Primary Cilia in the Bovine Mammary Gland

Characterising Ciliary Distribution and Morphology During Lactation and Involution

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Primary cilia are small antenna-like sensory organelles, found on virtually all vertebrate cell types. They can express specialised receptor profiles, which define their key mechano- and chemo-sensory functions within a wide range of cells and tissues. Primary cilia coordinate extracellular signals by activating specific transduction pathways, vital for the appropriate function of their resident tissue. Their distribution in bovine mammary tissue has not yet been assessed, despite the prospect that they could attribute specialised perceptive and regulatory functions to this acutely-responsive gland. Therefore, the objectives of this study were to develop a procedure for detecting primary cilia within bovine mammary tissue, and subsequently assess primary cilia distribution and morphology during active lactation, milk stasis and early involution.

A time-course of bovine mammary tissue samples was analysed, in which involution was induced by the abrupt cessation of milking in non-pregnant Friesian dairy cows at mid-lactation. Routinely fixed, wax-embedded and sectioned alveolar tissue (of 5 – 8 µm thickness) was obtained at intervals of 6, 12, 18, 24, 36, 72 and 192 hours after the last milking (n = 3 animals per group). Post-milking time points were assigned to active lactation (6 – 12 hours), milk stasis (18 – 36 hours) and early involution (72 – 192 hours) stages. Fluorescent immunohistochemistry was performed using antibodies to identify primary cilia (anti-acetylated α-tubulin), centrioles (anti-γ-tubulin) and myoepithelial cells (anti-α-smooth muscle actin (SMA)). Primary cilia cell-type distribution and morphology were examined by confocal microscopy across the entire range of time points, and ciliary incidence was assessed at active lactation.

Primary cilia were distributed on the apical aspect of luminal alveolar and ductal epithelia, on the alveolar-facing cell membrane of sub-luminal SMA-positive myoepithelial cells, and on stromal cells of inter-alveolar and inter-lobular regions. Primary cilia in all three cell types were short, often tapered at the distal tip, and projected in varying orientations in relation to the alveolar lumen. In secretory epithelial cells (SECs) some primary cilia appeared deflected around the apical cell membrane, and the proportion of those deflected was greatest in sections at milk stasis (72.7%) compared to active lactation (52.4%) (p = 0.0027), suggesting their orientation may be influenced by alveolar events.
relating to milk stasis. Ciliary length remained consistent throughout the time-course sections in SECs and SMA-positive myoepithelial cells \((p > 0.05)\). Primary cilia alveolar incidence during active lactation was between 11% and 44% in luminal SECs, and between 14% and 46% in total alveolar cells (myoepithelial cells and SECs).

Findings show that, during active lactation, milk stasis and early involution, all cell types involved in the regulation of bovine lactation can express a primary cilium. Primary cilia were suitably-placed in three important cell types to potentially coordinate various forms of ciliary transduction relying on both mechano- and chemo-sensation according to the physical and physiological state of the gland. Their presence provides new research directions in the study of mammary regulation within the dairy cow and other mammalian species, to enhance the understanding of how various mammary-specific cellular responses may be influenced by local factors.
Preface

Funding

This project is the result of a successful collaboration between research groups at the University of Otago, Dunedin, and AgResearch, Ruakura, and was made possible by funding provided by the University of Otago, Dunedin, through a Priming Partnerships Pilot Projects initiative. This funding has met both resource and study-related costs and has provided the valuable opportunity to connect core biological research with industry-related research. As a result, the following research thesis presents background, experiments, and findings of study undertaken at Master’s level into aspects of mammary gland regulation in the dairy cow. It addresses the potential for primary cilia (small cellular sensory organelles) to play a role in the regulation of lactation, and in relaying signals to initiate glandular regression as milk removal ceases – an area of regulation where gaps in the current knowledge still remain. The research undertaken in this project provides fundamental insights into primary cilia distribution in the mammary gland of dairy cows as they enter the dry period. It will provide new research directions for future studies on the regulation of lactation, which will be of relevance for the both the agricultural industry and various disciplines of cell biology.

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Having a baby during the course of this study has had its challenges, although has also helped in many ways, especially since during my maternity leave I was constantly immersed in the topic of my thesis: lactation! I hope any findings made through this study may also, in future, shed light on any clinical aspects of lactation and potentially contribute to studies that may be of benefit to lactating mothers.
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## Abbreviations

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<tr>
<td>Akt</td>
<td>(Also interchangeable with protein kinase B (PKB))</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>BBS</td>
<td>Bardet-Biedl syndrome</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>Calcium ion</td>
</tr>
<tr>
<td>CDK</td>
<td>Cyclin-dependent kinase</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>FIL</td>
<td>Feedback inhibitor of lactation</td>
</tr>
<tr>
<td>GAG</td>
<td>Glycosaminoglycan</td>
</tr>
<tr>
<td>Gli</td>
<td>Glioma (transcription factor)</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Haematoxylin and eosin</td>
</tr>
<tr>
<td>IFT</td>
<td>Intra-flagellar transport</td>
</tr>
<tr>
<td>IGF-1</td>
<td>Insulin-like growth factor-1</td>
</tr>
<tr>
<td>IGFBP-5</td>
<td>Insulin-like growth factor binding protein 5</td>
</tr>
<tr>
<td>K⁺</td>
<td>Potassium ion</td>
</tr>
<tr>
<td>LIF</td>
<td>Leukaemia inhibitory factor</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>mTOR</td>
<td>Mammalian target of rapamycin</td>
</tr>
<tr>
<td>PA</td>
<td>Plasminogen activator</td>
</tr>
<tr>
<td>PAS</td>
<td>Plasminogen activator system</td>
</tr>
<tr>
<td>PC1</td>
<td>Polycystin 1</td>
</tr>
<tr>
<td>PC2</td>
<td>Polycystin 2</td>
</tr>
<tr>
<td>PCP</td>
<td>Planar cell polarity</td>
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PDGF  Platelet-derived growth factor
PDGFR  Platelet-derived growth factor receptor
PKB  Protein kinase B (also interchangeable with Akt)
Ptc  Patched
qPCR  Quantitative polymerase chain reaction
rER  Rough endoplasmic reticulum
RTK  Receptor tyrosine kinase
SAC  Stretch-activated cation channel
SEC  Secretory epithelial cell
SEM  Standard error of the mean
Shh  Sonic Hedgehog
STAT  Signal transducer and activator of transcription
TDLU  Terminal ductule lobule unit
TEM  Transmission electron microscopy
TGF  Transforming growth factor
TRPV4  Transient receptor potential V4
Vngl2  Van gogh-like 2
α-SMA  α-Smooth muscle actin
β-gal  β-galactosidase

Abbreviations used only once are listed with the full term in the text, and not included in the above list.
Chapter 1 - Introduction

1.1 Overview

Mammary involution is the transformation of the mammary gland from a milk-producing state to a dormant state, where the tissue progressively loses its ability to synthesise and secrete milk components. The process is regulated through a host of cellular signals in response to biological and external stimuli, and exemplifies the acutely responsive nature of the mammary gland and its ability to react fluidly to changing requirements.

Involution is initiated by lack of milk removal, which causes an accumulation of milk products within the mammary alveoli and ductal branches, and a resulting physical distension within the gland. Prolonged milk stasis promotes various hormonal, cell signalling and mechanical stimuli, which act in unison to prevent further milk production and return the gland to its pre-lactation state. The functional transition within the gland is predominantly due to a dramatic decrease in secretory epithelial cell function and proliferation, in parallel with an increase in the number of those milk-producing cells dying or being removed from the gland due to the process of apoptosis. An accompanying tissue transformation also arises following remodelling of the surrounding stroma. These events effectively enable the mammary gland to revert to a less metabolically-demanding state where it will remain until subsequent gestation and lactation cycles. Many aspects of involution have been identified, however the specific cellular mechanism enabling the tissue to respond to the change in intra-mammary pressure and relay signals that initiate key events - such as the cessation of milk production and acceleration of apoptosis - remains elusive.

A promising line of investigation into a potential cellular trigger is the primary cilium. The existence of this cellular appendage was initially documented over a century ago, yet its functions have only recently become apparent. This solitary organelle acts as a finely-tuned sensor in virtually all vertebrate cell types, and performs an essential role in many signalling pathways. Numerous studies have highlighted its importance by showing that the absence of a correctly functioning primary cilium interrupts the cell’s signal transduction process, and is severely detrimental in most species and cell systems. To date, only a small number of investigators have demonstrated the presence of primary cilia in
mammary tissue, but their particular functions in lactation and involution have yet to be examined.

The following chapter summarises the current knowledge regarding bovine mammary gland biology, the regulation of lactation and involution, the importance of primary cilia as cellular sensory devices, and the evidence for primary cilia within mammary tissue. This background information and current knowledge provide a foundation for the subject of this research thesis, where the aim is to assess various aspects of primary cilia expression during lactation, and at progressive intervals following forced involution in the dairy cow.

1.2 Features of the lactating mammary gland

1.2.1 Alveoli and ducts

The anatomy of the lactating mammary gland has been extensively characterised, and its most defining aspect is the branched alveolar system. An alveolus is essentially a single milk-producing unit of between 50 – 250 µm in diameter, and clusters of sac-like alveoli surrounded by stromal connective tissue form a lobule. Many thousands of lobulo-alveolar units form the glandular structure, which is supported by bands of fibrous connective tissue and constitutes the terminal end of the mammary ductal network. During milk production, milk products are first secreted into the alveolus from the cells lining its surface. The lumen contained within the alveolus enables extracellular storage of milk secretions and, during suckling or milking, its contents are expelled into the adjoining intra-lobular duct. These ducts, which drain milk from each lobule, form the smallest branches within the ductal system, and multiple intra-lobular ducts connect to a larger, common inter-lobular duct. Each of these ducts in turn propel milk distally towards larger, higher capacity cisternal spaces, from where it is expelled directly via the teat canal during suckling or milking. A schematic of the mammary gland structure, and the basic composition of an alveolus is shown in Figure 1.2.

Mammary alveoli – the production centres of the gland – are the main focus of this research, and are essentially formed from three functionally-distinct cellular components: the secretory epithelial layer and the myoepithelial cell layer - which together form the alveolar wall, and the surrounding tissue stroma which contains a collection of diverse cell types and extracellular matrix components.
1.2.2 Secretory epithelial cells

Secretory epithelial cells (SECs) are joined in a single layer by apically-located, paracelluar tight junctions to form the alveolar lining. They are generally columnar during milk production, and flattened to a cuboidal shape when the alveolus is maximally extended with secreted milk products. Their prime functions are to produce, assemble, modify and secrete milk constituents into the luminal space within the alveolus. Effectively, these cells are the milk manufacturers of the mammary gland and their high production demands during lactation are reflected in their ultrastructure. Hallmarks of a milk-producing SEC
include abundant mitochondria, plentiful rough endoplasmic reticulum (rER), and a prominent supra-nuclear Golgi apparatus, indicative of the cell’s high protein production and modification requirements. Small, apically-located secretory vesicles and lipid droplets are also evident ultrastructurally.³

Secretory epithelial cells extract milk precursors from the underlying interstitial fluid and internally transport, process and modify them before secreting them into the alveolus. The specialised processes that occur in the cell produce a nutritionally-balanced and generally consistent secretory product that contains three essential groups of nutrients – milk fats, proteins and lactose – in addition to trace minerals, immune components and water.⁴

In a process that is unique to mammary SECs, milk fatty acids are secreted following assembly by both the intra-cellular de novo synthesis of short-chain fatty acids, and the cleavage of pre-formed long-chain fatty acids that are derived from the blood supply. Modification of most fatty acids to produce a high proportion of triacylglycerols occurs in the rER prior to their transport to, and secretion from, the apical membrane. Small membrane-bound microlipid droplets fuse within the cytoplasm to create larger globules, and these acquire a plasma membrane coating upon secretion known as the milk fat globule membrane.⁵; ⁶; ⁷

Milk proteins are generally classed in two fractions – casein (α-, β-, κ-, and γ-caseins) and whey (predominantly comprised of α-lactalbumin and β-lactoglobulin). The amino acid precursors are transported through specific transport channels on the basolateral membrane and, following protein synthesis, folding, and the addition of other molecules or ions, the proteins are packaged with lactose in secretory vesicles which bud off from the Golgi and exocytose at the apical plasma membrane. Additionally, a variety of proteins that originate elsewhere, including immunoglobulins, are incorporated into milk by transcytosis, which involves endocytosis of fully-formed proteins from the interstitial fluid at the basal cell surface, and subsequent vesicular transport through the cell and secretion at the apical surface.⁵; ⁸

The lactose component of milk relies solely on glucose as the sugar precursor, and in the dairy cow at peak lactation, up to 85% of circulating blood glucose is utilised within mammary alveoli to assemble this essential disaccharide, for its incorporation into milk.⁹ Transmembrane glucose transporters at the basal surface, in combination with sodium/glucose co-transporters, enable glucose entry, and lactose is enzymatically formed in the Golgi apparatus prior to its secretion with proteins. Being an effective osmotic agent,
lactose also contributes to the total milk volume, as its presence in secretory vesicles draws in water from the cytosol,\(^9\) which gains entry into the cell through specific aquaporin channels.\(^{10}\)

### 1.2.3 Myoepithelial cells

A layer of myoepithelial cells adhering basally to a basement membrane constitute the thin sub-secretory layer of the alveolar wall. These specialised epithelial cells contain contractile components and, when a layer of such cells surrounding the alveolus contract in response to oxytocin stimulation,\(^{11}\) the luminal area is compressed to propel milk into the ductal system. They can be visualised immunohistologically by using an antibody against α-smooth muscle actin (αSMA), which selectively binds to the contractile apparatus in the myoepithelial cell cytoplasm. This technique was first used in lactating bovine mammary tissue to demonstrate myoepithelial cell distribution in a continuous wicker basket-like arrangement that envelops the luminal αSMA-negative SECs, and similarly within the sub-luminal layer surrounding the intra-lobular ducts. They have been shown to disperse within the basal layer of the inter-lobular ducts and more distally are seen as single, discrete cells until the most distal teat cisterns where they are absent.\(^{12}\) The main role for αSMA is to create contractile force within the myoepithelial cell,\(^{13}\) through the interaction of actin microfilaments with smooth muscle myosin (heavy chain).\(^{14}\) These contractile events facilitate milk clearance from the alveoli, and assist its movement through the ductal network.

The effectiveness and completeness of milk expulsion from the alveolus is due to the synchronised action of multiple myoepithelial cells within the basal layer of the alveolus, much like a multi-cell syncytium. Gap junctions form connections between myoepithelial cells at adjacent ends of their tapered stellate processes. Using western blotting and quantitative polymerase chain reaction (qPCR) analyses, studies on human breast tissue have proven these connections to be rich in the smooth muscle cell-specific protein connexin 43 (Cx43),\(^{15}\) which facilitates effective milk expulsion by coordinating cell-to-cell communication and cohesive contractile action.\(^{16}\)

In addition to their smooth-muscle functionality, the myoepithelial layer is also important in the completion of ductal and terminal alveolar development during pregnancy to achieve a fully differentiated secretory phenotype. As reviewed by Adriance et al., 2005,\(^{17}\) specific attachments are present between themselves and cells in their neighbouring
layers: desmosomes provide linkages to luminal SECs, while hemi desmosomes anchor them in a stud-like fashion to the underlying basal lamina in the basement membrane zone. The reviewers surmise that these highly-specialised junctions provide both structural and regulatory elements, to enhance the distinctive polarity of SECs and to facilitate paracrine functions within the alveolus.

1.2.4 Stromal tissue

The assortment of cells and associated extracellular components that surround the alveoli – both within and between alveolar lobules - collectively constitute the stromal component of the mammary gland. The nature, composition, and function of this connective stromal tissue vary depending on the state of the gland. During active lactation, fibroblasts are most often seen in the inter-lobular region surrounding lobulo-alveolar structures; while they are less often viewed in inter-alveolar regions, which can be as thin as 10 µm. Ductal structures, however, tend to have a more identifiable surrounding of connective tissue than alveoli, as do involuting alveoli and lobules. Additionally, endothelial cells associated with capillaries and some white blood cells (predominantly neutrophils and macrophages) are present in both intra- and inter-lobular stromal regions. Resident tissue macrophages, derived from blood monocytes, are present in the stromal component and can associate with the connective tissue structures and epithelial components. Their roles in the mammary gland are not well understood, however they are thought to contribute to developmental and regulatory processes, as observations in other tissue systems show they can secrete cytokines and growth factors.

Fibroblasts within the connective stromal tissue maintain the extracellular matrix (ECM), which contributes structure and a supportive architecture to the mammary gland and its associated network of capillaries, blood vessels and lymphatic components. The stromal fibroblasts, however, not only contribute to the supportive framework surrounding the alveoli – recent investigations into the interactions between the ECM of the stromal component and the epithelium have shed light on the significant regulatory roles of the stromal cells. Predominant fibrillar proteins identified in mammary stromal ECM are collagens I, III and V. Additionally, an array of glycoproteins - including fibronectin, tenascins, osteonectin, decorin and small leucine-rich proteoglycans - have also been detected (reviewed by Maller et al., 2010). These ECM proteins provide a means of physical and molecular interaction between the extracellular environment and cells that
either form contacts with them, or that are influenced by signals that are selectively released by them. The mesh-like ECM structure provides sites for binding and sequestering of growth factors and cytokines, mediated through the adherent properties of certain glycosaminoglycans (GAGs). Proteolytic cleavage has been shown to enable growth factor release from within the ECM, and more recently, studies in cell cultures have suggested that the degree of mechanical tension within the ECM fibrils may also determine whether such molecules are bound or released.\textsuperscript{22, 23} Directed proteolysis of ECM components and the accompanying release of growth factors provide an environment conducive to stromal remodelling, and will be discussed later in relation to involution. Although the finer details of some regulatory actions are still unknown, the mammary stromal tissue and its associated ECM are clearly involved in mammary tissue development and regulation beyond the merely supportive function they had been known to perform previously.

1.3. Mammary development and alveolar differentiation

The development of the mammary gland involves the establishment of the mature alveolar architecture and the acquisition of the specific cellular machinery and processes within each cell type. Certain phases of development occur in the foetus, however, a significant portion of growth and differentiation occur in the mammary gland of the postnatal, pubertal, and pregnant animal to ensure the gland is capable of initiating and maintaining the complex process of lactation. At the onset of lactation, physiological changes in other tissues also arise to maintain and support the process including increases in blood volume, cardiac output, mammary blood flow, and the flow of blood through the digestive system.\textsuperscript{24}

1.3.1 Early mammary growth and branching morphogenesis

The mammary gland begins to develop in the foetus within structures known as mammary buds. These ectodermal cell clusters are present from the second month post-conception and, during gestational development, cells within the mammary buds give rise to the teat structures, the mammary fat pad and the primary sprouts. The primary sprouts further develop into cords of epithelial cells called secondary and tertiary sprouts that will, in later development, form the template for the branched parenchymal mammary structures. At birth, the secondary sprouts have canalised to form a lumen, and other mammary
components including well-formed teats and surrounding blood vessels and lymphatics are present. White adipose tissue forms the majority of the mammary gland at birth, and this continues to grow and develop until puberty.  

The ductal epithelial structures elongate into the mammary fat pad prior to puberty - a development that is mediated through stimulation by the signals derived from the surrounding adipocytes. Following the onset of puberty, alternate bursts of ductal elongation and branching occur in parallel with levels of cycling oestrogen and progesterone hormones. Receptors for these reproductive hormones are also up-regulated accordingly on the surface of stromal and epithelial cells. This stage in mammary morphogenesis results in the formation of terminal ductule lobular units (TDLUs), which only undergo further growth and differentiation if the animal becomes pregnant. Pregnancy produces a number of changes to circulating hormones, and these changes drive further mammary parenchymal development and differentiation, known as lactogenesis.  

This phase is characterised by additional elongation and branching of TDLUs to form lobulo-alveolar units as the parenchymal structures advance into the fat pad between sheaths of fibrous connective tissue, replacing the adipose tissue and mobilising their lipid components.  

Branching morphogenesis in the TDLUs during late pregnancy results from constantly high levels of circulating progesterone, which enhances epithelial DNA synthesis, and contributes to the formation of alveoli. The exponential increase in mammary parenchymal tissue immediately prior to parturition is due to a surge in oestrogen levels, and is also correlated with increased levels of prolactin and a host of metabolic hormones that stimulate the production of colostrum.  

1.3.2 Mammary cell differentiation  

Alveolar differentiation, or lactogenesis, begins during pregnancy and is the process by which the cells gain their full functionality to enable them to undertake the highly specialised functions involved in lactation. In SECs it is evidenced by certain gene expression and protein production, and involves the maintenance of the high degree of cell polarity that is established during early development. Although a population of putative progenitor cells remain within the luminal layer in the actively lactating gland, most of the cells develop to become highly specialised for milk synthesis and secretion. The initial phase of lactogenesis occurs in response to pregnancy hormones, and results in the
production of some milk proteins. Throughout late pregnancy and at parturition, the second phase of lactogenesis involves further expression of milk protein genes, the closure of tight junctions between the luminal epithelial cells, and the initiation of secretion. During this phase, the abrupt reduction in progesterone, in parallel with an increase in prolactin, stimulates the increase in immunoglobulin uptake by the SECs for the formation of colostrum. Once suckling begins, lactogenesis culminates in further increases in milk production and secretory genes, and a final expansion of the alveoli due to cell proliferation. The role of pregnancy-related hormones is essential during lactogenesis, however, there is also growing evidence for the involvement of paracrine factors between the stromal and epithelial components in the regulation of certain aspects of mammary cell differentiation.

Myoepithelial cells acquire a smooth-muscle phenotype and distinctive shape during mammary development and differentiation. Studies in rodent myoepithelial cells during mammary development highlight the progressive differentiation process that is also likely to occur in the bovine mammary gland. Such studies show that the defining and selective expression of myoepithelial cell markers is dependent on the stage of mammary gland development, and is not fully complete until the onset of lactation. The earliest muscle-specific proteins that are expressed are α-SMA and SM-myosin heavy chain, which are evident in rat myoepithelial cells at birth, while significant integrin and β2-laminin expression is not seen until the later stages of lactogenesis. These observations suggest that modifications to the cell adhesion system mark the completion of myoepithelial cell differentiation, presumably in response to ECM interactions. During alveolar differentiation and proliferation, myoepithelial cells increase in size, and in the length and number of cytoplasmic processes to form stellate-shaped contractile cells. The original spindle-shaped myoepithelial cells remain surrounding the ductal structures, where they are oriented parallel to the long axis of the duct.

Mature stromal fibroblasts contribute significantly to the connective ECM of the mammary gland, and the synthesis and secretion of fibrillar ECM components is known to be subject to regulation by endocrine factors and local growth factors, such as transforming growth factor β (TGF-β). A study using a slow-release implant of TGF-β1 in pre-pubertal heifers was shown to selectively increase mammary stromal proliferation (through cyclin-associated cell cycle progression) and transcription of fibronectin mRNA, while transiently inhibiting proliferation in nearby epithelial cells. This growth factor can also stimulate the production of collagen type I, GAG, and chondroitin sulphate, which are secreted into
the stroma surrounding developing ductal structures at puberty. Additional differentiation of fibroblasts and myofibroblasts within the developing mammary gland relies on multiple factors - including endocrine stimulation, interaction with specific proteases, molecular signals from the surrounding adipocytes or resident mammary macrophages, and interaction with components of the ECM. Together, a complex network of external and local regulatory signals, and sensitive response mechanisms within stromal cells, drive the formation of the mature, dynamic and intricate mammary stroma that is itself capable of coordinating aspects of mammary parenchymal growth.

