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CHAPTER

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Lactation and its Hormonal Control

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s0005

INTRODUCTION

p0010   Breastfeeding offers significant advantages to both infants and their mothers as elegantly reviewed in a recent article by Mohammed and Haymond.[1] However, breast milk production is often compromised in those mothers whose children might benefit most by breastfeeding according to the recommendations of the American Academy for Pediatrics, the Centers for Disease Control and the World Health Organization. These organizations all recommend exclusive breastfeeding for the first six months of infant life with supplementation with appropriate complementary foods up to one year of age.[2] Breast milk has significant benefits for at-risk populations such as preterm infants and those born to mothers with diabetes, metabolic syndrome, and obesity,[2,3] as well as for mothers with gestational diabetes mellitus.[4] Breastfeeding is critical in developing nations where clean water and resources to purchase formula are in short supply. We are beginning to understand that nonnutritive components of breast milk offer significant benefits to both the infant and the mothers that exceed the nutritional components present in this complex fluid.

p0015   The nutritional status of the mother prior to conception and during pregnancy has profound effects upon mammary gland development and function, as well as neonatal development. While infants born to nutritionally deprived mothers have long been a focus of public health policy, it is clear that at-risk infants also include preterm infants, and those born to mothers with diabetes, metabolic syndrome, and obesity. Given the importance of lactation to human health, it is increasingly important that we understand mammary gland development and the physiology of lactation in a manner that can guide public health policy. In this chapter we will examine lactation physiology from a variety of perspectives, with an initial focus on the understanding of molecular

mechanisms gained by in-depth analysis of experimental models and protein interactions. We will proceed to metabolic interactions that lead to the increased flux of substrate to the mammary gland for milk synthesis and end with a summary of milk ejection mechanisms. We will also describe recent approaches to lactation research in humans, emphasizing some of the unique components of human milk and some new technologies that are beginning to provide molecular insight into human lactation.

p0020   Much of the early physiological research in lactation was carried out in dairy animals. Sometime in the 1950s, Jim Linzell, working at the Agricultural Research Council of the Institute of Animal Physiology at Babraham, Cambridge, UK, began to examine the blood flow in the mammary gland of the goat. Along with a young researcher, Malcolm Peaker, he soon began to combine blood flow measurements with new techniques that allowed metabolic reactions to be traced with radioactive isotopes in the animal. These experiments, summarized in *Physiological Reviews* in 1971,[5] provided insights into the mechanisms of milk secretion that had been undreamed of even a decade earlier. Around the same time, a world away in Berkeley, California, another researcher, Dorothy Pitelka, was applying her skills with the new miracle of microscopy, the electron microscope, to an examination of the morphology of the mammary gland at the minutest level.[6] Pitelka used her skills with transmission and freeze fracture electron microscopy to publish a series of papers that essentially defined cell–cell contacts in the mammary gland. She then went on to establish tissue culture models that would allow study of the regulation of these contacts and other aspects of the molecular control of the synthesis of milk proteins even to the present.[7] One of the exciting moments in the field was when Linzell and Pitelka both presented their work at a Gordon Conference in 1973, where each discovered that the other was working on complementary aspects of

1

tight junction regulation in the mammary gland, providing a framework for the work on tight junction regulation in the mammary gland that continues today. Many other researchers, too numerous to mention in this introduction, have contributed to our current understanding of the physiology of lactation, and some of their work will be described later in this chapter.

p0025   We are once again at a strategic junction in our understanding of the biology of milk secretion. During the decades leading up to the 1970s, biochemists identified and characterized the numerous enzymes that underlie the ability of the mammary gland to make milk. The application of molecular techniques during the 1980s allowed the cloning of genes for proteins known to be present in milk or to participate in synthesis and secretion of milk as well as identification of the promoter elements that regulate expression of these genes. The 1990s brought the identification of signal transduction pathways that control expression of specific genes and the differentiation of the mammary gland. The use of genetically modified mice confirmed the importance of these signaling molecules and pathways in regulating developmental processes during puberty, pregnancy, lactation, and involution, providing a better understanding of mammary gland development at the molecular level. In the last decade gene expression profiling, comparative genomics, and analysis of single nucleotide polymorphisms have produced a more complex picture of lactation that includes an appreciation of cross species differences and variation. The use of metabolomics is allowing a new appreciation of mammary physiology in a whole body context. Finally, the ability to localize specific molecules within the mammary cell using immunohistochemistry is greatly expanding our knowledge of the mechanisms of milk secretion. Our goal in this chapter is to make available a scaffold composed of past and current knowledge of the mammary gland providing a foundation for future advances that utilize biochemical, molecular, cell biological, nutritional, and genetic approaches in an integrated manner and advance our understanding of lactation and its importance to neonatal development.

s0010   # DEVELOPMENTAL ASPECTS OF MILK SECRETION

s0015   ## Methods for Studying Mammary Gland Development and Function

s0020   ### Histological Methods

p0030   Examination of the primary structure of the mammary gland has long been the first approach to analysis of developmental alterations that affect glandular function. In fact, characterization of glandular function without examining the morphology of the gland for changes that underlie changes of function is likely to lead in erroneous directions. The first standard approach is preparation of whole mounts of the mammary gland allowing determination of whether ductal elongation, alveolar budding, and alveolar expansion have taken place (Figure 31.1). To do this, the whole mammary gland is excised, fixed, stained with either carmine alum or hematoxylin, and clarified to remove excess stain in such a way that the ductal tree can be appreciated (for review of methods see Ref. 10). Under low power it is possible to discern many aspects of mammary gland development particularly during puberty and early pregnancy before the florid development of late pregnancy and lactation make it difficult to discern individual ducts and alveolar units.

p0035   Conventional examination of thin tissue sections that have been stained with hematoxylin and eosin remains highly informative as the fine details of terminal end buds (TEB) and secretory alveoli are readily evident. While lipid droplets are readily identifiable in sections stained in this manner, greater detail is revealed when a combination of stains is used to identify various structures. For example, the use of anti-adipophilin antibody to outline cytoplasmic lipid droplets (CLDs), DAPI (4′, 6-siamidino-2-phenylindole) to stain nuclei, and fluorescent tagged-wheat germ agglutinin that binds to the luminal surface of secretory alveoli has allowed investigators to demonstrate that the size and position of CLDs changes during the transition from pregnancy to lactation[11] (see following). The combined use of antibodies to specific proteins with cytological markers for the nucleus, mitochondria, or other organelles will continue to provide in-depth understanding of cellular and signaling events in mammary gland development and lactation.

s0025   ### Transplantation Methods

p0040   The fact that the majority of mammary gland development occurs postnatally offers the unique opportunity to use transplantation to study the development of mammary epithelia containing targeted mutations.[12,13] In this method the mammary fat pad of a three-week-old prepubertal mouse is cleared by removal of the region of the gland proximal to the nipple, leaving a fat pad devoid of precursor cells capable of developing into the ductal tree (Figure 31.2). In the majority of studies a section of mammary gland from another mouse is placed into the cleared fat pad to develop in this new environment. Development is assessed at varying times following transplantation by whole mount analysis using contralateral glands on the same mouse as a control. Numerous variations on this basic technique have been developed over the years, including transplantation of tissue into non-cleared glands, transplantation of partially purified mammary "stem cells", and transplantation of "marked" cells expressing different fluorescent proteins or other
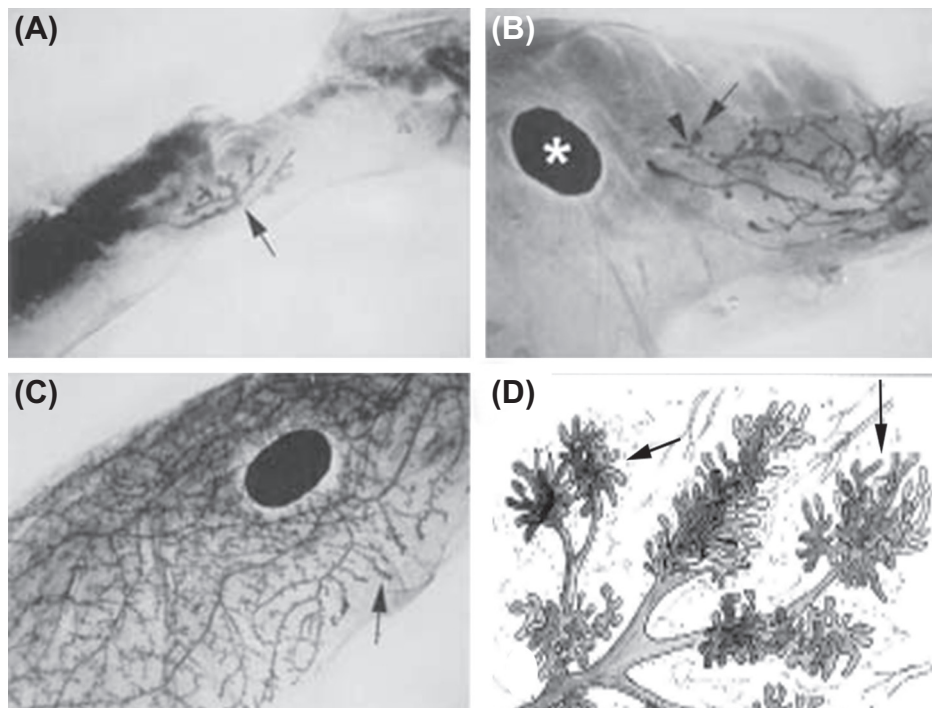
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FIGURE 31.1 **Whole mounts of mammary glands from virgin mice and humans.** (A)–(C) The fourth inguinal mammary glands were dissected from female mice at the indicated stages and stained with hematoxylin. (A) 3-week virgin. The arrow indicates the nipple region and the primary duct of the epithelial structure. 10× magnification. (B) 5-week virgin. Asterisk marks the lymph node, commonly used as a marker in whole-mount analysis. Ductal growth is indicated by the TEBs (arrow) and branch points (arrowhead). 45× magnification. (C) 10-week virgin. Alveolar buds are forming along the ducts (arrow). 45× magnification. (D) Human, drawing of a subgross preparation of a mammary gland from a 22-year-old nulliparous female. Arrows point to terminal duct lobular units (TDLUs). *Source: (A)–(C) From Ref. 8. (D) From Ref. 9.* (For a color version of this figure, the reader is referred to the online version of this book.)

detectable markers such as β-galactosidase. Transplantation studies have been critical in the identification of cell autonomous features, and in determining the role of the surrounding stromal environment in mammary gland development.

s0030 *Methods to Assess Lactation Competency*

p0045 Since the primary purpose of lactation is to support the growth of neonates, neonatal growth remains the best indication of lactation competency in most experimental species, although milk production can easily be assessed in dairy animals. Comments here are directed toward rodent experiments. Although it is simple to measure the weight of the litter and track its increase on a daily basis, several considerations should be given to this process so that meaningful data are obtained. First, it is important to standardize all litters to the same number of offspring so that each dam experiences an equivalent demand for milk; in the mouse, many investigators have standardized litters to eight pups, although for some lower producing strains six pups are more relevant.[15] Second, it is important to utilize pups without a developmental defect, often achieved by cross-fostering litters from a normal dam. When pups are well fed, the milk-filled stomach is readily apparent. Pups are either weighed individually or as a group, and the weights plotted over time. Numerous genetically modified mice display lactation defects when pup growth is used to access lactation competency; these include transgenic dams overexpressing constitutively activated AKT1,[16] *Akt1* null mice,[17] thyroid hormone responsive protein (THRSP or SPOT14) null mice,[18] and mice lacking tissue-specific expression of SREBP cleavage-activating protein (SCAP),[19] among many others.

p0050 Another approach is the "weigh-suckle-weigh" method in which pups are withdrawn from the dam for a defined period of time, such as 4 h, the pups weighed immediately before being returned to the dam, and then weighed again after 1–2 h of suckling. This method allows analysis over a defined period of time, sometimes in response to a specific treatment or intervention (for example, treatment with bromocriptine to disrupt prolactin (PRL) secretion[20]). Yet another approach is to weigh the excised mammary glands; however, results of this approach are likely to be influenced by the amount of time since suckling has occurred.[15]

p0055 In addition to analysis of pup growth, there is much to be learned by analysis of milk or milk curd itself.[15] Milk can be obtained from a lactating mouse with use of a suction device and oxytocin (OT).[21] The amount of

Mammary epithelial transplants
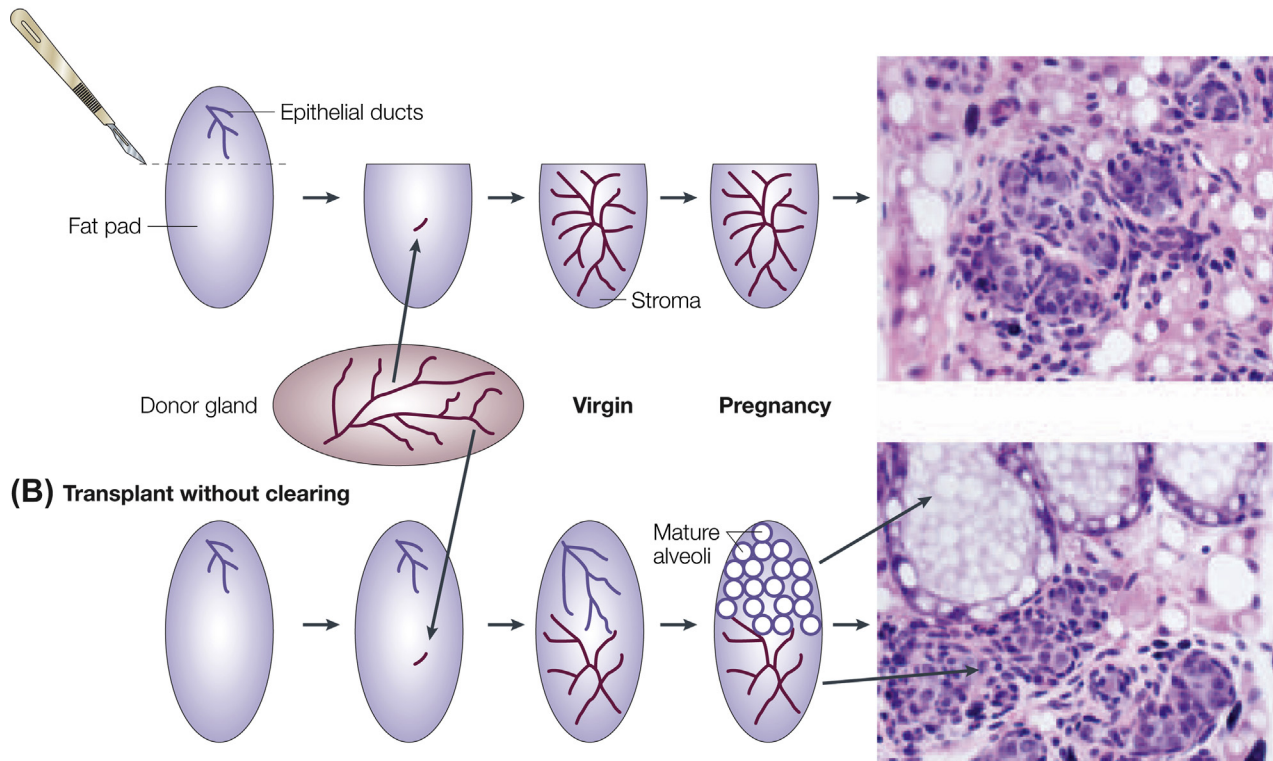
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FIGURE 31.2 **Use of mammary gland transplantation to assess developmental potential.** (A) The area containing the ductal anlage (purple) in a 3-week-old mouse is surgically removed to generate a "cleared fat pad" into which donor tissue (dark pink) is transplanted. The development of the transplanted tissue is monitored at intervals posttransplant by either whole mount or standard histological analysis. (B) An alternative approach involves direct transplantation into a non-cleared mammary gland. Transplanted cells are frequently marked with either immune-fluorescent markers, genetic markers, or infected with viruses expressing detectable markers. *Source: Used by permission from Macmillan Publishing Company; Ref. 14.* (For interpretation of the references to color in this figure legend, the reader is referred to the online version of this book.)

