcription before e12 as well (Figure 1D). C6 transcription levels in pregnant samples e3, e9, e10, and e11 were significantly less than in non-pregnant samples (Figure 1D). Transcript abundance for C7 was higher in earlier pregnancy than for other complement components measured (Figure 1E). The changes in transcription levels between consecutive time points were not large

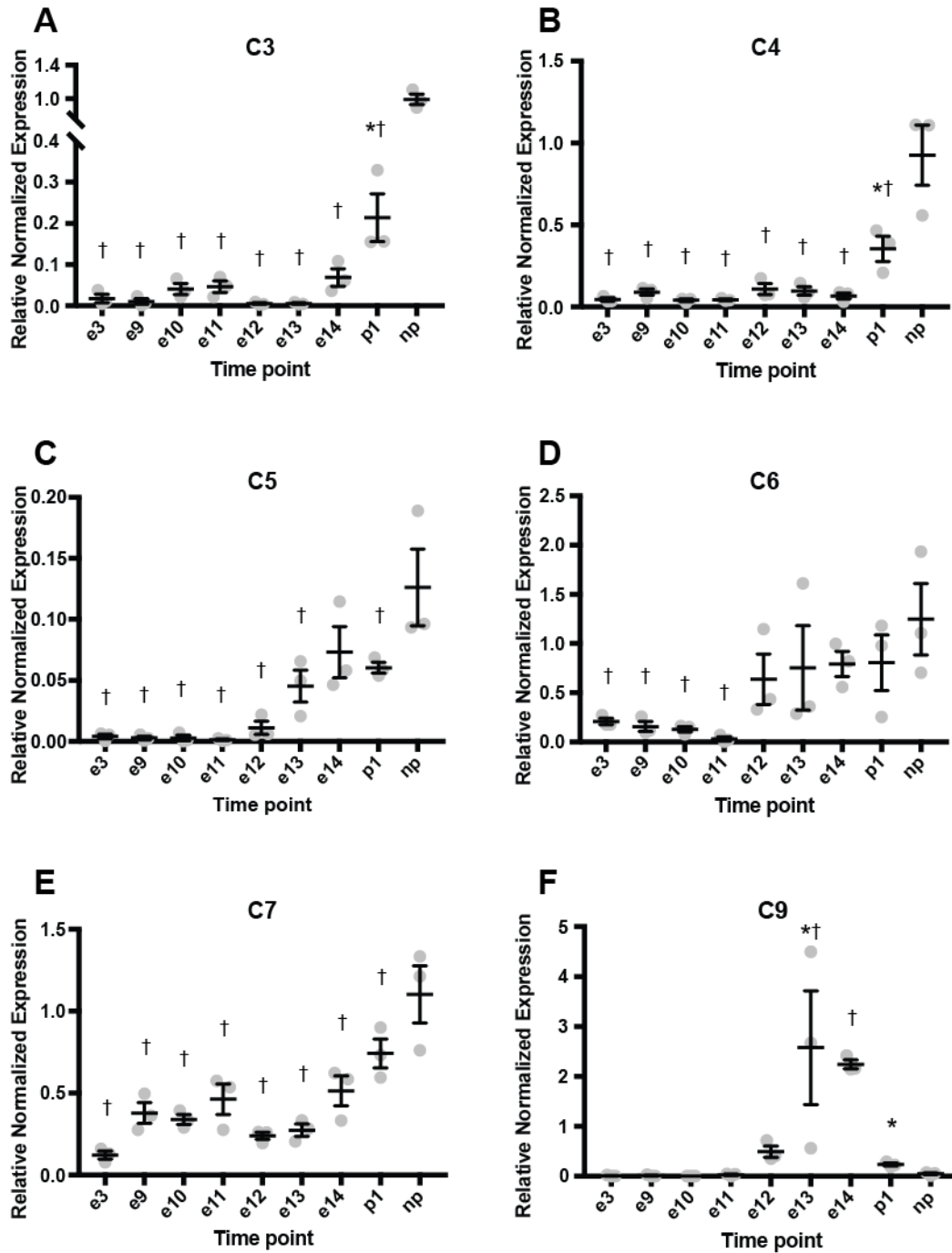


Figure 1. Relative normalized expression levels of complement components as measured by qPCR. Lines and bars represent mean and upper and lower SEM. Gray dots represent expression levels of individual samples. * $p < 0.05$ for difference from previous time point according to ANOVA with Sidak's test of multiple comparisons. † $p < 0.05$ for difference from non-pregnant (np) according to ANOVA with Dunnett's multiple comparisons test.

enough to be significant. However the non-pregnant transcription levels of *C7* were significantly higher than at all other time points. *C8* was not assessed because a working efficient qPCR primer was not successfully produced. *C9* transcription levels at the opossum fetomaternal interface remained negligible until e12 and significantly increased on e13 (Figure 1F). There was an unusually high amount of variation between individuals for *C9* transcription levels on e13. Transcription levels did not significantly change between e13 and e14, but they did drop significantly after parturition on p1 (Figure 1F). Transcription of *C9* in non-pregnant samples was significantly lower than e13 and e14.

C' regulators are important during pregnancy in humans and mice to protect fetal placental membranes from damage by the C' cascade (Girardi et al. 2009; Xu et al. 2000). Previous analyses of the opossum uterine transcriptome at terminal pregnancy revealed that transcripts for Decay Accelerating Factor (*DAF*), an inhibitor of C3 convertase, were relatively low compared to non-pregnant (Hansen et al., 2016). This was unexpected given the importance in *DAF* in regulating C' activity in eutherians. To determine if this result was due to the transcriptome analysis focusing on a terminal pregnancy time point alone, we tested for *DAF* transcripts in the other time points. Since *CD46* and *CD59* are additional C' regulators that protect human pregnancy we tested these as well (Holmes et al., 1992).

In opossum uterine tissue there was no significant change in the transcription of either *DAF* or *CD46* over the course of pregnancy and parturition (Figure 2A,B). The result of *DAF* having significantly higher transcription in non-pregnant samples than all other time points assessed was unexpected (Figure 2B). Non-pregnant levels of *CD46* transcription were higher than those at e3 and e10 but did not significantly differ from any other time points (Figure 2B). *CD59* transcription did not have any statistically significant

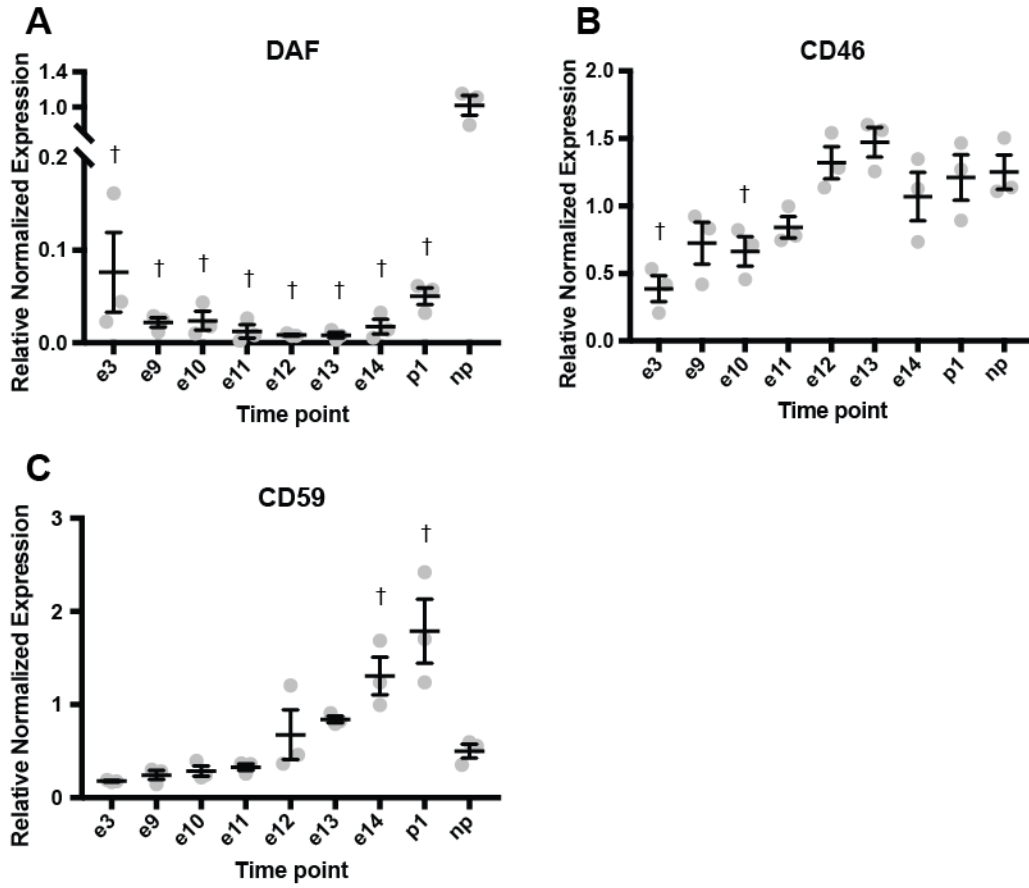


Figure 2. Relative normalized expression levels of complement regulators as measured by qPCR. Lines and bars represent mean and upper and lower SEM. Gray dots represent expression levels of individual samples. * $p < 0.05$ for difference from previous time point according to ANOVA with Sidak's test of multiple comparisons. † $p < 0.05$ for difference from non-pregnant (np) according to ANOVA with Dunnett's multiple comparisons test.

changes between time points (Figure 2C). Non-pregnant *CD59* transcription levels were significantly lower than those measured around parturition at e14 and p1.

DISCUSSION

Pregnancy complications resulting from immunological problems are still not fully understood despite extensive research in humans and mice. Abnormal C' action during pregnancy is associated with complications such as RSA, APS, and preeclampsia (Girardi et al., 2006; Lynch & Salmon, 2010; Tincani et al., 2010). However, systemic increase in C' activation is also common in normal human pregnancy (Richani et al., 2005). By studying C' action during pregnancy in a more diverse set of viviparous species, there is the potential to gain insight into the molecular mechanisms of C'-related pregnancy complications.