1.4 Regulation of milk production

There are many known regulators of lactation, and factors that influence potential milk yield have been well studied in the dairy cow, owing to the importance of persistent high-volume lactation to the dairy industry. The mammary gland responds to both external cues and mammary-derived signals to alter milk synthesis and secretion according to the demands from the animal’s offspring (or the particular milking regime, in the context of the dairy industry). The various sources of stimulation and regulation provide an elegant control system, whereby the mammary gland is meeting the nutritional requirements of the young, without unnecessarily exceeding them. The vast array of cellular processes occurring throughout milk synthesis and secretion, as detailed in the previous section, place constant demands on the lactating animal: there is a steady supply of nutrients for milk production, and the metabolic energy required for nutrient uptake, secretory functions and smooth muscle contraction is significantly higher than in a non-lactating gland. The regulators of milk production discussed below provide the mammary gland with ultimate control over the balance between milk production and energy conservation, and are also important for the gland during initiation of involution.

1.4.1 Endocrine regulation

Endocrine stimulation of the mammary gland is vital for successful lactation, and the release of certain mammary specific hormones is triggered via sensory input to the brain. During suckling or milking, the terminations of sensory nerves that are abundant in the proximal end of the teat and close to the surface of the udder transmit pressure, stretch and touch signals to the hypothalamus to trigger the release of hormones from the pituitary
gland. Galactopoietic hormones are those required for the maintenance of lactation, and include the reproductive hormones oxytocin and prolactin, and various metabolic hormones.

Oxytocin promotes myoepithelial cell contraction, and is essential for milk removal from the alveoli. The release of this nonapeptide from the posterior pituitary is triggered by teat stimulation, and circulating levels remain high throughout suckling or milking. Due to the fact that approximately 80% of milk is stored within alveoli between milkings, the contraction-mediated removal of these stores, and propulsion towards the teat cisterns are key processes in transferring nutrients from mother to offspring. The role of oxytocin, and its various regulatory aspects in the context of dairy milking is the subject of a review by Bruckmaier and Blum, 1998. These authors summarise the roles attributed to oxytocin in creating constant milk flow during milking, and the negative effects resulting from inhibition of oxytocin signalling. As is the case with most galactopoietic hormones, the secretion and efficacy of oxytocin is modulated according to both external influences and interactions with other hormones. Increased stress and the presence of elevated levels of catecholamines have been shown to interfere with oxytocin release and oxytocin receptor binding respectively, whereas a dairy cow in a relaxed state is able to fully eject milk from alveolar stores during milking.

Prolactin derived from the anterior pituitary gland has been recognised as an essential galactopoietic hormone. Circulating prolactin levels are controlled by a myriad of regulatory factors, some as yet not fully understood. Prolactin secretion is known to be partly regulated in a classic neuroendocrine reflex whereby circulating levels rise during suckling of offspring and return to pre-lactation levels when suckling ends. Prolactin levels are correlated with the intensity and length of suckling, and are also influenced by various external factors such as stress, circadian patterns and additional auditory and olfactory input.

Prolactin receptors are located on the basal surface of secretory epithelial cells through the interaction of sodium/hydrogen exchanger regulatory factor 1, which coordinates the polarity and distribution of basal membrane components during secretion. Binding of prolactin leads to the formation of receptor dimers, which mediate various cellular events. These include the activation of signal transduction pathways - particularly those involving specific signal transducer and activator of transcription (STAT) proteins and the anti-apoptotic protein kinase B (PKB)/Akt. Further effects include the modification of intra-cellular ion levels through the activation of potassium (K+) and
calcium (Ca^{2+}) ion channels.\textsuperscript{30} The resulting downstream cellular responses lead to increased transcription of the milk-specific proteins α-lactalbumin and caseins, maintenance of the differentiated secretory phenotype and promotion of cell survival.\textsuperscript{25}

During lactation, additional metabolic hormonal regulation facilitates the processes involved in milk production and secretion either directly or indirectly. The glucocorticoid hormone cortisol is important during lactation, and in addition to its metabolic effects of mobilising energy stores, it appears to be involved in maintaining the tight junction integrity between SECs.\textsuperscript{33} Growth hormone has also been shown to be important for the maintenance of lactation by stimulating the release of insulin-like growth factor-1 (IGF-1) from the liver or mammary stromal cells to promote epithelial cell survival. Many other growth factors including TGF-α, TGF-β, insulin and thyroid hormone act either by binding directly to cell receptors to regulate aspects of lactation, or by facilitating systemic body responses to indirectly maintain lactation.\textsuperscript{24}

1.4.2 Local mammary regulation

Inherent regulatory mechanisms provide the mammary gland with local control over milk production and secretion. It has long been noted that increased frequency of milk removal or longer suckling leads to increased milk synthesis, resulting in higher milk yield. These effects can be mediated unilaterally by altering the frequency or amount of milk removal in only one gland in the udder. This observation led researchers to speculate that this dynamic mammary response was beyond the scope of merely hormone-related control since all glands are exposed to uniform circulating hormone levels.\textsuperscript{34}

Research to identify potential milk-related regulatory compounds detected a glycoprotein present in alveolar milk stores that could inhibit milk synthesis.\textsuperscript{35} This previously uncharacterised glycoprotein was termed feedback inhibitor of lactation (FIL), and was shown to be synthesised in SECs, secreted into the alveolus, and act in a direct feedback manner on milk synthesis by reducing protein trafficking through the Golgi.\textsuperscript{36, 37} As such, FIL was the first purely autocrine compound defined as a regulator of synthetic activity, yet subsequent studies to date have been unsuccessful in identifying a putative receptor on the SEC apical membrane. The rate of FIL synthesis and secretion is assumed to be equivalent to that of milk constituents, and its presence in alveolar milk stores enables the SEC to detect and respond to the levels of accumulated milk. Its inhibitory effects are
correlated with FIL concentration, are mediated within hours, and are reversible when FIL-containing milk stores are removed.\textsuperscript{36}

In a review by Knight \textit{et al.}, 1998,\textsuperscript{38} various findings and theories regarding local control of lactation are discussed. In addition to the short term effects FIL clearly elicits, this paper also presents FIL as a potential modulator of longer term, indirect effects throughout lactation such as adapting SEC sensitivity to endocrine stimulation and altering cell number. These effects are proposed to be mediated through the reduced trafficking of galactopoietic-responsive hormone receptors through the Golgi in SECs which, under consistent FIL inhibition, would conceivably diminish the galactopoietic and anti-apoptotic influence of certain endocrine hormones.

The question of whether the physical distension or heightened intra-mammary pressure associated with milk accumulation is also able to influence lactation has not been as well defined. It is generally accepted that mechanical stretch can mediate medium term effects related to milk secretion,\textsuperscript{39} however, research is yet to uncover the pathways of any such mechanisms. It has been postulated that accumulated milk can stretch the affected alveolus to the extent where underlying capillaries become compressed, impeding the delivery of milk precursors to the SEC. Other theories on stretch-mediated effects involve the disruption of regulatory tight junctions and the resulting impact on ionic composition. However, in their overview of local mammary control, Knight \textit{et al.} discuss the various investigations to address these concepts, and highlight the lack of supporting evidence for specific mechanisms that directly link these events to mechanical stretch.\textsuperscript{38}

\section*{1.5 Mammary involution}

The myriad of signals, stimuli and feedback mechanisms that combine to regulate milk production and secretion are sensitive to the conditions where the demand for milk increases or decreases. When the demand for milk removal no longer exists they are also able to elicit a more dramatic response – to cease milk production completely. In such cases as weaning, offspring death, or the termination of milking, a resulting period of milk accumulation occurs in the mammary alveoli and the balance of lactation control is tipped completely in favour of a conservative, non-production state. In these circumstances the process of mammary involution ensues, where the gland is remodelled to a state resembling – but not identical to - the pre-lactation phenotype. In the dairy industry this is known as
the dry period, and it is an essential step in preparing the dairy cow for future productive lactation cycles.\textsuperscript{40}

Involution is a steady process which, in the bovine mammary gland, takes several weeks to complete.\textsuperscript{40,41} Throughout the course of involution, as the length of time increases since milk removal, the less likely it is to successfully re-establish milk production and secretion, however considerable inter-animal variation has been reported.\textsuperscript{41} The process effectively changes the state and appearance of the mammary gland and, when complete, the gland will either remain in a state of latency, or begin lactogenesis again if a new pregnancy occurs. Key events affecting alveolar appearance and function, apoptosis, tissue remodelling, and immune responses, have been well documented during bovine mammary involution and will be discussed in the following sections.

1.5.1 Histological observations during involution

The degree of milk stasis and the progression of involution in mammary tissue can be detected to some extent by microscopically examining certain features of alveolar architecture and SEC morphology. A study performed by Holst \textit{et al.}, 1987,\textsuperscript{3} on bovine tissue at incremental stages since last milk removal, showed typical features of mammary gland histology and ultrastructure both in pregnant and non-pregnant cows. Changes in the SEC ultrastructure throughout involution were observed, and indicate the modification in cell activity – these include a reduced Golgi presence and fewer mitochondria and rER. Certain histological features were observed, and can be used to gauge the progression of involution, however notable variation is apparent between animals and also within tissue samples from the same animal.\textsuperscript{3,19}

Significant tissue changes are seen within 56 hours of cessation of milk removal, which are consistent with a halt in cellular synthetic and secretory activity. Bulging of epithelial cells into the lumen occurs at this point, and results from a build-up of large vacuoles and secretory vesicles apparently formed by the fusion of smaller protein or lipid-filled secretory components within the cell. These can compact and sometimes distort or displace the remaining cytoplasmic organelles, and are present to some extent throughout the first two weeks of involution, becoming less evident after three weeks. Throughout this time the luminal space, which occupies the majority of the tissue in actively lactating lobules, initially distends with accumulated milk product and causes stretching of alveolar epithelial cells. Following maximal distension, the lumen becomes gradually smaller and
luminal cells narrow in shape and condense, while inter-alveolar tissue becomes gradually more predominant. This creates the appearance of collapsed alveoli with a thick surrounding of fibrous connective tissue from about three weeks onwards. The invasion of phagocytic macrophages and leukocytes in the sub-epithelial and luminal spaces also occurs throughout this process to aid removal of excess milk components.

General alveolar integrity, the prevalence of tight junctions (albeit with altered properties), and an intact basement membrane are maintained throughout involution in cows and other studied ruminants, which contrasts with the events of rodent involution. In rat or mouse studies, involution occurs swiftly and is accompanied by significant sloughing of SECs, loss of basement membrane structure, and a substantial degree of apoptosis. Although markedly less dramatic, apoptosis and removal of SECs also occur in involuting bovine mammary tissue, but these events do not lead to the same extent of tissue regression as observed in rodents.

1.5.2 Gene transcription during involution

Changes in gene expression of milk proteins during this time are indicative of the changing function of mammary cells, and represent the de-differentiation occurring within the alveoli. Dramatic decreases have been observed in mRNA levels for caseins and α-lactalbumin following cessation of milk removal, closely followed by a decrease in β-lactoglobulin mRNA. Similarly reduced during the beginning of involution are the transcripts for integrins, which facilitate interaction between the cells and ECM components. The downstream effectors of integrin signalling are also reduced, as are certain proteins implicated in cell survival. Conversely, during this stage an increase is detected in the abundance of transcripts for certain, immune, antioxidant and apoptotic genes.

1.5.3 Proliferation, apoptosis and cell removal

Throughout lactation there is constant cell turnover, with the balance of proliferative and apoptotic SECs being regulated, in part, according to the pattern of milk removal. Both proliferation and apoptosis continue throughout involution, although following the cessation of milk removal the changing systemic and local environments permit a higher degree of apoptosis than during active lactation. Determinants of milk yield have been studied in relation to cell number and synthetic activity, with the consensus that cell number
at peak lactation is determined by the balance between cell proliferation and apoptosis at the onset of lactation. An increasing rate of apoptosis occurs in the SEC population following peak lactation and contributes to reduced milk production. Throughout active involution, this rate of cell death is further increased in relation to proliferation, leading to a net loss in alveolar cell number.

Apoptosis is a complex and highly-regulated process of programmed cell death that can be either positively or negatively influenced based on molecular stimuli and events. When induced by either active processes, or by the withdrawal of apoptotic inhibitors, a set of endopeptidases within the cell, called caspases, set in motion a cascade of molecular events that prevent cell homeostasis and viability by cleaving intra-cellular proteins and DNA, and enhancing the cells reception to phagocytic cells. During involution, it is thought to contribute to tissue remodelling to an extent that the mammary tissue can regenerate sufficiently during the dry period between lactation cycles. Research has shown that if involution (and the accompanying apoptotic events) does not occur between lactations, or if the dry period is too short, the resulting milk yield will be significantly reduced. It is thought apoptosis contributes to the removal of exhausted SECs, and by the end of involution leaves the condensed alveolar structures with a population of metabolically responsive, but non-productive SECs. These remaining cells include putative progenitor cells, which can sometimes be distinguished by their pale cytoplasm, and contribute to the bulk of SEC proliferation.

Although the specific mechanisms have not been fully defined, alveolar cell death during involution has been the subject of much investigation, and is associated with various intra-mammary events, and further influenced by changes in circulating hormones. The cumulative findings regarding apoptosis regulation in mice have been reviewed by Green and Streuli, 1994. Although a markedly more extensive degree of apoptosis is evident in rodents than ruminants, the insights provided in the review into various sources of controls and interactions indicate the potential myriad of influences on involution-associated apoptosis in ruminants. These authors suggest that multiple signalling pathways need to be activated simultaneously for apoptosis to be fully effective. Pathways that usually promote cell survival, such as IGF-mediated signalling, integrin signalling, and some types of Wnt signalling, become down-regulated, resulting in a reduction of inhibitory factors. In addition there is up-regulation of pro-apoptotic pathways involving TGF-β, interleukin-6, leukaemia inhibitory factor (LIF) and their downstream effectors, which further enhance the progression to cell death. Although changes in these pathways are correlated with
apoptotic events, the specific links between cessation of milking and the resulting modifications in these pathways have not been fully identified.

It has been shown by using teat-sealing studies in lactating mice that milk accumulation in one gland results in a significant degree of apoptosis compared to other milked glands, despite the levels of systemic galactopoietic hormones remaining steady. This implies that initial apoptotic influences in rodents can originate in the mammary gland itself, and are not dependent on a reduction in circulating hormones.58

Though there has been much less research into involution-associated cell death in ruminants, certain studies highlight the multifactorial processes that have also been observed in mice. In bovine studies, the up-regulation of apoptosis has been observed when SECs are subjected to reduced levels of suckling-induced galactopoietic hormones. In a study using mammary explants prepared from lactating cows, the effects of prolactin, growth hormone and IGF-1 hormones on apoptosis were assessed in vitro. When compared to cells that were cultured in the presence of these hormones, those that were hormone-deprived showed an increased level of pro-apoptotic IGF binding protein-5 (IGFBP-5) mRNA and considerable DNA laddering, characteristic of apoptotic DNA cleavage. This research indicates that prolactin and growth hormone can act as survival factors by inhibiting the effects of IGFBP-5, which would otherwise enhance the progression to apoptosis.59

The local effects that result from abrupt cessation of milking in dairy cows have also been implicated in promoting various apoptotic events. Following eight days without milk removal, Singh et al., 2005, 48 reported a significant increase in intra-mammary mRNA levels for the pro-apoptotic gene αBax. The corresponding rise in apoptosis was verified by using an in situ end-labelling technique, which showed a substantial increase in apoptosing cells both within the alveolar epithelial lining and in the lumen. These apoptotic events were preceded by mammary changes, presumably related to the engorgement of alveoli, and the down-regulation of transcription related to the interaction of cells with the ECM.

Another mode of cell death receiving some attention as a means of SEC loss is autophagy, or programmed cell death type II, which has been observed in some SECs during involution. It is typified by the degradation of cellular constituents as a cellular defence against starvation, resulting in the accumulation of cytoplasmic autophagic vacuoles, which undertake subsequent recycling and catabolism of their engulfed contents upon fusion with lysosomes. It is thought that the events involved in autophagy may also
trigger apoptotic events, as evidence for both processes have been observed in dying cells (as reviewed by Zarzynska and Motyl, 2008). The molecular regulators of autophagy are still being investigated, yet preliminary studies show the process is subject to regulation by IGF-1, epidermal growth factor (EGF) and reproductive hormones in cultured bovine mammary cells.

1.5.4 Involution associated tissue remodelling

Just as tissue remodelling paves the way for parenchymal advancement and growth during mammary development, similar mechanisms occur during involution to support alveolar regression and connective tissue restructuring. Although the alveoli regress and lose their synthetic capacity throughout bovine mammary involution, they still retain their architecture as a template for subsequent lactations. A significant degree of tissue remodelling also accompanies the alveolar de-differentiation and, as such, the increased presence of the inter-alveolar stromal tissue is immediately obvious in tissue samples examined by light microscopy. The molecular mechanisms that enable this change are still being investigated, in particular the roles of TGF-β and certain matrix metalloproteinases are the subject of on-going attention due to their well-established roles in connective tissue remodelling, and identification in involuting rodent mammary tissue. Transcription of TGF-β has been shown to increase during declining lactation and at the onset of involution, although its protein expression does not appear to be noticeably altered throughout this period in initial immunohistochemistry studies. Future analyses of these aspects may provide clearer insights into the fibroblast-regulated tissue changes and the involvement of particular proteases that enable tissue remodelling.

The highly-regulated plasminogen activator system (PAS) is also responsible for targeted tissue remodelling. In this system, plasminogen activator (PA) proteins convert the pro-enzyme plasminogen to its active plasmin form that is capable of directed proteolysis of ECM components. Research into the activity of the PAS during involution highlights its role in stromal ECM remodelling to enable reorganisation of the intra- and inter-lobular tissue component. Transient increases in the expression of PA and in the concentration of plasmin are observed during involution, and they have been shown to be positively responsive to the hormonal environment that promotes involution (reviewed by Politis, 1996). Targeted activation of this system also contributes to the proteolytic hydrolysis of excess milk proteins within alveoli during milk stasis.
1.5.5 Immune responses to involution

Following extended milk stasis and the onset of involution, an immune response is triggered to aid the removal and processing of accumulated milk products and cellular debris. Leukocytes including macrophages, lymphocytes and neutrophils are evident in tissue biopsies and secretions, and are more numerous between one and four weeks following milk cessation than at any other time (reviewed by Nickerson, 1989). Neutrophils are the most predominant leukocyte in the first week of involution, after which macrophages are generally more prevalent. Macrophages and neutrophils function to identify, phagocytose and digest foreign particles, including bacteria, during early involution – the stage that carries the greatest risk of exposure to bacterial infection due to the cessation of milk removal and the incomplete closure of the teat canal.

Additional immune agents have been detected at the onset of involution, one being the SEC-derived protein, lactoferrin, whose expression is inversely correlated with that of milk proteins. Lactoferrin is a multifunctional iron-binding glycoprotein that can act in a non-specific manner as a diffuse immune effector when secreted into alveoli. Through the binding of iron molecules, lactoferrin can sequester this mineral in milk secretions, making it unavailable for use by invading bacteria – which depend on iron for growth. In addition to enhancing disease resistance, it is thought to mediate a variety of involution-associated events following the observation the protein contains a nuclear localisation sequence. Details of such mechanisms are not clear, however lactoferrin has been shown in bovine mammary cells to interact with aspects of retinoid signalling, which involves the binding of vitamin A derivatives to nuclear retinoid receptors to regulate transcription.

1.5.6 Regulatory aspects of involution

Considerable interest has been directed at how various aspects of involution are triggered and regulated in the mammary gland, particularly because any knowledge of the cellular processes involved may ultimately reveal factors that may be manipulated to reduce the length of time the animals spend in the dry period. Animal health is paramount during this time, therefore a thorough understanding of the biology of involution is critical to any considerations of this aspect of dairy management.

Alveolar de-differentiation proceeds gradually following extended milk stasis, and lactation can be re-established with varying degrees of success in dairy cows after extended lengths of time since last milk removal. A study undertaken on twice-daily milked Holstein...
cows demonstrated that lactation can be partially recovered when milk removal is re-initiated after eleven days without milking.\textsuperscript{41} Under these conditions, the re-milked secretory volume could be recovered by up to nearly a third of the original milk volume (on average), and a partial recovery of lactose secretion and the transcription of some milk protein genes was seen in comparison to the contralateral udder half, which was not re-milked. Considerable variation was observed between different animals regarding these aspects of the re-initiation of milk removal.

The integrity of tight junctions (or zonula occludens) between SECs has been proposed as a determinant of secretory viability and, although intact junctions can still be observed throughout bovine involution, changes in their tension and molecular nature are thought to increase inter-cellular permeability and enhance the commitment to involution. During active lactation, tight junctions from a barrier between the baso-lateral interstitial fluid and the milk secretions at the apical surface. However, these become ‘leaky’ during involution and also during lactation if milk accumulates for more than 17 hours.\textsuperscript{33, 70} Increased permeability is associated with increased protease activity in milk secretions, coupled with reduced secretory activity, and can be indicated by the detection of lactose in plasma.\textsuperscript{33} Tight junction integrity is maintained by various systemic and galactopoietic factors, while the degree of physical distention in the alveolar wall may also contribute to increased permeability.\textsuperscript{43}

In order to study the effects of mechanical strain within the alveoli as milk accumulates, Quaglino et al., 2009,\textsuperscript{71} engineered a stretching device to subject mouse cell culture samples to mechanical tension, and subsequently observed an induction or up-regulation of various involution-associated protein levels, including LIF and STAT-3. Their work indicates that some early molecular events of involution may be mediated through local factors that are responsive to mechanical strain, although the mechanisms by which these events occur are not yet understood.

Much like the inherent regulation of milk synthesis and apoptosis during lactation, the initial triggers of involution-associated events appear to be intrinsic to the mammary gland, and their effects are compounded and reinforced when the galactopoietic effects of milk removal are lost for an extended time. During the progression to involution, the molecular mechanisms through which the initial local biochemical and biophysical influences are translated to the cell, are not well understood. Through the study of the cellular sensory organelle – the primary cilium – in bovine mammary tissue, new research avenues may be opened in this investigative process.
1.6 Primary cilia

Primary cilia are small, antennae-like cellular organelles that can be tailored to the specific sensory requirements of a cell, and a single cilium projects from the surface of virtually all cell types in vertebrate species. Despite once being considered a vestigial organelle, knowledge of the structure and function of the primary cilium has expanded at an exponential rate in recent years, due to its unforeseen involvement in a multitude of cellular processes. Primary cilia occupy a very small area on a cell’s surface, yet their intrinsic capabilities enable cells to sense, interpret, and respond to the dynamic extracellular environment, and contribute to overall cellular function.

The following sections describe the structural elements of the primary cilium, and highlight the growing body of research detailing the functions of these sensory organelles in a variety of organs.