protein present in the milk and the protein species present can be readily determined by gel electrophoresis. The milk lipid content can be determined on a volume basis using whole milk. Analysis of the milk clot from the stomach of pups after nursing is also possible, although effects of digestive enzymes make precise findings less reliable, or on dry weight basis using milk clot analysis. Furthermore, gas chromatography–mass spectroscopy can be used to determine the precise fatty acids present in the milk and their relative or exact concentrations.[19,22] New technologies are allowing precise determination of increasingly important components of milk, such as oligosaccharides, growth factors, cytokines, and glycosylated proteins.[23,24]

s0035 *Methods for Studying Human Lactation*

p0060   Studies of the physiology of lactation in humans have the advantage of direct clinical relevance. Further, milk is available in quantities that allow precise quantitation of nutrients and other molecules. They are made more difficult by the complex methods needed to accurately quantitate milk volume transfer to the infant and the near impossibility of obtaining samples of mammary tissue in a systematic fashion at various stages of mammary development. Our understanding of the histology of the human breast has come mostly from autopsy studies[25] and from samples of normal tissue taken in association with breast cancer diagnostic procedures. However, breast milk is available at all stages of lactation and offers considerable insight into both the complex composition of this fluid and its changes with time postpartum. Recently, insight into molecular mechanisms has been obtained from analysis of milk fat globule (MFG) membranes; these contain significant amounts of mammary cell cytoplasm, allowing assessment of mRNA expression.[26] In addition, the process of secretory activation takes place after birth rather than prepartum as in most species, allowing the molecular underpinnings of this process to be studied by careful analysis of milk components.

     Breast milk production can be assessed in three ways: p0065 (1) In mothers who are pumping their breasts to obtain

milk for a preterm or ill infant, the volume of milk pumped is easy to measure, most accurately by weighing the milk container before and after pumping. This technique allowed researchers to determine that retained placental fragments can inhibit secretory activation in women, most likely by their secretion of P4.[27] (2) Willing mothers can apply a version of the weigh-suckle-weigh technique to assess quantitative transfer of milk to the infant. If this procedure is carried out on a 24 h basis, the daily production of milk can be assessed as was done in a landmark study of secretory activation in the Neville laboratory.[28,29] (3) Finally, the rate of appearance of deuterated water in the saliva or urine of the infant after defined dosage of the mother provides an accurate measure of milk transfer once milk volume has stabilized.[30] While the doses of deuterium necessary are not harmful,[31] it is often difficult to obtain permission to use the technique in mothers who have an aversion to taking an "isotope" (albeit a stable one) while breastfeeding.

p0070   The concentration of macronutrients in human milk has been known for many decades.[32] However, recent advances in mass spectroscopic techniques have allowed a remarkable refinement both in the analysis of low abundance proteins and their structures, e.g., glycosylation and phosphorylation, as well as a detailed understanding of the structures of the numerous oligosaccharides present in milk. The importance of these compounds to intestinal microbiome development in the infant is just now being revealed.[33,34]

p0075   Analysis of mRNA associated with the MFG is being carried out in a number of species. Most importantly this technique is available for human milk and has been used effectively in the Haymond laboratory at Baylor College of Medicine (Texas) to examine the transcriptome of the human mammary gland during lactation[26] and the gene expression correlates of the onset of lactose synthesis during secretory activation.[35] Application of new technology such as RNA-Seq[36] and advanced bioinformatics technology[37] in situations where lactation is likely to be compromised such as obesity is an exciting prospect for advances in our understanding of human lactation.

s0040 Hormonal Control of Anatomical Development

s0045 Endocrine and Paracrine Regulation of Ductal Development

p0080   The mammary gland is unique since, unlike most secretory organs, the capacity for milk secretion develops in the adult in concert with other reproductive events and is directed by reproductive hormones.[38] The mammary anlagen and the adipose fat pad into which the gland will develop are specified during embryonic development, however, further growth and differentiation must await the hormones of puberty and pregnancy.[39–42] The molecular basis of the developmental process has been best described in the rodent, where the ductal development that occurs during early puberty has been extensively studied using both genetically modified mice and transplantation approaches. Although hormone ablation studies demonstrated a role for estradiol-17β (E2), P4, and PRL in ductal and alveolar development in the 1950s, recent studies have provided better definition of this process and indicate that insulin-like growth factor-1 (IGF-I), growth hormone (GH), and amphiregulin are also important. At birth the gland contains a rudimentary ductal tree that is attached to the teat (Figure 31.1(A)). During puberty the ducts elongate until they reach the margins of the fat pad (Figure 31.1(C)). Elongation of the ducts occurs through proliferation of mammary epithelial cells (MECs) present in bulb-like structures at the ends of the ducts, which are referred to as TEB and disappear by the end of puberty (Figure 31.1(B)). Ductal branching also occurs during puberty resulting in a structure composed of regularly spaced ducts that never cross each other. Spacing between ducts appears to be the result of growth inhibitory effects of transforming growth factor-β (TGFβ).[43] Cross-transplantation experiments by Naylor and Ormandy showed that strain specific patterns of ductal branching in the mouse are a function of the mammary stroma rather than the epithelium,[44] and the extent of branching also varies between different strains of mice. Side branches develop under the influence of P4 during the luteal phase of the estrous cycle. In the rat the side branches are generally more complex and tend to be variable.[45] In the human the lobular units that are derived from the side branches are complex in character. In this species the so-called terminal duct lobular unit (TDLU) consists of a straight extralobular duct that branches into several intralobular ducts terminating in acinar complexes (Figure 31.1(D)).[9,46] In most dairy species, the ductal structure does not reach the margin of the fat pad until the hormones of pregnancy stimulate both ductal elongation and branching.[47]

Estrogens are known to act through two variants of   p0085 the nuclear estrogen receptor, ERα and ERβ, which are both expressed in the mammary gland.[48] Mice in which expression of ERβ has been eliminated still show normal ductal elongation during puberty.[49] Initial studies demonstrating that ductal elongation was diminished in ERα knockout mice were complicated by the presence of a residual truncated ERα in these mice.[50,51] More recently, elegant studies by Cathrin Brisken and her colleagues have clearly demonstrated a role for ERα in elongation.[52] ERα null MECs transplanted into the cleared fat pad of three-week-old normal mice showed no ductal outgrowth. However, when ERα null MECs were co-transplanted with wild-type MECs, they were able to form ducts, suggesting the presence of a paracrine signaling pathway dependent upon ERα.[52] Further studies

identified amphiregulin as the paracrine growth factor and thereby implicated the epidermal growth factor receptor (EGFR), the only known receptor for amphiregulin, as a critical signaling molecule for ductal elongation.[53] EGFR is expressed in both MECs and the stromal cell compartment, however, only stromal expression of EGFR is required for ductal elongation,[52,54] suggesting the presence of a second paracrine signaling network regulating ductal elongation.

p0090   Increased production of circulating GH is coincident with the onset of puberty; formation of TEBs and ductal elongation have clearly been shown to require both GH and its primary transcriptional target IGF-I.[55,56] IGF-I, IGF-II, the IGF-I receptor (IGFIR), and IGF-binding proteins are all expressed in the murine mammary gland throughout puberty.[57] IGF-I is expressed in the mammary gland stroma throughout postnatal development and in the TEBs during puberty.[58] Loss of greater than 50% of IGF-I expression from MECs resulted in a decrease in both ductal complexity and the cyclins A2 and B1, which are important for S and G2 phase progression.[59] The role of circulating versus locally produced IGF-I in ductal elongation has been debated at length; recent studies by Cannata et al. demonstrate that elevated circulating IGF-I can stimulate development resulting in a more complex ductal network.[60] These data suggest roles for both circulating and locally produced IGF-I in pubertal development. However, it is clear that at least a portion of ductal elongation requires local production of IGF-I, which cannot be completely replaced by circulating IGF-I.[61]

p0095   In contrast to IGF-I, expression of IGF-II is induced by PRL and not GH.[62,63] During puberty IGF-II is coexpressed with IGF-I, however postpuberty and during early pregnancy IGF-II expression is restricted to ductal and alveolar epithelial cells in a nonuniform pattern reflecting that of the receptors for progesterone (PR) and PRL (PRLR).[64] The differential regulation of IGF-I and IGF-II suggests that they may each have unique roles in mammary gland development. MECs lacking PRLR transplanted into a wild-type mammary fat pad showed normal ductal elongation,[65] suggesting that neither PRL nor IGF-II is necessary for this process.

p0100   For a more complete description of these morphological changes the reader is referred to a number of excellent reviews[8,9,45,46] and an excellent online tutorial at http://mammary.nih.gov.

s0050 *Mammary Development in Pregnancy*

p0105   The hormones of pregnancy are critical in producing a mammary gland that is capable of producing milk. Although ductal elongation is normal in mice that lack PR, alveolar differentiation does not occur in PR knockout mice, or in wild-type fat pads transplanted with MECs lacking PR.[65,66] Deletion of PR from the stromal compartment does not alter alveolar differentiation during pregnancy. Two different isoforms of PR are produced from different promoters present in the same gene, and analysis of genetically modified mice lacking specific PR isoforms has revealed that only PR-B is required for alveolar development during pregnancy.[67,68]

p0110   In addition to P4, PRL is required for lactogenic differentiation during pregnancy. Genetically modified mice lacking either PRL or PRLR have been generated, and ductal elongation is normal in these mice.[69,70] However, analysis of lactogenic differentiation is complicated by the diminished fertility. Alveologenesis and differentiation of PRLR null MECs transplanted into wild-type mammary fat pads is completely blocked.[71] Consistent with a critical role for PRL and its receptor in lactogenic differentiation, MECs lacking either JAK2 or STAT5, the signaling molecules downstream of PRLR are also unable to undergo lactogenic differentiation,[72,73] further demonstrating the critical nature of the PRL pathway in pregnancy-induced mammary gland differentiation.

p0115   Understanding the role of IGFs in mammary gland development is confounded by the complex pattern of expression of both IGF-I and IGF-II during pregnancy; however, a role for IGF-I is suggested in recent studies by Sun et al.,[74] who examined lactogenic differentiation in genetically modified mice expressing a dominant-negative form of IGFIR in the mammary epithelium. Expression of this receptor delayed alveolar differentiation, luminal expansion, decreased epithelial cell proliferation, lipid droplet formation, activation of signaling molecules downstream of the IGFIR, and expression of milk protein genes.[74] Despite these dramatic changes, pup growth was not altered, suggesting that either the mammary gland is able to compensate for the observed defects, that complete loss of IGFIR is required, or that the insulin receptor (IR) takes over signaling through these pathways during lactation.[75,76]

p0120   In addition to IGFs, there is also increasing evidence that insulin plays an important role in mammary gland development and function in both pregnancy and lactation. The potential importance of insulin and its receptor in mammary gland development and lactation are at variance with previous thoughts that lactation would be insulated from the actions of insulin since insulin levels vary dramatically with food intake.[77] Berlato and Doppler analyzed the expression of both IGFIR and IR during lactation and observed that the levels of the IGFIR decreased at both the mRNA and protein levels following parturition, with an approximately 80% decrease in the amount of mRNA.[76] In contrast, the expression of total IR remained constant at both the mRNA and protein levels.[76] IR exists as the product of two different splice variants, referred to as IR-A and IR-B, which differ in the presence (IR-B) or absence (IR-A) of exon

11, which encodes 12 amino acids that are located at the C-terminus of the extracellular subunit of the receptor.[78,79] The major difference between IR-A and IR-B is that IR-A has a 10-fold higher affinity for IGF-II, making it a second physiological receptor for IGF-II.[78] Analysis of the expression pattern of IR-A and IR-B during pregnancy and lactation revealed that while the mRNA levels of IR-A remain the same over this developmental time course, the amount of IR-B mRNA increases three-to fourfold following parturition.[76] When the sixfold decrease in the amount of IGFIR mRNA that occurs following parturition is also taken into account,[76] one must conclude that there is a dramatic increase in insulin signaling during lactation.

p0125   The importance of insulin signaling in mammary gland development and lactation is indicated by recent analysis of mice bearing a floxed IR. Excision of the IR gene from MECs using Cre recombinase expressed specifically in these cells resulted in the formation of mammary glands that lack at least 50% of secretory alveoli by day 14 of pregnancy, and in a 75% decrease in pup growth over the first 10 days of lactation.[80] Despite this dramatic decrease in pup growth, the majority of pups do survive. A comparison of the genetically modified mice expressing the dominant-negative form of IGFIR and mice lacking expression of IR in the mammary epithelium clearly provides evidence that while IGF-I may be important for alveolar development during pregnancy, it is not the entire story in either pregnancy or lactation. These observations suggest that conditions that impair insulin signaling, such as obesity and diabetes, may have dramatic effects upon lactation.

s0055 Functional Differentiation

p0130   Functional differentiation of the gland can be divided into three phases that have been most thoroughly studied in the mouse: (1) the *proliferative* phase of early pregnancy, (2) *secretory differentiation* starting in mid-pregnancy, during which the gland becomes competent to secrete milk, and (3) *secretory activation* around parturition when the secretion of milk commences.