C' genes and proteins are extraordinarily well-conserved across vertebrates, and some components are even present in invertebrate species (Goshima et al., 2016; Smith et al., 1996; Zhu et al., 2005). Vertebrates with a viviparous lifecycle, such as mammals, gestate fetuses that are potentially vulnerable to attack by the maternal immune system. Due to fetuses being half non-self from the maternal perspective, it is generally thought that the adaptive immune system recognizing and responding to paternal alloantigens would be the primary source of conflict. However, pregnancy complications are often associated with abnormal C' action at the fetomaternal interface despite being part of the innate immune system (Denny et al., 2013).

Here we have assessed transcription of C' component and regulator genes at the fetomaternal interface during pregnancy in a marsupial. Marsupials are viviparous mammals that form the sister group to the eutherians (Bininda-Emonds et al., 2007). Mammalian viviparity arose prior to the marsupial-eutherian split (Baker et al., 2004).

Therefore, conserved elements in their reproductive immunology are likely to be essential in maintaining a viviparous lifecycle.

We assessed transcription of C' components genes *C3*, *C4*, *C5*, *C6*, *C7*, and *C9*. The earliest C' gene we targeted for assessment by qPCR was *C3* because it is considered to be one of the most integral C' components due to it being involved in the initiation of all three C' pathways (Sarma & Ward, 2011). C' components *C3* and *C4* were an exception with transcription remaining low throughout pregnancy and only significantly increasing after birth (Figures 1A,B). This may indicate that excessive transcription of *C3* and *C4* could harm the pregnancy. One of *C3*'s cleavage products, *C3a*, is an anaphylatoxin that can be as dangerous to fetal tissues as the MAC itself (Girardi et al., 2005, 2011). This is a good reason for C' regulators that inhibit C3 convertases being present at the fetomaternal interface.

Human trophoblast contacts the maternal blood directly and the presence of these C' regulators is thought to prevent attack by circulating maternal C' (Girardi et al., 2011). In human trophoblast C' regulators DAF, CD46, and CD59 are expressed during pregnancy starting as early as six weeks into gestation (Holmes et al., 1992). An unexpected result was that DAF had dramatically reduced transcription during pregnancy than non-pregnant controls (Figure 2A). DAF inhibits C3 and C5 convertases in the Classical and Lectin pathways (Liszewski et al., 1996). If there is little to no *C3* present during *M. domestica* pregnancy there may not be a need for DAF at the fetomaternal interface. In human trophoblast DAF is primarily expressed on cells that directly contact maternal circulation (Holmes et al., 1992). Given that in *M. domestica* the trophoblast invades maternal

endometrium but does not break into maternal vessels (Freyer et al., 2007), perhaps DAF is not necessary at the fetomaternal interface in opossums.

CD46 inhibits C3 convertases in all three C' pathways, including the Alternative Pathway (Liszewski et al., 1996). *CD46* is transcribed during later *M. domestica* pregnancy at similar transcription levels as non-pregnant samples (Figure 2B). Therefore, there is probably still a need for protection from C3 convertases to an extent at the fetomaternal interface. Significantly lower transcription of *CD46* in early pregnancy time points could be attributed to embryos not yet having implanted into the maternal endometrium. It is also important to note that what we have called the *CD46* gene in *M. domestica* is technically *CD46-like* and its nucleotide identity was not similar enough to human *CD46* to call it as such (Ong et al., 2016). Nucleotide sequence databases such as NCBI and Ensembl have the gene we assessed here as *CD46* listed as *CD46-like*.

In general, we observed that transcription of MAC-forming C' components remained low during pre-implantation pregnancy time points e3, e9, e10, and e11 (Figure 1). After implantation on e12 the transcription of *C5*, *C6*, *C7*, and *C9* increased (Figures 1C,D,E,F). *CD59*, like the downstream C' components assessed, also increased in transcription after implantation and through parturition (Figure 2C).

Anti-paternal antibodies capable of activating C' have not been found to be produced by marsupial mothers during pregnancy so the Classical Pathway may not be a danger to fetal tissues (van Oorschot & Cooper, 1988). In cross breedings between mouse strains that produce anti-paternal antibodies in maternal circulation, the antibodies produced did not induce the Classical Pathway of C' (Bell & Billington, 1980). However, in a mouse model of APS, anti-phospholipid (aPL) antibodies were observed activating the

Classical Pathway, which lead to pregnancy loss (Girardi et al., 2004). C5, but not C6, was required for aPL-mediated pregnancy loss in mice (Girardi et al., 2003; Redecha et al., 2007). This indicates damage was primarily due to inflammation induced by anaphylatoxin C5a, the cleavage product of C5 that does not become part of the MAC (Girardi et al., 2003; 2011). It is possible that *M. domestica* and other marsupials species do generate anti-paternal antibodies during normal pregnancy that simply do not activate the Classical Pathway.

In conclusion, relative to non-pregnant uterus, transcription of key components of the C' system appears to be kept relatively low in the opossum. This apparent active down-regulation of the C' genes appears to start early in gestation and is maintained throughout pregnancy. It also appears that immediately following parturition, transcription of many of the C' components appears to return to "normal" or non-pregnant state. All this implies that there is potential harm to the embryo and/or fetus by the C' system in marsupials and regulating C' was part of the evolutionary process leading to viviparity early in mammals.

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Editors

Azad K. Kaushik

Yfke Pasma



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Edited by

Azad K. Kaushik, DVM, DSc (Paris), and Yfke Pasman, PharmD, MSc
University of Guelph, Guelph, Ontario Canada



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CHAPTER 4

MARSUPIAL AND MONOTREME IMMUNOGLOBULIN GENETICS

ROBERT D. MILLER and VICTORIA L. HANSEN

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ABSTRACT

Much of the history of mammalian immunology has focused on only one of the three living lineages of mammals: the eutherians, more commonly called the placentals. This is not surprising given that humans and nearly all economically important mammals are eutherian. This chapter reviews what is known about immunoglobulin genetics in the other two lineages, the marsupials and the monotremes. Marsupials appear to have a fairly minimal complement of heavy chain isotypes and limited variable region diversity. Instead they appear to rely more on light chain complexity for generating a diverse antibody repertoire. Monotremes, in contrast have a more diverse collection of heavy chain isotypes and have retained more heavy chain variable region gene complexity than have the marsupials. Monotremes, more than the other two mammalian lineages have also retained more of the characteristics shared with our reptile-like ancestors. This is true of their immunoglobulin genes in addition as evidenced by a novel heavy chain isotype in the platypus with characteristics of both IgG and IgY.

4.1 INTRODUCTION

In the late 1970s, R. B. Ashman lamented the lack of progress on the study of immune systems of noneutherian mammals but predicted, or perhaps hoped for, a brighter future for this field (Ashman, 1977). He would be pleased to know that advances in comparative genetics, genomics, and whole genome sequencing has significantly advanced the fields of marsupial and monotreme immunology in the 21st century. Notable progress has been made in two representative species, the South American gray short-tailed opossum, *Monodelphis domestica*, and the duckbill platypus, *Ornithorhynchus anatinus*. This chapter summarizes what is known about marsupial and monotreme immunoglobulin (Ig) genetics and the corresponding relationships to B cell ontogeny and antibody repertoire development.

4.1.1 MAMMALIAN RELATIONSHIPS AND LIFE HISTORY

To put this chapter into perspective it is important to remember that there are two living subclasses of extant mammals: Subclass Prototheria, containing Order Monotremata (hereafter referred to as the monotremes), and Subclass Theria (Fig. 1). The Therians include two infraclasses: the Marsupialia and the Placentalia, hereafter referred to as the marsupials and eutherians, respectively. These lineages last shared a common ancestor at least 185 million years (MY) and perhaps greater than 200 MY ago (O'Leary et al., 2013). Research on the immune systems of monotremes and marsupials is well established and has been driven by interest in both evolutionary and veterinary immunology. Of particular interest has been the interplay between the immune system, maternal immunology, and differences in reproductive strategies among the monotremes, marsupials, and eutherians.

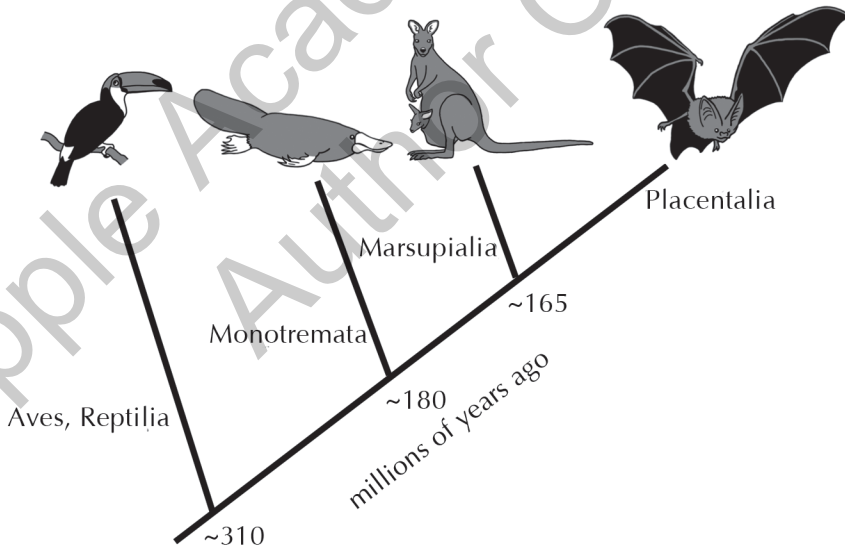


FIGURE 1 Phylogenetic relationships among the major amniote lineages. The numbers at each of the nodes represent time since the last common ancestor (based on Kumar and Hedges 1998; O'Leary et al., 2013).

Marsupials, like eutherians, are viviparous, however, they give birth to young that are in an extremely altricial state. Marsupials and eutherians last shared a common ancestor 145 to 165 MY ago. There is evidence that marsupials once inhabited a greater geographic range as fossils of mammals resembling metatherians have been found in North America and China (Luo et al., 2003). Today the greatest number and diversity of marsupial species is found in Australasia.