1.6.1 Structure and attributes of the primary cilium

There are two main components of the primary cilium - the basal body and the axoneme. The basal body is comprised of the most mature of the two centrioles in the centrosome, which also functions to anchor the mitotic spindle during cell division. The projecting axoneme is sheathed in the specialised membrane that is continuous with - but distinct from - the cell’s plasma membrane, and has an internal structure of rod-like microtubules arranged in a hollow cylinder of 9 doublet microtubules. Although this arrangement closely resembles the internal structure of motile cilia (such as those seen lining the trachea or in the fallopian tube) primary cilia lack the central microtubule doublet and radial spokes that create ATP-dependent motility and are, therefore, not capable of self-generated movement. This internal differentiation between the two types of cilia has led to primary cilia often being described as having a 9 + 0 microtubule arrangement, while that of motile cilia (including flagella) is termed 9 + 2.\textsuperscript{72}

Ultrastructural studies have provided information regarding the specialised site of projection of the ciliary axoneme from the cell surface, and various features have been described. In some cells, a ring-like depression in the cell membrane known as the ciliary pocket is noticeable around the base of the projection, and this area demarcates the transition from cell membrane to specialised ciliary membrane. The depth of this membrane invagination can vary, and in some cells the ciliary axoneme appears completely retracted into a deep ciliary pocket.\textsuperscript{73} The membrane associated with this pocket contains
clathrin-coated pits as targeted sites for endocytosis of secretory vesicles, and it also forms connections with the actin cytoskeleton. The area at the base of the cilium represents the transition zone and incorporates the circumferential ciliary necklace and transition fibres. The ciliary necklace links the base of the cilium to the ciliary membrane, while sheets of transition fibres emanating from the basal body form connections to the plasma membrane, and define the boundary between the cell and ciliary membrane.

It is generally accepted that this transition zone acts as a physical and molecular regulator for ciliary-specific targets. The arrangement of the ciliary necklace and transition fibres allows selective entry of ciliary membrane constituents into the shaft due to the restrictive spacing between the internal components. Additionally, septin proteins, which belong to a family of GTPases, have been shown to localise to the ciliary base and have been implicated in forming a diffusion barrier between the plasma membrane and the ciliary membrane. Various other proteins including centrosomal protein 290kDa (CEP290) and Meckelin have also been shown to localise to the transition zone, and may function to allow selective entry of proteins or lipids associated with the ciliary membrane or axoneme. Together, the elements of the transition zone maintain the distinctive and specialised ciliary membrane and contribute to the cell-specific ciliary expression profile.

1.6.2 Assembly and disassembly of the primary cilium

The initial formation of a primary cilium occurs within the cell during interphase when the mother centriole fuses with a specialised Golgi-derived vesicle. Following initial assembly and subsequent fusion with the plasma membrane, the axoneme elongates as the microtubules polymerise at the distal tip (reviewed by Satir, 2010). Further elongation is mediated by the intra-flagellar transport (IFT) mechanism, which involves the actions of motor proteins, kinesin 2 and dynein 2, for the respective anterograde and retrograde transport of IFT particles. These particles bind components and receptors destined for the ciliary membrane, and the system allows for constant modification of membrane constituents. Cilia length appears to be tightly controlled, and can vary in tissues from between 1 µm – as is typically seen in chondrocytes – and 5 µm in epithelial cells lining kidney nephrons, depending on the particular developmental stage. In some cultured cell preparations, longer cilia can be seen (up to 30 µm) depending on cell type and cell culture conditions. Throughout ciliary assembly and maintenance there is a high degree of protein targeting and trafficking involving Golgi-derived macromolecules
destined for microtubular polymerisation or membrane localisation, and the Golgi apparatus is typically found in close proximity to the primary cilium.\textsuperscript{84}

The specialisations and modifications that the centriole undergoes as it assumes the role of ciliary basal body have also been well studied, particularly because of the link with cell cycle regulation the centriole incorporation entails for the primary cilium. Recent findings have been summarised in reviews that address centriole development and ciliary formation and disassembly in regard to the cell cycle.\textsuperscript{85; 86} As highlighted in these recent reviews, the primary cilium cannot be formed in a mitotic cell since the centrioles become otherwise engaged in duplication and anchoring the mitotic spindle for cell division. Although they have been observed in cells in highly proliferative populations during the synthesis (S) phase, cilia are removed prior to mitosis, and may therefore regulate aspects of cell division.

The mechanisms involved in disassembly may occur either through IFT processes, or by as-yet unidentified IFT-independent processes where the axoneme is engulfed into the cytoplasm. Research regarding the initiation of ciliary disassembly is currently exploring the associations with the cell cycle protein Aurora A centrosomal kinase and its targets, tubulin deacetylase HDAC6 and cyclin dependent kinase 1 (CDK1). Preliminary findings suggest that destabilisation of the ciliary microtubules through the tubulin deacetylase protein may be coordinated with cell division.\textsuperscript{85; 86}

Following the completion of cell division, the mother centriole can undergo modifications to become the basal body and re-initiate ciliary assembly. The signals and processes involved in centriole conversion and axoneme assembly are still not well understood, but they are thought to involve the removal of inhibition by centriole-associated proteins, which would otherwise restrict axoneme assembly.\textsuperscript{85} The mature basal body undergoes post-translational acetylation, detyrosination\textsuperscript{80} and polyglutamation of tubulins to increase stability, as well as gaining additional proximal striated rootlets and laterally-projecting basal feet in some cells. These modifications serve to secure the basal body in position and maintain stability of the primary cilium in order for it to withstand extracellular forces.\textsuperscript{86}

The specialised tubulins that comprise the basal body and ciliary axoneme enable primary cilia to be imaged using specific antibodies and immunofluorescence techniques, procedures which have greatly enhanced the understanding of primary cilia distribution in many tissue types.\textsuperscript{80; 84}
1.6.3 Sensory functions

The extension of the cilium from the cell’s surface and its inner core of polymerised microtubule rods attribute a degree of flexibility to the ciliary axoneme. While still maintaining its overall structural integrity, the axoneme can bend in response to extracellular forces such as fluid flow or mechanical force. This feature enables cilia to act as mechano-sensors, and the key aspects that mediate these signals have been determined following the discovery of force-responsive transmembrane complexes located in the ciliary axoneme.

The first documented study of the mechano-sensory role of primary cilia detailed their ability to bend and create a rise in intra-cellular Ca\(^{2+}\) concentration in cultured canine kidney cells.\(^8^7\) Primary cilia were manipulated by being deflected by suction with a micropipette to mimic the effect of extracellular urine flow that they experience in the renal collecting ducts. In response to cilia bending, an initial calcium influx was observed followed by a secondary increase in intra-cellular Ca\(^{2+}\) levels, consistent with a resultant release from intra-cellular stores. The calcium response was proportionate to the degree of bending and was also detected in nearby cells, suggesting a tissue response mediated through gap junctions.

Further research has proposed a mechanism for how the extracellular fluid flow is translated into an intra-cellular response, and evidence highlights the involvement the polycystin protein complex of PC1 and PC2, which resides within the ciliary membrane. The large extracellular domain of PC1 reacts to ciliary bending in response to luminal flow shear stress in a manner that allows PC2 to become activated as an intra-cellular Ca\(^{2+}\) channel. Upon Ca\(^{2+}\) influx, intra-cellular Ca\(^{2+}\) stores are released through the activation of ryanodine receptors and the altered ionic composition mediates various molecular events within the cell that contribute to tissue maintenance and development.\(^8^8\)

In addition to their role in interpreting extracellular fluid flow in renal tubules, the mechano-sensory properties of primary cilia are essential in various other tissues for maintaining tissue homeostasis, and for eliciting appropriate cellular and tissue responses. Ciliary bending in endothelial cells subject to high vascular flow have been shown in culture to act through PC1 and PC2 to mediate the Ca\(^{2+}\)-induced release of nitric oxide to enable vasodilation.\(^8^9\)

Osteocytes, which reside in lacunae within the bone matrix, can contribute to bone remodelling in response to mechanical loading experienced by the bone. In a role analogous to that performed by the flow-sensing cilia in the renal tubules, the primary cilia in
osteocytes have been implicated in responding to changes in oscillatory fluid flow. The changes in fluid flow through the canalicular network reflect specific changes in bone matrix organisation according to the compressive forces associated with movement and exercise. Although the exact mechanisms have not yet been clarified, it is generally accepted that the mechano-transduction of bone remodelling is essentially undertaken by primary cilia. They are shown to be suitably placed on the osteocytes to detect the direction and degree of loading, and are thought to transmit this information to the cell through putative secondary messenger systems which ultimately lead to increased or decreased bone formation (reviewed by Anderson et al., 2008).

Another tissue that displays plasticity in response to mechanical stimuli is cartilage, and in chondrocytes the primary cilium is appropriately positioned to interpret any force-driven alterations in ECM architecture to enable subsequent targeted tissue remodelling. ECM fibres in hyaline cartilage are arranged according to the load they experience, and can undergo modifications that provide the connective tissue with optimal shock-absorbing capabilities. Poole et al., 1997, first highlighted the consistent presence of primary cilia immediately distal to the Golgi apparatus in chondrocytes using confocal imaging in intact cartilage tissue. Additional studies revealed that the ECM receptors, α2-, α3-, and β1-integrins, and NG2 are localised to the primary cilium in embryonic chick sternal tissue. These studies, in addition to subsequent reviews, indicate a likely function for primary cilia in cartilage is to bind to collagen fibres within the ECM and transduce signals from structural changes to the chondrocyte. The positioning directly adjacent to the Golgi apparatus may also allow the primary cilia to coordinate targeted secretion of Golgi-derived ECM macromolecules.

In addition to providing sensory information on extracellular biomechanical properties, certain cells can also utilise primary cilia to monitor highly-specific environmental signals. Primary cilia in olfactory neurons are designed for detection of olfactory ligands, and their internal secondary messenger systems relay signals to create odorant sensation. Specialised primary cilia in the rod and cone cells of the vertebrate retina possess photo-receptors, and the reception and transduction of light occurs in these cilia in a process that is dependent on IFT-mediated transport of specialised retinal photoreceptors, such as rhodopsin.

Depending on the cell type and ciliary specialisations, primary cilia possess chemosensory capabilities, and can interpret signals relating to extracellular biochemistry and osmolarity. In addition to activating specific signal transduction pathways, this
functionality is thought to contribute to cell migration during development or tissue formation, as primary cilia are perceptive to the direction of secreted chemokines and, in many cell types, can orientate parallel to the migratory path (reviewed by Christensen et al., 2012).95

1.6.4 Signal transduction pathways mediated within primary cilia

Within the shaft of the cilium, various signal transducers are located in the form of secondary messenger components and transcription factors. These relay receptor signals through the basal body region, which in turn contribute to cell maintenance or initiate events that alter cell behaviour. Cellular responses depend on the particular signal and can involve cell differentiation, migration, metabolism and cell cycle entry.95

Such signalling systems may also be present on the plasma membrane of the cell itself, however the projection of the cilia into the extracellular milieu, and the additional surface area provided by its cylindrical shape enable the primary cilium to provide increased perception. A ciliary location can increase the exposure of receptors to extracellular ligands, allowing a very sensitive detection system – especially of ligands that may be in very low concentrations, or undergo only subtle changes. The primary cilium can therefore serve to augment cellular responses to extracellular stimuli depending on their pattern of receptor expression.74

Tissue homeostasis and development is coordinated through ligand-receptor interactions and, increasingly, research has shown that specific receptors are either enriched within the ciliary membrane, or exclusively positioned there. This is the case for the one-pass cell-surface receptors that belong to the receptor tyrosine kinase (RTK) superfamily, and whose localisation to primary cilia is the subject of a recent review by Christensen et al., 2012.95 These classes of receptors are known to mediate cell maintenance and development through the high-affinity binding of growth factors, cytokines and hormones. Receptor activation of RTKs, in response to binding of the extracellular ligand, induces phosphorylation of an intra-cellular catalytic domain and also of tyrosine resides at the docking sites for signal transduction proteins. Well studied RTKs include platelet-derived growth factor receptor (PDGFR) isoforms α and β, and insulin-like growth factor 1 receptor (IGF-1R). As highlighted in the review, these can initiate signalling pathways which can cross-talk with separate signalling systems to influence multiple cellular processes.
PDGFRα has been well-studied in fibroblasts, where it has been demonstrated to localise almost exclusively to the primary cilium and provide specific binding for platelet-derived growth factor (PDGF) – a ligand that is released during wound-healing. Upon activation, PDGFRα dimerises and enables downstream activation of various signalling pathways, including Akt and Mek1/2 - Erk1/2 interactions. These pathways regulate processes involving protein synthesis (via specific transcription factors), the control of cell cycle entry (via inhibition of basal body-associated glycogen synthase kinase 3β (GSK3β) and CDK inhibitors). Aspects of cell migration are also controlled via such signalling cascades, partly through the involvement of targeted sodium-hydrogen exchange factor 1 (NHE1) translocation.95

IGF-1R is also localised on primary cilia, and its activation by insulin or insulin-like growth factors (IGFs) mediates signal transduction pathways involved in cell differentiation. Although they are also situated on the plasma membrane, there is evidence the ciliary-located IGF-1Rs generate the immediate signalling response, presumably due to their extracellular projection. Cellular responses to IGF-1R activation are transduced through similar secondary messengers to PDGFR, and activate the downstream signalling molecules Akt and insulin receptor substrate 1 (IRS-1) at the cilium basal body. Cellular differentiation is directly influenced by these molecules, and while not yet confirmed, they are also likely to interact with other signalling systems to elicit a range of processes similar to that of PDGFRα.95

Primary cilia are required for embryonic development, and they have been shown to play a pivotal role in certain developmental signalling following the discovery that the critical components in Hedgehog signalling and some types of Wnt signalling are localised to the ciliary membrane and within the axoneme. These pathways are key regulators of development, cell migration and tissue patterning, and their recent association with the primary cilium adds to the diverse roles that cilia perform, while considerably expanding the research possibilities of this once-forgotten organelle.

The primary cilium houses molecular components of Hedgehog signalling. Upon binding of a Hedgehog signalling ligand to the ciliary transmembrane protein patched (Ptc), the associated protein, smoothened (Smo), can regulate glioma (Gli) transcription factors to coordinate the expression of specific gene targets. Such genes depend on cell type and stage of differentiation or development, and they control factors such as left-right asymmetry, tissue patterning and aspects of organogenesis.96 As reviewed in relation to primary cilia, Satir and Christensen, 2007,72 provide a summary of findings from various
transgenic knock-out studies. These findings demonstrate ciliary membrane localisation of Hedgehog components is dependent on IFT, such that perturbations in essential IFT proteins lead to inefficient Hedgehog signalling, with detrimental developmental consequences similar to those observed in animals lacking Gli regulation.

Wnt signalling is mediated through two different pathways: the canonical pathway, which regulates β-catenin (and cell proliferation), and the non-canonical pathway (also known as the planar cell polarity pathway), which impedes the canonical pathway and coordinates planar cell polarity and cell differentiation. A key component of the non-canonical pathway is the transmembrane protein Van gogh-like 2 (Vngl2), which has been shown to localise to the primary cilium. The two Wnt pathways are activated independently, and have somewhat opposing downstream effects, however, there is interaction between them in certain cases, and this relies on coordination by primary cilia. In addition to specific ligand binding, recent research shows that the multifunctional protein, inversin, which resides in the primary cilium, can override canonical Wnt activation. Inversin is up-regulated in response to fluid flow in renal tubule epithelial cells during development, and its activity impedes the canonical pathway, thereby blocking the transcriptional activity of β-catenin and inhibiting proliferation.

Further studies have also linked primary cilia to a variety of other signalling pathways, including Notch signalling and mammalian target of rapamycin (mTOR) signalling. As more attention is directed at primary cilia in signal transduction studies, researchers are contributing to the discovery of a plethora of critical functions that the cilium coordinates and, as highlighted in the following section, the negative consequences of ciliary malfunction can impact on multiple developmental and physiological processes.

1.6.5 Pathologies related to ciliary dysfunction

Defects in the assembly or function of primary cilia cause disruption to normal ciliogenesis or ciliary maintenance and function, and lead to a variety of diseases now classed as ciliopathies. Depending on the manner in which the cilium is affected, the loss of ciliary regulation can affect many tissues and result in a range of pathologies including polycystic kidney disease, Bardet-Biedl syndrome and, possibly, cancerous processes. Many of the proteins now known to be associated with primary cilia were, in fact, discovered by analysis of the genetic aberrations that lead to the presentation of certain severe diseases.
Polycystic kidney disease (PKD) is characterised by multiple, large cysts in the kidney nephrons. The autosomal dominant form of this disease involves mutations in the genes encoding the polycystin proteins PC1 and PC2, which are selectively expressed on the ciliary membrane. Impaired polycystin function in the developing kidney leads to the loss of flow-generated Ca\textsuperscript{2+} signalling within the epithelial cells. In adequately-functioning cilia, this sensory function contributes to tissue homeostasis and regulated proliferation, therefore in polycystin-defective cilia this functionality is lost, and the resulting uncontrolled proliferation contributes to the formation of cysts.\textsuperscript{101; 102; 103}

The near-ubiquitous presence of primary cilia in various cell types in the body contribute to the array of cellular processes at particular developmental stages that depend on certain extracellular perception and signal transduction. A systemic defect in widely-conserved ciliary proteins results in ciliopathies that display a broad range of pathologies. Such disorders, through which the identification of genetic mutations led to the discovery of multiple ciliary proteins, include Bardet-Biedl (BBS), Meckel-Gruber and Joubert syndromes. Specific ciliary-related proteins are affected in these cases and, while clinical distinctions exist, these disorders display overlapping phenotypes. Tissue abnormalities that are common to these ciliopathies include renal cysts, developmental defects in the central nervous system (CNS), and polydactyly.\textsuperscript{101; 102}

At least fourteen distinct genes are affected in BBS patients, which encode proteins essential for the formation, maintenance and function of primary cilia, and also for establishing planar cell polarity (PCP).\textsuperscript{104} This type of tissue polarisation is mediated through the non-canonical Wnt/PCP pathway and enables the appropriate organisation of cells during developmental events such as ductal elongation and oriented cell division. The involvement of seven BBS proteins in this process has recently been established as they form a complex known as the BBSome, which directs specific secretory vesicles to the transition zone of the primary cilium to coordinate aspects of ciliary membrane biogenesis. Additionally, BBS proteins may genetically interact with Vngl2, a key component in the non-canonical Wnt pathway, a topic reviewed by Ross \textit{et al.}, 2005.\textsuperscript{105}

The close association that primary cilia have with the cell cycle has spurred many investigations into ciliary presence and function in various cancer cell lines and tissues. It has been speculated that primary cilia can act as cancer suppressors in some cell types by contributing to regulated cell proliferation. Due to their molecular complexity, and varied signalling potential in different cell types and stages, it has proved to be a complex issue to
investigate, however ongoing research may shed light on any direct causal relationships between ciliary malfunction, the cell cycle and cancer.\textsuperscript{85}

The varied and pleiotropic phenotypes associated with ciliopathies emphasise the crucial cellular processes this organelle contributes to. It has been through the assessment of such ciliopathies that the primary cilium has gained recent elevated status as a highly regulatory and multi-functional organelle.

1.7 Primary cilia in mammary tissue

The earliest documented observation of primary cilia in the bovine mammary gland was an ultrastructural study of lactating and involuting tissue just over two decades ago.\textsuperscript{106} In tissue obtained from both lactating and non-lactating cows, the research was intended to explore any possibility of ciliary involvement in augmenting the mechanical stimulation of milk secretion, particularly in myoepithelial cells. Transmission electron microscopy (TEM) results showed primary cilia, with an average length of 1.2 µm, on the apical aspect of very few myoepithelial cells and SECs. Whilst no numerical data is given, the author describes a very low frequency in either cell type, with marginally more in post-lactation, involuting samples than in actively lactating tissue. TEM images show primary cilia projecting out towards the lumen, and some in contact with luminal macrophages (though this occurrence is described as very rare).

Despite these early findings, apparently no other published research has addressed the role of primary cilia in bovine mammary tissue or sought to characterise their tissue distribution, although a small number of studies have explored these sensory organelles in mammary tissue of other species - particularly in a developmental context. In mouse mammary tissue, primary cilia have been linked to both canonical Wnt and Hedgehog signalling during glandular development.\textsuperscript{107, 108}

McDermott \textit{et al.}, 2010,\textsuperscript{107} published a comprehensive study which assessed primary cilia distribution in murine mammary tissue during development. Findings of immunofluorescence detection and confocal projections showed an incidence of ciliated cells between 30% and 50% for sub-luminal basal cells and stromal cells, which remained consistent throughout development until pregnancy. The frequency of ciliated luminal cells, however, was shown to decrease from just below 20% in early development to 4% in adulthood and pregnancy. To examine potential ciliary function, these researchers
compared branching morphogenesis in wild type mice with those harbouring IFT defects. The mammary tissue of both control and mutant types were transplanted into normal mice to overcome potentially confounding factors indirectly resulting from malfunctioning cilia in other organs. When assessing the tissue during mammary development they reported that mammary glands with malfunctioning cilia had significantly inhibited ductal extension and expansion within the mammary fat pad at the onset of puberty. Additionally, the ductal structures that did develop displayed structural anomalies in branching patterns and a lack of directionality. The defective developmental processes were further linked to significantly altered components of the canonical Wnt signalling pathway, and defects in Hedgehog signalling.

Further consolidating the connection between primary cilia and Hedgehog signalling, a recently-published study has shown the distribution of Hedgehog-responsive cells in mice are clustered around neighbouring secretory epithelial cells – suggesting a paracrine action – and that these cells also express primary cilia. To achieve this, transgenic mice containing mammary-specific promoters of Sonic Hedgehog (Shh) (which encodes a Hedgehog pathway ligand), were bred with mice that have a reporter construct associated with the hedgehog-responsive gene Ptc-1. This transgenic model enabled the researchers to stimulate Hedgehog signalling and subsequently identify and locate the cells which are responsive to the activated pathway. Double-labelling immunohistochemical staining was used to identify and locate the β-galactosidase (β-gal) hedgehog-responsive reporter with other proteins of interest. The primary cilium protein, acetylated tubulin, was demonstrated to co-localise with β-gal with very high frequency, although a small number of cilia were also observed in non- Hedgehog-responsive cells.

Together, findings from both studies indicate important roles for primary cilia during mammary ductal development in mice, particularly in mediating orientation signals through Wnt and Hedgehog signalling pathways. Primary cilia may also be important in the control of cell proliferation at this stage, as the Hedgehog-responsive cell populations that expressed cilia also expressed progenitor cell markers and displayed a slow rate of proliferation.

The increasing interest in cancer-related primary cilia investigations has also encompassed the mammary gland. In a study on human mammary tissue and cell lines, Yuan et al., 2010, assessed primary cilia abundance in normal and cancerous samples. Their results of primary cilia frequency were comparable to those shown by McDermott et al., 2010, as primary cilia were also most often seen in fibroblasts and myoepithelial...
cells, with a lower incidence observed in the luminal epithelial cells. In cancerous tissue, primary cilia were only seen in one cancer type from the twenty-six types analysed, with similar findings in certain cancer cell lines. The few cancer cell lines that did express cilia displayed a very low frequency compared to normal breast cell lines, and their frequency was inversely correlated with the rate of cell proliferation.

1.8 Aims of the research

Given the involvement of primary cilia in mammary gland development, it is clear they mediate signal transduction in certain stages of mammogenesis and lactogenesis. What is not clear, however, is whether their presence in mammary tissue during lactation or involution can attribute specialised regulatory functions to the gland, or coordinate any regulatory processes.

The roles primary cilia perform as extracellular sensors and signal transducers in other well-studied systems are indicative of their potential purpose in the mammary gland. Their critical functions in such other systems lend these organelles to being the target of this current experimental research, as they could conceivably mediate any of the regulatory elements involved in lactation or the progression to involution. Currently, questions remain regarding the significance of any stretch-mediated effects associated with milk accumulation, and there is still uncertainty surrounding the initial trigger for the distinctive apoptotic events of involution.