s0060 *The Proliferative Phase*

p0135   This phase starts immediately after conception, reaching a peak about day 5 of pregnancy, when an astonishing 25% of the mammary alveolar cells are labeled with ^{3}H-thymidine 1h after injection in the mouse (Figure 31.3(A)).[81,82] Cell proliferation tapers off gradually through the remainder of pregnancy until MECs reach quiescence just prior to parturition. During this period the content of mRNA for epithelial markers cytokeratin 19 and claudin 7 increases nearly three orders of magnitude (Figure 31.3(B)),[83] indicating a remarkable expansion of the alveolar compartment. Whether these markers reflect cell number, cell size, or a denser cytoplasm is not currently clear. Three paracrine factors—Rank ligand (RANKL), WNT 4, and amphiregulin—show an expression pattern that parallels proliferative activity (Figure 31.3(C)); expression of RankL is regulated by PR[68] while expression of amphiregulin is induced by IGF-II.[62,63] In the mouse a single, coordinated round of cell division takes place immediately after parturition (Figure 31.3(D)),[82] possibly resulting in the high proportion of binucleate cells present in the lactating gland[85]; it is currently unknown what growth factors stimulate this round of cell division as the three paracrine factors elevated during early pregnancy are not expressed at this time. It is also not clear whether the proliferative activity in early lactation is specific to the mouse or occurs in other species as well.

p0140   As mentioned before, studies using genetically modified mice have indicated that P4[86,87] and PRL[88] are the major hormones that promote side branching and the formation of alveoli.[39] It is surprising to note that the receptors for PR are expressed in only a subset of cells in the mammary epithelium[64,89–92] (Figure 31.4). Indirect evidence indicates that this is also the case for the PRLR. Analysis of normal human mammary epithelium has shown that 98% of the proliferating cells are negative for estrogen receptor (ER) and PR,[90] and, significantly, the absence of PR in proliferating cells has also been observed in the mammary epithelium of mice, rats, and cows.[94–96] The disparate localization of proliferating cells and PR+ epithelial cells could be explained by the rapid downregulation of steroid receptors following ligand binding; this hypothesis is supported by observations that ERα is rapidly degraded by the proteasome,[97] and that ERα is also rapidly lost in MECs following entry into the cell cycle.[98]

p0145   The generally accepted view is that PR is not expressed in proliferating cells and that P4 action induces expression of paracrine growth factors that stimulate proliferation of PR− epithelial cells. Thus, PR has been shown to induce expression of RANKL[68] and WNT-4,[99] while PRL induces expression of IGF-II.[62,63] WNTs are a family of signaling molecules similar to the *Drosophila* protein wingless and the mammalian INT. Overexpression of WNT-4 stimulates ductal branching and formation of alveoli following transplantation into cleared mammary fat pads in a manner that resembles development observed in pregnant mice.[100] Further studies have demonstrated that expression of WNT-4 is dependent upon activation of PR by P4, and that WNT-4 functions in a paracrine manner to induce ductal branching and alveologenesis during pregnancy.[99] It is not clear whether WNT-4 acts directly upon PR-negative MECs, or on other cells in the surrounding microenvironment.
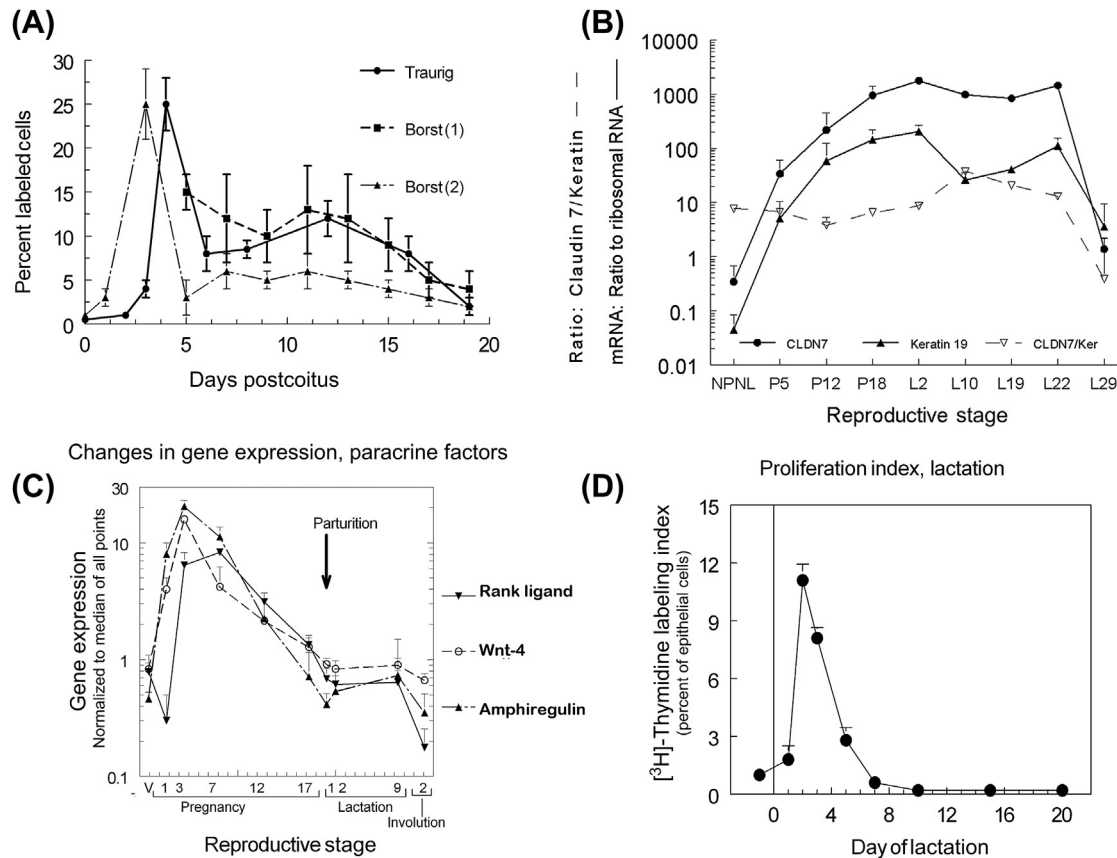
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FIGURE 31.3   Proliferative activity during pregnancy and lactation in the mouse. (A) Proliferation through pregnancy as measured by 1 h incorporation of ^{3}H-thymidine in vivo. (B) Expression of mRNA for keratin 19 and claudin 7, epithelial cell markers determined by real time RT-PCR. Dashed line is the ratio of claudin 7/keratin 19. These changes are indicative of the proportion of epithelial cells in the gland as pregnancy progresses. (C) Changes in expression of mRNA of the paracrine factors Rank ligand (RANKL), Wnt-4, and Amphiregulin over pregnancy and lactation. (D) One hour ^{3}H-thymidine labeling index during lactation. The synchronized round of DNA synthesis probably results in the production of the binucleate cells that are numerous in the mammary gland of the lactating mouse. *Source: (A) Data replotted from Refs 81,82; and Borst DW, Mahoney WB. Mouse mammary gland DNA synthesis during pregnancy. J Exp Zool 1982;22:245–50. (B) Reproduced from Ref. 83. (C) Unpublished data from Ref. 84. (D) Data replotted from Ref. 82.*

FIGURE 31.4   Role of paracrine signaling in alveologenesis. (A) As pregnancy progresses side branches develop at discreet intervals along the ducts; cells in these side branches proliferate to form alveoli. The progesterone receptor (PR) is expressed in a subset of alveolar cells that are stimulated by increasing P4 from the ovaries to secrete RANKL. This paracrine factor acts on neighboring cells to promote proliferation. (B) Expression of PR and proliferating cells after 2 days of treatment with E2 and P4 in wild-type (control) and PRL-null mice. Proliferating cells (BrDU labeled, green) and PR positive cells (red) are generally not coincident. Note that the number of proliferating cells is reduced in the absence of PRL. Scale bar, 50 μm. *Source: (A) Used by permission from Ref. 93. (B) Original figure from Ref. 64.* (For interpretation of the references to color in this figure legend, the reader is referred to the online version of this book.)

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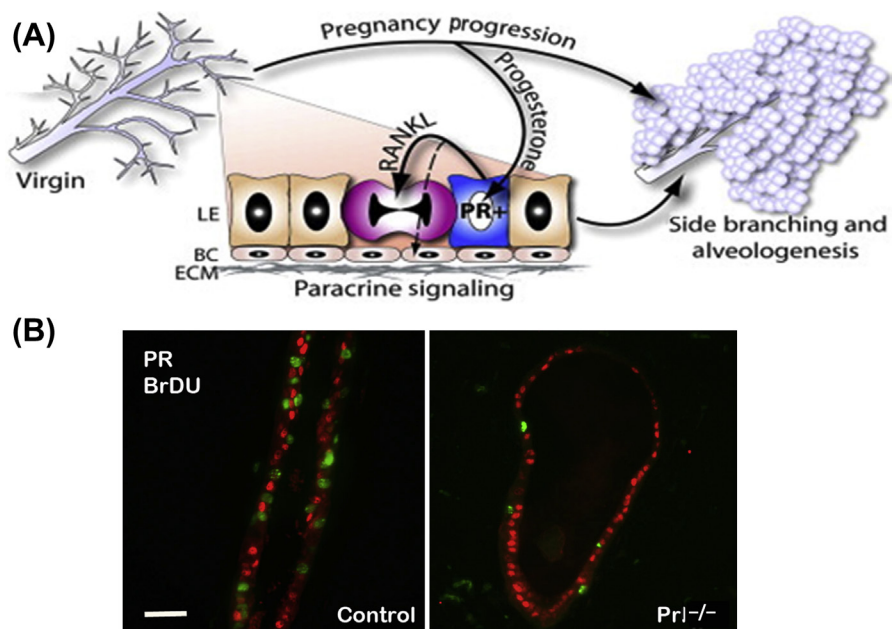
p0150   To observe the effects of overexpression of RANKL in MECs, Fernandez-Valdivia et al.[101] constructed transgenic mice in which expression of RANKL was driven by the mouse mammary tumor virus (MMTV) promoter, resulting in RANKL expression in the pubertal mammary gland when RANKL is not normally expressed. Transgenic mice expressing RANKL exhibited more TEBs, ductal side branching, and alveolar buds reminiscent of an early pregnant mammary gland, and there was a dramatic increase in the number of proliferating cells in both ductal and alveolar structures.[101] The florid proliferation and development observed in the RANKL transgenic mice is consistent with the attenuated alveologenesis and lactation defect observed in the RANKL knockout mice.[102] Expression of RANKL in MECs in vivo can be induced by treatment with either P4 or PRL.[62,67] Induction of RANKL is restricted to PR⁺ cells, which reside in close proximity to PR⁻ cells that express cyclin D1 and proliferate in response to P4.[68] Further details regarding both WNT-4 and RANKL can be found in excellent recent reviews by Rajaram and Brisken[103] and Fernandez-Valdivia and Lydon.[93] Future research is needed to reveal the cells targeted by WNT-4, the signaling pathway(s) activated by WNT-4, and the role of these ligands in maintaining mammary stem cells in the normal gland. How amphiregulin, whose expression pattern in early pregnancy parallels that of WNT-4 and RANKL (Figure 31.3(C)), fits into this picture is not yet clear.

p0155   Another interpretation comes from experiments in the Haslam laboratory where the mammary gland from early pregnant mice showed a decrease in expression of PR-A, while the expression of PR-B remained constant in alveolar epithelial cells. Further, and in contrast to the virgin gland, expression of PR-B co-localized with cells that label with 5-bromo-2′-deoxyuridine (BrDU) indicating that these cells are proliferating.[104,105] These authors concluded that P4 interacting with PR receptor directly stimulated proliferation of alveolar cells during pregnancy.

Secretory Differentiation
s0065

p0160   Secretory differentiation, previously referred to as lactogenesis I,[106] begins about mid-pregnancy and is signaled by changes that depend on the species and the experimental paradigm used for their study. For example, Akers[47] showed that α-lactalbumin increased in mid-pregnancy in the fluid extracted at this time from the bovine mammary gland; a similar phenomenon is also observed in the mouse. McManaman and colleagues[16,105] have documented the accumulation of lipid droplets in the mammary alveolar cells of the mouse starting around day 8–10 of pregnancy (Figure 31.5(A)). Mellenberger and Bauman[108] showed a biphasic increase in the enzymes of lipid synthesis

in the rabbit mammary gland, and Hartmann and colleagues[109] showed that lactose appeared in the plasma and urine in the women at mid-pregnancy. Gene expression profiling of whole murine mammary glands during pregnancy and lactation has revealed that expression of milk protein genes increases about fivefold during pregnancy and then increases a second time at parturition (Figure 31.5(B)).[84] Similar data are obtained when the expression of these genes is examined in preparations of adipocyte-depleted MECs (Rudolph and Anderson, unpublished data). Analysis of genes involved in de novo synthesis of fatty acids and β-oxidation of fatty acids reveals that the latter decrease over the course of pregnancy, while expression of genes involved in fatty acid biosynthesis increases sharply at parturition. Expression analysis of adipose-depleted MECs reveals that the increase in fatty acid biosynthetic genes is specific to the epithelium during pregnancy.[19] There is also a decrease in the proportion of adipose tissue observed in the mammary gland by microscopic examination, reflected by a proportionate decrease in expression of adipocyte-specific mRNAs. However, this decrease may reflect expansion of epithelial cells rather than loss of adipocytes (Figure 31.5(A)).[84] There is also a decrease in collagens expressed in the mammary gland as pregnancy progresses; it is not clear whether this change represents stromal remodeling or simply dilution of stromal components by the expanding epithelium. During late pregnancy the gland begins to produce small amounts of secretion product. This product can escape the gland through the junctional complexes between the alveolar cells, which are highly permeable during pregnancy.[110,111] Copious milk secretion in all species that have been examined is inhibited by the high concentrations of circulating P4 produced by the ovaries or placenta, depending on species, at this time.

