

Kevin Nicholas Laboratory for Comparative Genomics of Mammary Gland Development and Lactation

The research program in comparative functional genomics and bioinformatics focuses on gene and bioactives discovery in the mammary gland exploiting the unique lactation strategies of the tammar wallaby, fur seal, echidna and platypus as model systems to better understand mammary gland development and function. The research platform is comprised of microarray, bioinformatics, proteomics, metabolomics, molecular and cell biology, and captive colonies of tammar wallabies and opossums. Research targets include regulation of mammary cell fate by milk factors and non-coding RNA, endocrine and autocrine control of milk protein gene expression, the role of milk in regulating growth and development of the young, mature onset disease, stem cells in milk and milk proteins with the potential to regulate human breast cancer growth and metastasis.

Project 1. Comparative genomics of mammary gland development and function in marsupials.

The tammar wallaby (*M. eugenii*) and the opossum (*M. domestica*) have adopted a reproductive strategy that is very different to eutherians; it includes a short gestation, birth of an immature young and a relatively long lactation. Both the rate of production and the composition of milk, particularly the proteins (and milk protein genes), change progressively during the lactation cycle to meet the nutritional demands for investment in considerable development of the pouch young prior to weaning. We know that the lactating tammar regulates these changes in milk composition which in turn determines the rate of pouch young growth and development. Therefore it is reasonable to argue that many of the biological factors that regulate development of eutherian embryos *in utero* are delivered in the milk by the mammary gland of marsupials. This allows access to all the bioactives in milk and to examine the processes by which the mammary gland produces these factors. Therefore the marsupial provides exciting opportunities to better understand the regulation of milk and mammary gene expression during lactation and involution and genes regulating cell fate.

To address questions related to mammary development we have established a number of *in vitro* models including mammospheres (primary mammary epithelial cells that form a 3-dimensional mammary alveolus), mammary explants and a variety of epithelial cell lines that are responsive to both endocrine and autocrine (milk factors) signalling for differentiation and cell death. These models will be used to examine the endocrine and autocrine control of expression of milk protein and mammary genes, cell survival and apoptosis.

Other experiments will be directed to studying the promoter region of the major marsupial milk protein genes. The endocrine control of constructs prepared with the

wey protein gene promoter and a reporter gene will be examined in a range of cell lines to identify transcriptional regulators of these genes.

Project 2. Epigenetics; factors in milk, colostrum, placenta and uterine fluid that impact on growth and developmental of the young to regulate adult-onset disease.

The reproductive strategy of the tammar allows access to the bioactives in milk regulating growth and development of an altricial young and the opportunity to examine the processes by which the mammary gland produces these factors. It is well established that eutherian young that are born small have a higher incidence of disease (diabetes, hypertension etc) in adult life. Therefore the process by which these developmental clocks are set at the early stages of development is of primary importance. The marsupial may allow us to manipulate the external environment of an exposed foetus and therefore study milk factors which potentially play a role in setting these developmental clocks. In addition, there are very good experimental models available that produce small newborn and subsequently adults with a higher incidence of disease that are amenable to manipulation by oral delivery of putative milk bioactives to address this pattern of disease. This approach will be complimented by the development of in vitro models to screen milk components for putative reprogramming activity and effects on gene imprinting and epigenetics.

An additional factor that impacts on mature onset disease is babies born small for age who subsequently are treated with a program for accelerated “catch up” growth. We have published a number of studies showing that we can markedly accelerate the rate of growth of the immature tammar pouch young by cross fostering the young to a mother at a more advanced stage of lactation. However, these fostered animals have not been assessed for their health status in adulthood. This approach will address a number of questions; Is the tammar newborn representative of an exposed foetus? Is it likely that early stage milk plays a critical role in development? Are there specific factors in tammar amniotic fluid that prevent the pouch young from progressing to mature onset disease?

Project 3. Mammary stem cells isolated from milk; characterisation and potential to form mammary gland and other tissues.

Stem cells have now been identified in the mammary gland and provide new opportunities to understand the mammary lineage, and approaches to manipulate the mammary gland to improve milk composition and milk production. Recent reports have shown that stem cells can be isolated from human milk. This project will isolate and characterize stem cells from marsupial milk. These cells will be studied further in vitro examining the role of extracellular matrix and other local mammary factors (particularly mammary gland-derived proteins) on the formation of mammospheres. In vivo studies will examine stem cells transferred into the cleared fat pad of nude mice to assess progression to mammary tissue and subsequent epithelial cell function.

We are particularly interested to know if stem cells are stimulated to produce different mammary epithelial cells at specific phases of the lactation cycle in the marsupial.