The aim of this study is to provide novel and fundamental insights into the presence of primary cilia throughout active lactation and the stages leading to involution in the dairy cow. Tissue sections have been obtained from AgResearch, Ruakura, from a stock of slides relating to bovine mammary involution experiments. These will be used to:

1. Develop a suitable immunofluorescence staining technique to successfully image primary cilia. This technique has not previously been used for ciliary detection in this particular species/tissue.
2. Use an immunofluorescence staining technique to visually differentiate between primary cilia in myoepithelial cells and SECs by using co-immuno-detection for cilia and α-SMA.
3. To examine the distribution of primary cilia, using confocal microscopy, in the lactating bovine mammary tissue in order to gain a general understanding of the relationship between these sensory organelles and the glandular architecture. Additionally, information regarding ciliary morphology in certain cell types will be gathered during the course of milk stasis and the progression to involution to monitor the expression of primary cilia as their residing tissue undergoes a functional and structural transition.

4. To gain an understanding of ciliary incidence during active lactation in bovine mammary alveoli.

5. To provide a basis for subsequent, more focused research into specific aspects of ciliary involvement in the physiology of the bovine mammary gland.
Chapter 2 – Materials and Methods

2.1 Histological analysis

Animals

The tissue provided was collected by investigators at AgResearch, Ruakura, for other studies on bovine mammary involution, as published by Singh et al., 2005 and 2008.\textsuperscript{48–50} AgResearch ethics regulations for all animal experimental procedures were met, as stated in the publications: \textit{“All animal experimentation was conducted in compliance with the rules and guidelines...”} Briefly, non-pregnant, primiparous Friesian dairy cows that were accustomed to twice-daily milking (for 92 ± 3 days), were sacrificed at set times following the abrupt cessation of milking to initiate forced involution. Alveolar mammary tissue was obtained from the mid region of the upper-most third of the rear quarter from each animal.

Histological analysis

Tissue was fixed in 4% phosphate-buffered paraformaldehyde, routinely processed and embedded in paraffin, and sectioned at between 5 and 8 µm thickness on polysine glass slides by the Lactation Biology Team at AgResearch.\textsuperscript{48} Serial sections of approximately 15 X 25 mm\textsuperscript{2} cross-sectional area were received for this study (between 5 and 10 slides for each animal).

Tissue from three animals were selected at seven time points following last milking: 6, 12, 18, 24, 36\textsuperscript{*}, 72\textsuperscript{*} and 192\textsuperscript{*} hours. At the latter three time points (*) information regarding the relative levels of tissue α-lactalbumin mRNA expression measured by quantitative real-time reverse-transcription PCR (q RT-PCR) analysis was available,\textsuperscript{50} and samples had been classed according to low-, moderate-, or high- α-lactalbumin mRNA expression levels.

Sections from all groups were stained with haematoxylin and eosin (H&E) to assess histological features of lactation and involution prior to using adjacent sections for fluorescent immunohistochemistry detection of primary cilia and myoepithelial cells. Sections were viewed and photographed using an Olympus AX70 light microscope. All quadrants of the tissue section at various magnifications were viewed using objective lenses.
ranging from 4X to 60X, and observations regarding alveolar appearance and secretory epithelial cell (SEC) morphology were recorded.

The width of luminal SECs was measured from a minimum of seven alveoli in multiple micrographs using ImageJ imaging software (ImageJ 1.45s, National Institutes of Health, USA). For this calculation, alveolar circumference was measured, and divided by the number of luminal SECs. An average SEC width ± standard error of the mean (SEM) was obtained for each animal. A time point average for SEC width was obtained by averaging the mean SEC width from each animal classed in that time point. In this pooled calculation, the SEM was obtained by using the standard deviation of all measurements and the number of animals (n = 3) within each group. The degree of SEC engorgement was judged visually, from multiple micrographs, according to the size of cytoplasmic lipid vesicle accumulation.

A grading criteria was adopted to categorise the lactation state for each tissue section with a score of between 0 and 4, as devised by researchers at AgResearch. The entire tissue section was divided into ten sub-sections, and each was given a score according to the percentage of alveoli which had a lactating appearance: 4 ≥ 95%; 3 > 50%; 2 ≤ 50%; 1 ≤ 5%; 0 = 0%. Alveoli which showed features of significant milk stasis, (lipid-engorged SECs and darkly-stained luminal contents) or involution (condensed alveoli, increased inter-alveolar stromal area, shed SECs, and frequent leukocytes) were considered to have a non-lactating appearance. The average of the ten scores for each section (± SEM) was taken as the overall lactation grade for each animal. When combining data from animals at each time point, the SEM was obtained by using the standard deviation of all grades and the number of animals (n = 3) within each group.

Following preliminary histology assessments and earlier analyses, the seven post-milking time points were grouped into three lactation stages for analysis: 1) Active lactation (6 and 12 hours), 2) Milk stasis (18, 24 and 36 hours), and 3) Early involution (72 and 192 hours). Animals in active lactation were still within the routine milking time-frame, those in milk stasis were between six and twenty-four hours overdue for their next milking, and those in early involution had experienced a protracted period of milk stasis, with at least sixty hours since last milk removal.
2.2 Fluorescent immunohistochemical procedures

2.2.1 Optimisation of double-immunofluorescence primary cilia detection

The reagents and antibodies used during optimisation trials, and for all double-staining procedures are listed in Table 2.2.1, and the successfully-optimised protocol is summarised in Table 2.2.2.

<table>
<thead>
<tr>
<th>Reagent/antibody</th>
<th>Source</th>
<th>Working conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphate Buffered Saline (PBS) (Dulbecco A) tablets</td>
<td>Oxoid Ltd, UK</td>
<td>1 tablet/100 mL Milli-Q water</td>
</tr>
<tr>
<td>Anti-Acetylated α-Tubulin (clone C389) antibody</td>
<td>Produced in the Poole laboratory</td>
<td>1:10 in PBS/0.1% Tween 20/5% NGS</td>
</tr>
<tr>
<td>Anti-Acetylated α-Tubulin (clone 6-11B-1) antibody</td>
<td>Sigma, USA Product code #T7451</td>
<td>1:250 in PBS/0.1% Tween 20/5% NGS</td>
</tr>
<tr>
<td>Anti-γ (Gamma)-Tubulin (clone GTU-88) antibody</td>
<td>Sigma, USA Product code #T6557</td>
<td>1:250* in PBS/0.1% Tween 20/5% NGS (Trialled 1:250 – 1:1000)</td>
</tr>
<tr>
<td>Anti-Pericentrin antibody (rabbit polyclonal)</td>
<td>Abcam, UK Product code #ab4448</td>
<td>(Trialled at 1:150 – 1:4500 in PBS/0.1% Tween 20/5% NGS)</td>
</tr>
<tr>
<td>Anti-α-Smooth Muscle Actin antibody (mouse monoclonal)</td>
<td>DAKO, Denmark Product code #MO85129</td>
<td>1:100* in PBS/0.1% Tween 20/5% NGS (Trialled 1:50 – 1:100)</td>
</tr>
<tr>
<td>Alexa Fluor 488 goat anti-mouse IgG secondary antibody</td>
<td>Molecular Probes, Inc., USA Product code #A-11001</td>
<td>1:500 in PBS/0.1% Tween 20/5% NGS</td>
</tr>
<tr>
<td>Alexa Fluor 546 goat anti-mouse IgG secondary antibody</td>
<td>Molecular Probes, Inc., USA Product code #A-11003</td>
<td>1:500 in PBS/0.1% Tween 20/5% NGS</td>
</tr>
<tr>
<td>Hoechst nuclear dye</td>
<td>Molecular Probes, Inc., USA</td>
<td>1.3 µg/mL in PBS</td>
</tr>
<tr>
<td>Prolong Gold antifade reagent</td>
<td>Molecular Probes, Inc., USA</td>
<td>Undiluted</td>
</tr>
<tr>
<td>Tri-sodium citrate dihydrate</td>
<td>Merck, USA</td>
<td>0.01 M in Milli-Q water, pH adjusted to 6.0</td>
</tr>
<tr>
<td>Pepsin from porcine gastric mucosa lyophilised powder</td>
<td>Sigma, USA Product code #P7-012-1G</td>
<td>0.5% (w/v) in 10 mM HCl/Milli-Q water, pH 2.0 15 min* (Trialled 5–30 min)</td>
</tr>
<tr>
<td>Hydrochloric Acid (HCl)</td>
<td>BDH Chemicals, England</td>
<td>As required, for pH of solutions</td>
</tr>
<tr>
<td>UltraPure Glocine</td>
<td>Invitrogen, NZ Product code #15527-013</td>
<td>0.1 M in PBS overnight* (Trialled 30 min to 18 hr)</td>
</tr>
<tr>
<td>Bovine Serum Albumin (BSA)</td>
<td>GIBCO, Invitrogen, NZ Product code # 30036-578</td>
<td>10 mg/mL in PBS/10% NGS for blocking</td>
</tr>
<tr>
<td>Normal Goat Serum (NGS)</td>
<td>Invitrogen, NZ</td>
<td>As required in solutions</td>
</tr>
<tr>
<td>Tween 20</td>
<td>Sigma, USA</td>
<td>0.1% (v/v) in PBS for washing</td>
</tr>
<tr>
<td>Triton X-100</td>
<td>Sigma, USA</td>
<td>0.5% (v/v) in PBS for cell permeability</td>
</tr>
<tr>
<td>Proteinase K from Tririchium album</td>
<td>Sigma, USA Product code # P4850</td>
<td>(Trialled as per product manual)</td>
</tr>
</tbody>
</table>

Where applicable, brackets () indicate the range over which the dilution or time parameters were trialled during optimisation and (*) indicates the optimum working condition determined by experimentation.
To visualise primary cilia in the time-course bovine mammary tissue sections, it was necessary to develop a protocol for the satisfactory staining of these organelles. Optimisation of the fluorescent immunohistochemical detection protocol focused on the distinct and specific staining for both the centriole and the ciliary axoneme, and is described below. The identification of both the ciliary axoneme and basal body components, and their imaging in separate colour channels was an important step for ensuring that when the images of each component were overlaid, both the proximal and distal regions of the primary cilium could be determined. This was also necessary to minimise the likelihood of obtaining false-positive results attributable to intra-cellular microtubules.

Initial double-labelling immunohistochemical procedures used existing protocols developed for imaging primary cilia in cultured cells or murine mammary tissue,\textsuperscript{107} however, these methods proved unsatisfactory for the bovine mammary tissue sections in this study. Satisfactory imaging results were impaired by a high degree of endogenous tissue autofluorescence. This significant background fluorescence can, in some cases, be due to the nature of the highly-secretory lipid-rich mammary tissue and/or the tissue-bound free-aldehyde groups that often result from fixation in 4% paraformaldehyde.\textsuperscript{111} Such autofluorescence can reduce the quality of subsequent confocal imaging,\textsuperscript{112} therefore procedures were trialled to quench any fixative-induced autofluorescence, and focused on incubating the slides in glycine prior to antigen retrieval.\textsuperscript{111} Following slide de-waxing and rehydration, an incubation in two changes of glycine over 18 hours was used to substantially reduce background autofluorescence. Figure 2.2.1 shows micrographs taken throughout the course of immunofluorescence optimisation, in which the tissue sections without glycine pre-treatment (A and B) were considerably more autofluorescent than those with glycine pre-treatment (C and D).

Further issues encountered when using mammary tissue with existing staining protocols included the inadequate exposure of centriole-specific epitopes. Gamma-tubulin ($\gamma$-tubulin) is a highly-specific centriole marker,\textsuperscript{81, 113} although application of a titration series of anti-$\gamma$-tubulin antibody did not, initially, provide specific staining with standard citrate antigen retrieval treatment (Figure 2.2.1, A). Similar, non-specific results were obtained with anti-pericentrin, another widely-used centriole-specific antibody,\textsuperscript{114} when using citrate antigen retrieval (Figure 2.2.1, C). Alternative antigen retrieval with Proteinase K proved unsuccessful (results not shown). These initial problems were overcome for the anti-$\gamma$-tubulin antibody (which was optimal at 1:250 dilution) by adjusting the antigen retrieval protocol to include an incubation step in 0.5% pepsin at 37°C for 15
minutes, following heating in citrate buffer (Figure 2.2.1, D). Pepsin treatment alone did not successfully image centrioles (Figure 2.2.1, B).

When imaging the ciliary axoneme, the C3B9 clone antibody for acetylated-α-tubulin was trialled at 1:10 dilution, and produced specific staining of ciliary structures (Figure 2.2.1, A, C and D). For comparison, the 6-11B-1 clone was also trialled, and showed similar results, however, due to its tendency to produce a slightly punctate stain, it was not used in subsequent trials. The specific staining of the primary cilia axoneme with C3B9 was adequate with citrate buffer antigen retrieval (Figure 2.1.1, A), and still maintained the same specificity and staining intensity when used in conjunction with the pepsin antigen retrieval technique. Figure 2.2.1, D shows a colour-merged micrograph obtained following dual antigen retrieval and acetylated-α-tubulin/γ-tubulin double-labelling. Both the ciliary basal body and axoneme can be separately identified.
Figure 2.2.1

Optimisation trials for primary cillum axoneme and centriole detection

Representative composite micrographs

A: Ciliary axoneme detection but no obvious centriole detection, following citrate antigen retrieval (no glycine incubation); B: No obvious centriole detection, following pepsin antigen retrieval (no citrate antigen retrieval or glycine incubation); C: Ciliary axoneme detection but no centriole detection with anti-pericentrin, following citrate antigen retrieval and glycine incubation; D: Inset shows successful double-labelling of primary cilia centrioles (red) and axonemes (green) following glycine incubation and dual antigen retrieval with both pepsin and citrate.

Arrows indicate ciliary structures. Dashed box in D indicates zoomed, inset area.

All images taken using 100X objective, with an Olympus epi-fluorescence microscope.
2.2.2 Immunofluorescent detection of primary cilia

Two sets of tissue sections, which were adjacent to those stained with H&E, were stained for the detection of nuclei and the selective detection of specific cellular components. The first set was stained according to the protocol in Table 2.2.2 for double-labelling of acetylated-α-tubulin/γ-tubulin to enable dual identification of primary cilia. The second set was double-labelled for α-SMA/acetylated-α-tubulin to identify α-SMA-positive myoepithelial cells and ciliary axonemes. Sections for α-SMA/acetylated-α-tubulin detection were taken through the same preliminary steps as in Table 2.2.2, with the following changes: a) pepsin antigen retrieval (step 3.2) was omitted, b) step 5 was replaced with axoneme binding using anti-acetylated-α-tubulin primary antibody for 1 hr at 37°C, and c) step 8 was replaced with α-SMA binding using α-SMA primary antibody for 30 min at room temperature. Sets of sections at all seven post-milking time points outlined in Section 2.1 (n = 3 animals per group) were processed in this manner.

Negative controls, which excluded the primary antibody application, were run in all staining procedures. All negative controls were assessed, and none showed specific staining.

| Table 2.2.2 |
| Summarised sequence of steps used for fluorescent immunohistochemical double-labelling of primary cilia |

1. De-waxing and rehydrating tissue | Graded series of xylene and alcohol, to water |
2. Autofluorescence quenching | Two changes of glycine solution, over 18 hr |
3. Antigen retrieval | 1. Heat in citrate buffer, 25 min at 95°C  
2. Pepsin incubation, 15 min at 37°C |
4. Tissue preparation and blocking non-specific sites | 1. Triton X-100, 5 min  
2. 10% NGS/1%BSA in PBS, 1 hr |
5. Centriole binding with primary antibody | Anti-γ-tubulin primary antibody, overnight at 4°C |
6. Secondary detection | Alexa Fluor® 546 (red) goat anti-mouse IgG, 1 hr |
7. Additional blocking | BSA, 1 hr |
8. Axoneme binding with primary antibody | Anti-acetylated-α-tubulin, 1hr at 37°C |
9. Secondary detection | Alexa Fluor 488 (green) goat anti-mouse IgG, 1 hr |
10. Nuclei detection | Hoechst, 15 min |
11. Cover-slipping | Prolong Gold antifade reagent |

Notes: Slides were washed in multiple changes of wash buffer (PBS/0.1% Tween 20) following each antibody incubation. Unless otherwise stated, incubations were carried out in a humidity chamber at room temperature.
2.2.3 Confocal imaging and analysis of primary cilia

Confocal microscopy

Following immunohistochemistry, sections were initially checked for labelling quality using the fluorescence settings on an Olympus AX70 light microscope, equipped with epi-fluorescence capabilities. All subsequent imaging work was carried out using a Zeiss LSM 510 upright laser scanning confocal microscope with a 100X oil immersion objective (N.A = 1.40) at the Otago Centre for Confocal Microscopy. Detector gain and amplification settings were adjusted to reduce the signal-to-noise ratio, and at least ten alveoli, from all four quarters of each section were imaged. Cilia were imaged as a series of Z slices obtained using immunofluorescence specific laser excitation lines 488 nm and 543 nm, according to the staining protocol employed. Nuclei were imaged from the mid-section of the selected Z-slice using standard fluorescence excitation. In cases where digital zoom was used to enhance the view of individual cilia, the corresponding field view was recorded to maintain information of ciliary orientation in relation to lumina. All LSM image files were processed in ImageJ to create composite micrographs. Images from each of the red and green detector channels were stacked to create a single image. Where necessary, minor adjustments to colour threshold levels were made to optimise signal-to-noise ratio. The single-colour images were then merged to create single composite RBG micrographs, and scale bars added.

Ciliary length

Length measurements were made from composite micrographs of the α-SMA/acetylated-α-tubulin double-labelled sections. Since double-labelling studies with acetylated-α-tubulin/γ-tubulin showed a direct co-localisation, ciliary length was defined as the entire length of the fluorescently-labelled ciliary structure, which was assumed to include the ciliary basal body. Length measurements were made for cilia that were lying close to perpendicular to the emitted light path to ensure the entire length of the structure was measured as accurately as possible. For each suitably-imaged primary cilia the alveolar cell of origin was recorded, according to the positive or negative α-SMA staining associated with the Hoechst-stained nucleus. The number of cilia imaged per animal varied – those measured from each animal in SMA-positive cells were between one and ten, while those measured in SECs were between one and eighteen. Due to this factor, the weighted average ciliary length for each post-milking stage was calculated by averaging the mean ciliary
length from individual animals according to the number of measurements made for each animal. For each post-milking stage, where average data from individual animals were combined, the pooled standard deviation was used as an estimate of the combined within-group variance. These descriptors were used in subsequent comparative statistical tests.

**Ciliary deflection**

Ciliary deflection was based on observations of ciliary orientation and morphology. These characteristics were assessed using composite micrographs from both the acetylated-α-tubulin/α-SMA and acetylated-α-tubulin/γ-tubulin labelled image sets. Data was collected for luminal SECs by assessing images from the two immuno-detection sets, and for myoepithelial cells by assessing images from the acetylated-α-tubulin/α-SMA set. Deflected primary cilia were defined by their angle of orientation relative to an orientation directly perpendicular to the alveolar wall, i.e. projecting towards the centre of the lumen. A cilium was classed as ‘deflected’ if its axoneme lay at an angle greater than 45° in either direction from the perpendicular angle. Those that were within 45° of perpendicular, were classed as non-deflected. Of those classed as deflected, cilia were further categorised as ‘bent’ or ‘non-bent’. A bent cilium was defined as being bent in such a way that it could not be mistaken for a curved cilium. Count data for each post-milking stage was used in subsequent comparative statistical tests.

**Ciliary incidence**

Incidence data was gathered using suitable composite micrographs of alveolar structures from the acetylated-α-tubulin/γ-tubulin set of sections. Individual micrographs ranged in depth from 1.64 µm to 6.55 µm, with an average depth of 3.8 µm, according to the number and thickness of Z-slices used for image compilation. To eliminate potential bias relating to the inherent variability in alveolar appearance during milk stasis and involution, only sections from animals in the active lactation post-milking stage were assessed (n = 6 animals). Data was gathered for luminal cells (cells whose apical surface directly bordered the lumen) and total alveolar cells (cells from the entire epithelial alveolar structure). As a result of this definition, only SECs would be included in the luminal cell data, and both SECs and myoepithelial cells would be included in the total alveolar cell data. Hoechst-stained nuclei were counted, and information was recorded regarding their cellular association with centrioles and primary cilia. Data was obtained for the number of
nuclei, the number of centrioles (whether associated with primary cilia, or not) and the number of ciliary axonemes.

For each animal, multiple composite micrographs were assessed (between two and eleven), and the average percentages of (a) nuclei with cilia, (b) nuclei with centrioles, and (c) centriole pairs with cilia were obtained. The total post-milking stage average for each incidence assessment was obtained by averaging the data from all animals.

Statistics

GraphPad Prism v.6 statistical software (GraphPad Software Inc., USA) was used for all comparative statistical tests, and to generate graphs. *p*-values for all tests were defined as being statistically significant if *p* < 0.05.

For analysis of ciliary length, a two-way ANOVA was performed for between-post-milking-stage and between-cell-type comparisons. For analysis of the proportions of deflected and bent cilia and comparisons between post-milking stages, a Chi-Square analysis was used, based on total count data at each post-milking stage. In cases where a Chi-Square test returned a statistical level of significance across proportions in the three post-milking groups, each pair-wise comparison was re-run separately to identify which pair-wise comparison had returned the significant result.
Chapter 3 – Results

Findings from the analysis of bovine mammary tissue, sampled from animals following the abrupt cessation of milking, are divided into three sections. Initial histology assessments were made to define the appearance of the tissue across all post-milking time points, and these findings are presented in Section 3.1. The immunofluorescent imaging of primary cilia within the mammary tissue is detailed in Section 3.2, and further analytical assessments of the primary cilia are shown in Section 3.3.

3.1 Analysis of H&E-stained time-course slides

3.1.1 Qualitative histology assessment

6 hours post-milking (active lactation)

Figure 3.1.1, A – D shows representative micrographs from 6 hours post-milking animals. At this time point there were no significant inter-animal variations observed in the glandular histology. The majority of the tissue section from all animals was dominated by open alveolar luminal space. At low magnification the alveoli appeared open and relaxed, and bands of connective tissue were seen surrounding lobules (A and C). A small amount of accumulated milk product was seen within some lumina, apparent as light to darkly-stained a-cellular material (C), and no obvious cellular material was seen. At higher magnification, the alveolar walls appeared narrow, very little stroma was apparent between neighbouring alveoli, and the luminal SECs were cuboidal in shape and defined the highly ordered alveolar structures (B). The alveoli displayed minimal variation in their size, and the thickness of alveolar walls was consistent across all alveoli seen. Occasionally, lipid vesicles were seen on the apical surface of SECs (not shown), although no cell distention or bulging was evident. Some ducts were seen within the glandular tissue and these were distinguished from the alveoli due to their thicker surrounding of connective tissue, and their lining of densely-packed, columnar epithelial cells – often in a double layer (D). Within the inter-lobular connective tissue, some adipose tissue was seen, and stromal structures including blood vessels were also apparent (not shown). For the most part, the
connective tissue components were sparse in relation to the alveolar structures, but a few very thick bands were seen, which encompassed multiple lobules.

12 hours post-milking (active lactation)

Figure 3.1.1, E – H shows representative micrographs from 12 hours post-milking animals. In comparison to observations of six hour post-milking sections, some lobules from the 12 hours post-milking time point showed a small degree of variation in alveolar appearance across the glandular tissue (E). At low magnification, the alveoli from all three animals appeared more dense than at 6 hours (F and H), and slightly thicker intra-lobular stromal areas were present, where larger ducts and adipose tissue could be seen (examples shown in G). Although not demonstrated in these micrographs, many lumina contained a-cellular material, indicating secreted, stored milk products. At higher magnification, lipid-filled vesicles were seen on the apical aspect of SECs within some alveoli (F and H). No significant cell sloughing was evident, although leukocytes were occasionally seen in vessels (F).