Hormonal Regulation of Secretory Differentiation
s0070

p0165   PRL, P4, and the lactogenic product of the placenta, placental lactogen (PL), have all been implicated in the regulation of secretory differentiation.[112] There are clearly species differences in the roles of these hormones, particularly PRL and PL, and even though genetically modified mice have allowed a good understanding of the role of PRL, the role of PL remains unclear. GH is lactogenic in many species,[113] but its role in differentiation has been controversial because it is not clear whether the growth hormone receptor (GHR) is expressed in stromal cells or epithelial cells.[114] Lactation occurs although it is not normal in the GHR⁻/⁻ mouse.[115] Furthermore, definition of the precise role of GH is complicated by its ability to induce expression of IGF-I, which has been shown to have direct effects upon secretory differentiation (see previous discussion).
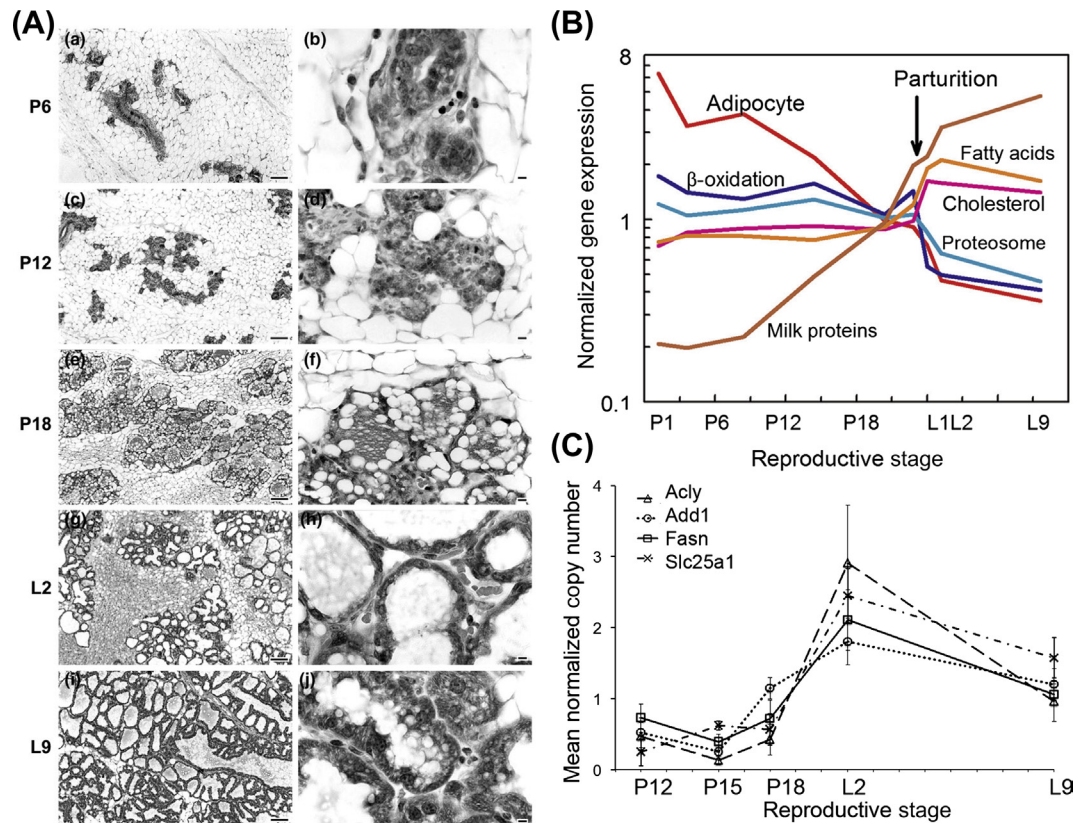
f0030

FIGURE 31.5   Changes in the differentiated activity of the mammary gland in pregnancy and lactation. (A) Morphology of secretory development in the mouse. Shown are histological sections of the mammary gland of FVB mice through pregnancy and lactation. Mammary glands were isolated on the days indicated, fixed, sectioned, and stained with hematoxylin and Eosin. Scale bars, 100 μm in (a, c, e, g, and i) and 10 μm in (b, d, f, h, and j). Note the intracellular lipid droplets in late pregnancy (panel (f)), the expansion of the lumens at the onset of lactation (panels (f) and (h)), and the diminution of adipocytes as lactation progresses (compare panel L2 and L9). (B) Changes in gene expression of different categories of genes in the mouse mammary gland from microarray studies. Expression of adipocyte-specific genes and collagens (not shown) decreases six- to eightfold during pregnancy and another twofold at parturition, whereas the genes for fatty acid degradation and many components of the proteosome are level during pregnancy and decrease about twofold at parturition. Milk protein genes, on average, increase about fivefold during pregnancy and another threefold around parturition, whereas the genes for fatty acid and cholesterol synthetic enzymes increase about twofold just after parturition. Normalized data for each class were averaged to produce the lines on this graph. (C) Time course of expression of genes important for de novo lipogenesis. Note that the major increase in expression occurs during secretory activation. *Source: (A, B) From Ref. 11. (C) Original data from Rudolph MC. University of Colorado, Denver.* (For a color version of this figure, the reader is referred to the online version of this book.)

s0075 **PROLACTIN**

p0170   PRL remains high throughout human pregnancy, and women with low PRL levels during pregnancy have difficulty in lactating,[116] suggesting that PRL is involved in secretory differentiation in humans.[117] The role of PRL in secretory differentiation has been more extensively studied in rodents, and although PRL is secreted in a pulsatile fashion early in pregnancy (Figure 31.6(A)), PRL itself is not necessary in mid-pregnancy in rodents and may even be deleterious by inducing premature secretory activation. Circulating PRL returns to high levels just prior to parturition[119] (Figure 31.6(B)). Furthermore, expression of the PRLR changes during pregnancy, and the long form, which activates downstream signaling pathways, is downregulated in pregnancy and increases markedly at parturition[118] (Figure 31.6(D)). Finally, if a constitutively active PRL receptor is expressed under the control of the β-lactoglobulin promoter, alveolar development is increased in late pregnancy, but the animals fail to lactate.[120] PRLR and the downstream signaling molecules JAK2 and STAT5 are required for secretory differentiation since mice lacking PRLR, JAK2, or STAT5 lack expression of milk protein genes.[69,72,73,121,122] PRLR, like PR, is expressed in only a subset of epithelial cells in early pregnancy,[64,123] and it is not yet clear whether these cells are coincident or not.

p0175   In the human serum, PRL and PL levels rise starting in early pregnancy reaching the very high levels of about 100 ng/ml (compared to ~10 ng/ml in the nonpregnant woman) and 2000 ng/ml (nonexistent in the nonpregnant animal), respectively, by the twentieth week of gestation; increases in PRL levels directly correspond to increases in lactose excreted into urine,[124,125] implying that PRL regulates activation of lactose synthesis.

FIGURE 31.6   **Hormone profiles during pregnancy in rats and mice.** (A) Pulsatile secretion of PRL during early pregnancy in the rat. At 8 days postcoitus, this pulsatile activity ceases. (B) Return of PRL secretion 24 h prior to parturition in the rat. (C) Steroid hormone profiles in the mouse during pregnancy. Corticosterone remains approximately constant with a slight elevation at parturition, possibly due to stress. P4 rises early in pregnancy and falls one day prior to parturition. Estradiol rises about threefold over the course of pregnancy. (D) Expression of mRNA for the long (PRLL) and short (PRLS) forms of the prolactin receptor during the transition from pregnancy to lactation. Note the increase in the ratio of total RNA to DNA at the onset of lactation. *Source: (A, B) Modified from Ref. 118. (C, D) Data replotted from Mizoguchi Y, et al. Corticosterone is required for the prolactin receptor gene expression in the late pregnant mouse mammary gland.* Mol Cell Endocrinol 1997;**132**:177–83.
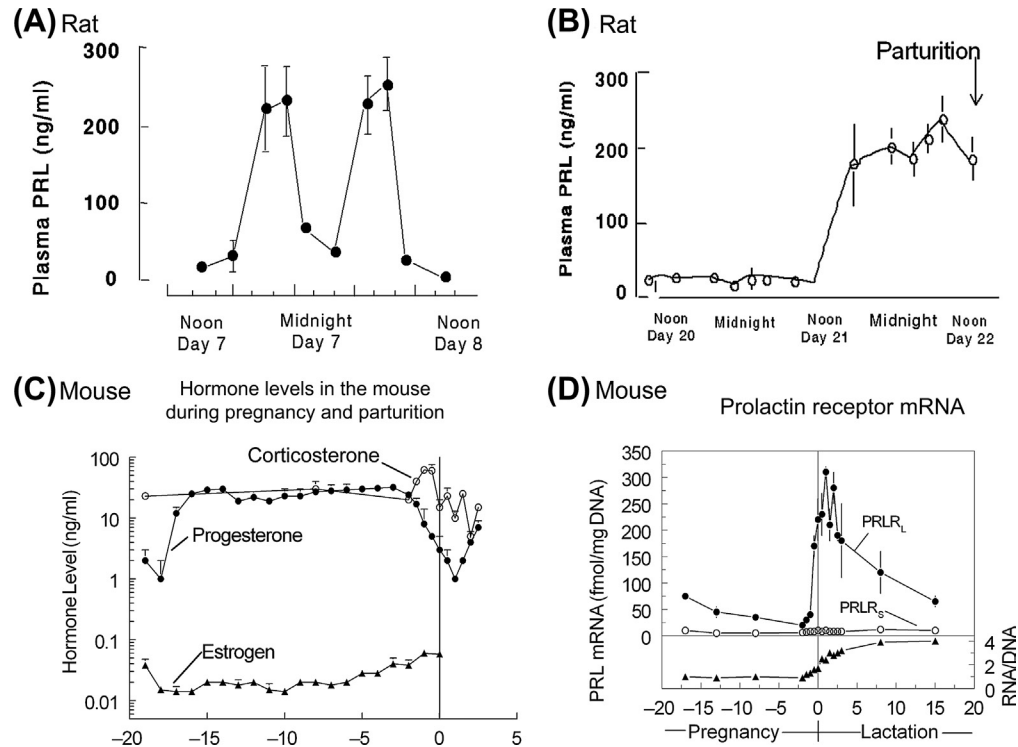
PLACENTAL LACTOGEN

PL is present at high levels only during secretory differentiation.[126–129] The hormone has evolved at least twice, in rodents and ruminants from the PRL gene and in primates from the GH gene.[112,130,131] Women with deletions in the GH/PL gene complex and undetectable PL concentrations in plasma have been reported to have quite normal lactations,[132,133] suggesting that PRL is the hormone responsible for secretory differentiation in humans. Several rodent PLs have been fully characterized (mouse, rat, hamster) and their patterns of secretion in pregnancy measured. The twice-daily surges of pituitary PRL in rats are replaced in mid-pregnancy by, first, a surge in PL I and then by PL II, which continue to increase until term. There is currently no evidence for a specific PL receptor, and PL is thought to act through either the GHR, the PRLR, or both.[134]

PROGESTERONE

P4 increases early in pregnancy (Figure 31.6(C)) secreted by the ovaries. As discussed previously, there is good evidence that it drives proliferation during early pregnancy; however, it is not clear whether it plays a role in secretory differentiation during mid- to late pregnancy. In most species ovarian secretion of P4 continues through pregnancy. However, in women ovarian P4 is replaced by placental P4 after the first trimester. There is incontrovertible evidence that it does inhibit secretory activation in late pregnancy[135,136] and prevents premature lactation in all species that have been studied. P4

withdrawal triggers both secretion and tight junction closure. Thus these processes are triggered in the mammary epithelium of late pregnant mice by injection of the PR antagonist RU486; PRL and glucocorticoids are required.[136] This observation, which has been repeated in many species, indicates that P4 is able to suppress secretory activation through the small number of P4 receptors present in late pregnancy, although it is not clear whether an unidentified paracrine factor could be involved in a manner similar to that responsible for alveolar proliferation in early pregnancy or whether myoepithelial cells could be involved.

Secretory Activation

Secretory activation is the onset of copious milk secretion; it is sometimes referred to as lactogenesis II or the initiation of lactation.[106] As described before, this process is set in motion by the fall of P4 around parturition in all species that have been studied[135,137–142]; while in mice the fall in P4 occurs at parturition, in humans P4 levels fall two orders of magnitude over the first four days postpartum, resulting in the production of approximately 500 ml/day of milk by day 5.[143] In both the mouse and human, PRL levels are high at parturition. In the mouse the fall in P4 is accompanied by profound changes in the expression and metabolic activities of many classes of molecules including milk proteins and the enzymes of lipid and cholesterol synthesis (Figure 31.5(B)). The expression of milk protein genes is largely regulated by PRL via the PRLR/JAK2/STAT5 signaling pathway due

to the presence of specific promoters that bind STAT5,[14] however, the regulation of fatty acid biosynthesis is less well defined.

p0195   Among potential regulators of fatty acid biosynthesis are PRL, sterol-response element binding protein (SREBP), AKT1, and SPOT14 (also known as THRSP). Experimental evidence supports roles for both SREBP and PRL in regulating enzymes involved in fatty acid and cholesterol biosynthesis[19,20] (Figure 31.7). Analysis of gene expression profiles in adipose-depleted MECs from lactating mice that were treated with bromocriptine for 8 h to block PRL-dependent signaling demonstrated a two- to fourfold decrease in the expression of genes involved in glycolysis and the pentose shunt, a three- to sixfold decrease of genes encoding enzymes involved in de novo fatty acid biosynthesis, and a two- to twentyfold decrease in the expression of genes involved in

triacylglycerol synthesis.[20] When SCAP was deleted from MECs, SREBP function was lost and pup weight gain decreased from day four of lactation on. Expression of fatty acid synthase (FASN), the mitochondrial citrate transporter SLC25A1, and stearoyl-CoA-desaturase (SCD2) were all decreased in MECs from lactating mice lacking SCAP, however, the levels of other enzymes involved in de novo fatty acid biosynthesis were unchanged.[19] These findings indicate that PRL has a more significant role in upregulating the expression of enzymes involved in de novo fatty acid biosynthesis at secretory activation than SREBP, which regulates this process in the liver and adipose tissue. However, both molecules do contribute to the regulation of these genes (Figure 31.7). It is very likely that other levels of control remain to be discovered as de novo fatty acid biosynthetic enzymes are likely to be regulated at both the
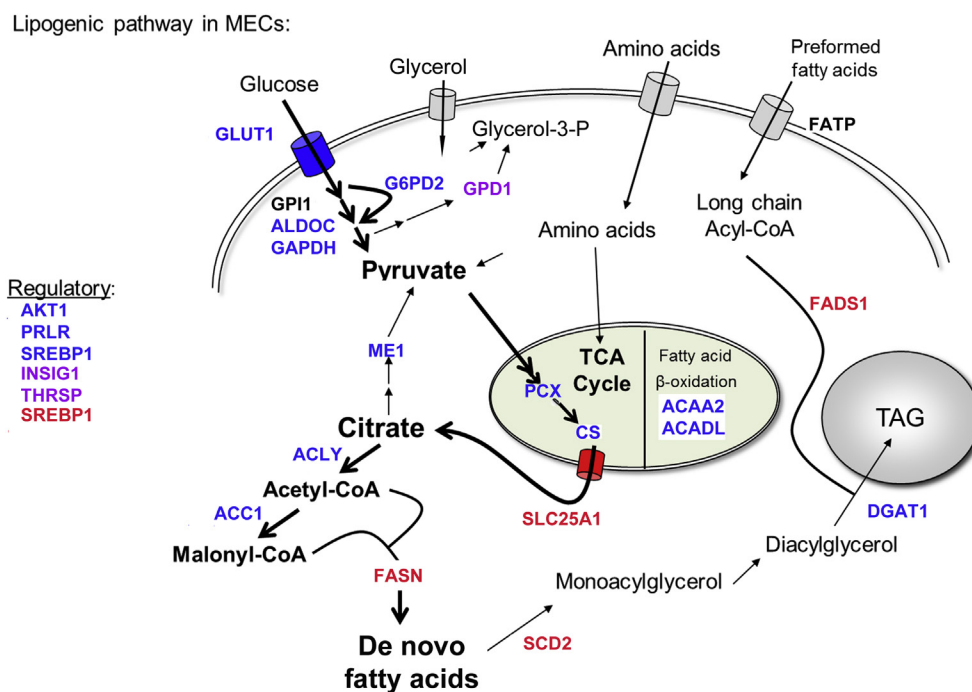
Blue = Regulated by PRL; Magenta = Regulated by SREBP; Red = Regulated by both PRL and SREBP