Monotreme biogeography is currently limited to Australia and Indonesia although, as with marsupials, the fossil record reveals a once larger geographic range as well (Pascual et al., 1992). In addition to the platypus there are four living recognized species of echidna (spiny anteaters) belonging to the genera *Tachyglossus* and *Zaglossus*. Monotremes have retained many characteristics shared with mammals' reptile-like ancestors, the most distinctive of which is the laying of eggs. Egg-laying mammals raise a number of questions regarding the evolution of transmission of maternal immunity to offspring, as well as providing models to investigate the origins of features of the immune system that are mammal specific: (e.g., the root origins of IgG and IgE) (Vernersson et al., 2002). Lamentably, information on the development and function of monotreme immune systems remains sparse. No monotreme species is currently bred in captivity for research purposes resulting in limited access to material for these species. However, probably due to their iconic place in the fauna of Australia, the platypus has played a more prominent role in monotreme research and immunology. The platypus is, so far, the only monotreme species for which there is currently a whole genome sequence available.

4.2 MARSUPIAL POSTNATAL IMMUNOLOGY

At birth most marsupial species are developmentally similar to fetal eutherians (Deane and Cooper, 1988). Upon leaving the birth canal neonatal marsupials crawl up their mother's abdomen to find a teat. Neonates latch on by their mouths and begin to suckle for several weeks, a period of time that varies between species. The teats are usually, but not always, inside a pouch structure referred to as a marsupium. The opossum *M. domestica*, for example, is a species that lacks a pouch and the young are exposed

while attached to the teats. Marsupials essentially complete the equivalent of fetal development while continuously attached to the teats, after which they will begin to leave the teat but remain in the pouch or nest, suckling intermittently as necessary similar to a newborn eutherian.

The newborn marsupial thymus is primarily undifferentiated epithelium, and neonatal marsupials are generally unable to generate humoral or cellular adaptive immune responses until late in the first or second postnatal week (Kalmutz, 1962, La Via et al., 1963, Rowlands et al., 1972, Hubbard et al., 1991, Old and Deane, 2000, Wang et al., 2012a). Not surprisingly neonatal marsupials are dependent entirely on maternal factors for immune protection for a sustained period of time after birth. Milk plays an obvious important role in conferring protection.

Marsupial lactation is more complicated than that of eutherians (Adamski and Demmer 2000, Daly et al., 2007, Kuruppath et al., 2012). Following a colostrum phase, there are three distinct phases referred to as early, switch, and late. Some investigators refer to these phases as 2A, 2B, and 3; where phase 1 corresponds to prenatal changes in the mammarys during gestation. The early lactation phase (phase 2A) produces milk that is generally high in carbohydrates but low in fat content. This early phase corresponds to when the newborn is continuously attached to the teat. Late phase milk (phase 3) is lower in carbohydrates but increased fat content and its production correspond to when the neonates are not yet weaned but are independent of the teats. Some species of marsupials, members of the kangaroo family in particular can have more than one neonate (joeys) at different stages of development. Hence there can be two mammary glands side-by-side producing different phases of milk.

Although marsupials do have some form of placental structure, typically late in gestation, most marsupials do not obtain maternal Ig *in utero*. Only one species, the tammar wallaby *Macropus eugenii*, has been shown to transfer maternal IgG to the fetus via the yolk sac (Deane et al., 1990). All other marsupials studied depend entirely on milk for maternal IgG and IgA. Maternal IgG in the newborn opossum, for example, can be detected by the end of the first postnatal day (Samples et al., 1986). The transfer of maternal Ig through milk follows a pattern that mirrors the different phases of the milk. At the start of the early milk phase there is a spike of IgA that rapidly declines. There is a second peak of IgA transfer during

the switch (phase 2B) when the neonate is transitioning to leaving the teat (Adamski and Demmer, 1999). Presumably the early transfer of IgA may play an important role in the early establishment of a gut microbiome. The second transfer may be preparing the neonate for greater exposure to ingested antigens and potential pathogens in solid food. There appears to be a peak of IgG detected in the early lactation phase, which declines, but is followed by a second increase in IgG in the late phase as well (Adamski and Demmer, 2000). Mammary expression of the poly Ig receptor (pIgR) and the neonatal IgG Fc receptor (FcRN) appears to mirror the expression of the different Ig isotypes. It is intriguing to speculate that, in addition to varying the nutrient content of milk to suit the stage of development of the suckling young, the mammary glands are also adjusting the class of antibody transferred to meet the needs for protection.

4.3 MARSUPIAL IMMUNOGLOBULIN GENETICS

4.3.1 CONSTANT REGION ISOTYPES

IgH chain constant region sequences have been reported for a variety of marsupial species. However, the exact genomic content and organization of the *IgH* locus has only been determined for one marsupial species, the opossum *M. domestica*. *M. domestica* may have the simplest, or most generic, of all mammalian *IgH* loci (Fig. 2). There are four functional IgH constant region genes, IgM, IgG, IgE and IgA. The IgG, IgE and IgA are single copy genes. There is a second pseudogene copy of C μ upstream of the functional C μ . Both copies of C μ have three J $_H$ gene segments just upstream, one of which is a pseudogene; an arrangement that appears to be the result of an [ψ J $_H$ - J $_H$ - J $_H$ - C μ] duplication (Wang et al., 2009). The J $_H$ gene segments upstream of the pseudogene copy of C μ are not used during V(D)J recombination (Wang et al., 2012a).



FIGURE 2 Diagram of the opossum *IgH* constant region (not to scale). The ψ symbol designates pseudogenes. Gray boxes represent J $_H$ gene segments and solid black boxes designate identified switch regions. It should be noted that sequences resembling canonical mammalian switch regions were only identified upstream of the two C μ genes. The box labeled ERV indicates the expected location of C δ exons but they appear to have been replaced with ERV and LINE retroelements.

IgD is absent from the opossum genome and has not been reported in any marsupial species. The region of the IgH locus downstream from the C μ exons where one would expect to find the C δ genes contains a stretch of inserted retro-elements including endogenous retrovirus (ERV) and LINE elements, that appear to have deleted or replaced IgD from the opossum germline (Fig. 3) (Wang et al., 2009). Whether this is the case for other marsupial species is not yet known.

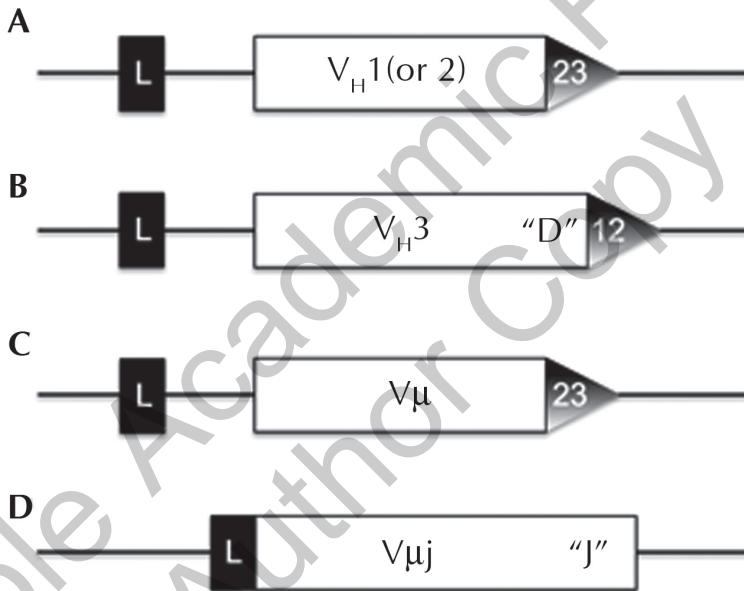


FIGURE 3 Diagram illustrating the basic structure of (A) a typical opossum germline V_H gene, (B) the partially germline joined opossum $V_H 3$ gene, (C) a typical opossum germline V_μ gene, and (D) the germline joined opossum $V_\mu j$ gene.

4.3.2 GERMLINE VARIABLE REGION DIVERSITY

What emerged from one of the first studies of expressed antibody diversity was the presence of limited heavy chain V gene (V_H) complexity (Miller et al., 1998). All amniote V_H genes group into one of three Clans (I, II, and III) based on nucleotide sequence identity. In analyzes of opossum Ig heavy chain cDNAs, only two V_H gene subgroups were found and both

were Clan III members. As it turns out, all marsupial V_H genes described so far are Clan III members; furthermore, all marsupial V_H genes practically form a single major subclade within Clan III (Baker et al., 2005). During the evolution of the *IgH* locus in marsupials there appears to have been a major bottleneck in the diversity of V_H genes. It is not unusual to find limited germline V_H gene diversity in individual mammalian species (e.g., rabbits), however, in this case it is an entire class of diverse mammals. This suggests that the genetic bottleneck in V_H gene diversity occurred very early in marsupial evolution (Baker et al., 2005).

In the opossum, genomic analysis revealed three V_H gene subgroups. V_H1 is the only multimember family, makes up the majority of germline V_H genes, and contributes to the majority of expressed heavy chain V_H domains (Table 1, Wang et al., 2012a). The $V_H2.1$ subgroup, composed of a single member, is also expressed early in development at a frequency proportional to its representation in the germline genome (Baker et al., 2005, Wang et al., 2012a). The third subgroup, $V_H3.1$, also a singleton, is unusual in that it is partially germline joined and will be discussed in its own evolutionary context later in this chapter.

TABLE 1 Number and complexity of V, D, and J gene segments at the Ig heavy and light chain loci in the opossum, *Monodelphis domestica*.