18 hours post-milking (milk stasis)

Figure 3.1.2, A – C shows representative micrographs from 18 hours post-milking animals. A common feature of alveoli among the animals sampled at this time point was the darkly-stained proteinaceous material, which was present in many lumina, and is shown in all micrographs at this time point. An obvious degree of heterogeneity was seen within the tissue from some animals, and is demonstrated in the two high magnification images (B and C). As in C, some alveoli appeared more condensed, with a more pronounced surrounding of connective tissue, while others, as seen in B, were more characteristic of active lactation, and their lining SECs were flattened. In some alveoli, (particularly those that were more condensed) the SECs appeared vesicle-engorged and sometimes caused the cells to bulge into the lumen. Additionally, shed SECs were occasionally seen within, or at the edges of some lumina (C). Intra-lobular connective tissue was increased slightly in some areas compared to the 12 hours post-milking samples.
24 hours post-milking (milk stasis)

Figure 3.1.2, D – F shows representative micrographs from 24 hours post-milking animals. Significant inter-animal variation was observed at this time point, and more histological features associated with the cessation of milk removal are noted in some regions. Tissue sections from all three animals showed evidence of darkly-stained accumulated milk product in the luminal spaces (evident in D and F). The separation of lobules by inter-lobular connective tissue bands was particularly obvious in one animal examined at this time point, which also had wider inter-alveolar stromal, with more connective tissue between adjacent alveoli (E). All sections displayed inter-alveolar variation, and alveoli that were more condensed had rounder SECs, which were engorged with large lipid vesicles. Thinner-walled alveoli tended to have more open lumina, which were generally lined with flattened cuboidal SECs, and some accumulated lipid material was still evident (F). Occasionally, shed SECs were seen in some lumina (E). Small areas of adipose tissue and small ductal structures were seen within inter-lobular stromal areas (ducts shown in D). No significant leukocyte infiltration was observed among the three sections.

36 hours post-milking (milk stasis)

Figure 3.1.2, G – I shows representative micrographs from 36 hours post-milking animals. The three samples analysed at this time point were from animals with low, moderate, and high α-lactalbumin classifications. The inter-lobular variation in the appearance of alveoli was quite marked at this stage, as shown in G. The most condensed alveoli were separated by a more established inter-alveolar stroma than was noted in sections from earlier time points (G). Accumulated milk products were evident in some more open alveoli, evident as darkly-stained a-cellular material. Ductal structures were also seen within some areas of inter-lobular connective tissue. This was the earliest time post-milking that a consistent leukocyte presence was observed within some lumina (H and I), however there was considerable inter-animal variation regarding their numbers. Tissue from the animal with the low α-lactalbumin mRNA expression displayed more signs of advanced involution, such as thicker connective tissue surrounding lobules and alveoli, occasional sloughed epithelial cells within lumina, and abundant leukocytes (G and H). At high magnification, in all three animals, large lipid vesicles engorged the SECs in the more condensed alveoli. These were not only confined to apical cytoplasmic areas, and in some cases were seen to occupy the majority of the cytoplasm. In SECs with particularly lipid-
engorged cytoplasmic contents, the nuclei often appeared slightly distorted and sometimes assumed a lateral or apical cellular position (H).

72 hours post-milking (early involution)

Figure 3.1.3, A – D show representative micrographs from 72 hours post-milking animals. Sections from one of each low, moderate, and high α-lactalbumin animal were examined at this time point. The alveoli displayed similar features to those observed in 36 hours post-milking. Observations included some darkly-stained luminal contents, large lipid-engorged SECs, and some areas of inter-alveolar variability. The epithelial lining of some larger alveoli appeared stretched, with flattened cuboidal SECs. At high magnification, lipid vesicles were seen in the cytoplasm of these flattened cells (B). The SECs lining the more condensed alveoli were often seen to bulge into the lumen due to the large accumulated lipid vesicles (D). Although not shown in the images, leukocytes were also apparent at higher magnification in some lumina, and sloughed SECs were occasionally seen (D). Tissue from the low α-lac animal showed the greatest evidence of involution, with a visibly higher proportion of condensed alveoli, little open luminal space, and a more established inter-alveolar stromal component (C and D).

192 hours post-milking (early involution)

Figure 3.1.3, E – H shows representative micrographs from 192 hours post-milking animals. Tissue from two moderate, and one high α-lactalbumin animals were assessed, and significant inter-animal variations were observed, even between the two sections from the moderately-classed animals (E/F and H). Of all three sections, one examined from a moderate α-lactalbumin animal showed the most striking degree of milk stasis, and the greatest degree of involution (E and F). Tissue sections from this animal showed a large amount of accumulated luminal material, such that almost no white space was visible at low magnification. When viewed at higher magnification (F), there was an abundance of luminal leukocytes, and sloughed SECs in most alveoli. SECs bulged with accumulated large lipid vesicles, and many nuclei had been pushed out to the lateral or apical margins of the cell. These signs were also seen in the other moderate α-lactalbumin animal – but to a lesser extent. In this animal there was less accumulated luminal material, and comparatively fewer leukocytes and shed SECs (H). The sample from the high α-lactalbumin animal displayed inter-alveolar variation in the degree of involution observed,
with lobules of condensed alveoli seen beside those containing more open alveoli (G). In all animals, as noted at previous time points, the more open, stretched alveoli were lined by flattened, cuboidal SECs, while those of the more condensed alveoli displayed bulging, more closely-packed SECs, and lipid vesicle accumulation was evident among the different shaped SECs.
Micrograph image plates

Figure 3.1.1 .......................... 50
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Figure 3.1.1 (facing page)

H&E-stained sections of mammary alveolar tissue from animals in active lactation

A – D: representative micrographs from animals 6 hours post-lactation; E – H: representative micrographs from animals 12 hours post-lactation.

Lac: Lactating area; Inv: Involuting area; V: Blood vessel; L: Leukocyte
Inter-alv Stroma: Inter-alveolar stroma; Ad: Adipose tissue

Arrowheads indicate leukocytes, Arrows indicate lipid vesicles in SECs

Dashed lines define lobules, an alveolus, and areas inter-lobular variation, as labelled.

Images A, C, E and G taken using 4X objective; B, D, F and H taken using 60X objective.
Fig 3.1.2 (facing page)

H&E-stained sections of mammary alveolar tissue from animals in milk stasis

A – C: representative micrographs from animals 18 hours post-lactation; D – F: representative micrographs from animals 24 hours post-lactation. G – I: representative micrographs from animals 36 hours post-lactation.

M: Accumulated milk products;  Lac: Lactating area; Inv: Involuting area; S: Shed SEC; L: leukocyte

Arrowheads indicate a leukocyte or shed SEC—as labelled, Arrows indicate apically-located nuclei in SECs

Dashed lines define areas of inter-lobular variation, as labelled.

Images A, F and G taken using 4X objective; B—E, H and I taken using 60X objective.
Fig 3.1.3 (facing page)

H&E-stained sections of mammary alveolar tissue from animals in early involution

A – D: representative micrographs from animals 72 hours post-lactation; E – H: representative micrographs from animals 192 hours post-lactation.

Lac: Lactating area; Inv: Involuting area; S: Shed SEC; L: leukocyte;
L/S: A leukocyte engulfing a shed SEC

Arrowheads indicate a leukocyte, shed SEC, or both — as labelled, Arrows indicate apically-located nuclei in SECs

Dashed lines define areas of inter-lobular variation, as labelled.

Images A, C, E and G taken using 4X objective; B, D, F and H taken using 60X objective.
3.1.2 Semi-quantitative analysis

The averaged lactation grades (± SEM) for each post-milking time point (obtained from \( n = 3 \) animals in each group) are shown in Figure 3.1.4. These were allocated according to the grading criteria described in Materials and Methods 2.1. The highest average grade was seen in sections from 6 hours post-milked animals, which received an average grade of 3.97 ± 0.11. Based on a maximum grade of 4.0, this indicates that very few, if any, signs of milk stasis or involution were observed amongst the sections from animals grouped in this time point. Following 18 hours post-milking, the averaged lactation grades reduced considerably to 2.47 ± 0.29, indicating the appearance of features associated with milk stasis or involution. The lowest average grading was observed within 192 hours post-milked sections (1.17 ± 0.53), indicating greater signs of milk stasis and involution. The SEM associated with the averaged grade at 192 hours post-milking was the largest amongst all post-milking time points, which reflects both the inter-lobular variation and the inter-animal variation observed in the tissue.

![Figure 3.1.4](image_url)

**Figure 3.1.4**

Lactation grades (± SEM) within mammary tissue sections from dairy cows after the abrupt cessation of milk removal.

Maximum score = 4 (≥ 95% lactating alveoli), minimum score = 0 (0% lactating alveoli)

\( n = 3 \) animals per time point. x-axis not to scale
The histological findings of individual animals regarding lactation grades and two aspects of SEC size (width and cytoplasmic engorgement) are displayed in Table 3.1.

<table>
<thead>
<tr>
<th>Time since last lactation</th>
<th>Animal</th>
<th>Histological grade of lactation (± SEM)</th>
<th>Individual animal SEC width (μm) (± SEM)</th>
<th>Grouped animal SEC width (± SEM)</th>
<th>Degree of SEC engorgement</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 hours</td>
<td>1</td>
<td>4.0 (± 0.0)</td>
<td>10.1 (± 0.34)</td>
<td>11.4 (± 0.86)</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>3.9 (± 0.1)</td>
<td>12.0 (± 0.50)</td>
<td>12.1 (± 0.29)</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>4.0 (± 0.0)</td>
<td>12.0 (± 0.50)</td>
<td>12.1 (± 0.29)</td>
<td>—</td>
</tr>
<tr>
<td>Active Lactation</td>
<td>4</td>
<td>3.6 (± 0.16)</td>
<td>13.1 (± 0.54)</td>
<td>12.6 (± 0.99)</td>
<td>+/—</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>3.4 (± 0.16)</td>
<td>12.4 (± 0.52)</td>
<td>12.2 (± 0.38)</td>
<td>+/—</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>3.8 (± 0.13)</td>
<td>12.2 (± 0.38)</td>
<td>12.2 (± 0.38)</td>
<td>+/—</td>
</tr>
<tr>
<td>12 hours</td>
<td>7</td>
<td>2.6 (± 0.16)</td>
<td>12.2 (± 0.46)</td>
<td>12.6 (± 1.60)</td>
<td>+/—</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>2.6 (± 0.17)</td>
<td>11.1 (± 0.34)</td>
<td>12.6 (± 1.60)</td>
<td>+/—</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>2.3 (± 0.15)</td>
<td>14.6 (± 0.91)</td>
<td>14.6 (± 0.91)</td>
<td>+/—</td>
</tr>
<tr>
<td>Milk Stasis</td>
<td>10</td>
<td>3.1 (± 0.18)</td>
<td>11.1 (± 0.42)</td>
<td>10.8 (± 1.03)</td>
<td>+/—</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>1.7 (± 0.15)</td>
<td>10.1 (± 0.24)</td>
<td>10.8 (± 1.03)</td>
<td>+/—</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>2.5 (± 0.17)</td>
<td>11.4 (± 0.54)</td>
<td>11.4 (± 0.54)</td>
<td>+/—</td>
</tr>
<tr>
<td>24 hours</td>
<td>13* m</td>
<td>2.5 (± 0.17)</td>
<td>11.3 (± 0.55)</td>
<td>10.9 (± 0.37)</td>
<td>++/—</td>
</tr>
<tr>
<td></td>
<td>14* h</td>
<td>1.6 (± 0.16)</td>
<td>10.3 (± 0.41)</td>
<td>10.9 (± 0.37)</td>
<td>++/—</td>
</tr>
<tr>
<td></td>
<td>15* l</td>
<td>1.3 (± 0.15)</td>
<td>10.9 (± 0.37)</td>
<td>10.9 (± 0.37)</td>
<td>++/—</td>
</tr>
<tr>
<td>36 hours</td>
<td>16* m</td>
<td>2.0 (± 0.0)</td>
<td>11.8 (± 0.39)</td>
<td>11.3 (± 0.57)</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>17* l</td>
<td>1.0 (± 0.0)</td>
<td>11.3 (± 0.57)</td>
<td>11.3 (± 0.57)</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>18* h</td>
<td>2.5 (± 0.17)</td>
<td>10.9 (± 0.84)</td>
<td>10.9 (± 0.84)</td>
<td>++/—</td>
</tr>
<tr>
<td>Early Involution</td>
<td>19* m</td>
<td>1.7 (± 0.15)</td>
<td>11.7 (± 0.50)</td>
<td>11.7 (± 0.50)</td>
<td>++/—</td>
</tr>
<tr>
<td></td>
<td>20* m</td>
<td>0.0 (± 0.0)</td>
<td>11.9 (± 0.34)</td>
<td>11.9 (± 0.34)</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>21* h</td>
<td>1.8 (± 0.13)</td>
<td>11.3 (± 0.76)</td>
<td>11.3 (± 0.76)</td>
<td>++/—</td>
</tr>
</tbody>
</table>

Notes:
- Histological grades are based on a minimum score of 0 (no lactating alveoli) and a maximum score of 4 (≥95% lactating alveoli), as described in Materials and Methods 2.1
- SEC engorgement score criteria: (−) = no engorgement; (+) = small to moderate lipid vesicles seen; (++) = large lipid vesicles seen; (+++) = very large vesicles seen, and cells bulge into lumen.
- Combinations of observations within a section are indicated by (/)
- (*) denotes independently-obtained α-lactalbumin mRNA levels: l = low, m = moderate, h = high

Table 3.1
Histological features of bovine mammary alveoli and secretory epithelial cells (SECs) following abrupt cessation of milking

<table>
<thead>
<tr>
<th>Time since last lactation</th>
<th>Animal</th>
<th>Histological grade of lactation (± SEM)</th>
<th>Individual animal SEC width (μm) (± SEM)</th>
<th>Grouped animal SEC width (± SEM)</th>
<th>Degree of SEC engorgement</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 hours</td>
<td>1</td>
<td>4.0 (± 0.0)</td>
<td>10.1 (± 0.34)</td>
<td>11.4 (± 0.86)</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>3.9 (± 0.1)</td>
<td>12.0 (± 0.50)</td>
<td>12.1 (± 0.29)</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>4.0 (± 0.0)</td>
<td>12.0 (± 0.50)</td>
<td>12.1 (± 0.29)</td>
<td>—</td>
</tr>
<tr>
<td>Active Lactation</td>
<td>4</td>
<td>3.6 (± 0.16)</td>
<td>13.1 (± 0.54)</td>
<td>12.6 (± 0.99)</td>
<td>+/—</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>3.4 (± 0.16)</td>
<td>12.4 (± 0.52)</td>
<td>12.2 (± 0.38)</td>
<td>+/—</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>3.8 (± 0.13)</td>
<td>12.2 (± 0.38)</td>
<td>12.2 (± 0.38)</td>
<td>+/—</td>
</tr>
<tr>
<td>12 hours</td>
<td>7</td>
<td>2.6 (± 0.16)</td>
<td>12.2 (± 0.46)</td>
<td>12.6 (± 1.60)</td>
<td>+/—</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>2.6 (± 0.17)</td>
<td>11.1 (± 0.34)</td>
<td>12.6 (± 1.60)</td>
<td>+/—</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>2.3 (± 0.15)</td>
<td>14.6 (± 0.91)</td>
<td>14.6 (± 0.91)</td>
<td>+/—</td>
</tr>
<tr>
<td>Milk Stasis</td>
<td>10</td>
<td>3.1 (± 0.18)</td>
<td>11.1 (± 0.42)</td>
<td>10.8 (± 1.03)</td>
<td>+/—</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>1.7 (± 0.15)</td>
<td>10.1 (± 0.24)</td>
<td>10.8 (± 1.03)</td>
<td>+/—</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>2.5 (± 0.17)</td>
<td>11.4 (± 0.54)</td>
<td>11.4 (± 0.54)</td>
<td>+/—</td>
</tr>
<tr>
<td>24 hours</td>
<td>13* m</td>
<td>2.5 (± 0.17)</td>
<td>11.3 (± 0.55)</td>
<td>10.9 (± 0.37)</td>
<td>++/—</td>
</tr>
<tr>
<td></td>
<td>14* h</td>
<td>1.6 (± 0.16)</td>
<td>10.3 (± 0.41)</td>
<td>10.9 (± 0.37)</td>
<td>++/—</td>
</tr>
<tr>
<td></td>
<td>15* l</td>
<td>1.3 (± 0.15)</td>
<td>10.9 (± 0.37)</td>
<td>10.9 (± 0.37)</td>
<td>++/—</td>
</tr>
<tr>
<td>36 hours</td>
<td>16* m</td>
<td>2.0 (± 0.0)</td>
<td>11.8 (± 0.39)</td>
<td>11.3 (± 0.57)</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>17* l</td>
<td>1.0 (± 0.0)</td>
<td>11.3 (± 0.57)</td>
<td>11.3 (± 0.57)</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>18* h</td>
<td>2.5 (± 0.17)</td>
<td>10.9 (± 0.84)</td>
<td>10.9 (± 0.84)</td>
<td>++/—</td>
</tr>
<tr>
<td>Early Involution</td>
<td>19* m</td>
<td>1.7 (± 0.15)</td>
<td>11.7 (± 0.50)</td>
<td>11.7 (± 0.50)</td>
<td>++/—</td>
</tr>
<tr>
<td></td>
<td>20* m</td>
<td>0.0 (± 0.0)</td>
<td>11.9 (± 0.34)</td>
<td>11.9 (± 0.34)</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>21* h</td>
<td>1.8 (± 0.13)</td>
<td>11.3 (± 0.76)</td>
<td>11.3 (± 0.76)</td>
<td>++/—</td>
</tr>
</tbody>
</table>
Among all three post-milking stages, the lactation grades were the highest in tissue from actively lactating animals, where they ranged from 3.4 to 4.0. Within this stage, the grouped mean SEC lateral width was $11.4 \pm 0.86 \, \mu m$ at 6 hours post-milking, and $12.6 \pm 0.99 \, \mu m$ at 12 hours post-milking. Individual animal SEC widths ranged from $10.1 \pm 0.34 \, \mu m$ to $13.1 \pm 0.54 \, \mu m$. The degree of SEC cell engorgement in this group ranged from no engorgement to areas of moderate engorgement.

At milk stasis, the lactation grades ranged from 1.3 to 3.1. Grouped mean SEC widths were the greatest in 18 hours post-milking animals ($12.6 \pm 1.60 \, \mu m$), and the lowest at 24 hours post-milking ($10.8 \pm 1.03 \, \mu m$). Individual animal SEC widths at this stage ranged between $10.1 \pm 0.24 \, \mu m$ and $14.6 \pm 0.91 \, \mu m$. The widest average SEC measurement at this stage was also the widest among all individual animals in this study, and recorded in an 18 hours post-milking animal. During milk stasis, the degree of SEC engorgement was greater than the previous active lactation stage, with larger lipid vesicles seen more frequently.

Lactation grades at early involution ranged from 0 to 2.5, which were the lowest rankings observed between the three post-milking stages. The grouped mean SEC width was $11.3 \pm 1.21 \, \mu m$ at 72 hours post-milking, and $11.6 \pm 0.95 \, \mu m$ at 192 hours post-milking. Individual animal SEC width was between $10.9 \pm 0.84 \, \mu m$ to $11.9 \pm 0.76 \, \mu m$. At this most-extended post-milking stage, a greater degree of SEC engorgement was observed, with most animals displaying large to very large accumulated lipid vesicles in most areas of the section.

Expression levels of $\alpha$-lactalbumin mRNA were generally correlated with histological grades. Within time points, tissue from animals defined as having high or moderate $\alpha$-lactalbumin levels consistently received a higher lactation grade than the tissue from animals with low $\alpha$-lactalbumin levels. Except for at 36 hours post-milking, tissue from animals with the highest $\alpha$-lactalbumin levels received higher lactation grades than those classed as moderate, among animals at the same time point.
3.2 Immunofluorescent identification of primary cilia components

3.2.1 Confocal imaging of primary cilia in mammary tissue

Representative composite micrographs of the acetylated-α-tubulin/γ-tubulin primary cilia detection series, were obtained using the optimised immuno-detection procedures and imaging methods detailed in Materials and Methods, (Figures 3.2.1 – 3.2.3). These are grouped according to the three previously established post-milking stages: active lactation, milk stasis, and early involution. Primary cilia were identified by green axoneme staining and co-localisation with red basal body/centriole staining in relation to the blue-stained nuclei of the alveoli and surrounding structures. Images where primary cilia could be detected are shown, however some alveoli appeared to have no (or very few) obvious primary cilia.

Often, the red blood cells were imaged as red or orange cells (eg. Figure 3.2.1, F, G and J) due to their intrinsic auto-fluorescence in these preparations. These cells were not analysed, and could be easily distinguished from the specific immuno-positive centrioles based on their considerably larger size.

Active lactation

Figure 3.2.1 shows representative composite micrographs of primary cilia distribution within alveolar mammary tissue of animals at active lactation. A – E show 6 hours post-milking images, F – J show 12 hours post milking images. In those alveoli where primary cilia were evident, they were seen projecting from the apical aspect of luminal SECs. Most primary cilia were short (between 1 µm and 2 µm) and frequently their distal tips were slightly tapered, and these projected into the luminal space at varying orientations (G). Some primary cilia appeared to have bent axonemes (F), while others were generally straight, and projected either obliquely (A and C), or perpendicular to the alveolar wall (D). Their associated centriole was most often seen in close proximity to the nuclei, such that their cell of origin could generally be identified. However, in cases where alveoli had been sectioned close to a margin, the alveolar epithelial cell layers were more difficult to interpret (H). In alveoli comprised of widely-spaced nuclei (indicating alveolar distention), the cilia of SECs were often deflected to lie more parallel to the alveolar wall (A), suggesting deflection against the apical surface. Primary cilia were also detected in the
small ducts, where they projected into the ductal lumen (I), and in fibroblasts in the interlobular connective tissue (E).

**Milk stasis**

Figure 3.2.2 shows representative composite micrographs of primary cilia distribution within alveolar mammary tissue of animals during milk stasis. A and B show 18 hours post-milking images, C and D show 24 hours post milking images, and E and F show 36 hours post-milking images. The immunofluorescent double-labelling of primary cilia in mammary tissue from animals in milk stasis gave similar results to those from animals at the active lactation post-milking stage. A degree of variability was seen in the shape of the ciliary axoneme, and in the direction of ciliary projection at milk stasis (evident throughout all images shown). Upon viewing individual cilia in relation to their cell of origin, some appeared to be wrapped around the apical aspect of the SECs (B, D, and E), with a small number deflected completely between the lateral cell surfaces, away from the lumen (F). This may be in response to the increase in SEC engorgement, as seen in the H&E analysis (as shown in Figures 3.1.2 and 3.1.3). Primary cilia were also seen on fibroblasts in the inter-alveolar stroma, and similar ciliary morphology was seen in these cells that was seen in alveoli (C).