FIGURE 31.7 **Lipid synthesis pathways in mammary epithelial cells during lactation.** Substrates for lipid synthesis enter the cells via the glucose transporter (GLUT1), a glycerol transporter, as amino acids, or as preformed fatty acids via a fatty acid transport protein (FATP). Glycolysis leads to the production of both glycerol-3-phosphate and pyruvate from glucose. The genes for several enzymes in this pathway, fructose bis-phosphate aldolase (ALDOC) and glycerol-3-phosphate dehydrogenase (GAPDH), as well as glycose-6-phosphate dehydrogenase (G6PD2 in the mammary gland) are all upregulated by prolactin along with mitochondrial genes for pyruvate carboxylase (PCX) and citrate synthase (CS). Glycerol-3-phosphate is formed from dihydroxyacetone phosphate, a product of glycolysis, by glycerol-3-phosphate dehydrogenase (GPD1) to be used as a backbone for triacylglyceride (TAG) synthesis. GPD1 is regulated by SREBP. Amino acids are transformed into pyruvate and other substrates that enter the mitochondria to be transformed into citrate, which is exported via the tricarboxylic acid transporter, SLC25A1. Citrate is the major substrate for de novo synthesis of fatty acids in species other than ruminants, which utilize acetate for this purpose. Citrate is transformed into acetyl-CoA by ATP citrate lyase (ACLY), then to malonyl CoA by acetyl-CoA carboxylase (ACC1), and finally to saturated fatty acids with 8–16 carbons (C:8–C:16) by fatty acid synthase (FASN). Cytosolic malic enzyme (ME1) and the enzymes of the pentose phosphate shunt both provide the necessary reducing molecule NADPH that is required for activity of FASN. C:16 fatty acids can be desaturated by stearoyl-CoA desaturase (SCD2) prior to being esterified into monoacylglycerol, then diacylglycerol, and finally into triacylglycerols, with subsequent integration of the fatty acids derived from preformed sources. The final step in the TAG synthesis pathway is catalyzed by diglyceride acyltransferase (DGAT1). The TAG coalesce into lipid droplets. Both prolactin and SREBP have been shown to regulate the genes for FASN, SLC25A1, SCD2, and FADS1. *Source: Diagram derived from data in Refs 19,20.* (For a color version of this figure, the reader is referred to the online version of this book.)

posttranscriptional and posttranslational level by diet and other factors.

p0200 Lactose is the main osmotic agent present in milk, and the levels of lactose are a major determinant of the amount of water drawn into milk in most species. The synthesis of lactose in the mammary gland requires two proteins present in the Golgi compartment, galactosyltransferase and α-lactalbumin; these combine to produce an enzyme capable of producing lactose at physiological concentrations of glucose.[144] It should be noted, however, that milk from fur seals, sea lions, and walruses does not contain lactose apparently because of a mutation in their α-lactalbumin gene.[145] It has also been suggested that the presence of this mutated form of α-lactalbumin may allow the mammary glands of these species to maintain lactation even in the presence of long interruptions that occur during foraging.[145] Synthesis of lactose has been examined in rodents, dairy cows, and humans,[134,144,146–150] and it is clear that P4 inhibits high level synthesis of lactose during pregnancy. Thus robust expression of the requisite enzymes and high level synthesis/secretion occurs around parturition, the timing depending on the timing of the fall in P4.

s0095 ## Lactation

p0205 The continuous production of milk is known as lactation; in earlier literature it was referred to as galactopoiesis. As noted before, lactation is set into motion by a decrease in serum levels of P4 and either a rise in PRL levels (rodents) or maintained high PRL levels (humans). In rodents and dairy animals the decrease in P4 occurs the day before parturition, also setting parturition in motion. In humans, where most P4 is made by the placenta, this decrease takes place over four days after removal of the placenta. Levels of PRL rise at parturition in rodents (Figure 31.6(B)), coincident with a rise in the PRL receptor (Figure 31.6(D)). Further rises in humans and dairy animals after parturition are dependent on stimulation by suckling.[124,151,152] Once lactation is initiated, it is sustained by two different hormones, PRL and OT.[151] During lactation, PRL secretion is stimulated by suckling or milking; in seasonal animals the plasma concentration of PRL is influenced by the day length,[153] the time since feeding/eating, and the time postpartum (reviewed in Ref. 154). Treatment of women,[155] rats,[156] and mice[20] with bromocriptine, a dopamine analog that suppresses PRL release from the pituitary, blocks lactation. In addition to being required for expression of milk protein genes, studies with bromocriptine demonstrate that PRL is required for expression of genes involved in glucose metabolism and de novo synthesis of fatty acids. Furthermore, loss of PRL results in apoptosis of MECs, indicating that it activates survival pathways. Circulating levels of PRL generally decrease as lactation proceeds[96,157] despite continued high production of milk, providing evidence that PRL does not control day-to-day milk volume production.

p0210 As described in further detail in the sections Conclusion Milk Ejection, OT is critical during lactation as it stimulates the contraction of myoepithelial cells resulting in the ejection of milk from the alveolus into the ducts. If this function is impeded in any way, involution ensues.

p0215 Lactation consists of two phases in placental mammals, a colostral phase during which the secretion product contains large concentrations of immunoglobulins and other protective substances[158] and a mature secretion phase during which large quantities of milk are produced. Colostrum is critical in species such as dairy animals, which do not transport immunoglobulins across the placenta. In these species colostrum contains particularly large quantities of immunoglobulins, which are transported across the intestinal epithelium, providing immunoprotection to the young, which suffices until maturation of their own immune systems.[158] It has been suggested that the delay in the production of mature milk in humans is beneficial in exposing the infant to the immune-protective components of colostrum,[159] which is, however, produced in very small quantities. Mature milk provides all the nutrients required by the offspring of the particular species for growth during a period of time that varies from 7 days in the guinea pig to 4 to 6 months in the human. Some adjustments in milk composition occur during this process.[28] For example, in humans the concentrations of protein, Na^+, K^+, Cl^-, and Ca^{++} decline significantly between 3 weeks and 6 months postpartum, whereas lipid, lactose, and glucose increase.[28] In general these changes are small, usually not more than 20%.

p0220 When both the concentration of milk constituents and milk volume were monitored in 12 very cooperative women, a dramatic illustration of the changes in the activity of the gland over the period of secretory activation was obtained (Figure 31.8).[29,160,161] The increase in milk volume, which takes place primarily between days 2 and 4 postpartum in women and earlier in many other species,[29,160–162] is brought about by the coordinated increase in the activity of pathways for ion (citrate, $PO_4^=$, K^+, Mg^{++}, Ca^{++}, Na^+ and Cl^+) secretion and for lipid, lactose, and protein synthesis and secretion. A number of other changes take place prior to this coordinated increase in milk secretion. As detailed in the section on cell–cell interactions, although present between alveolar cells throughout pregnancy, tight junctions are highly permeable, closing only at the onset of lactation, leading to a decrease in the Na^+ and Cl^- flux from the interstitial space into the lumen as a well as a decrease in lactose flux out into the interstitium; the result is a rapid increase in lactose concentration and fall in milk Na^+ and Cl^-.

p0225 One of the remarkable aspects of lactation is that milk production can expand to meet increased demands, and
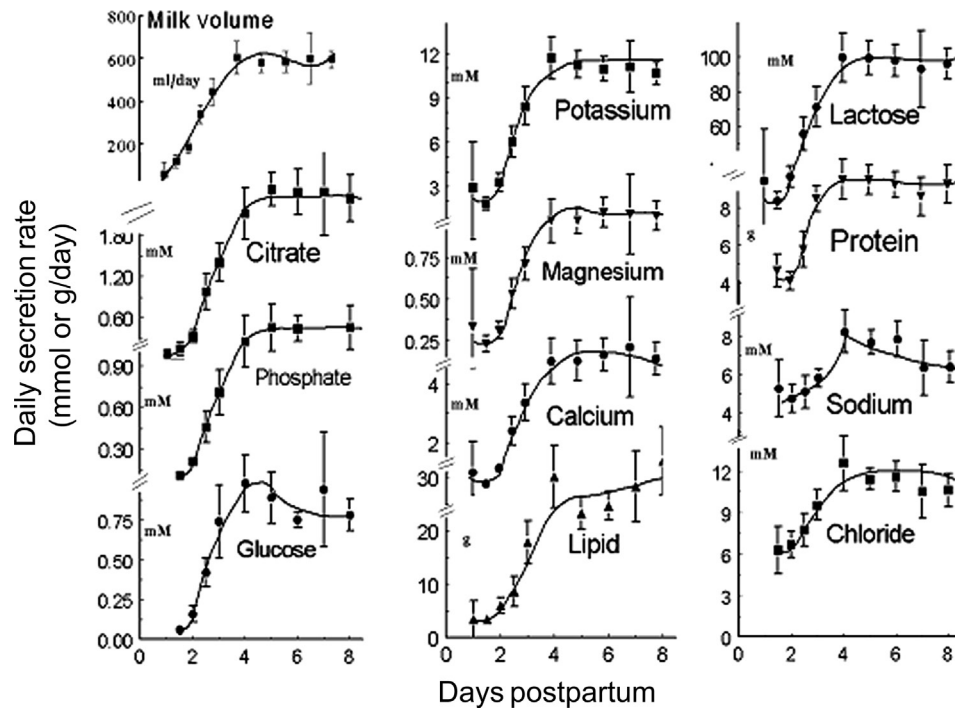
FIGURE 31.8 Milk volume secretion and the rate of secretion of several milk components during the first week postpartum in women. Twelve subjects weighed their infants before and after every feed for the first week postpartum and mid-feed milk samples were taken twice a day from each breast. *Source: Reproduced by permission from Ref. 160.*

f0045

can cease when milk is no longer needed. The precise feedback mechanisms that control the dynamics of lactation have been the topic of great speculation for a long time, specifically around the topic of what are the sensors for milk stasis that lead to loss of milk secretion. The long hypothesized "feedback inhibitor of lactation"[163] has never been purified or proven to exist, however, in recent years attention has focused on the possibility that serotonin may play this role. While searching for PRL-induced transcripts, Matsuda et al. identified the enzyme tryptophan hydroxylase (TPH) as a target for PRL that was induced during pregnancy and lactation.[164] TPH is the rate-limiting enzyme in the synthesis of serotonin (5-hydroxytryptamine, 5-HT), a neurotransmitter regulating mood and cognition. Expression of TPH was induced by PRL in a time- and concentration-dependent manner, and its expression was highest in mammary glands whose teats were sealed producing milk stasis. Further studies showed that treatment of mammary tissue slices or MECs with 5-HT suppressed expression of milk protein genes and induced histology similar to that of involuting mammary glands.[164] Further study demonstrated that 5-HT regulated tight junctions and milk secretion,[165] suggesting that accumulation of 5-HT in the interstitial fluid surrounding secretory alveoli during milk stasis could lead both to cessation of milk secretion and apoptosis of epithelial cells. Treatment of bovine MECs and lactating cows with the selective 5-HT uptake inhibitors (SSRI) such as fluoxetine increased plasma lactose and increased the ratio of Na^+/K^+ in the milk, decreased expression of milk protein genes, and decreased milk volume secretion in lactating cows.[166] These studies were confirmed in mice; further, Tph1 (tryptophan hydroxylase 1) null mice were resistant to these effects.[167] SSRIs are a highly prescribed class of drugs that are commonly used to treat depression, and therefore it should be of little surprise that pregnant women taking SSRIs were found to be more likely to experience delayed secretory activation.[167] It has been long appreciated that there is a delay in the onset of lactation in obese women, and recent studies have suggested that a high fat (HF) diet significantly increases the expression of TPH1 and the mammary receptor for 5-HT (HTR7).[168] These findings produce tantalizing evidence that the serotonin pathway may contribute to the effects of diet and obesity upon lactation.

For a relatively complete description of milk composition in various species, see works by Jenness[169,170] and Jensen.[171] In marsupials, in which the young are born at an extremely immature stage and develop within an abdominal pouch, the changes in milk composition during lactation are much more extreme.[172] A new area of investigation is how the composition of milk from primates may change with the gender of the infant, as well as psychological/emotional factors,[173–176] raising fascinating questions in evolutionary biology.