<i>IgH</i>										
Numbers of Gene Segments (Total/Functional ¹)										
V_H subgroups	Total V_H	V_H1	V_H2	V_H3	D	J_H				
3	26/21	24/19	1/1	1/1	9	6/4				
<i>Igk</i>										
Numbers of Gene Segments (Total/Functional ¹)										
V_K subgroups	Total V_K	V_K1	V_K2	V_K3	V_K4	V_K5	V_K6	V_K7	J_K	C_K
7	122/89	32/25	45/33	22/17	5/3	5/5	1/1	12/5	4	1
<i>Igλ</i>										
Numbers of Gene Segments (Total/Functional ¹)										
$V_λ$ subgroups	Total $V_λ$	$V_λ1$	$V_λ2$	$V_λ3$	$V_λ4$	$J_λ$	$C_λ$			
4	64/58	54/48	7/7	2/2	1/1	8	8			

¹Functional includes gene or gene segments that have an open reading frame and appear to have all the necessary components to be expressed based on genomic sequence, independent of whether they have been found in a cDNA sequence or not.

There are nine recognized D_H gene segments in the opossum, and all are both functional and used for V(D)J recombination (Wang et al., 2012). They vary in length and repertoire analysis revealed that the shorter D_H gene segments are overrepresented in productive rearrangements. As stated earlier in the chapter, of the four functional J_H gene segments only two are used and these are the two immediately upstream of the functional C_H exons (Wang et al., 2012a).

4.3.3 GERMLINE JOINED V GENES IN THE MARSUPIAL GENOMES

One of the more unusual features of the opossum *IgH* locus is the presence of a single, partially germline joined V_H gene (Wang et al., 2009). Called $V_H3.1$, it is so far the only such case reported in a mammal and is a V_H gene with a pre-joined D_H (Fig. 3). $V_H3.1$ was recognized as being a partially germline joined V_H due to its extended length and the presence of a recombination signal sequence (RSS) containing a 12 base pair spacer, typical of D_H gene segments, rather than a 23 bp RSS at the end of V_H genes (Wang et al., 2009). $V_H3.1$ was only discovered through the analysis of the whole genome sequence and had been absent from previous analyzes of expressed *IgH* cDNA sequences (Miller et al., 1998). However, its use and germline joined nature were confirmed recently when transcripts containing recombined $V_H3.1$ segments were detected in mature, young opossums (Wang and Miller, 2012). These transcripts contained evidence that when $V_H3.1$ undergoes V(D)J recombination it is joined directly to a J_H gene segment. The late detection of $V_H3.1$ recombinants most likely reflects its rare use in the *IgH* repertoire. This may be due to a direct V_H to J_H recombination being unusual and out of order for normal V(D)J recombination during B cell development. Alternatively, B cells rearranging $V_H3.1$ are at a functional disadvantage due to having a long complementarity-determining-region-3 (CDR3) resulting from the fixed D_H segment at the end of this V gene (Wang and Miller, 2012). $V_H3.1$ or its equivalent has not been reported for any other marsupial species and may be unique to *M. domestica*.

The presence of a germline joined V_H in the opossum raises a number of interesting questions regarding the origin of such genes. This has been studied in sharks where germline joined V_H are more common and it is clearly due to ectopic expression of the RAG recombination activating genes in germ cells (Lee et al., 2000). A similar explanation for the origin of $V_H3.1$ in the opossum is likely, although it resulted in only a partial rearrangement of a V_H and D_H . This is a more straightforward scenario than has been proposed for the only other known germline joined V genes found in mammals. Marsupials and monotremes are unique in having an extra T cell receptor (TCR) chain called $TCR\mu$ (Parra et al., 2007). In the marsupials $TCR\mu$ is unusual in that it uses a complete germline joined V gene called $V\mu j$ to encode one of the V domains (Fig. 3). $V\mu j$ is found in all marsupial species and apparently the germline joining event has a single ancient common origin for all species (Parra et al., 2007, 2012). $V\mu j$ is unique from other germline joined V genes in that it is missing the intron separating the leader exon from the V exon (Fig. 3). This has led to speculation that the origin of $V\mu j$ also involved a retro-transposition event (Parra et al., 2007, 2012). It is noteworthy that monotremes have a homologue of $TCR\mu$, however, the gene segments corresponding to $V\mu j$ are not germline joined and still require V(D)J recombination for expression (Wang et al., 2011). This indicates that the $V\mu j$ germline joining event was unique and early in the marsupial lineage.

Although $V\mu j$ and $V_H3.1$ appear to have followed different evolutionary origins, the presence of both in at least *M. domestica* indicates the role that ectopic RAG expression in germ cells has shaped the germline Ig and TCR repertoires in marsupials. The functional role or advantage to the presence of the partially germline joined $V_H3.1$ remains unclear. In sharks, germline joined V_H genes are expressed early in ontogeny and may play a functional role in young animals (Rumfelt et al., 2001). This does not seem to be the case for opossum $V_H3.1$ as it appears late and sparingly in the developing antibody repertoire (Wang et al., 2012a).

4.3.4 MARSUPIAL LIGHT CHAINS

Marsupials, like all mammals, have both the $Ig\kappa$ and $Ig\lambda$ light chains (Lucero et al., 1998; Miller et al., 1999; Belov et al., 2001, 2002). Like

the *IgH* locus, the complete genomic composition and complexity of the light chain loci has only been established for a single marsupial species, the opossum *M. domestica* (Wang et al., 2009). One of the more noteworthy observations from this analysis was the greater germline V gene complexity for light chains as compared to that of heavy chains (Table 1, Baker et al., 2005; Wang et al., 2009). This appears to be a pattern common to all marsupial species studied so far (Baker et al., 2005). Indeed it has been proposed that light chains may make a greater contribution to marsupial antibody repertoire diversity than does the heavy chain (Baker et al., 2005). The limited *IgH* but complex *Igκ* and *Igλ* diversity found in early developing B cells in the opossum supports this hypothesis (Wang et al., 2012).

The overall organization of the *Igκ* and *Igλ* loci in the opossum are well conserved for a mammal. The *Igκ* locus contains a single *Cκ* gene located at the 3' end of the locus. There are four *Jκ* genes and all are used in the expressed repertoire (Wang et al., 2009, 2012). The *Igκ* locus is the most complex with regards to V genes, containing seven different subgroups (Miller et al., 1999; Wang et al., 2009). The *Igλ* locus is also well conserved and contains eight *Cλ* genes, each with its own *Jλ* gene segment upstream (Lucero et al., 1998; Wang et al., 2012). The repeated *Jλ*-*Cλ* organization is conserved in *Igλ* loci in all therian mammals. Phylogenetic analyzes of *Cλ* from marsupials and other mammalian species implicate that these genes cluster by species (Lucero et al., 1998). Some investigators have explained this pattern of evolution as being the result of gene duplication, deletion, and replacement following speciation (Bengten et al., 2000). Concerted evolution may provide a preferable alternative explanation. Since the *Cλ* genes in each species function redundantly and must interact with the same heavy chains, there would be selection to homogenize the sequences through some gene conversion-like mechanism.

4.3.5 POSTNATAL B CELL ONTOGENY AND ANTIBODY REPERTOIRE DEVELOPMENT

As was stated earlier in this chapter, much of the immune system development in marsupials appears to take place postnatally. One of the more

thorough analysis of B cell ontogeny in a marsupial was recently published for the opossum *M. domestica* (Wang et al., 2012a). Based on expression of early B cell markers such as CD79a and CD79b, initiation of B cell ontogeny could be detected in *M. domestica* embryos within the final 24 hours of gestation. Birth in the opossum takes place approximately 14.5 days post copulation and within the first 24 hours following birth there is evidence of V(D)J recombination. The diversity of V gene recombinants is relatively low, most likely due to the low number of pre-B cells at this stage. The majority of heavy chain V(D)J recombinants lack N region additions to their junctions in spite of concomitant Terminal deoxynucleotidyl transferase (Tdt) expression (Wang et al., 2012a). Indeed, for the first two postnatal weeks the endogenous Ig heavy chain repertoire is fairly limited.

The next developmental B cell stage detected is defined by the expression of the VpreB surrogate light chain. VpreB can be identified on postnatal day 6 in the opossum (Wang et al., 2012b). Unlike eutherian mammals, which can have up to three VpreB genes (VpreB1, 2, and 3), the opossum only has a VpreB3 homologue. The function of VpreB3 is not completely understood and evidence from the expression of this gene in chicken B cells suggests that it is not expressed on the cell surface. The opossum genome also appears to lack a gene for the $\lambda 5$ surrogate light chain. It is possible, therefore, that the marsupials do not have a cell surface pre-B cell receptor (BCR) similar to eutherians, but rather are more like nonmammalian vertebrates such as birds. How quality control of heavy chain V(D)J recombination and subsequent initiation of light chain rearrangement is regulated during marsupial B cell development, without the “conventional” pre-BCR as it is known from humans and mice, is not fully understood. However, likely VpreB3 expression does play a role on stimulating light chain gene rearrangement in the opossum due to the close timing of these two events (Wang et al., 2012a, b).

Light chain gene rearrangement, beginning with the *Ig λ* locus can be first detected on postnatal day 7, followed one day later by *Ig κ* rearrangements. Unlike the heavy chain repertoire the light chain repertoire is diverse from the start. An 8- to 9-day-old opossum has similar light chain diversity to what is found in the adult repertoire (Wang et al., 2012a). This observation is consistent with an earlier hypothesis based on germline V

gene complexity that light chains may contribute more to the antibody diversity than do heavy chains (Baker et al., 2005).

One conclusion that can be drawn from B cell ontogeny studies in the opossum is that it is unlikely that this species is able to generate endogenous, antigen specific antibody responses until at least the second postnatal week of life. This conclusion is entirely consistent with previous studies of antibody responses in other newborn marsupial species. In most cases where it has been tested, neonatal marsupials are unable to generate antigen specific T-dependent antibody responses until the 7th postnatal day or later (Kalmutz 1962; La Via et al., 1963).