**Early involution**

Figure 3.2.3 shows representative composite micrographs of primary cilia distribution within alveolar mammary tissue of animals during milk stasis. A – E show 72 hours post-milking images, F – J show 192 hours post milking images. Although not illustrated in these images, primary cilia became difficult to identify in some sections due to the increased background autofluorescence in comparison to the earlier two post-milking stages. Of the clearly-interpretable images, primary cilia were seen in alveolar cells, although fewer were seen to project into the luminal space compared to the previous two stages. Ciliary morphology at this extended post-milking stage resembled that seen in the earlier two stages. Primary cilia were most often shown to gently curve around the apical aspect of SECs (A, B and D). Cells in the inter-lobular, and inter-alveolar stromal areas (J) were shown to have primary cilia, which were also of a similar morphology to those seen in these regions at the earlier stages. Occasionally, the cell of origin of a primary cilium could not be identified (F and I). In these cases, the cilia may have been either associated with inter-alveolar cells or myoepithelial cells at a further distance from the nucleus than
is typically seen, resulting in the nucleus being absent from the focal plane during microscopy.
Micrograph image plates

Figure 3.2.1 .......................  60
Figure 3.2.2 .......................  61
Figure 3.2.3 .......................  62
Figure 3.2.1 (facing page)
Primary cilia in alveolar mammary tissue sections from actively lactating animals
Representative confocal composite micrographs

A – E: 6 hours post-milking; F – J: 12 hours post-milking; E: primary cilium in an inter-lobular stromal cell; I: ductal epithelium showing primary cilia distributed along the luminal surface.

Lu: Lumen;  Bb: Basal body;  Ax: Axoneme;  Inter-lob Stroma: Inter-lobular stromal tissue

Arrows indicate examples of primary cilia
Green: acetylated-α-tubulin; Red: γ-tubulin; Blue: nuclei

Scale bars are 20 μm, except those in enlarged, inserted images, where scale bars are 5 μm. Dotted lines indicate areas of enlargement.
Figure 3.2.2 (facing page)

Primary cilia in alveolar mammary tissue sections from animals in milk stasis

Representative confocal composite micrographs

A and B: 18 hours post-milking; C and D: 24 hours post-milking; E and F: 36 hours post-milking.

Lu: Lumen; Bb: Basal body; Inter-alv Stroma: Inter-alveolar stromal tissue

Green: acetylated-α-tubulin; Red: γ-tubulin; Blue: nuclei

Scale bars are 20 μm, except those in enlarged, inserted images, where scale bars are 5 μm. Dotted lines indicate areas of enlargement.
**Figure 3.2.3 (facing page)**

Primary cilia in alveolar mammary tissue sections from animals in early involution

Representative confocal composite micrographs


Lu: Lumen;  Inter-alv Stroma: Inter-alveolar stromal tissue

Green: acetylated-α-tubulin; Red: γ-tubulin; Blue: nuclei

Scale bars are 20 μm (unless otherwise stated), except those in enlarged, inserted images, where scale bars are 5 μm. Dotted lines indicate areas of enlargement.
3.2.2 Primary cilia in myoepithelial cells

Primary cilia were selectively identified in myoepithelial cells of mammary alveoli by the co-localisation of a ciliary structure and the corresponding cytoplasmic presence of α-SMA immuno-reactivity. Based on the positive dual identification of ciliary components in adjacent sections (as described in Section 3.2.1), primary cilia could be convincingly identified in these sections, despite only relying on acetylated-α-tubulin detection. Representative examples of the composite micrographs are shown in Figures 3.2.4 – 3.2.6, and grouped according to the three post-milking stages. According to the positive α-SMA staining surrounding some sub-luminal nuclei, these were identified as belonging to myoepithelial cells. Thin bands of α-SMA-positive staining were also evident underlying the luminal alveolar cell layer. These were inferred to be cytoplasmic processes, which typically extend from alveolar myoepithelial cells. The bands of α-SMA-positive staining delineated the circumference of one alveolus from an adjacent alveolus, but were not completely continuous. This was indicative of the wicker-basket-like arrangement that networks myoepithelial cell cytoplasmic processes in mammary alveoli. As seen previously in the immunofluorescent double-labelling of primary cilia, the fluorescent imaging also detects autofluorescent red blood cells, however these were not analysed in any way.

Active lactation

Figure 3.2.4 shows representative composite micrographs of primary cilia and α-SMA distribution within mammary alveoli of animals in active lactation. A – E show 6 hours post-milking images, F – J show 12 hours post-milking images. As shown throughout the selected images, α-SMA could be readily identified in cytoplasmic areas of cells underlying the layer of luminal, un-stained (α-SMA negative) cells, designated as SECs. Primary cilia could be identified in close proximity to the nuclei of myoepithelial cells, and projected from the apical aspect of these cells (A, B and D). They were visually identical to those observed in SECs and displayed a degree of between-cell variability in their orientation in relation to the alveolar lumen. Some were virtually parallel to the alveolar wall (D), while others projected at various oblique angles towards the lumen (A and B). Occasionally, the distinction between SECs and myoepithelial cells was not immediately obvious due to cells being imaged in a cross-section close to an alveolar margin (H). Stromal areas showed α-SMA negative fibroblasts, where primary cilia were evident (J).
Milk Stasis

Figure 3.2.5 shows representative composite micrographs of primary cilia and α-SMA distribution within mammary alveoli of animals in milk stasis. A – C show 18 hours post-milking images, D – F show 24 hours post milking images, and G – I show 36 hours post-milking images. As in sections from actively lactating bovine mammary tissue, the α-SMA/acetylated-α-tubulin double immunofluorescence detection provided good visualisation of primary cilia in myoepithelial cells during milk stasis. They projected from the apical aspect of the cell at a range of orientations (A, C – F). In some cases, primary cilia projected from myoepithelial cells directly towards the lumen within the baso-lateral spaces between SECs (H). An α-SMA-negative fibroblast in the inter-lobular stroma can be seen in image I.

Early involution

Figure 3.2.6 shows representative composite micrographs of primary cilia and α-SMA distribution within mammary alveoli of animals in early involution. A – E show 72 hours post-milking images, F – H show 192 hours post milking images.

In some alveolar areas at this extended post-milking stage, the α-SMA-positive bands in the sub-luminal regions appeared more condensed in comparison to those observed at the earlier post-milking stages (C and F). Primary cilia were identified in myoepithelial cells (A, C, E and F), SECs (B and D), and stromal cells from inter-alveolar and inter-lobular areas (G and H). No obvious differences in the appearance or orientation of the primary cilia were observed between tissue sections at this time point and those at earlier stages. As noted with the set of slides stained for acetylated-α-tubulin/γ-tubulin primary cilia detection (detailed in the previous section), there was a noticeable increase in the degree of background and tissue autofluorescence in comparison to the earlier time points, obvious to a small degree in B.
Micrograph image plates

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Figure 3.2.4 (facing page)

**Primary cilia in myoepithelial cells and SECs in tissue sections from actively lactating animals**

Representative confocal composite micrographs

A – E: 6 hours post-milking; F – J: 12 hours post-milking.

**SE:** Secretory epithelial cell; **ME:** Myoepithelial cell; **Lu:** Lumen;
**Inter-lob Stroma:** Inter-lobular stromal tissue

Long arrows indicate examples of cilia on myoepithelial cells; Arrowheads indicate examples of cilia on secretory epithelial cells in alveolar areas, or fibroblasts in stromal areas

**Green:** α-SMA; **Red:** acetylated-α-tubulin; **Blue:** nuclei

Scale bars are 20 μm, except those in enlarged, inserted images, where scale bars are 5 μm
Dotted lines indicate areas of enlargement.
Figure 3.2.5 (facing page)

Primary cilia in myoepithelial cells and SECs in tissue sections from animals in milk stasis

Representative confocal composite micrographs

A – C: 18 hours post-milking; D – F: 24 hours post-milking; G – I: 36 hours post-milking.

SE: Secretory epithelial cell; ME: Myoepithelial cell; Lu: Lumen;
Inter-lob Stroma: Inter-lobular stromal tissue

Long arrows indicate examples of cilia on myoepithelial cells; Arrowheads indicate examples of cilia on secretory epithelial cells in alveolar areas, or fibroblasts in stromal areas

Green: α-SMA; Red: acetylated-α-tubulin; Blue: nuclei

Scale bars are 20 μm
Figure 3.2.6 (facing page)

Primary cilia in myoepithelial cells and SECs in tissue sections from animals in early involution

Representative confocal composite micrographs


SE: Secretory epithelial cell; ME: Myoepithelial cell; Lu: Lumen;
Inter-lob Stroma: Inter-lobular stromal tissue; Inter-alv stroma: Inter-alveolar stromal tissue

Long arrows indicate examples of cilia on myoepithelial cells; Arrowheads indicate examples of cilia on secretory epithelial cells in alveolar areas, or fibroblasts in stromal areas

Green: α-SMA; Red: acetylated-α-tubulin; Blue: nuclei

Scale bars are 20 μm
3.3 Primary cilia analysis

3.3.1 Primary cilium length

Mean primary ciliary length measured in SECs and α-SMA-positive myoepithelial cells is shown in Figure 3.3.1. The mean length of primary cilia in myoepithelial cells (± SEM) was 1.59 (± 0.22) µm at active lactation, 1.45 (± 0.17) µm at milk stasis and 1.36 (± 0.51) µm at early involution. In SECs, mean ciliary lengths were 1.44 (± 0.18) µm at active lactation, 1.48 (± 0.15) µm at milk stasis, and 1.41 (± 0.19) µm at early involution. No significant differences in primary ciliary length were detected when comparing either within (p > 0.05) or between (p > 0.05) post-milking stages for any cell type.

![Ciliary length in bovine mammary tissue after the abrupt cessation of milk removal](image)

**Figure 3.3.1**

*Primary ciliary length in bovine mammary tissue after the abrupt cessation of milk removal*

Mean ciliary length measurements ± SEM are displayed for myoepithelial cells and secretory epithelial cells (SECs) at each post-milking stage. Active lactation: 6 and 12 hours post-milking, milk stasis: 18 – 36 hours post-milking, early involution: 72 and 192 hours post-milking. Ciliary length is defined as basal body and ciliary axoneme. Alveolar cell types were identified by the presence (myoepithelial cells) or absence (SECs) of cytoplasmic α-smooth muscle actin (SMA) fluorescent immuno-staining.
3.3.2 Primary cilium morphology and orientation

In SECs, all cilia projected from the apical cell surface into the alveolar lumen (Figures 3.2.1 – 3.2.6). Variations in the shape and orientation of primary cilia were observed throughout all composite micrographs in the post-milking time-course tissue sections. Often, there was no uniform appearance of primary cilia within the same alveolus. However, some alveoli, particularly those which appeared distended (with flattened cuboidal cells), contained primary cilia on the luminal surface of SECs that all appeared flattened parallel to the alveolar wall (Figure 3.2.2, E). As noted in Section 3.2.2, some primary cilia appeared deflected beyond parallel to the alveolar wall, and projected toward the lateral aspect of the cell (Figure 3.2.2, F). Judging by the distance of the centriole from the nucleus (which was further than in many other cells), these overtly-deflected cilia were assumed to be associated with engorged SECs. In α-SMA-positive myoepithelial cells, varying ciliary orientations were also observed. Following these observations, primary cilia were classified according to their orientation relative to the alveolar wall, as described in Materials and Methods 2.2.3.

3.3.3 Examples of bent and deflected primary cilia

The variations in primary cilium morphology, and examples of cilia that were classified as ‘deflected’ and ‘bent’ from luminal SECs, are shown in Figure 3.3.2. Examples of the varying extents of deflection that were observed throughout the time-course tissue sections are shown in A – C, with these examples being classified as ‘non-bent’ cilia. C shows a primary cilium that appears completely deflected, given the apical location of the centriole in relation to the axoneme. D shows an example of a ‘non-deflected’ primary cilium, projecting from the apical aspect of a luminal SEC, virtually perpendicular to the alveolar wall. E and F show deflected primary cilia that were classed as ‘bent’.
Figure 3.3.2 (facing page)

Variations in primary cilium morphology

Confocal composite micrographs showing deflected, straight and bent primary cilia throughout time-course post-milking bovine tissue sections

A: A 12 hours post-milked section showing a deflected, non-bent primary cilium, with dashed lines indicating how the angle of the ciliary axoneme in relation to the lumen was determined for all deflection assessments. B: A 12 hours post-milked section showing a deflected, non-bent primary cilium. C: 36 hours post-milked section, the primary cilium is completely deflected. D: An 18 hours post-milked section, the dotted line indicates the alveolar wall, and the primary cilium is classed as straight. E: A 12 hours post-milked section, with a deflected cilium, classed as ‘bent’. F: An 18 hours post-milked section, with a deflected cilium, classed as ‘bent’.

Lu: Lumen; Bb: Basal body

Green: acetylated-α-tubulin; Red: γ-tubulin; Blue: nuclei

Scale bars are 5 μm
3.3.4 Primary cilia orientations in luminal SECs

Ciliary deflection data obtained in luminal SECs are shown in Figure 3.3.3. Primary cilia observed at active lactation showed the lowest proportion of deflected cilia, out of the total number of cilia counted (52%). The highest proportion was seen in sections from milk stasis (73%), and the proportion deflected in early involution fell in between these two groups (60%). The difference of 21% in the proportion of deflected cilia in the actively lactating tissue sections, compared to those in milk stasis was statistically significant ($p = 0.0027$). There was no statistical significance for the ciliary deflection comparisons between early involution and either active lactation or milk stasis ($p > 0.05$).

![Figure 3.3.3](image)

Proportion of primary cilia classed as ‘deflected’ or ‘non-deflected’ in luminal SECs.

Primary cilium morphology assessed following the abrupt cessation of milk removal in dairy cows

Lactation: 6 and 12 hours post-milking ($n = 6$ animals), milk stasis: 18 – 36 hours post-milking ($n = 7$ animals), early involution: 72 and 192 hours post-milking ($n = 5$ animals)

(* $p < 0.01$)
Cilia with acutely bent axonemes

The greatest proportion of those deflected cilia that were acutely bent in luminal SECs were in tissue sections from actively lactating animals (17%). Fewer were observed in animals in milk stasis (5%), and none of the deflected cilia seen in early involuting animals were bent. Results are displayed in Figure 3.3.4. There was a statistically significant difference in the proportion of those that were bent between active lactation and milk stasis ($p = 0.027$), and between active lactation and early involution ($p = 0.015$). The proportions of bent/non-bent cilia were similar between milk stasis and early involution ($p > 0.05$). Primary cilia proved more difficult to image in tissue sections from some early involuting animals due to increased background autofluorescence. This is reflected in the total number of cilia included within the early involution post-milking stage.

![Figure 3.3.4](image-url)

**Figure 3.3.4**

Proportion of primary cilia classed as ‘bent’ and ‘non-bent’, of total deflected cilia in luminal SECs

Primary cilia morphology assessed following the abrupt cessation of milk removal in dairy cows

Lactation: 6 and 12 hours post-milking ($n = 6$ animals), milk stasis: 18 – 36 hours post-milking ($n = 7$ animals), early involution: 72 and 192 hours post-milking ($n = 5$ animals)

(*$p < 0.05$)
3.3.5 Primary cilia orientations in myoepithelial cells

The primary cilia which projected from myoepithelial cells all appeared to originate from the cell surface closest to the lumen. There were variations in their angle of projection in relation to the alveolar lumen which they were associated with, although the majority of cilia at each post-milking stage were ‘deflected’. The proportions of ‘deflected’ and ‘non-deflected’ cilia at each post-milking stage are graphically presented in Figure 3.3.5. Of total primary cilia belonging to myoepithelial cells, the proportion of ‘deflected’ were similar among post-milking stages ($p > 0.05$). These were 75% at active lactation, 72% at milk stasis and 88% at early involution. Primary cilia were occasionally classed as ‘bent’, although since this definition included no more than two for any post-milking stage, a comparative analysis of the proportion of bent cilia in myoepithelial cells was not performed.

![Figure 3.3.5](image)

**Figure 3.3.5**

Proportion of primary cilia classed as ‘deflected’ and ‘non-deflected' in myoepithelial cells at each post-milking stage

Primary cilia morphology assessed following the abrupt cessation of milk removal in dairy cows

Lactation: 6 and 12 hours post-milking ($n = 4$ animals), milk stasis: 18 – 36 hours post-milking ($n = 6$ animals), early involution: 72 and 192 hours post-milking ($n = 4$ animals)
3.3.6 Primary cilia in stromal fibroblasts

Primary cilia were associated with cells in inter-lobular and inter-alveolar stromal regions at all three post-milking stages. According to their location and lack of α-SMA immuno-reactivity, these were assumed to be fibroblasts. Representative images are shown among those presented in the composite micrograph plates in Figures 3.2.1 – 3.2.6 (see 3.2.1 E, 3.2.2 C, 3.2.3 J, 3.2.4 J, 3.2.5 I, and 3.2.6 F and H). Stromal cilia ranged in length from 0.87 µm to 2.01 µm, and were sometimes seen oriented parallel to the collagen fibres of the stroma (which were dimly visible prior to optimising the signal-to-noise ratio for the positive staining). Due to the nature of connective tissues, the cells were more widely spread than those of epithelial structures and, therefore, were less frequently imaged than alveolar cells. For this reason very few primary cilia were imaged in the stromal cells from inter-lobular or inter-alveolar regions, and data between post-milking stages in these cells was not analysed.
3.3.7 Ciliary incidence in mammary alveoli during active lactation

The incidence data at active lactation were collected and averaged for two cell categories: luminal cells, and total alveolar cells, as described in Materials and Methods 2.2. Results are shown in Figure 3.3.6, which displays the mean incidence ± SEM, as observed within multiple micrographs (for n = 6 animals).

During the active lactation post-milking stage, primary cilia were detected in an average of 11% (± 4%) of luminal cells and 14% (± 3%) of total alveolar cells. Centrioles (whether associated with a cilium or not) were seen in 25% (± 6%) of luminal cells, and 28% (± 3%) of total alveolar cells. Not all centrioles observed formed a ciliary basal body, and an identifiable cilium was associated with 44% (± 7%) of centriole pairs on luminal cells, and 46% (± 2%) of centriole pairs in total alveolar cells. These incidence data will most likely be underestimates of the actual biological incidence, as both the thickness of the tissue sections (5 – 8 µm) and the depth of Z-stacks (1.64 – 6.55 µm) were considerably less than the average width of a luminal SEC, and these issues are further detailed in Discussion 4.1.5. Raw data from individual animals ranged between 3% and 30% for ciliated cells, between 15% and 48% for cells with centrioles, and between 13% and 65% for centrioles with cilia.
<table>
<thead>
<tr>
<th>Incidence observation</th>
<th>Mean (± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Luminal cells</td>
<td></td>
</tr>
<tr>
<td>Primary cilia per 100 nuclei</td>
<td>11 (± 4)</td>
</tr>
<tr>
<td>Centrioles per 100 nuclei</td>
<td>25 (± 6)</td>
</tr>
<tr>
<td>Primary cilia per 100 centriole pairs</td>
<td>44 (± 7)</td>
</tr>
<tr>
<td>Alveolar cells</td>
<td></td>
</tr>
<tr>
<td>Primary cilia per 100 nuclei</td>
<td>14 (± 3)</td>
</tr>
<tr>
<td>Centrioles per 100 nuclei</td>
<td>28 (± 3)</td>
</tr>
<tr>
<td>Primary cilia per 100 centriole pairs</td>
<td>46 (± 2)</td>
</tr>
</tbody>
</table>

**Figure 3.3.6**

Incidence of primary cilia and centrioles observed in actively lactating bovine mammary tissue in luminal cells and total alveolar cells

n = 6 animals from 6 hours and 12 hours post-milking time points. Total nuclei counted = 1042, including 701 luminal nuclei. Data compiled from the analysis of 36 composite confocal micrographs.
Chapter 4 – Discussion

The balance of lactation and involution – and the spectrum of mammary function that lies between these two opposing physiological states – is under constant regulation by a multitude of interacting factors. Many regulatory aspects have been previously determined, and much is now known about the endocrinology of milk production, the maintenance of cell survival, and the cellular and tissue responses to local factors, such as accumulated milk.\textsuperscript{40; 44; 50; 59} Despite a vast and growing body of knowledge of many regulatory aspects, questions still remain as to how certain stimulatory factors mediate a cellular response – especially regarding the local control of milk production.\textsuperscript{34; 38; 58; 71}

Introductory sections in this research thesis have provided a broad profile of primary cilia and their various sensory capabilities. The recent surge in primary cilia-related research has provided evidence that these organelles can act as highly-sensitive mediators of various signal transduction events in a range of tissues and organs. Primary cilia have not previously been extensively imaged or studied in the bovine mammary gland during lactation. Therefore, this study into their expression and distribution at various lactation stages has provided novel information, which could indicate how they might contribute to the regulation of mammary gland function.

According to the aims outlined for this investigation, primary cilia have been successfully imaged in the glandular alveoli of bovine mammary tissue sections from a selection of dairy cows throughout the course of lactation, milk stasis, and early involution. Primary cilia were expressed in the two main cell types that comprise the alveolar wall: secretory epithelial cells (SECs) and myoepithelial cells, and in stromal fibroblasts within the inter-alveolar and inter-lobular stroma. Primary cilia were also identified in the epithelial cells lining both small and large ducts, which are interspersed within and between the lobules. This chapter discusses these results in relation to current published findings of primary cilia in the mammary gland (Section 4.1), and postulates what roles these sensory organelles may play in various mammary regulatory processes, given their known capabilities in other tissue types (Section 4.2). The implications of these findings for future studies of mammary regulation is also discussed (Section 4.3), and overall conclusions are outlined (Section 4.4).
4.1 Lactating and involuting bovine mammary tissue

4.1.1 Cellular and tissue changes following the abrupt cessation of milking

Following the abrupt cessation of milking, a post-milking time-course was tracked, by assessing mammary sections from three animals at each of seven time points ranging from 6 hours to 192 hours post-milking. These time points were grouped into three post-milking stages: 1) active lactation (6 and 12 hours post-milking), 2) milk stasis (18, 24 and 36 hours post-milking) and 3) early involution (72 and 192 hours post-milking). In order to gain an understanding of the general histology, and to assess the degree of involution within tissue sections, all were imaged and viewed for qualitative and semi-quantitative assessments prior to undertaking primary cilia detection studies on tissue in adjacent sections. This provided the opportunity to determine suitable post-milking time-point groupings, according to particular assessments of the tissue architecture and SEC appearance. Although variation was evident, the tissue observed within the post-milking groups was relatively consistent in histological appearance, and the time-frame within each group was biologically relevant in relation to the twice-daily milking schedule.

As documented by other researchers, there are general features of the alveolar histology that can indicate the secretory capacity of the alveoli, or the progression of involution. There is also a natural degree of variability within and between lobules, and between different animals at the same post-milking stage. The range of biological variability between animals and within tissue sections (inter-lobular variability) is shown throughout Figures 3.1.1 – 3.1.3, as described in Section 3.1.

Within the sections, the level of lactation capacity within alveoli was judged according to the histological features of alveoli and their resident SECs. Generally open, relaxed alveoli with a thin surrounding of stroma, and lined with cuboidal SECs (often containing small amounts of apical lipid content) were defined as ‘lactating’, when assigning lactation grades. Alveoli with signs of milk stasis or involution were indicated by the accumulation of darkly-stained luminal material, their more condensed appearance, and a more prominent surrounding of connective tissue. Their lining SECs were either stretched to a flattened cuboidal shape around a distended lumen, or bulged with large accumulated lipid vesicles into the lumen. Within these lumina, shed SECs and leukocytes were often seen.
An overall increase in the degree and number of features associated with milk stasis and involution, as the time since milk cessation increased, can be seen throughout the micrographs in Figures 3.1.1 – 3.1.3. As shown in Table 3.1, the semi-quantitative data obtained from the tissue also indicate a steady progression to involution – as expected, and lactation grades decreased as time post-milking increased (Figure 3.1.4). Comparing grades between animals at each time point (Table 3.1) also illustrates the overlap between stages, and the variation between animals, as reported in previous studies. Generally, the capacity for the alveoli to synthesise milk components at a given post-milking stage (as indicated by the level of α-lactalbumin expression) was correlated with the lactation grades (Table 3.1). As tissue was assessed from animals with a range of α-lactalbumin expression levels at each of the three latter time points, this minimised any potential bias relating to the inherent secretory capacity of individual animals in subsequent comparative studies.