Involution

s0100

After weaning, most of the secretory cells as well as their stromal environment undergo a process of remodeling, and the mammary gland returns to a state similar

p0230

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to that of the virgin through a process referred to as involution.[177,178] This process is complex, highly regulated, and occurs in two distinct phases: the first phase is reversible and protease independent, while the second phase is not reversible, depends on expression of proteases, and involves considerable remodeling of connective tissue.[179] The use of genetically modified mice and global gene expression profiling over the last decade has greatly advanced our understanding of the changes that occur during the first six days of involution. The vast majority of these studies have used a model of forced involution in which the pups are removed from the lactating dam on day ten of lactation. Although this model is artificial when compared to normal weaning, which is more gradual, it does allow for a very reproducible series of events, which have been well characterized at the cellular and molecular levels. An alternative approach involves sealing of teats on one or more glands to induce milk stasis and involution in the affected gland. This less utilized approach has the advantage that functional and nonfunctional glands can be compared in the same animal with the maintenance of the hormonal environment of lactation.

p0240   The original definition of the two phases described the time, approximately 48 h after pup removal, at which the process becomes irreversible: if pups are returned to the dam within 48 h, lactation resumes.[179] In the human this period is about three days. Interestingly, there are species such as fur seals in which lactation can be interrupted by at least 3 weeks when the mothers feed offshore before returning to nurse.[180] A more functional definition might divide the 6-day involution interval into the period, usually 3 days in the rodent, during which alveolar volume is markedly decreased by removal of both milk and epithelium,[181] and a second phase starting after 3 days that involves stromal remodeling. Teat-sealing studies in the mouse clearly demonstrated that the first phase is regulated by local factors within the individual gland and not by circulating hormones.[179,182]

p0245   During the first day after pup removal the luminal spaces fill with milk (Figure 31.9); by 24 h shed, apoptotic cells appear within this expanded luminal space. It has been suggested that MECs die of an apoptotic process since activation of executioner caspases (caspase 3 and 8) and fragmentation of DNA typically associated with this process are readily detected.[184–187] However, the shed "apoptotic" cells do not have the classical appearance of apoptotic cells in other organs in that they are swollen, frequently have two hypercondensed regions of chromatin, and lack the membrane blebbing classically associated with apoptotic cells.[188] Monks et al.,[181] in an elegant study that for the first time took into account the volume of the gland, found that the peak of apoptosis occurs between 2.5 and 3 days following pup withdrawal and that almost all of the apoptotic cells as
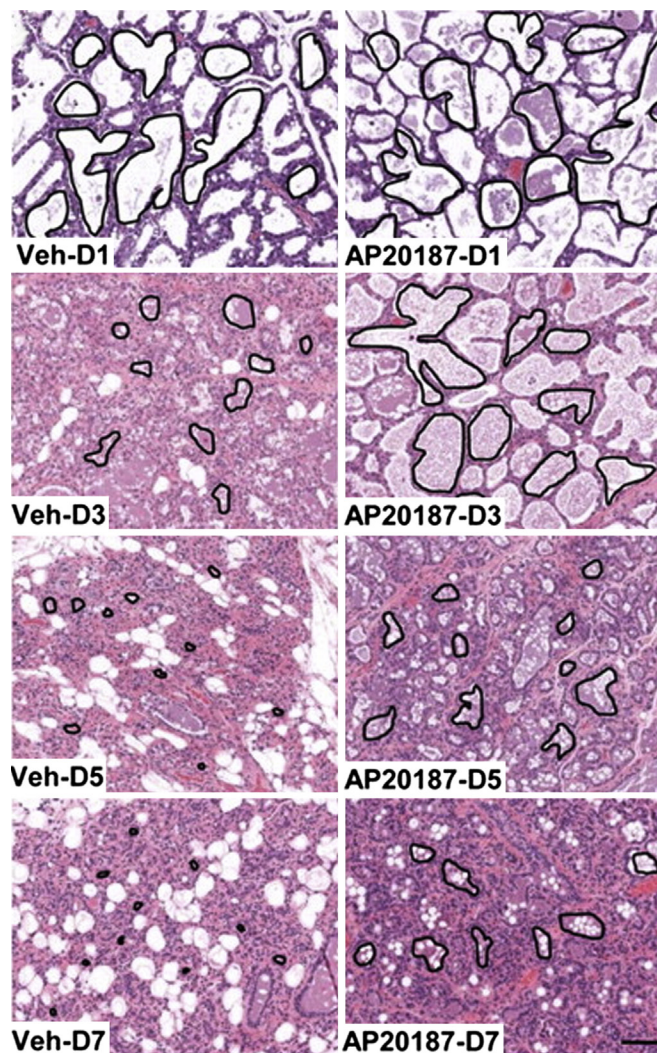
FIGURE 31.9   **The role of macrophages in mouse mammary gland involution; histological analysis.** Glands from Mafia mice were analyzed on days 1, 3, 5, and 7 after pup removal from a 10-day lactating mouse suckling five to six pups. Three days prior to pup withdrawal experimental dams were given a dose of AP20187, which depletes macrophages in this strain. Left-hand images, vehicle only (macrophages present); right-hand images, AP20187 (macrophages depleted). In both control and experimental mice marked luminal expansion is evident 1 day after removal of pups (some lumens outlined in black for illustration). In vehicle-only mice a marked decrease in lumen size by day three is observed as milk is resorbed. Very small lumens remain on day 7, however significant numbers of lipid filled adipocytes are evident between the alveoli starting on day three and increasing to day 7. These changes occur much more slowly in the glands of AP20197-treated mice, providing evidence that macrophages are required for several aspects of normal involution. *Source: Reproduced by permission from Ref. 183; link at Development: dev.biologists.org.* (For a color version of this figure, the reader is referred to the online version of this book.)

[AU1]

well as the milk are cleared in this interval. In this study MECs were thought to be responsible for the removal of apoptotic bodies because they did not detect markers of macrophages in the mammary gland during the first 3 days following pup withdrawal.[181] These authors

proposed that clearance of shed (apoptotic) epithelial cells during the first 3 days after pup removal is accomplished by the remaining resident MECs by a process they refer to as efferocytosis from the Greek for "to take to the grave" or "to bury". Their elegant images depict apoptotic cells clearly being engulfed by epithelial cells in the monolayer.

p0250 However, in a subsequent study O'Brien et al.[183] clearly showed involvement of M2 macrophages in several aspects of involution, including clearance of apoptotic epithelial cells during the first 3 days following pup withdrawal. Further, in the absence of M2 macrophages involution was dramatically delayed and the rate of milk clearance was drastically reduced (Figure 31.9). Macrophages bearing markers of M2 development were clearly present and stained with anti-F4/80 antibodies in normal mice. When mice sensitive to a treatment that depletes macrophages were used, all aspects of involution were drastically slowed, and milk was not cleared from the luminal space 3 days after pup withdrawal. The sum of the evidence leads to the conclusion that both MECs and macrophages are involved in clearance of milk and shed cells.

p0255 An alternative hypothesis was put forth by Watson and her colleagues who suggested that a nonapoptotic form of programmed cell death was activated during involution; they proposed that lysosome-mediated cell death occurs during involution.[189] This process would occur independently of caspases 3, 6, and 7; however, it requires STAT3, which upregulates the expression of cathepsin B and L. Cathepsins B and L are dramatically activated during the first 3 days of involution corresponding to the first phase of apoptosis. Current evidence suggests that activation of STAT3 alone may not be sufficient to induce programmed cell death during involution since loss of M2-differentiated macrophages prevented this type of cell death even in the face of tyrosine-phosphorylated, activated STAT3.[183] Perhaps both activated STAT3 and M2 macrophages are necessary for involution to proceed, but neither alone is sufficient to support this process. It also remains possible that other processes are involved in this cell death, including autophagy, which appears to be involved in cell death in three-dimensional culture models of the mammary epithelium.[190,191]

p0260 Numerous molecules have been implicated in the apoptotic process. Some investigators have suggested that the relative balance between pro- and anti-apoptotic members of the BCL2 family of proteins is important in regulating apoptosis and involution.[192–195] Consistent with this hypothesis, overexpression of *Bcl2* delays involution,[193] as does overexpression of an activated form of AKT1, which is capable of phosphorylating and inactivating pro-apoptotic BCL2 proteins.[196] The relation of anti-apoptotic BCL2 family members to the formation of apoptotic mammary cells is currently unclear.

Milk stasis appears to be the major trigger for involu- p0265 tion,[182] possibly involving increased mechanical stretch of alveolar epithelial cells due to expansion of the luminal space.[197] Two different groups have used global gene expression profiling to independently identify a group of genes whose expression changes during the first 12 h following pup withdrawal.[198,199] Both groups noted increased expression of genes encoding death receptors, and their ligands, proteins associated with the acute phase response, and most notably the transcription factor STAT3 and its targets.[198,199] Death receptor ligands included leukemia-inducing factor (LIF), tumor necrosis factor, tumor necrosis-like weak inducer of apoptosis, Fas Ligand, and TGFβ.[198,199] LIF activates STAT3, which in turns enhances expression of pro-death molecules; consistent with a critical role for STAT3 is the observation that cell death is delayed in STAT3-deficient mice and that the first phase of involution is extended for at least 6 days in the forced involution model in these mice.[200–202] Other critical changes include decreased expression of both AKT1 and insulin-regulated substrate-1,[196,203,204] which would eliminate the ability of AKT1 to suppress apoptosis. In addition to STAT3, the nuclear factor kappa B-signaling pathway is also likely to be an important component of the immediate signaling response to pup withdrawal.[205,206] It has also been suggested that decreased expression of the plasma membrane Ca^{++}ATPase 2, which is important in transport of Ca^{++} into milk,[207] results in an increase in intracellular Ca^{++} thereby stimulating apoptosis within the cell by a direct mechanism.[208]

Another important regulator of involution that has p0270 received extensive study is TGFβ. Although all three isoforms of TGFβ are expressed during pregnancy, expression of all three is downregulated at parturition, and only low levels of TGFβ3 are detected in alveolar cells during lactation.[209–212] Expression of TGFβ3 increases dramatically during milk stasis and involution.[198,199,211,213] Studies with transplanted MECs from TGFβ3 null mice support a paracrine role for this growth factor in mammary involution.[211] Expression of TGFβ from the whey acidic protein (WAP) promoter, which turns on in late pregnancy, inhibited alveolar development,[214] and it appeared that the stem cell pool available for alveolar morphogenesis was reduced.[215] Moses and others found that targeting a dominant-negative TGFβ Type II receptor (MMTV-DNTGRII) to the mammary gland inhibited TGFβ signaling and resulted in alveolar hyperplasia and premature secretory activation in pregnancy, followed by lactation failure.[216–218] This result is expected if TGFβ is inhibiting both alveolar proliferation and secretory activation. Expression of a constitutively active subunit of the Type I receptor leads to apoptosis in pregnancy and decreased proliferation in a transgenic mouse model.[219] More recently, in studies in which TGFβ type

II receptor was deleted from the mammary epithelium using Cre recombinase driven by the WAP promoter, the first phase of involution occurred normally, but TGFβ appeared to be required for commitment to the second phase of involution.[220] MECs lacking TGFβ-RII continued to express markers typical of a cell from a lactating mouse even 7 to 10 days after induction of involution.[220] These data all suggest a critical role for TGFβ in suppression of ductal and alveolar proliferation, suppression of PRL-induced gene programs, and programmed cell death of MECs particularly during the second phase of involution.

p0275 In contrast to the first phase of involution, commitment to the second phase of involution is dependent upon factors such as TGFβ as described earlier, and can be halted by treatment with glucocorticoid;[179] it has been speculated that glucocorticoid may act through maintenance of tight junctions.[221] The activation of matrix metalloproteases (MMPs) is critical in the remodeling of the tissue that occurs in the second phase, and although MMPs are expressed during the first phase of involution, their activation is held in check by tissue inhibitors of metalloproteases (TIMPs).[222,223] Substrates for MMPs include collagen IV, chemokine, and E-cadherin, and loss of these proteins causes detachment of epithelial cells and death by anoikis (detachment-induced cell death). The importance of MMP activation in the second phase is demonstrated by the observation that involution proceeds more rapidly in TIMP-3-deficient mice.[224] Recently it has been shown that MFG epidermal growth factor 8 (MFGE8) is essential for the second phase of involution as it binds to phosphatidylserine on the plasma membrane of apoptotic cells and enhances their phagocytosis.[225] Gene expression profiling studies by Stein et al.[199] suggest that the increase in pro-inflammatory signals during phase 1 of involution is important in the infiltration of inflammatory cells during the second phase,[199] but these signals could actually be important in attracting macrophages to the gland in the first phase.

p0280 Further details of the two phases of involution are available in numerous excellent reviews.[226–228] Future research should show how all these somewhat disparate pathways work together and also provide further understanding of the aspects of the microenvironment of the involuting mammary gland that promote mammary tumorigenesis.

s0105 CELL–CELL INTERACTIONS IN THE MAMMARY EPITHELIUM

p0285 Epithelial cells are joined to their neighbors by four types of junctional complex.[6] Starting from the apical pole of the cell these are: (1) the tight junctions (*zonula occludens*), which regulate passage of molecules through the paracellular pathway; (2) the adherens junctions (*zonula adherens*), thought to provide structural stability to the epithelium as a whole; (3) desmosomes (*macula adherens*), specialized for cell-to-cell structural adhesion; and (4) gap junctions made up of hexamers of connexin proteins that permit passage of molecules between cells. We will summarize the role of these junctions in turn as they apply to alveolar development and lactation.

Mammary Tight Junctions and Their Regulation s0110

p0290 Production of milk of a defined composition requires that the milk space be firmly isolated from the fluids of the interstitial space, such that milk composition is entirely determined by the secretory activity of the mammary epithelial cell. By injecting a marked compound that could not cross cell membranes into the milk space and determining its appearance in the blood stream, Linzell and Peaker[229,230] showed in the 1970s that this isolation is complete in the lactating goat. Specifically they measured the transfer of injected $[^{14}C]$-sucrose from the lumen into the blood stream and could detect no transfer of the tracer during lactation. When a similar tracer was injected into the blood, it did not appear in the milk. Further, potential differences of −20 to −35 mV were found between the blood and the milk in lactating goats[231] and mice.[232] All these findings provide very strong evidence that the paracellular pathway of the mammary epithelium is highly impermeable during lactation.

p0295 The same is not true of the mammary epithelium during pregnancy. $[^{14}C]$-sucrose has consistently been observed to move from the blood stream into the milk at this stage of development,[136,229,230] and no electrical potential difference can be measured between electrodes in the blood stream and milk space. Moreover, transfer of macromolecules labeled with fluorescent tracers across the epithelium, either from the lumen to the interstitial space or in the opposite direction,[136,233] has been observed. These experiments, illustrated in Figure 31.10, show that during pregnancy molecules as large as albumin and IgA permeate the junctional complexes;[111] such molecules do not pass through the paracellular pathway in the lactating gland. When such large plasma proteins are present in milk, they must be transferred across the epithelium of the lactating gland by transcytosis as described in the section Transcytosis (Pathway III). Taken together these findings from isotope tracer, blood-milk potential, and fluorescent tracer experiments indicate that the mammary tight junctions, particularly in the alveoli, are leaky during pregnancy and close around parturition to form a tight barrier that prevents paracellular movement of molecules across the mammary epithelium.