Project 4. Autocrine regulation of cell fate in the mammary gland

Our current experiments have shown that mammary epithelial cells have an extraordinary capacity for survival under certain culture conditions. A population of mammary epithelial cells do not require hormones or growth factors to survive. Their survival is most likely dependent on the presence of survival factors in the mammary gland and the presence of kill factors produced by the mammary gland and secreted in the milk. Microarray studies have identified specific genes that play a role in mammary cell survival and these genes and the pathways leading to their expression will be examined in a number of cell culture models. Approaches will include over expression and knock out of these genes to examine subsequent effects on cell fate.

In addition, we have identified a naturally occurring mammary protein that induces apoptosis of primary mammary epithelial cells, immortal HC11, AGS, Hek293 cell lines and breast cancer cell lines. The cells become irreversibly programmed into apoptosis within 1-3 hours of exposure to this protein. Microarray analysis indicated that this protein acts by the classic apoptosis pathway, which includes caspase-8, Bid and cytochrome c. The protein is present in the whey fraction of milk, particularly during the drying off period, when mammary cells are undergoing apoptosis due to involution and remodelling of the mammary gland. Experiments will characterize this protein and the mechanism by which it signals cells to induce apoptosis.

Project 5. The role of non-coding RNA in the control of mammary cell fate and function during the lactation cycle

Marsupials such as the tammar wallaby have adopted a reproductive strategy that includes a short gestation, birth of an immature young and a relatively long lactation. Both the rate of production and the composition of milk, particularly the proteins, change progressively during the lactation cycle to meet the nutritional demands for investment in considerable development of the pouch young prior to weaning. It is likely conserved non-coding RNAs (nc RNAs) play a significant role in the control of cell fate and mammary function during the lactation cycle.

These studies will focus on the opossum (a small marsupial mouse) to exploit the availability of the sequenced genome for this species. Monodelphis mammary gland will be transcript profiled across the lactation cycle using 454 sequencing. Bioinformatic and other approaches will be used to analyse the transcriptome and to identify candidate ncRNAs. The ncRNAs will be assessed for their role in cell fate and regulating synthesis of milk composition (particularly gene expression) in a number of cell culture models.

Project 6. Characterisation of milk factors with the potential to reduce tumour size using mouse models of human breast cancer growth and metastasis.

We have identified a naturally occurring bovine mammary protein that induces apoptosis of primary mammary epithelial cells, immortal mammary cell lines, gut and kidney cell lines, and breast cancer cell lines. The cells become irreversibly programmed into apoptosis within 1-3 hours of exposure to this protein. Microarray analysis indicated that this protein acts by the classic apoptosis pathway, which includes caspase-8, Bid and cytochrome c. The protein is present in the whey fraction of milk, particularly during the drying off period, when mammary cells in the udder are undergoing apoptosis and remodelling due to involution of the mammary gland.

This protein has potential as a topical application for skin cancer and other skin lesions. Understanding the mechanism by which this protein, or possibly an active fragment of the protein elicits an apoptotic response in the cells may open new possibilities for broader treatment. This will be examined using a number of cancer models in transgenic mice. In addition, we propose examining alternative approaches to deliver the protein specifically to mammary tumour cells to target outcomes for cancer therapy.

Project 7. The role of milk in stomach development

Parietal cells are the targets for anti-ulcer drugs because these cells contain the $H^+K^+ATPase$ proton pump responsible for acid secretion. However, little is known about the factors influencing parietal cell development. The tammar wallaby (*Macropus eugenii*) offers a good model to investigate the role of milk on the development of these cells. The immature wallaby pouch young depends totally on its mother's milk for growth for the first 200 days post partum and the progressive changes in milk composition, particularly the milk proteins, regulates growth and development of the suckled young. We have observed that the forestomach and hindstomach of the wallaby young have uniform morphology with parietal cells distributed in the mucosal layer in the first 180 days post-partum. During the subsequent transition of the young's diet from milk exclusively to milk and herbage, glands in the forestomach and hindstomach begin to specialize into cardia and acid-secreting gastric glands, respectively. However, by 230 days post-partum, parietal cells are no longer detected specifically in the forestomach. Preliminary data suggests that milk components most likely program the loss of parietal cells, possibly by ceasing programming of stem cells to parietal cells. The role of milk in this process will be examined.

Gene expression in the monodelphis mammary gland and gut will be transcript profiled across the lactation cycle. These data will provide the fundamental information to assess this marsupial model for studies on gut development and function and for ongoing studies on the regulation of the milk protein genes. Additional experiments will examine the effect of milk fractions/components to program stomach development in vivo using cross fostered young, stomach explant and cell cultures, and stimulating stem cells down the lineage towards gut cells.