Analyzes of B cells in developing opossums also enabled investigation into the role of somatic mutation in diversifying the primary antibody repertoire. Species with limited germline V gene diversity, as in marsupials, often rely on somatic mutation to contribute additional diversity not achieved through V(D)J recombination alone (reviewed in Butler, 1997). This process typically involves a gut associated lymphoid organ like the appendix in rabbits or Peyer's patches in sheep. Reports of early detection of B cell markers in gut tissues of neonates lead to speculation that a similar process may be going on in marsupials (Old and Deane, 2003). However, there is little evidence in the opossum that their primary antibody repertoire is being diversified through additional somatic mutation (Wang et al., 2012a). Analyzes of large sequence databases of expressed Ig heavy and light chain V(D)J recombinants when compared with a reasonably high quality germline genomic sequence reveal no evidence of mutation. Indeed, the extent and importance of affinity maturation in marsupial memory B cell responses is not yet clear.

Lastly, when the analysis of B cell development in marsupials was extended to later developmental time-points, the timing of the appearance of isotype switch recombined B cells was assessed. One observation is that IgA production could not be detected in the gut or other organs until the animals were 8 weeks of age (Wang et al., 2012a). This is noteworthy as it corresponds with the time point when opossums are fully weaned. In other words, although they have been off the teats for nearly 4 weeks, with the possibility of ingesting antigens and have a well established gut microbiome, there has been little stimulation of IgA production in the gut associated lymphoid tissue. This observation suggests that maternal

antibodies, likely IgA, in the milk are playing an important role on regulating gut microbes and pathogens throughout the lactation period.

4.4 MONOTREME IMMUNGLOBULIN GENETICS

Monotremes are a puzzling lineage of mammals because they have retained a number of characteristics usually associated with nonmammalian vertebrates. This was made evident when in 1884 Caldwell sent from Australia to the British Royal Society the four-word telegram “Monotremes oviparous, ovum meroblastic.” His message was that they were egg laying and the egg is structured more like that of a bird, reptile or fish, rather than the holoblastic egg of a marsupial or eutherian (Caldwell, 1887). In addition to being egg-laying, they expel waste through a cloaca like a bird or reptile and the males have venom glands that are active during breeding season in the platypus, but may be vestigial in the echidnas. Monotreme genomics has also revealed a number of ancestral characteristics. Their sex chromosomes (which are unusual in there being 10 of them per diploid cell!) have conserved synteny with both the XY system found in marsupials and eutherians, as well as the WZ system found in birds (Grützner et al., 2004). Curiously the MHC region is located on the sex chromosomes in monotremes as well (Dohm et al., 2007).

Monotreme immunology has been greatly enhanced over the past decade with the development of molecular resources including a whole genome sequence of the duckbill platypus, *O. anatinus* (Warren et al., 2008). Fortunately this has included the study of monotreme immunoglobulins. Like the rest of monotreme anatomy and physiology, monotreme immune systems share characteristics with nonmammalian tetrapods. For example like marsupials, the monotremes have a clear homologue of the TCR μ T cell receptor chain, but the platypus TCR δ locus also contains V_H-related V genes similar to what has been found in birds and amphibians (Parra et al., 2010, 2011, 2012a, b, Wang et al., 2011).

4.4.1 MONOTREME IG HEAVY CHAIN GENETICS

4.4.1.1 MONOTREME CONSTANT REGIONS AND ISOTYPES

The discovery that platypus and echidna have both IgG and IgE pushed the origin of these uniquely mammalian heavy chain isotypes back as far as the earliest mammals (Vernersson et al., 2002, 2004). The most complete view of the antibody isotypes found in monotremes comes from genomic analyzes in the platypus. The platypus *IgH* locus contains exons for a single IgM and IgD, two IgG subclasses, two IgA subclasses, and a single IgE (Zhao et al., 2009). Nested between the IgD and IgG₂ exons are the genes encoding a novel isotype. Zhao and colleagues named this isotype IgO (for *Ornithorhynchus*), whereas Gambón-Deza and colleagues described these genes as being IgY (Gambón-Deza et al., 2009; Zhao et al., 2009). The reason for the different designations is understandable. This isotype had not been seen before in mammals and appeared unique to the platypus, hence the IgO. On the other hand, both groups recognized that IgO shared similarity to IgG, IgE and the IgY found in birds and reptiles. Platypus IgO/IgY appears to be an immunoglobulin missing link, although phylogenetic analyzes put it much closer to mammalian IgG than to avian IgY (Zhao et al., 2009). Platypus IgD is also unusual for a mammal. It shares more structural similarity to the IgD of reptiles and fish by having 10 C_H domains, rather than the typical three found in eutherian IgD (Gambón-Deza et al., 2009; Zhao et al., 2009).

4.4.1.2 VARIABLE REGION GENES AND ANTIBODY DIVERSITY

Earlier in this chapter it was noted that all marsupial V_H genes are similar, even across distantly related species (Baker et al., 2006). This is not true of monotremes. The platypus *IgH* locus contains only a single V_H subgroup that belongs to Clan III (Johansson et al., 2002). Genomic analysis estimated there to be 44 total V_H genes (Gambón-Deza et al., 2009). V(D)J recombination in the platypus produces among the longest and most diverse CDR3 found in any mammal, which is thought to compensate for limited

germline V_H diversity (Johansson et al., 2002). In contrast to platypus, the short-beaked echidna *T. aculeatus* germline V_H repertoire is highly diverse (Belov and Hellman, 2003). The echidna uses at least seven different subgroups that are derived from all three V_H Clans (Wong et al., 2009).

4.4.1.3 MONOTREME IGH LIGHT CHAIN GENETICS

Like all mammals, the monotremes have both $Ig\kappa$ and $Ig\lambda$ (Nowak et al., 2004, Johansson et al., 2005). $Ig\lambda$ V region diversity has been analyzed in the platypus, where there are only two $V\lambda$ subgroups. As in the platypus antibody heavy chain CDR diversity, both long and sequence, is thought to compensate for limited germline $V\lambda$ diversity (Johansson et al., 2005). The platypus $J\lambda$ - $C\lambda$ genes are organized in tandem pairs like in other mammals. The exact number of $J\lambda$ - $C\lambda$ pairs has not been determined for the platypus but based on sequence it appeared to be at least four.

The monotreme $Ig\kappa$ locus also has typical mammalian organization. There appears to be a single $C\kappa$ gene and, although the exact number is not known, there seems to be at least eight to ten $J\kappa$ gene segments (Nowak et al., 2004). The platypus and echidna $Ig\kappa$ loci appear to contain at least four and nine $V\kappa$ subgroups, respectively (Nowak et al., 2004). Therefore the monotreme $Ig\kappa$ diversity likely relies more on germline complexity for generating antibody diversity than does the $Ig\lambda$ locus.

4.5 CONCLUSIONS

In conclusion for the marsupial section of this chapter, much as been learned about antibody genetics and B cell ontogeny in marsupials, taking advantage of a limited number of model species for which resources have been developed. These resources include a high quality genome sequence for at least one species, the gray short-tailed opossum, and captive bred, pedigreed colonies for research (Samollow, 2006). However, most comparative analyzes of the immune systems within the marsupial lineage have found them to be remarkably homogeneous, particularly for immunoglobulins and T cell receptors (Baker et al., 2005, 2010; Parra et al., 2007,

2010). The opossum Ig loci are, with the possible exception of missing IgD, among the most generic and simplified of Ig genes among mammals. It is tempting to speculate that much of what has been discovered in the opossum may also be true for other marsupials, which is a bit surprising given the diversity of organization and complexity that exists within the eutherian mammals (Butler, 1997). In the end, the appearance of homogeneity may yet be an artifact due to the limited number of species examined.

Monotremes hold a special position in mammalian evolution and the results that emerge from their study never ceases to astound. Several important conclusions have emerged from the analysis of monotreme Ig genetics. The evolution of uniquely mammalian antibody classes such as IgG and IgE clearly occurred very early in mammalian evolution and the relationship these two antibody isotypes have to non-mammalian IgY is most evident from genes found in the platypus. Monotremes also continue to illustrate how closely related lineages are often on their own evolutionary paths towards generating antibody diversity. The platypus and the echidna have retained very different levels of germline diversity.

KEYWORDS

- B cells
- development
- diversity
- immunoglobulins
- marsupials
- monotremes

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Ellen Hsu

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rdmiller@unm.edu

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Department of Physiology and
Pharmacology
SUNY Downstate Medical Center
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rdmiller@unm.edu

The Evolution and Structure of Atypical T Cell Receptors

Victoria L. Hansen and Robert D. Miller

Abstract The T cell receptor structure and genetic organization have been thought to have been stable in vertebrate evolution relative to the immunoglobulins. For the most part, this has been true and the content and organization of T cell receptor genes has been fairly conserved over the past 400 million years of gnathostome evolution. Analyses of TCR δ chains in a broad range of vertebrate lineages over the past decade have revealed a remarkable and previously unrealized degree of plasticity. This plasticity can generally be described in two forms. The first is broad use of antibody heavy chain variable genes in place of the conventional V δ . The second form containing an unusual three extracellular domain structures has evolved independently in both cartilaginous fishes and mammals. Two well-studied vertebrate lineages, the eutherian mammals such as mice and humans and teleost fishes, lack any of these alternative TCR forms, contributing to why they went undiscovered for so long after the initial description of the conventional TCR chains three decades ago. This chapter describes the state of knowledge of these unusual TCR forms, both their structure and genetics, and current ideas on their function.

1 Introduction

At the center of adaptive immune responses are the receptors that specifically recognize epitopes or antigens associated with pathogens. In the jawed vertebrates, or gnathostomes, the first of these receptors to be discovered were the antibodies, later also called immunoglobulins (Ig). Antibodies were discovered as a soluble activity in the bloodstream of immune animals in the late nineteenth century (von Behring 1901). The structure of Ig and the use of Ig as an antigen receptor on the surface of antibody producing B cells had been established by the end of the 1960s (Raff 1970; Porter 1973). It would not be until the early 1980s, nearly a century

V.L. Hansen • R.D. Miller (✉)

Department of Biology, Center for Evolutionary and Theoretical Immunology, University of New Mexico, Albuquerque, NM 87131, USA

e-mail: rdmiller@unm.edu

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after the discovery of antibodies, that the antigen receptors used by a second lineage of gnathostome lymphocytes, the T cells, would be discovered (Hedrick et al. 1984).