Overall, the histological evaluations validated the assumptions that the sections provided for the study would reflect the progression towards mammary involution expected following the abrupt cessation of milk removal. Allowing for inter-animal and within-sample variation, the range in times post-milking, which defined each lactation stage, were also appropriately assigned for subsequent comparative analyses.

When assessing luminal cell width, the results indicate that between 12 and 18 hours post-milking SECs are, on average, wider than at any other post-milking time points (Table 3.1). This relates to the time period that extends from when the animals would be scheduled for the next milking until the time when the animals are six hours overdue for the next milking. From 36 hours until 192 hours post-milking, SECs show a greater degree of bulging and engorgement which, presumably, creates increased tension within the apical cell membrane. These observations relate to the time period when the animals are between one to seven days overdue for milking. The indicators of both stretch and tension are more prevalent in animals that have experienced at least twelve hours without milk removal compared to only six hours. Such physical changes, which are observed within a considerably short time-frame since last milk removal, provide a potential for changes in the tension or stretch within the SEC plasma membrane to act as a signal for the initiation of certain involution-associated events.

In many involution studies, the altered physical characteristics of SECs, and concurrent change in secretory behaviour, have been well-characterised and more recently, cell culture investigations are beginning to focus on manipulating
cell stretch to induce involution. Such studies indicate increased tension within the mammary epithelial cell membrane is associated with the initiation of certain events of involution, although the mechanisms by which these events occur have not yet been defined. In Section 4.2, the possibility the primary cilium plays a role in transducing signals associated with cell compression or stretch is considered, in relation to known cellular events occurring at this time.

4.1.2 Primary cilia in luminal secretory epithelial cells

Within post-milking stages, mean SEC primary cilium length was 1.44 ± 0.18 µm at active lactation, 1.48 ± 0.15 µm at milk stasis, and 1.41 ± 0.19 µm at early involution (Figure 3.3.1), where the length was defined as the length of the basal body and the axoneme. No statistically significant differences were seen in ciliary length comparisons between either the two alveolar cell types (SECs and myoepithelial cells) or any of the different post-milking stages. Although care was taken to only measure those cilia where the full length could be completely seen in the x-y axis, it is likely that some of those measured would be oriented tangentially to this axis. For this reason the length measurements may be slightly less than the actual length. However, for comparisons between cell types and post-milking stages, it was assumed any potential underestimation of ciliary length would apply to the entire set of measurements, without creating bias for any particular comparisons.

In SECs lining the alveolar lumen, primary cilia projected from the apical aspect of the cell into the lumen at a variety of orientations. As described in Section 3.2.1, and in association with the micrographs shown in Figures 3.2.1 – 3.2.3, primary cilia were seen throughout alveoli and stromal tissue. Overall, double-labelled cilia were seen in some – but not all – luminal cells, where they were most often tapered slightly at the distal tip. Often, primary cilia appeared gently curved around the apical aspect of the cell, a feature which suggests deflection of the cilia against the cell surface.

Ciliary deflection was most often observed in animals in milk stasis compared to those in active lactation or early involution (Figure 3.3.3). SEC primary cilia from all animals seen at milk stasis totalled 126 (n = 7 animals), and included 73% which were deflected. This proportion was considerably higher than at active lactation where 88 were counted (n = 6 animals) and 52% were deflected. These figures suggest that increased ciliary deflection may be correlated with factors specifically relating to the
events of milk stasis, as observed in the H&E sections (Figure 3.1.2). Such factors include the compression and stretch of some luminal SECs in response to a build-up of luminal material or the cellular engorgement seen, due to increased cytoplasmic lipid content (Table 3.1).

As luminal primary cilia are exposed to a fluid extracellular environment, where the pressure dynamics are considerable, it was not surprising to see cilia deflected in any direction around the luminal surface. Having identified this feature, it now remains to be considered whether ciliary deflection is biologically-relevant in the regulation of lactation or mammary involution. Primary cilia have been defined as mechano-sensors in a variety of tissue types, based on their bending in response to extracellular forces, their specific expression of mechano-receptors, and their inherent signal transduction functions. There is potential for this functionality to also translate to mammary tissue, and such mechanisms could possibly be considered in cellular homeostatic or regulatory processes, which are important for the local control of mammary function. This prospect is further discussed in Section 4.2.

Most SEC cilia appeared to have slightly curved axonemes, however the axoneme of some deflected cilia appeared to be acutely bent (Figure 3.3.2, E and F). The proportion of deflected cilia with bent axonemes was greater at active lactation (17%) than at either milk stasis (5%) or early involution (0%). Whether ciliary bending is more biologically relevant during active lactation than milk stasis or early involution is not known, although bending of the axoneme could result from the forces exerted by the accumulation of luminal contents. Given the reasonably low number of deflected cilia on which these observations are based (between 31 and 66 within post-milking stages), and the low number of bent cilia seen (between 0 and 11), broad interpretations may be more appropriate than comparing post-milking stages within these data. Further investigations are required to reliably quantitate aspects of ciliary axoneme bending for comparative purposes.

Another consideration when interpreting the data on SEC cilia is that the cilia of some small ducts are most likely included in these analyses. When using fluorescently-labelled sections, a certain amount of the detail regarding tissue architecture is not visible. During data collection, those cilia imaged in epithelial structures that were assumed to be alveoli, may in fact have been small ducts and, while these two structures are continuous, the cilia lining these structures are likely to be exposed to different extracellular conditions according to their distinct functions. Specifically, the
mammary alveoli function as milk storage structures and the ducts function as conduits for milk to flow through. For the purposes of this study, it was assumed that due to the method of sampling from varied areas throughout the sections, small ducts would be most likely imaged across sections from all animals, therefore limiting potential bias at any given stage. This provides scope for any future studies – particularly for those specifically relating to either ciliary involvement in ductal milk flow or intra-alveolar pressure, in which case small ducts and alveoli will need to be differentiated.

4.1.3 Primary cilia in myoepithelial cells

Average ciliary length in myoepithelial cells showed a tendency to decrease as time was extended post-milking, although considerable variability was seen in sections from animals at early involution, and no statistically significant comparisons were observed (Figure 3.3.1). Overall, average myoepithelial ciliary length ranged from 1.59 ± 0.22 µm at active lactation to 1.36 ± 0.51 µm at early involution.

Primary cilia were seen in myoepithelial cells of alveolar structures by using α-SMA/acetylated-α-tubulin double-detection fluorescent immunohistochemistry (see Figures 3.2.4 – 3.2.6). Cilia were seen on the alveolar-facing aspect of these cells in animals at all three post-milking stages, and projected into the intercellular space underlying SECs. They projected at varying orientations in relation to the alveolar wall, and were often seen in close proximity to the nucleus, suggesting they might be invaginated within a ciliary pocket, as was evident in previously published ultrastructural findings.106

The proportion of deflected myoepithelial cilia were recorded across the three post-milking stages, and no statistically significant differences were seen between-stage comparisons, possibly reflecting the low numbers counted. At active lactation, 75% of cilia were deflected (of 12 counted), at milk stasis 72% were deflected (of 32 counted), and at early involution, the highest proportion of myoepithelial cilia were deflected, at 88% (of 8 counted). The low number of cilia counted for this cell type reflects the fact that they are more widely-spaced in their distribution than SECs, therefore they will not always be imaged in an alveolar cross-section. Occasionally, bent cilia were seen, although no analysis was performed due to low numbers of observations (1 at active lactation, 2 at milk stasis, and 2 at early involution).
The fact that cilia were identified in myoepithelial cells raises many possibilities as to their role in these cells during mammary function and regulation. Given their known paracrine signalling potential,\textsuperscript{17, 26} and regulatory functions in SEC development and differentiation,\textsuperscript{17, 120} it is possible that the primary cilium could initiate signals in a myoepithelial cell in response to external cues, which could in turn modulate SEC activity. The potential for such ciliary-mediated signalling in this cell layer during lactation and involution is further discussed in Section 4.2.

4.1.4 Primary cilia in stromal fibroblasts

At all post-milking stages, primary cilia were detected in fibroblasts in both inter-alveolar and inter-lobular stromal areas. Individual ciliary length measurements in these cells ranged from 0.89 µm to 2.01 µm, however they were not grouped for analysis due to the very small numbers observed. The inter-alveolar stromal areas in lactating animals is often very scant, as observed in the H&E micrographs in Figure 3.1.1. Except for isolated areas of alveolar condensation (E), the inter-alveolar stroma is not of sufficient size to frequently see nuclei until the later post-milking stages (Figure 3.1.2). The less-frequent identification of stromal fibroblasts in inter-lobular areas is attributed to the nature of this tissue, in which the majority of the area is filled with secreted collagen fibres, and individual cells are usually widely-spaced. Due to the less-frequent sightings of fibroblasts in either of these areas and the potential for a nucleus to be included in a section without the primary cilium being in the same cut section, it was difficult to determine if every fibroblast was ciliated. Regardless of this detail, the fact primary cilia were seen in fibroblasts in stromal areas, and were variable in size, introduces many possibilities as to their potential roles, particularly in their interaction with ECM components, and this will be discussed in Section 4.2.

4.1.5 Ciliary incidence in lactating mammary alveoli

Primary cilia incidence assessments (Section 3.3.3 and Figure 3.3.6) were intended to provide preliminary incidence data to give an overall impression of their distribution. For sections belonging to the active lactation post-milking stage, the tissue set stained for double-labelling of primary cilia was used for analysis (n = 6 animals) to ensure information regarding centrioles could also be collected. Within alveoli, the
average incidence of primary cilia (± SEM) was 14 ± 3% for total alveolar epithelial cells (both SECs and myoepithelial cells), and 11 ± 4% for luminal SECs alone.

Alveolar cells spanned a wider region than could be completely seen in a single section. Therefore, centriole incidence was assessed to indicate whether the portion of a cell with its nucleus included in the incidence observations, would be imaged in the appropriate location to detect a primary cilium, if present. Centrioles were imaged in an average (± SEM) of 28 ± 3% of total alveolar cells and 25 ± 6% of luminal SECs. Given every cell will have a centriole, this observed incidence is slightly lower than the expected incidence, when allowing for imaging parameters. According to the depth of the tissues imaged (at an average of 3.8 µm) and the average width of SECs within post-milking time points (between 10.8 ± 1.0 µm and 12.6 ± 1.6 µm, Table 3.1), an incidence of between 30% and 35% would be expected. This difference between observed and expected centriole incidence may reflect the tendency for the fluorescent label bound to anti-γ-tubulin to bleach easily following exposure to laser light, as its target antigen is only present in very small quantities. There is also the possibility that the confocal imaging in some areas may not have captured images from a wide-enough focal plane, effectively omitting some centrioles. To allow for these factors, the percentage of centriole pairs with associated primary cilia were calculated, and found to be 46 ± 2% in total alveolar cells and 44 ± 7% in luminal SECs.

Considering all incidence data, a conservative, yet valid, interpretation is that in lactating bovine mammary alveoli, primary cilia were seen in some – but not all – epithelial cells, at an incidence of between 11% and 46%. In this case, ciliary expression among SECs could reflect differential expression in distinct sub-populations of cells within this layer. For instance, a known sub-population of mammary epithelial cells have been identified as putative progenitor cells by their light-staining cytoplasm.56 This idea is further explored in Section 4.2.

For the incidence assessments, data was only gathered from acetylated-α-tubulin/γ-tubulin-labelled sections, so as to allow for the inclusion of centriole data. It was not possible to selectively determine primary cilia incidence in myoepithelial cells, as cell types could not be separately identified. Furthermore, there was not enough information to make an accurate estimate of how many (or what proportion) of nuclei in the total alveolar count were myoepithelial cells. Based on the results of this present study, the possibility all myoepithelial cells express a primary cilium cannot be entirely ruled out. It is possible that the depth of the imaged section, from which the assessments
were made, in relation to the more extended width of the myoepithelial cell, precluded visualisation of a cilium in a given section. For a more accurate incidence assessment, further investigation is needed, and section thickness will be an important aspect of any future study design. Triple-labelling of tissue sections to include dual ciliary identification in conjunction with myoepithelial identification would also allow for a more thorough investigation into primary cilia incidence in myoepithelial cells.

4.1.6 Fluorescent immuno-detection procedure considerations

Generally, the optimised procedure gave consistent imaging results, although an increase in background auto-fluorescence was noted in tissues during early involution. This is most likely due to the changing nature of the gland following abrupt cessation of milking, and could be considered as an area for further optimisation in any future studies, particularly if more extended time points post-milking are studied. This factor may have impaired the detection of primary cilia in some cases from early involuting animals, and is reflected in the lower count data obtained at the most extended post-milking stages. This, however, did not negatively impact on the ability to measure and assess morphological aspects of primary cilia. Future immunofluorescent studies on mammary tissue, particularly following extended times post-milking, may require additional optimisation.

4.1.7 Comparisons with existing findings

As yet, mammary tissue has not undergone the same rigorous investigations into the regulatory roles of primary cilia as many other organs and tissues – particularly the kidney, CNS, articular cartilage and bone. This is especially applicable in the dairy cow, which has been the subject of only one such published study to date regarding primary cilia in the mammary gland. In that ultrastructural study from 1989, observations of primary cilia were reported in myoepithelial cells and luminal SECs in lactating and non-lactating (7 – 90 days post-milking) bovine mammary tissue. In the brief analysis detailed for this study, primary cilia on luminal epithelial cells projected 1.2 µm into the lumen. Length measurements in this present study showed similar results. Considerable advances in primary cilia detection methods and confocal microscopy have evolved in the last two decades, affording improved interpretations of primary cilia morphology and distribution in relation to the tissue architecture – as has
been demonstrated in the imaging results of this research thesis. For this reason, aside from further re-validating their presence in the same species, it is difficult to compare aspects of this current study with the published ultrastructural findings.

More recently, other researchers have investigated primary cilia in the mammary tissue of mice,\textsuperscript{107, 108} as discussed in \textbf{Introduction 1.7}. In these investigations, cilia have been implicated in coordinating aspects of ductal branching morphogenesis during development, through their involvement in Wnt and Hedgehog signalling, as reported by McDermott \textit{et al.}, 2010.\textsuperscript{107} Primary cilia are also thought to be important for regulating the differentiation of alveolar and ductal progenitor cells in a paracrine manner following the onset of lactation, through their involvement in the coordination of Hedgehog signalling.\textsuperscript{108} In a study of mammary Shh signalling in mice at early lactation, reported by García-Zaragoza \textit{et al.}, 2012,\textsuperscript{108} the majority of alveolar cilia were seen in sub-luminal, Hedgehog-responsive epithelial cells. Conversely, fewer were seen in non-Hedgehog responsive epithelial cells, or in myoepithelial cells or stromal areas.\textsuperscript{108} Although no overall incidence data in control animals was included in the publication, there are similarities between both those findings in mouse tissue and the present findings of this bovine study. Both demonstrate that primary cilia can be seen in all alveolar cell types, although not necessarily in every cell. An important consideration when comparing such findings with the results of this present study, is that well-established differences exist between certain aspects of rodent and ruminant mammary regulation.\textsuperscript{19, 46} Allowing for these considerations, studies in other species undoubtedly provide potential insights into ciliary coordination of cell differentiation and tissue development, due to the conserved structure and function of primary cilia between species.\textsuperscript{77}

In the study published by McDermott \textit{et al.}, 2010,\textsuperscript{107} the incidence of primary cilia expression was tracked from early development until mid-pregnancy in the mammary alveolar tissue of mice. During this time, the ciliary incidence decreased in the luminal epithelial cells from 17\% to 4\%. Primary cilia were, however, observed more frequently in sub-luminal alveolar cells, where their incidence remained relatively steady over the same period.\textsuperscript{107} The observed alveolar ciliary incidence within the lactating tissue sections from this present study is not easily comparable to those published findings, due to the disparity in the species and mammary function between the two animal models used. Despite these differences, it can be inferred that the results
obtained in this present study also reflect the published observations, in that the incidence of primary cilia in alveolar epithelial cells was also under 50%.

During branching morphogenesis in mice, some cilia of myoepithelial cells projected towards the stromal tissue, from the basal cell membrane. However, observations of ciliary projection from myoepithelial cells in this current study showed cilia only on the aspect of the myoepithelial cells closest to the lumen. This may indicate differences in ciliary expression between the species or developmental stages, or alternatively it may reflect the need for increased sampling to ensure higher numbers of myoepithelial cells are included in the analyses. Another possibility is certain sub-populations may also exist within the myoepithelial layer, in which case some cells may be exclusively involved in stromal interactions during development.

4.2 Interpretations of the present findings

The distribution of primary cilia throughout all alveolar cell types, as seen in the mammary tissue sections in this study, raises a multitude of questions and possibilities regarding the roles of these organelles in lactation, and following the cessation of milk removal. The findings described in Section 4.1 do not immediately provide information about the biological or physiological significance of primary cilia distribution within each cell type, nor are they able to directly identify specific ciliary functions, but they do provide a basis for further investigation. There is certainly scope to consider their involvement in coordinating signalling cues that contribute to tissue homeostasis, or that trigger certain de-differentiative processes which are associated with the onset of involution. As discussed in this section, the presence of primary cilia in mammary tissue, and their known functionality, may open up new investigative avenues in the study of mammary gland regulation.

The mere presence of a primary cilium does not denote ciliary function, rather the conserved ciliary structure and core molecular architecture provides a template for the cilia to be modified according to the specifications of a particular cell. In many cases, the cell-type specific expression of ciliary receptors is tightly regulated, and the cell can effectively tailor ciliary receptors according to its sensory requirements. Such requirements can change depending on the nature of the extracellular
environment or altered temporal and spatial parameters, which in turn can alter the molecular profile of the ciliary membrane.\textsuperscript{123}

Previously-identified functions of primary cilia in other organs and cell types have begun to define their often exclusive roles in coordinating a variety of signalling processes. Ciliary-mediated processes rely on specific stimuli, including mechanical, biochemical, hormonal and osmotic extracellular events.\textsuperscript{76} This knowledge can indicate the potential range of signalling functions the primary cilium could coordinate within the mammary gland – specifically during the onset of involution, during which time the tissue can respond very sensitively to acute milk stasis and decreased lactogenic demands.\textsuperscript{40} Such tissue responses include the dramatic reduction in milk protein transcription and biosynthesis within SECs,\textsuperscript{47, 50} and the stromal remodelling, resulting in increased inter-alveolar and inter-lobular stromal tissue.\textsuperscript{63}

As previously mentioned, recent studies point towards ciliary involvement in the coordination of Wnt and Hedgehog signalling pathways during branching morphogenesis.\textsuperscript{107, 108} In the mature, lactating mammary gland, the cells would most likely rely on primary cilia to transduce different (or additional) types of sensory interpretation. During galactopoiesis, signalling pathways within SECs would need to effect the homeostatic mechanisms, or to detect regulatory cues. This is perhaps highlighted by the observation that primary cilia have been identified in both Hedgehog-responsive and non-Hedgehog-responsive cells within the same alveolus.\textsuperscript{108} Once the developmental stages of mammmogenesis and lactogenesis are complete, different cell types may either shed their primary cilium or employ different forms of ciliary signalling according to ciliary receptor expression, and the specific functions and regulatory mechanisms of the cell.

Possible areas where primary cilia could interpret signals, and transduce this extracellular information to initiate the distinctive events of involution, are indicated in \textbf{Table 4.2}. The pathways requiring potential ciliary involvement, as summarised in the table, and further detailed in this section, highlight an area of mammary regulation where there is a current gap in the knowledge of specific cellular processes. The local mechanisms, through which primary cilia could coordinate signals to trigger the cellular responses to accumulated milk, can be classed into two broad groups. Firstly, the local mechanical changes relating to milk accumulation and alveolar distention, and secondly, the changes in biochemical composition within the alveolus. These two modes of sensory detection could theoretically apply to all cell types in the alveoli. As
detailed in Introduction 1.4, systemic endocrine regulation is maintained during lactation. Once milk removal ceases, this impacts on circulating levels of galactopoietic and metabolic hormones. Changes in this endocrine balance have been shown to significantly influence mammary involution following the initial locally-mediated events. Such hormonal changes may further enhance the pro-apoptotic signals, to fully commit the tissue to involution if the cessation of milk removal is sustained. 38; 58 57
### Table 4.2
Mechanisms and pathways coordinated by primary cilia in various tissues, and their theoretical involvement in mammary cells for the regulation of mammary function

<table>
<thead>
<tr>
<th>Potential translation of primary cilia function to cell-type specific mammary regulation</th>
<th>Primary cilia sensory functions, as determined in various tissue/cell types</th>
<th>Chemo-transduction roles</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mechano-transduction roles</strong></td>
<td><strong>Chemo-transduction roles</strong></td>
<td></td>
</tr>
</tbody>
</table>
| Luminal epithelial cells | - Ciliary deflection in response to external compression: Activation of intracellular \([Ca^{2+}]\) signalling cascades via mechano-sensitive receptors targeted to the ciliary membrane\(^{79}\)
- Cytoskeletal prestress (isometric tension) and cell shape: Alterations can create indirect modulation of ciliary transduction\(^{124}\)
- • Modification of gene transcription and secretory profile according to the events of milk stasis eg. Increased lactoferrin, immune agents, pro-apoptotic molecules and decreased milk proteins in response to cell stretch or forced involution\(^{50, 71}\)
- • Increased tight junction permeability\(^{29, 43, 70}\)
- • Osmotic sensation and signal transduction: Activation of TRPV4-mediated signalling to alter downstream biosynthetic activity and secretion\(^{125}\)
- • Specific ligand-receptor interactions: Recession and transduction of extracellular hormonal or chemical signals to effect downstream signal amplification and gene transcription\(^{89, 93, 95}\)
- • Control of cell division\(^{126}\)
- • Regulation of luminal toxicity or composition\(^{1}\)
- • Secretory response to hormonal stimuli\(^{124}\)
- • Progenitor cell proliferation\(^{56, 127}\)
- • Altered gene transcription in response to stasis biomolecules\(^{36}\)
- • IGF-1 signalling\(^{59}\)
| Ducts | - Ciliary deflection in response to fluid shear stress: Activation of intracellular \([Ca^{2+}]\) signalling cascades via PC1/PC2 signalling and/or other mechano-sensitive ciliary receptors\(^{87, 128}\)
- • Cellular interpretation of ductal fluid shear stress\(^{1}\)
- • Specific ligand-receptor interactions: Reception and transduction of extracellular hormonal or chemical signals to effect downstream signalling composition of ductal contents\(^{1}\)
- • Various modes of biochemical signalling in response to biochemical composition of ductal contents\(^{1}\)
| Myoepithelial cells | - Ciliary deflection in response to extracellular compression: Activation of intracellular \([Ca^{2+}]\) signalling cascades via mechano-sensitive ciliary receptors\(^{79}\)
- • Cytoskeletal prestress (isometric tension) and cell shape: Alterations can create indirect modulation of ciliary transduction\(^{124}\)
- • Modification of gene transcription in response to involution associated events\(^{1}\)
- • Specific ligand-receptor interactions: Reception and transduction of extracellular hormonal or chemical signals to effect downstream signal amplification and gene transcription\(^{89, 93, 95}\)
- • Altered gene transcription • Status of differentiation and paracrine signalling in response to extracellular biochemical signals\(^{17, 90, 120}\)
| Stromal cells | - Ciliary deflection in response to external compression: Activation of mechano-sensitive ciliary receptors via integrin-mediated ECM interactions\(^{39}\)
- • Detecting structural changes in ECM tension following stasis influenced changes in alveolar size\(^{1}\)
- • Specific ligand-receptor interactions: Reception and transduction of extracellular hormonal or chemical signals to effect downstream signal amplification and gene transcription\(^{95, 85, 89}\)
- • TGF-β signalling during stromal proliferation and remodelling\(^{28}\)

Black text denotes known ciliary functionality, blue text denotes mammary-specific processes, + denotes theoretical cellular responses based on regulatory mechanisms in other organs.
4.2.1 Potential regulatory cues coordinated by primary cilia in mammary cells

Luminal alveolar and ductal mammary epithelial cells

Luminal SECs within alveoli are well positioned to detect initial compressive forces associated with intra-alveolar milk accumulation and changes in luminal hydrostatic pressure. The axoneme of the projecting cilium, when equipped with mechano-sensitive receptors, is capable of transducing extracellular compressive signals to initiate various cellular responses. Aspects of the ciliary mediated response to mechanical loading have been studied in articular cartilage. The chondrocyte primary cilium, which extends into the ECM, can initiate signalling events in response to ECM compression, which ultimately lead to increased levels of intra-cellular calcium. The extracellular interpretation and ciliary involvement in signal transduction may also be employed in SECs of mammary alveoli. In these milk storage structures, the effects of accumulated milk are evident upon routine histological assessment, and the flattening of SECs in some alveoli, which is more notable following prolonged milk stasis, may induce signalling mechanisms analogous to those of the chondrocyte. Alternatively, mechanisms similar to renal epithelial cell signal transduction in response to fluid shear stress may also apply to alterations of the volume of accumulated alveolar contents. In these more well-studied systems, the mechano-sensitive ciliary receptors PC1 and PC2 are activated in response to ciliary deflection. If such sensory detection occurs in alveolar SECs, any downstream cellular events may lead to known involution-associated molecular changes, such as an inhibition of survival factors, or altered gene transcription.