p0300 This transition has a profound effect on the composition of the mammary secretion. During pregnancy, the
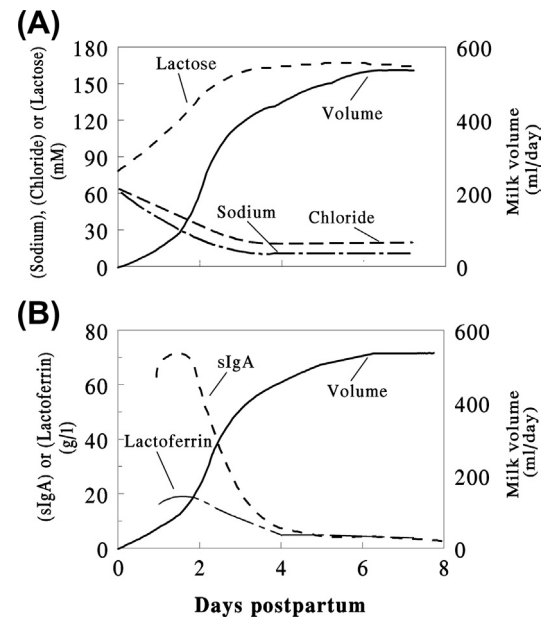
Pregnant   **Lactating**
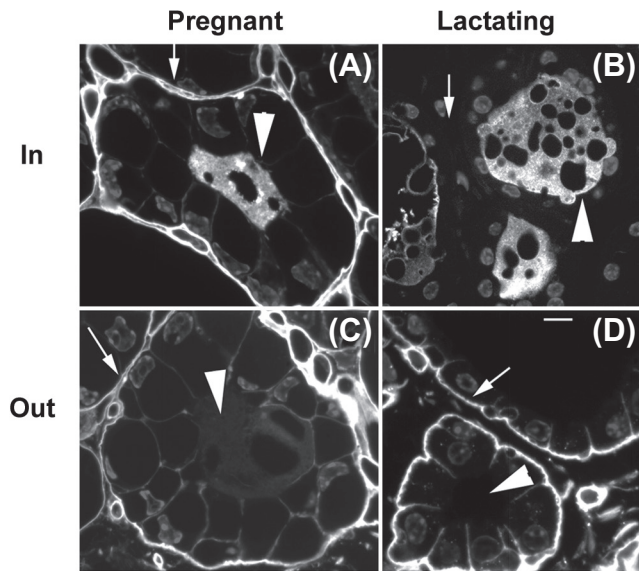
f0055

FIGURE 31.10 Paracellular permeability to FITC-albumin during pregnancy and lactation. (A, C) Pregnant mice. (B, D) Lactating mice. (A, B) FITC-albumin was injected intraductally (designated "In") and fixed within 5 min. The excised glands were embedded, sectioned, and visualized with the fluorescent microscope. During pregnancy, tracer can be seen in both lumen (arrowhead) and interstitial space (arrows); in lactation it is confined to the lumen. (C, D) The in situ gland was incubated with tracer for 1 h to expose the basolateral surface of the alveoli (designated "Out") and visualized as in (A) and (B). In pregnancy, tracer can be seen throughout the interstitial space and in the lumen; during lactation tracer is confined to the interstitial space extending just up to the tight junctions. Magnification bar (D) is 10 μm and applies to all panels. *Source: Reproduced from Ref. 136.*

FIGURE 31.11   Changes in human milk composition during the colostrum-forming stage. (A) Na^+ and Cl^- concentrations fall rapidly and lactose increases as the tight junctions between the epithelial cells close. Note that these changes are well underway prior to the increase in milk volume secretion beginning on day 2. (B) Secretory IgA (sIgA) and lactoferrin are found at very high concentrations in the mammary secretion during the first three days postpartum, the period when colostrum formation is at its peak. *Source: Reproduced with permission from Ref. 160.*

f0060

milk has high Na^+ and Cl^- and low K^+ as the result of ions moving through the paracellular space down their electrochemical gradients. The low Na^+ and Cl^- and high K^+ of true milk is produced by the activity of ion pumps in the basolateral and apical membranes of the cells. The transition between the two states is well illustrated for human milk in Figure 31.11. Thus Na^+ and Cl^- fall and lactose rises (Figure 31.11(A)) as the tight junctions close prior to the increase in milk volume illustrated in both panels of Figure 31.11. Freeze fracture studies carried out by Pitelka[6] showed that the tight junction strands in the gland during pregnancy are highly disordered, suggesting that molecules permeate between the strands. In lactation the strands are highly ordered and show no breaks that might allow passage of large or small molecules.

s0115 *Role of Claudins, Occludin and Tricellulin in Mammary Tight Junctions*

p0305   Although claudins in low resistance epithelia permit the passage of monovalent ions,[234,235] the high transepithelial potential maintained in the lactating mammary gland despite substantial transcellular permeability to these ions suggests that the complement of claudins prevents paracellular movement of both large and small molecules. Our unpublished studies show a large increase in claudin 8 gene expression at parturition in the mouse mammary gland from both microarray and real time PCR analysis (Rudolph MC and Neville MC, unpublished). This claudin does not permit ion flux between lumen and interstitial space in other epithelia. Recently claudin 3 was localized to the apical junctions in the lactating mammary gland,[236] and the authors suggested that it is responsible for the low permeability of the mammary epithelium.

p0310   It is worth mentioning that claudins are also present in large numbers of small vesicles that appear never to interact with junctional complexes in the cell. Claudin 7 has been carefully studied in this regard,[83] but claudins 3 and 4 are also found in such vesicles in both normal and tumorous mammary cells in locations that suggest they are not merely serving as reservoirs for tight junction proteins (Baumgartner Wilson, personal communication). The function of these very common nonjunctional claudins is currently unknown, but they may play a role in interactions with the extracellular matrix.[237] Occludin is also present at mammary tight junctions and is considered to play a role in epithelial permeability; recent studies also implicate occludin as a signaling molecule that helps initiate apoptosis when tight junction integrity is compromised.[238]

p0315 The structure of the tight junctions at positions where three cells come together in the formation of the epithelial sheet appears to differ from that of the bicellular junctional complexes. Here a novel tetraspanin protein, tricellulin, is concentrated at the vertically oriented TJ strands of tricellular contacts. RNA interference studies of tricellulin depletion suggest that this protein is necessary for complete sealing of the epithelial barrier.[239] Recently another protein has been identified as a component of the tricellular junction, the lipolysis-stimulated lipoprotein receptor. This immunoglobulin receptor-like protein was actually found to recruit tricellulin to tricellular junction and again appeared necessary for full tight junction sealing.[240] In the liver it is involved in lipoprotein metabolism. Whether it serves this function in the mammary gland remains for future research.

s0120 **Hormone Dependence of the Change in Tight Junction Permeability between Pregnancy and Lactation**

p0320 When mice were ovariectomized on day 17 of pregnancy, the subsequent decrease in circulating P4 set the transition between pregnancy and lactation in motion, and the sucrose permeability of the epithelium progressively decreased over the 20h subsequent to ovariectomy[136]; the effect could be delayed by injection of P4 at intervals. Injection of a P4 antagonist, RU486, produced the same effect as ovariectomy. These experiments firmly establish that P4 withdrawal is the trigger for tight junction closure. A series of endocrine ablation experiments showed that both PRL and glucocorticoid are necessary for mammary tight junction closure[241]; these molecules also alter tight junction permeability in tissue culture models.[242] These are the hormones that promote secretory activation in the mammary gland, providing evidence that tight junction closure is closely linked to secretory activation. Although insulin also promotes milk secretion in mice,[75,76] its role in tight junction closure is unknown.

s0125 **Epithelial Permeability during Mastitis and Involution**

p0325 The direct passage of interstitial molecules such as Na^+ and Cl^- across the mammary epithelium during pregnancy leads to the higher concentration of these ions in colostrum and the appearance of milk components such as lactose and α-lactalbumin in the blood stream as illustrated by the schematic in Figure 31.12. As discussed earlier, such changes in milk composition have been fully documented during the onset of lactation but have also been used as an indicator of tight junction status under conditions where direct tracer measurement of the permeability of the mammary epithelium is not possible such as during involution[243–245] and with infections such as mastitis.[246,247] Stelwagen and colleagues showed that increased milk components such as lactose and α-lactalbumin were found in the blood stream if goats and cows were left unmilked for 18h.[245,248] The authors interpreted these results as showing an effect of milk stasis on tight junction permeability. However, in none of these studies were tracers injected into the gland or blood stream to distinguish between opening of tight junctions and aberrant basally directed exocytosis in the stretched mammary epithelium. Thus it is not clear whether the changes in ion concentrations in the milk result from a return of the tight junction strands to the disordered condition of pregnancy, a loss of integrity of the epithelium as epithelial cells are lost by apoptosis, or
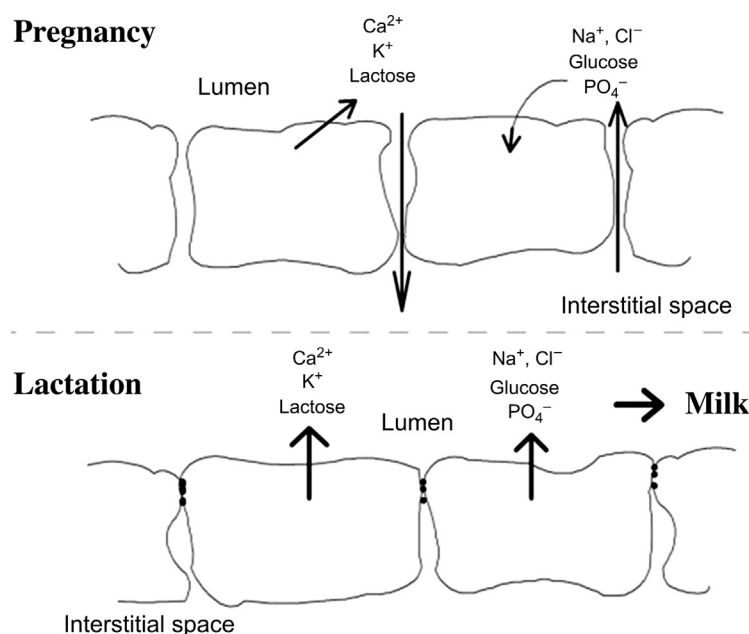
FIGURE 31.12 **The paracellular pathway is open in pregnancy and closed in lactation.** The schematic illustrates the net flux of several small molecules during pregnancy when the junctional complexes are very leaky and in lactation when they are tightly closed. As shown in Figure 31.10 large molecules such as albumin and α-lactalbumin are able to pass through the junctions during pregnancy. *Source: From Neville MC. Lactogenesis in women: evidence for a cascade of cellular events. In: Jensen RG, editor.* Handbook of composition of milks. *1st ed. San Diego: Academic Press; 1995. p. 87–98. Used with permission from Elsevier.*

activation of purinergic receptors in the mammary epithelium by mechanical or other disruptive stimulation.

p0330   Interestingly, it has been shown that cultured MECs release ATP, UTP, and UDP when stressed; these nucleotides interact with purinergic receptors to bring about an increase in cell Ca^{++} and activate Ca^{++}-sensitive apical Cl^- transporters[249] as well as alter Na^+ and K^+ transport.[250] It is possible that closure of junctions at the initiation of lactation is an irreversible process and that changes in both directional secretion of ions and milk components across the mammary epithelium may be responsible for alterations both in milk composition and the appearance of milk components in the blood when the gland is stressed by milk stasis or infection. The matter clearly requires additional investigation.

s0130 *Regulation of Tight Junction Formation*

p0335   Biochemically, tight junctions are complex structures, with claudins, occludin, and the junctional adhesion complex interacting across the intercellular space to form a tight apical band that restricts permeability through this space.[251] Intracellularly these molecules interact with scaffolding molecules immediately below the membrane called ZO-1, ZO-2, and ZO-3, which in turn interact with actin as well as members of the polarity-regulating complex PAR6/PAR3/aPKC.[252] A variety of regulatory molecules including PKCzeta[253] and the molecular complex CRUMBS3/PALS1/PATJ[252] have been shown to interact with the PAR complex and alter tight junction permeability.[254] A complete listing of tight junction proteins with their functional context is available,[255] with the suggestion that many of them may have a regulatory function in mammary development. Recently it has been shown that cytoplasmic polyadenylation element binding protein is necessary for claudin 3 and ZO-1 localization to both MECs in the mouse and in cell culture models of mammary acini.[256] Horseman and his group have implicated serotonergic signaling in tight junction integrity,[165] and work by Fischer et al. implicates the RHO pathway as well.[242] Occludin, a component of the tight junction, also plays a role in initiating apoptosis of MECs when tight junctions are perturbed.[238] At this writing a comprehensive picture of the interactions of all these signaling pathways in establishing and maintaining tight junction integrity in the lactating mammary gland is not yet available.

s0135 Adherens Junctions in the Mammary Epithelium

p0340   Adherens junctions encircle epithelial cells, usually basal to the tight junctions, and provide both cell–cell adhesion and linkage to the cytoskeleton through a group of molecules, the catenins. A transmembrane molecule, E-cadherin that interacts with the catenins, forms a Ca^{++}-dependent bridge from one cell to its neighbor, stabilizing cell–cell interactions. Disruption of these linkages by infusion of EGTA intraductally into the lactating mammary gland both decreases milk production and leads to apoptosis of MECs.[257] E-cadherin is essential for the self-organization of epithelial monolayers in culture models of the mammary epithelium[258] and was found to be necessary for establishment of the lactating phenotype in a Cre/loxP model directed to the mammary epithelium.[259] One of the catenin molecules, β-catenin, acts not only to stabilize the adherens junction but it is also a transcription factor, acting through the TCF/LEF pathway to regulate transcription and tumorigenesis by mechanisms well reviewed by Carraway and colleagues.[260]

p0345   Beta-catenin also functions as a transcriptional regulator in the WNT-signaling pathway;[261] its normal function was found to be essential for lobular-alveolar development.[261] Overexpression of the cytoplasmic domain of E-cadherin promoted precocious mammary development in mice but interfered with development of polarity in the fully differentiated gland.[262] Because loss of cadherin is considered to be a hallmark of the epithelial-to-mesenchymal transition that occurs during tumorigenesis,[263] much of the work on mammary adherens junctions is part of the breast cancer literature, which will not be reviewed here.

Desmosomes   s0140

p0350   Desmosomes are localized spot adhesions on the lateral sides of cells that use desmoglein and desmocolin to bind across the intercellular space possibly to prevent shearing forces from disrupting the epithelial monolayer. These structures have been found to be essential for branching morphogenesis of the mammary gland[264] and for formation of acinar cultures from purified MECs.[265] However, Pitelka and her colleagues showed almost 40 years ago that as the alveoli become secretory the desmosomes disappear,[6] likely to allow the profound shape changes in the epithelial cells as the lumens expand and contract with the accumulation and ejection of milk.