The discovery of the T cell receptors (TCRs) was the result of a series of elegant experiments by Mark Davis and colleagues. They identified T cell-specific transcripts that appeared to be from germ-line genes undergoing somatic DNA recombination and encoding proteins with homology to immunoglobulins (Ig) (Hedrick et al. 1984). We now know that those first transcripts encoded the TCR β chain, and within the next 2 years the Davis laboratory and other investigators would identify three additional TCR chains: TCR α , TCR γ , and TCR δ (Saito et al. 1984; Waldmann et al. 1985; Brenner et al. 1986; Loh et al. 1987). The discovery of four distinct TCR chains quickly leads to the recognition of two distinct T cell subpopulations defined by the composition of their TCR heterodimers: the $\alpha\beta$ T cell and the $\gamma\delta$ T cell (Havran and Allison 1988). Over the next decade it was firmly established that all jawed vertebrates had $\alpha\beta$ and $\gamma\delta$ T cells and that the genes encoding the TCR chains were well conserved from mammals to sharks (Rast et al. 1995, 1997).

The conservation of α , β , γ , and δ TCR chains and their pairing rules across gnathostomes is in contrast to the immunoglobulins (Ig) where there has been lineage-specific evolution of novel isotypes, such as IgG and IgE being unique to mammals, IgX in the amphibians, IgA in amniotes, IgT in teleost fish, etc. (Hsu et al. 1985; Warr et al. 1995; Danilova et al. 2005; Hansen et al. 2005). This contrast between Ig and TCR may not be surprising since TCRs primarily perform antigen recognition and signaling roles, whereas the Igs are both recognition/signaling cell surface receptors and effector molecules in their secreted or soluble forms (Raff 1970). Pressure to evolve isotypes with different functions or characteristics has acted on the Ig molecules in ways that are not exerted on TCR. For example, all bony vertebrates, from teleosts to mammals, have evolved an Ig specialized for mucosal surfaces. This role is performed by IgT in teleosts, IgX in amphibians, and IgA in amniotes (Mußmann et al. 1996; Zhang et al. 2010). Some isotypes have been lost in different lineages. The IgD/IgW family, for example, appears to have been retained and lost independently many times in evolution (Ota et al. 2003; Ohta and Flajnik 2006).

In contrast to the Ig isotypes, the $\alpha\beta$ and $\gamma\delta$ TCR appear to have been retained throughout gnathostome evolution without much modification. Indeed, some basic “rules” to the immune system appear to have been established for T or T-like cells early in vertebrate evolution (Criscitiello and de Figueiredo 2013). All vertebrates have one lymphocyte type that can convert its antigen receptor to a secreted effector molecule called the antibody, and two other cell types that do not secrete their receptor. B cells and $\alpha\beta$ and $\gamma\delta$ T cells fulfill this function in gnathostomes (Reviewed in Criscitiello and de Figueiredo 2013). Agnathans, such as the lamprey and hagfish, have variable lymphocyte receptor B (VLRB) expressing cells that secrete VLRB molecules as antibody. They also have VLRA+ and VLRC+ cells that do not secrete their receptor and may have functions analogous to $\alpha\beta$ and $\gamma\delta$ T cells, respectively (Guo et al. 2009; Kasamatsu et al. 2010). This rule evolved independently of the protein structure used to form the receptors. Gnathostomes use

Ig domain-based receptors, whereas agnathans use leucine-rich repeat (LRR) domain-based receptors (Pancer et al. 2004).

Although Ig and TCR are independent gene families in extant vertebrates, their common evolutionary origin is unquestioned (Marchalonis et al. 1997). The conservation of the enzymatic machinery for DNA rearrangement, the recombination recognition sequences that flank the gene segments, and the conserved gene organization all speak to a single evolutionary origin of Ig and TCR.

An early theory on the nature of the TCR predicted that T cells were using a different set of constant regions but the same variable (V), diversity (D), and joining (J) gene segments as antibodies. This hypothesis predicted that there would be constant region genes uniquely expressed in T cells that would be linked to the constant regions of the Ig heavy (*IgH*) chain locus. Testing this hypothesis, Frances Owen and Roy Riblet generated monoclonal antibodies (mAb) against mouse T cells and selected for those that recognized epitopes linked to the *IgH* locus on chromosome 12. They used cross-immunizations between *IgH* congenic strains to generate allotype-specific mAb (Owen and Riblet 1984). While successful in identifying *IgH*-linked T cell epitopes, these studies did not lead to the discovery of the TCR as hoped. Nonetheless, today their ideas seem prescient considering the recent discoveries of how Ig and TCR genes have teamed up to produce novel TCR forms in some vertebrate lineages. These and other novel TCRs found in non-eutherian mammals and non-mammals are the topic of this chapter.

Rather than present the discoveries made over the past decade chronologically, this chapter will attempt to present these findings in a way that aids in understanding of how the TCR δ locus may have evolved to produce a new TCR loci. As will be discussed, the TCR δ locus appears to have demonstrated a degree of plasticity not found in other TCR genes.

2 The Link Between Ig and TCR

In all sarcopterygian vertebrates examined, from coelacanths to humans, the genes encoding the TCR δ chain are nested within, or tightly linked to, those encoding TCR α (Isobe et al. 1988; Parra et al. 2008, 2012a, b; Saha et al. 2014). In some teleost fishes, TCR α and TCR δ also appear to be tightly linked, making a case for this being an ancient relationship (Fischer et al. 2002; Nam et al. 2003). One feature of the nested arrangement is an overlapping pool of V gene segments by TCR δ and TCR α during V(D)J recombination. Some, if not all, V δ gene segments can be recombined either to a D segment when used to encode a TCR δ chain or a J segment in the case of TCR α (Olaru et al. 2005; Pellicci et al. 2014).

A more interesting example of this plasticity was demonstrated by Criscitiello and colleagues, when they found translocus V(D)J recombination between Ig and TCR loci in sharks (Criscitiello et al. 2010). The cartilaginous fish organize their antibody genes in clusters rather than the translocon organization found in bony vertebrates (Hinds and Litman 1986). Shark Ig loci are organized as clusters of

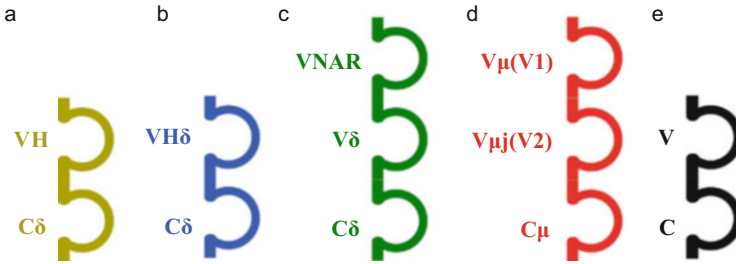


Fig. 1 Diagrams of the various novel TCR chains described in this chapter. (a) TCR δ chains found in sharks that are the result of VH genes from neighboring *IgH* loci recombining with D δ and J δ genes in the TCR δ locus. (b) TCR δ chains found in coelacanth, frog, birds, and prototherians that are the result of VH genes translocating into a TCR δ locus to become VH δ genes. (c) NAR-TCR found in cartilaginous fish. The N-terminal Ig domain is related to those found in IgNAR antibodies. The second or supporting V domain is a specialized V δ . (d) TCR μ is found in the prototherian and metatherian mammals. In metatherians the second or supporting V domain, V μ_j , is encoded by a germ-line joined exon. In the prototherians the two V domains have been designated V1 and V2 and both are encoded by exons that are the product of somatic V(D)J recombination. (e) A generic, two-domain TCR chain for comparison

genes containing V, D, J, and C gene segments necessary to encode IgH chains, and these clusters are dispersed around the shark genome. This cluster arrangement has allowed V(D)J recombination between V gene segments in an IgH locus (VH) to D and J gene segments in the TCR δ locus to encode TCR δ chains (Criscitiello et al. 2010). These are diagrammed in the figures as TCR δ -VH chains and have only been described in sharks (Figs. 1a and 2).

Among the Teleostomi, and specifically the Sarcopterygii, a different gene organization has evolved to allow TCR δ chains to utilize VH genes. This appears to have involved the direct translocation of VH gene segments into the germ-line TCR α /TCR δ locus. This was first found in the amphibian *Xenopus* but has since been described in amniotes such as birds and prototherians, as well as in sarcopterygian fish, the coelacanth (Parra et al. 2010, 2012a, b; Saha et al. 2014). These gene segments have been designated VH δ genes and in all cases appear indistinguishable from VH found in *IgH* loci but are recombined and used to encode TCR δ chains (Figs. 1b and 2). In the case of the coelacanth TCR δ locus, VH δ genes are present in the germ-line TCR α / δ locus, but so far no transcripts containing this gene segment have been reported (Saha et al. 2014). This is likely due to an absence of data given that in all other species where VH δ are present, they are somatically recombined, expressed, and contribute to the expressed repertoire (Parra et al. 2010, 2012a, b).