Luminal SECs of the mammary ductal network are frequently exposed to extracellular fluid flow and fluid shear stress as milk is ejected through the ductal network. The primary cilia of these cells could be more suitably placed than alveolar SECs to function via flow-sensitive mechano-sensitive receptors such as the PC1/PC2 flow-activated receptor channels (as studied in renal epithelial cells), or receptors which detect flow sensation in osteocyte cilia. Downstream cellular responses of flow-mediated ciliary signalling in these cell types govern many homeostatic processes which are critical for appropriate renal or bone function. In this way, analogous mechanisms may exist for ductal epithelial cells to sense changes in milk flow in response to altered frequency of milk removal. Downstream responses may conceivably contribute to changes in cell behaviour – which could be relayed via paracrine interactions to nearby alveolar cells to affect mammary regulation.
There are also many reasons to consider that additionally, various forms of ciliary chemo-transduction may occur in luminal epithelia. Within mammary alveoli, the stored milk contents contain a plethora of biofactors, some of which can act in a negative feedback manner on milk biosynthesis and secretion according to their concentration and the length of time since alveolar clearance.\textsuperscript{34; 36; 38} Additionally, these cells may detect certain pro-apoptotic signals in accumulated milk. The primary cilium of alveolar SECs, as imaged in this study, may be enriched for certain receptors to detect such factors, and theoretically could transduce any signals to create molecular events in the activation of apoptosis or in the regulation of milk protein gene transcription. Recently, the control of IGF-I activity has been shown to be important in mammary cell survival,\textsuperscript{59} and in other tissues the membrane receptors for IGF-I (IGF-1R) have been shown to localise to primary cilia,\textsuperscript{95} therefore this organelle may also serve to coordinate aspects of IGF signalling in mammary epithelial cells. Such signalling may be relevant when considering the balance of apoptosis and proliferation, which is altered in favour of cell loss during involution.\textsuperscript{52; 54; 129}

Certain forms of ligand-receptor interactions may be of specific relevance for certain sub-populations of SECs, especially if there is a requirement for putative progenitor cells to be more sensitive to cues for cell division. The absence of primary cilia in some SECs may possibly be explained in terms of their differentiative state, particularly if some high-capacity terminally-differentiated secretory cells were not able to support a primary cilium on their apical aspect. The secretory activity of some SECs at peak lactation is particularly high,\textsuperscript{5} and the constant binding and exocytosis of milk constituents from the apical cell membrane into the lumen may interfere with ciliary maintenance. Reviews of primary cilia distribution \textit{in situ} have found that in fully-differentiated, highly-secretory cells of the pancreatic acini and liver, primary cilia are not seen.\textsuperscript{85} The idea that the primary cilium may be required to achieve a polarised, differentiated cell, but no longer required during normal cell function has been supported by research in other cell types. In the auditory hair cells within the cochlea, a modified primary cilium (known as kinocilium) is only present during cell differentiation and maturation, and is subsequently shed once the cell is fully differentiated.\textsuperscript{130; 131} Recent findings also show that in murine embryonic heart endothelial cells, the selective loss of primary cilia in response to high cardiac flow rates is an essential step in terminal cell differentiation and overall cardiac function.\textsuperscript{132}

In mammary tissue, putative progenitor cells contribute to 10% of the overall alveolar epithelial population and 50% of overall cell proliferation in pre-pubertal Holstein heifers, up until eight months of age.\textsuperscript{56} Furthermore, certain delineations within this sub-population
are emerging, as recent research is beginning to define the particular lineage commitment and variations in pluripotent capabilities within these cells. Investigations into markers of gene transcription and protein expression have shown that levels of CD24 and CD49f can indicate the cell’s pluripotent capacity, and that certain locations within the alveolus are enriched for certain lineages. In recent equine studies, the capacity for self-renewal and proliferation within progenitor cells was shown to be greater in the lactating mammary gland than the non-lactating gland. Given the regulation of cell division that is intrinsic to these cells, primary cilia may coordinate aspects of these processes in response to physiological cues. The incidence data gathered in this present research enhances this possibility. In a theoretical model, primary cilia could be required for appropriate alveolar cell division and differentiation, but not required or maintained in terminally differentiated, highly polarised cells, hence not all cells are ciliated.

The precise and consistent composition of milk, which is continually produced in lactating animals, is undoubtedly under constant regulation to ensure the end-product contains the appropriate nutritional profile – a profile which is specific to individual species. In this way, a possible mechanism may exist to allow SECs to sense aspects of osmolarity or the concentrations of sugars, fats and proteins within alveolar secretions. In cholangiocytes, as well as functioning as flow-sensors, the primary cilia have been shown to function as chemo- and osmo-sensors to very sensitively detect certain osmotic and biochemical information within bile ducts. In these cells, they can effectively modify secretion according to the nature of the extracellular composition of bile, via the action of ciliary-located transient receptor potential V4 (TRPV4) cation channels. While it is not yet known if SEC primary cilia express TRPV4, similar sensory perception and secretory control or modification may be provided via the primary cilia of these cells. Biosynthesis of lactoferrin has been detected in cells dispersed throughout mammary ducts, and the expression of casein has been detected in ductal cells, particularly within the transition zone, in the region of the duct closest to the alveolus. (Personal communication: A. Molenaar, AgResearch). It is a possibility that primary cilia within these cells may further serve to sample and modify milk secretions in the control of milk production, or in the up-regulation of defence proteins during involution.

**Alveolar myoepithelial cells**

Throughout lactation and involution, myoepithelial cell function is crucial for appropriate mammary function. These cells serve to expel alveolar luminal contents via
their specialised and coordinated contractile properties, and furthermore have been implicated in contributing to the maintenance of overall alveolar differentiation. There is potential for this cell layer to react to increased luminal contents, which could likely be mediated via cell compression and stretch – as discussed in relation to alveolar SECs. Increased alveolar volume, as is evident between 12 and 18 hours post-milking, will translate to altered compressive and tensile forces experienced by these cells, in contact with a fully distended basement membrane. The myoepithelial primary cilia may be responsible for transducing compressive signals to effect distinct cellular responses. As already discussed in relation to alveolar SECs, compressive mechano-transduction has been studied in chondrocyte cilia of articular cartilage, and similar mechanisms may also be coordinated by the primary cilia of myoepithelial cells. They may function to detect and respond to the local physical changes relating to alveolar engorgement following the cessation of milk removal.

In this study, myoepithelial primary cilia were seen to project into the inter-cellular space underlying SECs. Given their location in contact with the interstitial fluid, they are also suitably-placed to sensitively detect extracellular ligands, such as those that function as survival signals or pro-apoptotic signals. In this way, a putative chemo-transduction role may be coordinated by the primary cilium of the myoepithelial cell to trigger downstream cellular processes which regulate aspects of lactation or involution. This may perhaps apply to the detection of altered interstitial fluid composition. In cases where the tight junction permeability between overlying SECs is altered in response to milk stasis, some alveolar contents are effectively allowed to flow in the basal direction. In a possible detection-response mechanism, the myoepithelial primary cilia may detect and coordinate cellular responses to the altered biochemical composition. Additionally, ultrastructural observations have shown alveolar myoepithelial cilia can be invaginated in a deep ciliary pocket, and the present confocal imaging findings show cilia frequently in close proximity to the nucleus - therefore possibly invaginated within a pocket. These observations introduce the possibility that this membrane domain may possess specialised functionality in the myoepithelial cell. Recent findings have shown the ciliary pocket contains clathrin-coated pits, and is essential for endocytosis of extracellular molecules.
Changes in alveolar cell size and stretch

There is a vast array of regulatory processes occurring during mammary regulation and involution, and additionally many opportunities for interactions between various processes. Certain parallel processes, either directly or indirectly involving primary cilia, may contribute to alter the physiological state of the gland. A cellular event that could potentially trigger a variety of indirect ciliary-related mechanisms, ultimately affecting cell behaviour in both SECs and myoepithelial cells, is that of cell stretch. Between 12 and 24 hours post-milking, SECs showed the greatest degree of lateral stretch among all post-milking time points (Table 3.1), which would likely also stretch the underlying myoepithelial cells, or alter their interactions with the underlying basement membrane. Furthermore, between 36 and 192 hours post-milking, some alveoli contained considerably engorged SECs (Table 3.1). The tensile forces experienced by these cells following prolonged periods of milk stasis will likely be altered in comparison to routinely lactating cells. According to published research, the tight junctions between luminal SECs will be expected to increase in permeability following 17 hours post-milking, pointing towards alterations in cell-to-cell interactions.\(^{33,70}\) Furthermore, research by Quaglino et al., 2009,\(^{71}\) has linked cell stretch to certain involution-associated events in cultured mammary cells.

Studies in various cells have shed light on the mechanisms through which changes in intra-luminal pressure, rather than flow, can mediate cellular responses. Alenghat et al., 2004,\(^{124}\) showed that the classical polycystin-2 based mechano-sensation, which is initiated on the primary cilium membrane, is dependent on complete cytoskeletal integrity in murine kidney epithelial cells, and is influenced by isometric tension (prestress) within the cell. These studies revealed an absence of flow-induced PC1/PC2-mediated intra-cellular calcium release when selective cytoskeletal elements were disrupted. This suggests that this form of ciliary-initiated signalling requires an interaction with multifactorial cellular processes affecting isometric tension.\(^{124}\) Such processes also appeared to include appropriate cell-to-cell and cell-to-ECM connections, and cell shape.

Subsequently, Sharif-Naeini et al., 2009,\(^{136}\) showed that in arterial myocytes, plasma membrane-associated PC2 can inhibit myocyte constriction, which occurs in response to the activation of non-selective stretch-activated cation channels (SACs) in the plasma membrane. This inhibition is dependent on the association of PC2 with elements of the cortical cytoskeleton, whereby it theoretically acts as a tether to alter cytoskeletal tension and reduce the stretch-response. SAC activation in response to extracellular pressure is regained when PC1 is co-expressed with PC2, which suggests that the PC1/PC2 ratio also
governs SAC sensitivity. No direct ciliary involvement is inferred in that study, however taken with evidence from the previously mentioned study, isometric tension within the cortical cytoskeleton appears to affect both SAC activation and certain ciliary function.\textsuperscript{124} \textsuperscript{136} Such overlapping control in the regulation of both ciliary function, and in the mechano-transduction of compressive force via SAC activation, points towards complex, multifactorial processes that may interact to effect appropriate cellular responses to changes in extracellular compression and cell shape.

**Stromal fibroblasts**

Stromal tissue encapsulates individual alveoli, lobules and lobes of the mammary parenchyma. Its intricate, fibrous ECM effectively restrains continual distention of the alveoli during milk accumulation. The biomechanical flexibility experienced by the ECM fibrillar components can sustain a degree of tensile strain, until a point at which no further give is possible.\textsuperscript{137} According to these properties, alveolar distention occurs until the limits of the surrounding connective tissue are reached, and any further milk accumulation results in increased luminal hydrostatic pressure. The change in tensile and biomechanical properties experienced by the ECM during alveolar distention, and the likely sustained nature of these events during milk stasis, may act as a trigger for stromal-related processes which occur during involution. Within inter-alveolar and inter-lobular stromal regions, considerable tissue remodelling events occur during mammary development, lactogenesis and involution.\textsuperscript{63} Cues to initiate any remodelling processes will need to be detected and relayed to the resident stromal cells.

Stromal fibroblasts reside within the ECM, which provides a means for physical transmission of mechanical compression signals originating within the neighbouring alveoli.\textsuperscript{22} The presence of primary cilia in stromal fibroblasts in both inter-alveolar and inter-lobular stromal regions, as detected in this present study, provide a possible mechanism for these cells to sense tensile or compressive changes in the mesh-like collagen network of the ECM.\textsuperscript{22} This form of mechano-transduction is assumed to occur in articular cartilage, in which the chondrocyte primary cilia form integrin-mediated connections with the ECM components.\textsuperscript{92} These connections are thought to be partly responsible for initiating responses to mechanical loading, as is experienced in joints during movement and load-bearing.\textsuperscript{79; 91; 138} Following cellular responses, the chondrocytes are presumed to alter the degree or directional targeting of secreted ECM components in a reactive manner.\textsuperscript{91; 138} Similar mechano-transduction roles are also being investigated in the primary
cilia of rat tail tendon cells, in which ciliary length appears to be sensitive to cyclic tensile loading in vitro.\textsuperscript{139} If a similar form of mechano-transduction occurs in mammary stromal fibroblasts, it would provide an opportunity for these cells to sense acute changes in local alveolar volume and pressure, and respond in a regulatory manner. Such responses may contribute to the increased secretion of fibrous collagenous components during early involution. In certain physiological states, such signalling mechanisms may also be enhanced or modified to influence other involution-associated events, such as increased stromal proliferation\textsuperscript{63} or the altered paracrine signalling profile which may influence alveolar de-differentiation or survival.\textsuperscript{18}

The stromal areas of mammary tissue have been less extensively studied than the parenchymal tissue, however certain hormones are known to affect fibroblast activity at different stages. A hormonal regulator of stromal tissue that has received some attention is TGF-\(\beta\), which has been shown to undergo increased transcription at the onset of involution. Its effects are inhibitory on epithelial cell proliferation, but stimulatory on fibroblast proliferation, and on secretion of certain ECM components.\textsuperscript{29; 63} For this reason, it is thought to act as a key regulator of stromal remodelling during involution.\textsuperscript{29} Recently, the presence of TGF-\(\beta\) has been shown to modulate activity of the primary cilium-coordinated Hedgehog pathway in studies of human lung fibroblasts.\textsuperscript{140} Additionally, for TGF-\(\beta\) to initiate differentiation in these cells, a fully functional Hedgehog pathway was required. In light of these findings, the researchers reported a link between TGF-\(\beta\) activity and components of the Hedgehog pathway, which are integral to the primary cilium.\textsuperscript{140} If such interactions also exist in the fibroblasts of mammary stromal tissue, this could infer a role for primary cilia in the coordination of TGF-\(\beta\) signalling and, by correlation, the initiation of certain involution-associated stromal remodelling processes. In addition, an increase in activated TGF-\(\beta\) may occur via its release from fibrillar proteins of the ECM in response to altered matrix tension,\textsuperscript{23} therefore allowing a mechanism in which stromal remodelling is enhanced by the concurrent physical ECM properties and lactogenic state.

\section*{4.3 Future Research directions}

It is important to consider the fact that although there are valid reasons to presume primary cilia may function throughout mammary regulation in similar ways to those
determined in other systems, these theories need to be experimentally assessed. Suitable follow-on studies from this work may involve both \textit{in vivo} and \textit{in vitro} investigations in order to further define aspects of ciliary expression, and to elucidate their roles within mammary tissue.

\textit{In vivo studies}

- \textbf{Incidence:} Future studies could initially focus on a more comprehensive \textit{in vivo} characterisation of ciliary incidence throughout mammary tissue. As discussed in relation to the incidence assessments of this present study, the use of thicker tissue sections would have allowed a more accurate estimate of ciliary incidence, particularly in the myoepithelia and stromal areas. Future experimental design could allow for this, and also compare ciliary incidence and length between different quarters of the udder within the same animal following different milking schedules, or different post-milking intervals. In doing so, investigators could isolate any inherent ciliary responses to local mammary factors. Incidence studies could also target putative progenitor cell populations within the mammary tissue – determining whether cilia are selectively expressed in alveolar cells of particular differentiative states may be of benefit to characterise any ciliary involvement in cell turnover, proliferation and differentiation. This may be achieved by imaging cilia in association with known progenitor cell markers or known markers of fully-differentiated cells.

- \textbf{Ciliary receptor expression:} In order to assess potential ciliary function, primary cilia could be imaged in association with known mammary receptors. Receptors of particular relevance to mammary regulation include IGF-1R, or prolactin receptors. Further investigations may also include whether FIL (which has been identified as an autocrine feedback molecule for SECs) binds to the ciliary membrane. The capacity of the primary cilium of certain mammary cells to provide similar sensory reception to that previously determined in other cell types, could also be assessed by determining if cilia express PC1/PC2 receptors or TRPV4 cation channels.

\textit{In vitro studies}

- \textbf{Cell stretch:} Isolated, cultured mammary cell populations could be used to manipulate biomechanical conditions, using cell stretch devices, to examine the
effect of tensile changes on certain cellular markers (eg. markers of apoptosis). Comparisons between ciliated cells, and cells in which ciliary function is selectively inhibited, would provide indications of the role of primary cilia in eliciting cellular responses.

- **Ductal flow**: Fluid flow studies, similar to those previously undertaken in the study of primary cilia in renal epithelia, could be designed to determine whether the primary cilia of mammary ductal epithelial surfaces respond to changes in flow through the ductal network.

- **Stromal compression**: Isolated stromal fibroblasts could be used in culture to study primary cilia responses to the presence or absence of compressive load, using a mechanical cell loading device. Changes in ciliary incidence or length may indicate they are sensitive to such conditions (as has been shown for chondrocytes and tenocytes)\(^79; 138; 139\) and further study may isolate any specific ciliary-dependent cellular responses.

- **Receptor-ligand responses**: Cell culture conditions can be manipulated by the selective addition of known regulatory ligands (eg. TGF-β, IGF-1). Ciliary-dependent responses in mammary cells could be isolated by studying cellular responses in cultures where ciliary function is selectively inhibited compared to those with normal ciliary function.

**Implications of future research**

The balance of apoptosis and proliferation in the mammary gland is of particular importance to dairy researchers, as the potential milk yield has been shown to be determined by the extent of proliferation at the onset of lactation, and a gradual increase in the rate of apoptosis following peak lactation.\(^51; 52\) When drawing parallels with research findings in rodents, various studies indicate the regulation of mammary epithelial cell survival in the dairy cow is determined by many different stimuli, derived from both local and systemic factors.\(^51; 57\) A more thorough understanding of how proliferation and apoptosis are controlled during lactation would lead to opportunities to target specific processes in order to maximise lactation productivity. Recognising the potential for primary cilia to coordinate certain molecular mechanisms contributing to cell survival in the mammary gland may enhance future research in this field, particularly relating to IGF-1 signalling. The emergence of findings that cell-wide processes (such as changes in
cytoskeletal properties) can affect certain ciliary function may also be of relevance when considering potential ciliary transduction. For instance, cell size and stretch characteristics of mammary epithelial cells, which can alter with lactation state, may indirectly modify cellular sensory mechanisms.

Further understanding primary cilia functionality in mammary tissue may ultimately improve knowledge of mammary regulation, in the same way the elucidation of their roles in kidney epithelia have greatly enhanced the understanding of renal function and pathology. The implications of this research may also translate to human studies. Primary cilia have become the focus of some human mammary gland studies, particularly in relation to the development of breast cancer. If they prove to provide crucial sensory functions throughout lactation, primary cilia may also provide new directions for studies to investigate factors which influence milk supply in humans. An increased understanding of how lactation is regulated may lead to improved therapies or feeding strategies for lactating mothers who, for various reasons, have insufficient milk supply.

4.4 Conclusions

At the outset of this study, very little information regarding the expression of primary cilia in the bovine mammary gland was available. Given the importance of lactation to mammalian species and to the dairy industry, the potential for these highly-sensitive organelles to interpret certain biochemical or biomechanical stimuli during mammary regulation is of great significance. The experimental research and findings detailed in this thesis have begun to define the distribution of the primary cilium within this dynamic secretory gland in the lactating dairy cow.

The most significant information obtained throughout this research is that primary cilia were expressed in three important mammary cell types: SECs, myoepithelial cells, and stromal fibroblasts. As demonstrated in this study, the use of samples obtained from forced-involution animal studies allowed certain morphological features of primary cilia to be assessed and correlated with the time-dependent mammary tissue changes, which occur during the abrupt cessation of milk removal. The current findings demonstrated the presence of primary cilia in mammary alveolar and stromal cell types during active lactation, throughout milk stasis, and in early involution. Their presence was detected in 11% – 46% of alveolar epithelial cells, and in some luminal epithelial cells of small ducts, where they projected into the lumen. SEC primary cilia were sometimes deflected, a
morphological feature which was observed more often in tissue from cows in milk stasis than in active lactation. The data collected about deflected primary cilia suggests that in SECs, the orientation and morphology of these projecting organelles could well be under the influence of the extracellular micro-environment to which they are exposed.

Primary cilia can provide crucial mechano-detection and transduction roles to effect cellular responses in many other tissue types. It is also possible to consider this form of signalling in the stromal cells, where the surrounding ECM may translate the altered pressure dynamics into an altered tensile state, which in turn may provide cues for ciliary signalling. These ECM dynamics have been shown in other tissue (eg. articular cartilage)\textsuperscript{79; 92} to create primary cilia-dependent cellular responses. Analogous responses may occur in mammary stromal tissue to contribute to the increased stromal remodelling that is evident in early involution.

The presence of primary cilia in cells following prolonged cessation of milk removal, as observed in this study, also suggests that these organelles may act as specialised osmotic or biochemical sensors to interpret changing physiological requirements. Their cell-type specific locations indicate primary cilia of mammary alveoli can contact luminal or ductal contents (on luminal or ductal SECs), or interstitial fluid (on myoepithelial cells). According to their chemo-sensory roles in many other tissue types,\textsuperscript{76; 89; 93} primary cilia may coordinate downstream cell type-specific events upon ligand-receptor interactions. Such cellular events may contribute to the appropriate function of the tissue at a particular stage of lactation, milk stasis or involution by eliciting targeted gene transcription or secretory activity.

In mammalian species, a remarkable range of complex mechanisms contribute to successful mammary function. Hormonal, metabolic and local biophysical mechanisms are involved throughout lactation cycles, and the initiation of specific processes may, in part, be coordinated by the primary cilium. Overall, the present findings provide fundamental knowledge on which to base future investigations and further the understanding of the physiological processes that regulate lactation in the exquisitely adaptive mammary gland.
References