Genomic organization information is available for the TCR α /TCR δ locus in the amphibian *Xenopus tropicalis*, where the genes encoding the TCR δ chains are in an inverted orientation relative to those encoding TCR α (Parra et al. 2010). The VH δ gene segments are in the same orientation as the rest of the TCR δ genes and outnumber the conventional V δ gene segments. In *X. laevis*, the *IgH* and TCR δ loci are tightly linked, an arrangement that may have facilitated the translocation of

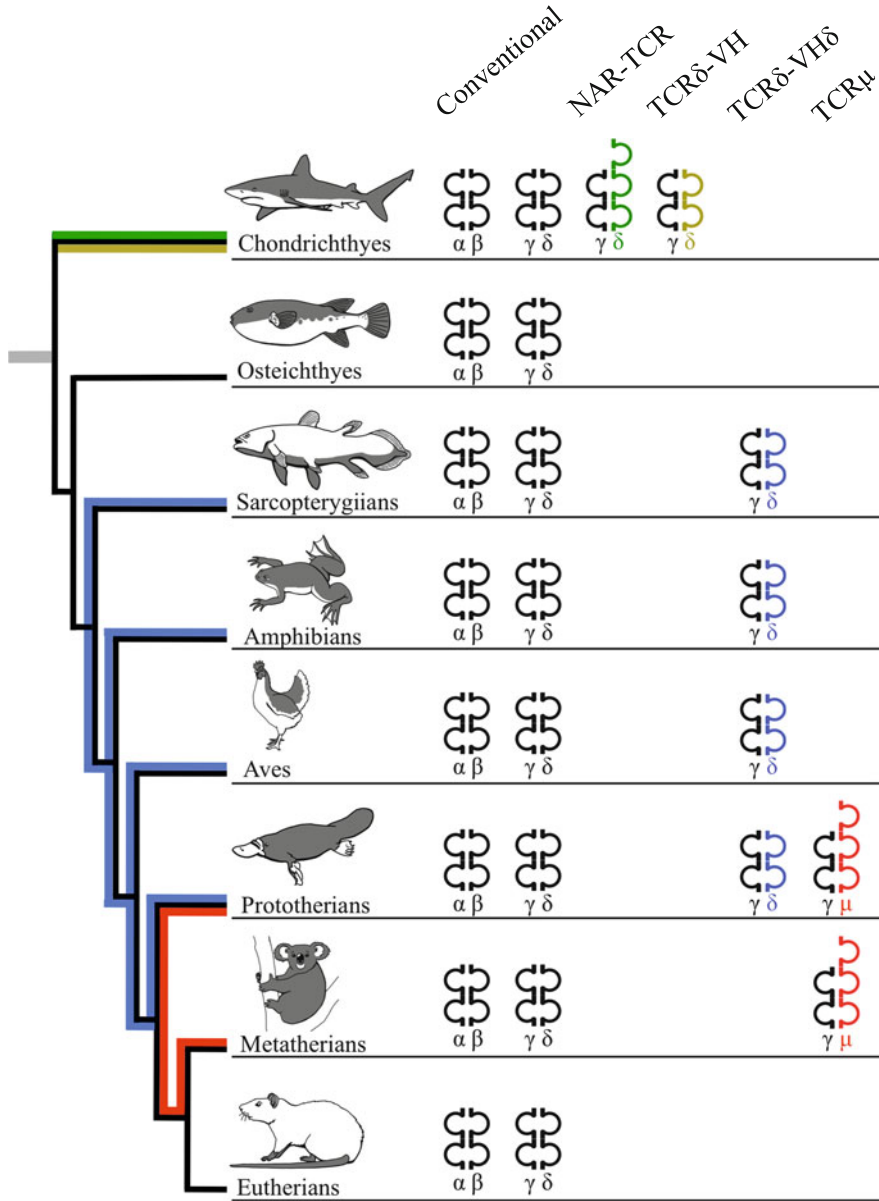


Fig. 2 Phylogenetic tree of the vertebrate lineages discussed in this chapter and their TCR repertoires. NAR-TCR and TCR δ using VH from *IgH* loci are only found so far in cartilaginous fishes, the Chondrichthyes. NAR-TCR has been described in both the Holocephali and the Elasmobranchii (for review, see Flajnik et al. 2011). TCR δ using VH have been described so far only in the Elasmobranchii. TCR δ using VH δ located within the locus encoding TCR δ chains have been described in the Sarcopterygii, from coelacanth to prototherian mammals. TCR μ is mammal specific and is found in both prototherians and metatherians but was lost in the eutherians

VH genes into the *TCR δ* locus creating the VH δ (Parra et al. 2010). There was no evidence for translocus V(D)J recombination between the frog VH genes in the *IgH* locus and genes in the *TCR δ* locus, as was reported for sharks (Parra et al. 2010; Criscitiello et al. 2010). It is noteworthy that the *X. tropicalis* *TCR α /TCR δ* locus contains only a single C δ gene. As we move across the evolutionary tree to the amniotes, we find more complex evolution having occurred in the birds involving gene duplication of the TCR δ genes.

In the passerine bird, *Taeniopygia guttata* (zebra finch), the *TCR α /TCR δ* locus is much like that of *X. tropicalis* by having V α , V δ , and VH δ gene segments (Parra et al. 2012b). A duplication event in the birds has resulted in the presence of two C δ genes, however, and a reduction to only a single VH δ . This single VH δ is recombined and transcribed to encode TCR δ chains (Parra et al. 2012b). In the distantly related Galliformes, such as chicken and turkey, the VH δ , along with D δ , J δ , and C δ gene segments, have trans-located to create a second unlinked locus encoding TCR δ chains using only VH δ (Parra et al. 2012b). Galliform birds, therefore, have evolved two TCR δ loci. One is within the conventional *TCR α /TCR δ* locus and is fairly typical in structure relative to mouse and human standards. This locus encodes both TCR α and TCR δ chains using V α and V δ gene segments only (Parra and Miller 2012). The second locus is dedicated to encoding TCR δ chains using only VH δ (Parra et al. 2012b). The translocation of genes encoding TCR δ –VH δ chains to a separate locus is likely a galliform-specific event since it is not found in the passerines and has not been found outside of birds.

The duckbill platypus, *Ornithorhynchus anatinus*, is the only other species for which VH δ have been described, demonstrating that these genes persisted into the mammalian lineage (Wang et al. 2011). The platypus holds a special place in the story of TCR δ and will be discussed in more detail later in this chapter.

3 Why Have Two Ig Domains when You Can Have Three?

In 2006, Criscitiello and colleagues reported finding transcripts in sharks that would encode a form of TCR δ chain predicted to have three extracellular Ig domains (Criscitiello et al. 2006). The N-terminal domain was most related to the V domains found in an unusual form of antibody unique to sharks called Ig novel antigen receptor (IgNAR) (Greenberg et al. 1995; Dooley et al. 2003). This new TCR form was named NAR-TCR, and, although yet to be proven, it is thought to pair with TCR γ like a conventional TCR δ . This creates an unusual predicted structure where the N-terminal V-NAR domain is extended in an unpaired fashion beyond the TCR γ V domain (Fig. 1c). The shark TCR δ locus encodes conventional TCR δ chains along with NAR-TCR (Criscitiello et al. 2006). The middle or supporting V domain in a NAR-TCR chain appears to be encoded by a V δ gene that is conventional in sequence structure. These V δ genes, however, lack the exon encoding the leader peptide. This is an informative change consistent with evolution of a specialized subset of V δ encoding the supporting V domain; these V δ will not be N-terminal in the final protein and do not need a leader peptide (Criscitiello et al. 2006).

To encode a complete NAR-TCR chain, multiple V(D)J recombination events need to occur successfully. A NAR-like V gene that is upstream of the locus needs to be recombined to D and J segments to encode the complete N-terminal V-NAR domain (Fig. 1c). In addition one of the specialized V δ genes is recombined to downstream D δ and J δ segments to encode the supporting or second V δ domain. Following transcription the V-NAR exon is spliced in the mRNA to the supporting V δ exon, presumably as splice uncomplicated by the presence of an unnecessary leader exon (Criscitiello et al. 2006).

The discovery of an atypical three-Ig domain TCR in sharks was quickly followed by an analogous TCR chain in marsupials (Figs. 1d and 2). Marsupials, or metatherians, are one of the three living branches of the mammalian tree, which also includes prototherians (egg-laying monotremes) and eutherians (the so-called placental mammals like you, me, and the mouse) (Fig. 2). The first evidence that there may be something unusual going on in metatherians came from the observation that they had a second TCR δ locus (Baker et al. 2005). Further genomic and transcriptomic analyses, primarily using the gray short-tailed opossum as the model marsupial, revealed that this was not just a second TCR δ locus, but a highly divergent TCR with atypical features shared with NAR-TCR (Parra et al. 2007). The locus and the TCR chains they encode were named TCR μ (μ or M for marsupial), and they were found to contain a number of features that were at first for a TCR. It was apparent early on that the structural similarity which TCR μ had with NAR-TCR was the product of convergent evolution. The overall genomic organization and sequence structure were too dissimilar to be due to homology by dissent (Parra et al. 2007).

The marsupial TCR μ locus is arranged in a tandem array of clusters (Parra et al. 2008). Not all the clusters are complete. Those that are complete contain one V μ , three to five D μ , and one J μ gene segment that are somatically recombined to create a diverse V μ domain that is N-terminal in the mature chain. Downstream from these V, D, and J genes is a complete exon encoding a V domain. This exon does not require V(D)J recombination to be assembled. Rather the exon encodes framework (FR) 1 through 4 in its germ-line state (Parra et al. 2007). The exon and the protein domain it encodes have been labeled V μ j due to it appearing to be an already germ-line “joined” V gene (Parra et al. 2007). Insight into the origin of the V μ j exon comes from the lack of an intron separating sequence encoding a leader peptide and the start of the exon encoding the V domain. V μ j appears to have been generated by a retro-transposition event as it is a processed gene, one that lacks introns due to reverse transcription from a mature mRNA transcript and inserted into the germ-line genome by site-specific recombination (Parra et al. 2008). Both V μ and V μ j gene segments are more related to antibody VH genes than to conventional TCR V genes, consistent with genes with the *IgH* locus being involved in the evolution of TCR μ (Parra et al. 2007).

To generate a full-length TCR μ chain, the V μ , D μ , and J μ gene segments are somatically recombined. In the RNA transcript, the rearranged V μ exon is spliced to the V μ j exon using a splice site between the leader and FR1 sequence (Parra et al. 2006). Only 60 % of thymus TCR μ transcripts have productive

rearrangements, whereas 100 % of splenic transcripts are productive. This supports the thymus being the site of $\text{TCR}\mu^+$ T cell development and there is some as-yet unknown mechanism of selecting productive rearrangements prior to those cells entering the peripheral tissues (Parra et al. 2007). The region corresponding to the complementarity determining region 3 (CDR3) of $\text{V}\mu\text{j}$ is highly conserved across marsupial species, consistent with the exon being from a single retro-transcription event early in the evolution of the metatherian lineage (Parra et al. 2007). The $\text{V}\mu\text{j}$ CDR3 is also relatively short, likely due to the absence of D segments in the original recombination event. This hypothesis is supported by the genomic organization of the platypus $\text{TCR}\mu$ locus (Wang et al. 2011).

Relative to the conventional TCR chains, the constant (C) region of $\text{TCR}\mu$ is most related to $\text{TCR}\delta$ C regions, and $\text{TCR}\mu$, like $\text{TCR}\delta$, is predicted to pair with $\text{TCR}\gamma$ (Parra et al. 2007). Recent analysis of opossum thymocytes has revealed that probes for $\text{TCR}\mu$ and $\text{TCR}\gamma$ co-stain the same cells using fluorescence in situ hybridization (unpublished). If these initial observations hold up, a third lineage of T cells, the $\gamma\mu$ T cell, is added to the repertoire along with $\alpha\beta$ and $\gamma\delta$ T cells. The genomic organization of the opossum $\text{TCR}\gamma$ genes is fairly conventional and all $\text{TCR}\gamma$ transcripts characterized would encode a typical V and C two-domain structure (Fig. 1e). It is unlikely that there is a three-domain version of $\text{TCR}\gamma$ in marsupials and, like NAR-TCR, the N-terminal $\text{V}\mu$ domain is probably unpaired (Figs. 1d and 2). This prediction is supported by the observation that when a single $\text{V}\mu$ domain is expressed as recombinant protein, it is soluble (unpublished observation). This result is consistent with the $\text{V}\mu$ domain being normally in an unpaired state in the extracellular protein.

The discovery of $\text{TCR}\mu$ raised the possibility of a novel T cell subset that might be a unique adaptation to metatherian reproductive strategies. Metatherians give birth to highly altricial young and most immune system development occurs postnatally (Old and Deane 2000; Parra et al 2009; Wang et al 2012). An early working hypothesis for why there would be a novel TCR chain in metatherians (that is lost in eutherian mammals) is that it provided protection to early developing young. This hypothesis predicted that $\gamma\mu$ T cells would appear early in development. Unfortunately that was not the case. Rather, $\gamma\mu$ T cells are the last of the three subsets to appear in development. $\alpha\beta$ T cells are the first to be detected in neonatal opossums, followed by $\gamma\delta$ T cells, and later $\gamma\mu$ T cells when the neonates are almost 2 weeks' old (Parra et al. 2009).

4 The Duckbill Platypus: A “Missing Link” in TCR Evolution?

The Australian duckbill platypus, *Ornithorhynchus anatinus*, was thought to be a hoax when first brought to the attention of European science (Moyal 2001). An animal with the fur of a mammal and the bill and webbed feet of a duck stretched

credulity for early nineteenth-century naturalists. Once they were accepted as reality, platypuses continued to surprise. The Scottish zoologist William Hay Caldwell, while a Cambridge University student, alerted the scientific world in the 1880s with his tersely worded telegram to the Royal Society, “*Monotremes oviparous, ovum meroblastic*” (Caldwell 1887). He was telling the scientific community that monotremes were mammals that laid eggs and the eggs were more like that of reptiles than metatherian or eutherian mammals. Even more delightful, male platypuses are venomous, although the venom glands are only active during mating season, the purpose of the venom probably being to chase off rival males and control uncooperative females. Genomic analysis of the platypus has done nothing to quell a sense that the platypus has retained features from our reptile-like ancestors that have been lost in the marsupials and eutherian mammals. Monotreme sex chromosomes, of which there are 5 Xs and 5 Ys, share gene content with both the XY system of other mammals and the WZ system of birds (Grützner et al. 2004). The platypus *IgH* locus encodes a constant region isotype, designated IgO, that is similar to both mammalian IgG and avian/reptilian IgY while also containing the genes for true orthologues of mammalian IgG and IgE (Zhao et al. 2009).

Analyses of the platypus TCR loci have yielded a wealth of information used to develop a model for the evolution of the TCR μ locus in mammals. The platypus TCR α /TCR δ locus is the only known mammalian locus to contain VH δ genes. The platypus VH δ are recombined and used in the TCR δ repertoire (Parra et al. 2012a). Hence, the platypus can generate TCR δ chains resembling those of birds, frogs, and coelacanths, something marsupials and eutherian mammal are unable to do (Fig. 2). The platypus and echidna genomes also contain clear homologues of the TCR μ loci found in metatherians (Wang et al. 2011). In the platypus, where some genomic information is available, the TCR μ locus appears to have a cluster organization much like that of opossum TCR μ . As in metatherians, the genes encoding the N-terminal most V domain undergo V(D)J recombination and are highly diversified in the expressed repertoire. Unlike metatherians, the genes encoding the second V domain also require V(D)J recombination to be assembled from the germ-line state (Wang et al. 2011). Single V and J gene segments are recombined with little junctional diversity to produce a nearly invariant second V domain (Wang et al. 2011). The V and J segments encoding the second or supporting V domain (V2 in Fig. 1d) are closely related to those encoding the N-terminal V1 domain, suggesting that the second V evolved by internal gene duplication (Wang et al. 2011). There are no D segments among the gene segments encoding V2, consistent with their deletion early in the evolution of TCR μ (Wang et al. 2011).

In both the opossum and the platypus, the N-terminal TCR μ V domains are diverse primarily due to junctional diversity from V(D)J recombination. The second or supporting V domain lacks diversity in the case of the marsupial or has limited diversity in the case of the monotreme. Presumably, the supporting V domain does not require diversity for function since its role is to extend the length of the protein chain for the terminal V domain to be sticking out where it can contact antigen. In the marsupials, this limited diversity is maintained by using a germ-line joined V domain with no recombination required. In the platypus, it is maintained by having

deleted the D segments and only recombining a V to a J to encode a CDR3 of very limited diversity (Wang et al. 2011).

The discovery that the platypus has both VH δ gene segments in its TCR α /TCR δ locus and the genes for TCR μ provided the opportunity to build a model of the evolution of these loci. It is clear that the monotreme and marsupial TCR μ loci are orthologous and are products of a single evolutionary origin (Wang et al. 2011). The platypus V μ gene segments are most related to the platypus VH δ genes found in the TCR α /TCR δ locus (Parra et al. 2012a). The most parsimonious explanation for the origin of TCR μ appears to be a duplication of a cluster of genes containing VH δ -D δ -J δ -C δ to a genomic location outside the TCR α /TCR δ locus, followed by divergence of these genes to become TCR μ (Parra et al. 2012a). This evolutionary model was less clear from analysis of metatherian genomes alone as they lacked the VH δ genes for comparison. The ever-remarkable platypus provided the “missing link” in TCR δ evolution (Parra et al. 2012a).

5 What Is the Function of $\gamma\mu$ T Cells and Why Were They Lost in Eutherians?

The current model or expectation of the function of the $\gamma\mu$ TCR is that, like conventional TCR, it is an antigen recognition receptor. This hypothesis is supported by the N-terminal domain undergoing somatic diversification through V(D)J recombination like a conventional Ig or TCR. One current hypothesis is that the TCR μ chain binds antigen directly, much like an antibody, and does this using the N-terminal V domain as a single binding domain. This hypothesis is supported by two observations. First, the V μ domains are more antibody-like than TCR-like. Similar to antibodies, they may bind native antigens or epitopes directly. Second, the best model for antigen binding by TCR μ may be the heavy-chain-only antibodies such as IgNAR and forms of IgG found in camelid species (Flajnik et al. 2011; Hamers-Casterman et al. 1993). It has already been speculated that shark NAR-TCR most likely binds antigen similarly to IgNAR and camel IgG, with the unpaired domain acting as a single antigen binding domain (Flajnik et al. 2011). This model seems logical for TCR μ as well.

The functional phenotype of $\gamma\mu$ T cells requires further analysis and many questions remain. Amino acid residues in the transmembrane and cytoplasmic regions that interact with CD3 chains are conserved in TCR μ consistent with much of the signaling machinery being similar to conventional T cells (Parra et al. 2007). Whether $\gamma\mu$ T cells are using CD4 or CD8 is unknown, as is their effector function.

The phylogenetic relationship among the three living mammalian lineages is no longer a matter of debate (Huxley 1880; Killian et al. 2001; Baker et al. 2004). The marsupials and eutherians sharing a more recent common ancestor over 140 million years ago and the monotremes splitting off earlier over 160 million years ago, as is shown in Fig. 2, are the broadly accepted relationship (Bininda-Emonds

et al. 2007). The presence of TCR μ in both marsupials and monotremes indicates that this locus evolved early in mammals and would have been present in the last common ancestor of marsupials and eutherians. Therefore, the eutherians lost TCR μ . The immunological consequences of having lost TCR μ will probably not be understood until there is better understanding of the role $\gamma\mu$ T cells play in the immune systems of prototherians and metatherians.

How TCR μ was lost in eutherians may have a relatively simple explanation. The genomic region where TCR μ is located in the opossum is one where there is little conserved synteny with other species (Parra et al. 2008). The disruption of syntenic blocks in this region not only exists between marsupials and eutherians but even among eutherians such as humans and mice. This is probably indicative of a region of the genome that has undergone significant rearrangement during speciation, a process that can facilitate genes loss.

6 Summary

The evolutionary story of the vertebrate T cell receptors is one of conservation. While immunoglobulins have continued to evolve new isotypes and new roles, the TCRs have remained relatively stable with all known gnathostomes having clearly homologous TCR α , TCR β , TCR γ , and TCR δ chains (Rast et al. 1997). There is strong evidence that the genomic organization of these genes has remained relatively stable (Chen et al. 2009). This is also a story of evolutionary plasticity, at least for one of the TCR chains, TCR δ . TCR δ has learned a lot of tricks to increase its diversity over the last 400 million years. TCR δ has spun off some interesting alternatives, such as TCR μ , while also retaining its basic structure and roles. NAR-TCR and TCR μ appear to have provided T cells with an alternative mechanism of antigen recognition. What roles these T cells may be performing remains to be determined. Whether or not $\gamma\delta$ T cells using VH δ genes are performing a distinct role, or if this is simply a way of adding diversity to the TCR repertoire, also remains to be determined.

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