Gap Junctions in the Mammary Epithelium   s0145

p0355   Direct cell-to-cell signaling is mediated in part by the passage of molecules with molecular weights less than 1000 kDa through gap junctions. These junctions are formed from six connexin molecules that aggregate to form a hemichannel or connexon in each cell; when these hemichannels in the opposing membranes of two cells are aligned, they form a channel with a

pore that allows intercellular passage of signaling molecules, metabolites, vitamins, and other substances up to 1.5 kDa,[266] but not macromolecules. Freeze fracture studies showed that gap junctions between epithelial cells in the mammary gland are composed of an aggregate of many connexons.[267] Studies using lucifer yellow dye showed extensive coupling between cells of the lactating mammary alveolus as well as suggested dye transfer between alveolar and myoepithelial cells.[232]

p0360 Four connexins have been shown to be expressed in rodent mammary glands.[268] The mRNAs for connexin 26 (*Cx26*) and connexin 32 (*Cx32*) are expressed at all stages of mammary development[268,269] but are markedly upregulated in pregnancy (*Cx26*[269–271]) and lactation (*Cx26* and *Cx32*[271,272]). The corresponding proteins are found in the junctional plaques where they form both homomeric and heteromeric channels.[273] Connexin 30 (CX30) was expressed after day 15 of pregnancy and peaked at the onset of lactation, disappearing thereafter.[268,273] Gusterson and his colleagues speculate that the heteromeric CX26/CX30 channels specify cell–cell communication at late pregnancy and are replaced by heteromeric CX26/CX32 channels with differing specificity during lactation.[273] Connexin 43 (CX43) is the only isoform that has been positively identified in the myoepithelium.[274] It switches to a hypophosphorylated form during lactation, a change that may by specified by a loss of WNT-5A signaling.[275] Serra and her colleagues found that overexpression of WNT-5A in the mammary epithelium altered CX43 phosphorylation and led to impaired lactation with no change in any other junctional complex proteins. Based on this and other evidence, the authors suggested that CX43 promotes communication between myoepithelial cells essential for milk ejection.[275]

p0365 Talhouk and his colleagues have presented convincing evidence that gap junction formation is essential to the differentiation of MECs.[268] Although part of this effect may be coordination of epithelial cell activities between members of the epithelial layer including both basal and luminal cells, there is considerable evidence that gap junction proteins interact with regulatory proteins more generally thought to be associated with the tight and adherens junctions. As shown in Figure 31.13, the proteins for which there is current evidence are α-catenin, β-catenin, and ZO-2.[266] The authors propose that as they form, maturing gap junctions recruit β-catenin away from the nucleus, promoting a switch from proliferation to differentiation. Since gap junctions are lost in breast cancer cells, it is also possible that functional gap junctions help prevent transformation of MECs into tumor precursors.[266] This is a story worth following into the future.
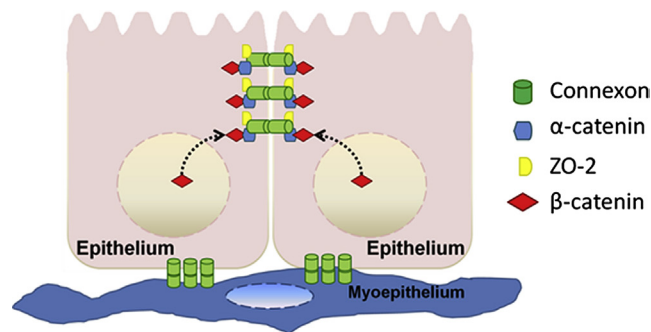
f0070
FIGURE 31.13 **Gap junction localization in the differentiated mammary epithelium.** Connexons interact as shown across the interstitial space between luminal epithelial cells as well as between luminal cells and myoepithelial cells. Luminal cell connexons also interact with signaling molecules α-catenin, β-catenin, and ZO2. As differentiation progresses, β-catenin may be recruited away from the nucleus to diminish the stimuli for proliferation. *Source: Reproduced with permission from Ref. 266.* (For a color version of this figure, the reader is referred to the online version of this book.) [AU2]

MILK COMPOSITION AND ITS REGULATION

s0150

p0370 Milk is qualitatively and quantitatively complex, containing proteins (mostly caseins), lipids (triglycerides), sugars (including oligosaccharides), vitamins, minerals, and growth factors, in addition to water.[169–171,276] The relative amounts of these substances vary significantly among species,[169,171] and the composition of milk can be influenced by the stage of lactation[28] and the mother's nutritional status.[277,278] Such variability implies that the mechanisms responsible for synthesis and/or secretion of milk substances are genetically determined and physiologically regulated. In this section we begin by summarizing the major milk components followed by a discussion of the major pathways for secretion of milk components and their regulation.

Major Milk Components

s0155

p0375 Genome-wide comparisons of mammalian taxa, including humans,[279] have led to greater understanding of how milk secretion evolved in mammals. These data suggest that the mechanisms of milk secretion developed over 160 million years ago and are highly conserved. Nevertheless, significant interspecies variability in genes encoding milk proteins as well as proteins involved in milk protein production have been detected. Such findings suggest that species variation in milk composition is likely to reflect differences in gene copy number and/or transcriptional or posttranscriptional mechanisms rather than major gene sequence differences.

s0160 *Proteins*

p0380   The protein content of human milk, initially about 3% (wt/vol), decreases to about 1.5% by the second week of lactation.[28] Caseins, which represent about 80% of total milk proteins,[280] form insoluble micelles containing high concentrations of calcium and phosphate. Other milk proteins are found in the soluble (whey) fraction, or are associated with the membrane that surrounds fat globules. A total of 285 distinct gene products have been identified in human milk; by comparing proteome data sets from human and bovine milk, a core of 106 conserved proteins has been identified.[280] Gene ontology analysis suggests that core mammary cell proteins fall into four general functional categories: cell proliferation, lipid metabolism, nutrient transport, and immune function.[280] Proteins enter milk through four distinct pathways as discussed in the section Milk Secretion Pathways, below.

s0165 *Lactose and Oligosaccharides*

p0385   Human milk is enriched in lactose (a disaccharide unique to milk) and oligosaccharides, relative to milk from most other species.[279,281,282] Total oligosaccharide concentration in human milk is higher in the colostral phase of lactation compared to that of mature milk. More than 200 different free oligosaccharides have been identified in human milk,[283] suggesting the presence of diverse mechanisms for oligosaccharide biosynthesis within human MECs. Analysis of oligosaccharides in the milk of other primates further suggests that primate milk oligosaccharides are generally more complex, and exhibit greater diversity, than those found in nonprimate milk.[284]

s0170 *Lipids*

p0390   Lipids in milk are primarily triglycerides (>98%),[285] which due to their high energy content provide the majority of the calories required for neonatal growth in most species.[286] Milk lipids also serve as a primary source of essential fatty acids needed for neonatal membrane synthesis, as substrates for synthesis of eicosanoids and other bioactive lipid signaling molecules, and they provide a mechanism for transfer of fat-soluble vitamins to infants.[276] The lipid content of milk is variable, with content differing among species and influenced by lactation stage.[287] In humans, the amount of lipid in mature milk is greater than that in colostrum, and it is positively affected by the degree of breast emptying.[288] It is thought that the total lipid content of milk is not significantly affected by diet for adequately nourished mothers.[77] However, more recent evidence from laboratory animals indicating that diet may influence milk consumption, and that maternal obesity may reduce milk lipid levels,[278] raises questions about possible negative influences of maternal overnutrition on human milk lipid content and offspring obesity risk.

Milk Secretion Pathways   s0175

p0395   Five general pathways are responsible for secreting the majority of milk products (Figure 31.14). Proteins, oligosaccharides, and some small molecules synthesized by MECs, as well as water are secreted by exocytosis (pathway I). Lipids, lipid-associated proteins, and membrane proteins are secreted by a unique membrane-envelopment process (pathway II). Externally derived macromolecular substances, including albumin, immunoglobulins, growth factors, cytokines, lipoproteins, and micronutrients enter milk by two pathways: transcytosis
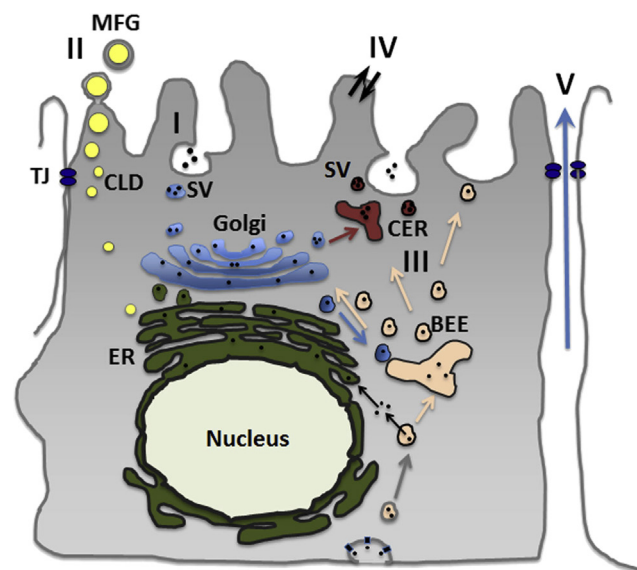
FIGURE 31.14   **Cellular pathways for the secretion of milk.** Five distinct pathways are responsible for the secretion of milk components. Major milk proteins, such as casein, and oligosaccharides, lactose, and water are packaged for secretion by exocytosis of secretory vesicles (pathway I) by processes originating in the Golgi complex. Lipids are synthesized and packaged into cytoplasmic lipid droplets (CLD) by enzymes in the endoplasmic reticulum. CLD are transported to the apical plasma membrane, where they are secreted by an apocrine process (pathway II) forming membrane-enveloped structures called milk fat globules (MFG). Immunoglobulins, and other macromolecules from the maternal circulation, are transported into milk by the transcytosis pathway (pathway III). In this pathway, substances taken up by either clathrin-dependent or clathrin-independent endocytosis at the basal plasma membrane initially enter into a basolateral early endosome (BEE) compartment where they are sorted to the trans-Golgi network for packaging into the secretory vesicles or to a common endosome recycling compartment (CER) for further sorting to apical or basolateral membranes. Direct movement of monovalent ions, water, and glucose across the apical and basal membranes of the cell occurs via membrane transporters (pathway IV). A paracellular pathway between epithelial cells, open during pregnancy, allows flux of plasma components into milk (pathway V). Tight junctions (TJ) close at the onset of lactation. *Source: From Monks J, Manaman JL. Secretion and fluid transport mechanisms in the mammary gland. In: Zibadi S, Watson RR, Preedy VR, editors.* Handbook of dietary and nutritional aspects of human breast milk. *Wageningen Academic Publishers, in press. Used with permission of J.L. McManaman.* (For a color version of this figure, the reader is referred to the online version of this book.)

(pathway III), which involves elements of endocytic recycling and exocytotic pathways; and paracellular transport between cells (pathway V) prior to tight junction closure. Ions and small molecules, such as glucose and amino acids, are transported into milk by specific membrane transport pathways (pathway IV). Each of these pathways is affected by the functional state of the mammary gland, and is directly or indirectly regulated by actions of hormones and growth factors. Information exists about the general features of these pathways, however, few details are available about their mechanisms, or how their activities are regulated. The properties and regulation of pathway V are discussed in the section Cell–Cell Interactions in the Mammary Epithelium.

s0180 *Exocytotosis (Pathway I)*

p0400 During the secretory activation phase of mammary development, there is significant expansion of the rough endoplasmic reticulum and the Golgi complex, the organelles responsible for synthesis and packaging of proteins, lactose, and oligosaccharides into secretory vesicles for secretion into milk.[289–291] In species where it has been studied, the Golgi complex accounts for between 5% and 15%, and the rough endoplasmic reticulum accounts for approximately 25%, of the total volume of milk secreting cells at mid-lactation.[292] In addition to protein and oligosaccharide cargo, the Golgi complex packages nutrients, such as lactose and citrate, into secretory vesicles. The basic mechanisms by which secretory vesicle cargo is released during exocytosis were established over 40 years ago in studies of the pancreas.[293] The same mechanisms are thought to apply to essentially all exocrine cells, including those that secrete milk.

p0405 The Golgi complex is composed of seven cisternae that are classified as cis-, medial-, or trans-compartments based on their structural and functional characteristics.[294] Newly synthesized proteins are transported from the endoplasmic reticulum to the Golgi in vesicles that dock at the cis-compartment, where they are incorporated into new cisternae. The protein cargo remains within the cisternal lumen of the Golgi as cisternae progress to trans-portions of the complex.[295] Here they are sorted into transport vesicles and exit the Golgi complex. High-resolution 3D-electron micrograph tomography, in combination with rapid freezing procedures that stop cellular processes without disrupting cellular structural integrity,[296–299] has demonstrated that the trans-Golgi compartment is structurally and functionally distinct from other compartments. Vesicles destined for the endosomes or lysosomes are derived exclusively from the trans-most cisternae,[294,298] whereas vesicles destined for the apical and basolateral regions of the plasma membrane are derived from preceding cisternae.[294] Tomographic 3D reconstructions of Golgi in actively secreting cells in tissue culture[296,300] also demonstrate that direct

tubule connections can form between cisternae. Such connections may facilitate cargo transfer between individual cisternal elements of the Golgi and promote secretory vesicle formation. Similar structural changes, if they occur among Golgi cisternae in milk-secreting cells, provide a mechanism by which the rate of secretion adapts to changing lactational demands of nursing young.

p0410 Exocytotic secretion requires fusion of secretory vesicle membranes with the plasma membrane to achieve cargo release. Fusion occurs at specialized sites on the plasma membrane called porosomes[301] and depends on the formation of a fusion complex between *N*-ethylmaleimide (NEM)-sensitive fusion protein (NSF), soluble NSF-attachment proteins (SNAPs), and specific soluble NSF-attachment protein receptors (SNAREs) that are found on vesicle membranes and the plasma membrane.[302] Interactions among these proteins lead to fusion of vesicles with the plasma membrane, resulting in the release of the vesicle contents into the luminal space by an ATP-dependent process.[303] This general mechanism of exocytosis appears to be conserved among cell types,[304] and striking similarities exist in protein composition of the machinery that mediates regulated- and constitutive-exocytotic processes.[304] Members of the fusion complex are expressed in mammary glands of lactating mice,[305] and proteomic analysis of isolated Golgi from MECs has demonstrated that lactation leads to the upregulation of exocytotic machinery and vesicle trafficking proteins.[306] Localization studies have implicated SNAP-23, Syntaxin-2, and VAMP (vesicle associated membrane protein)-8 as possible mediators of casein secretion. However, the specific functional importance of these proteins to milk secretion processes has not been validated by gain or loss of function studies.

PROTEINS   s0185

p0415 Proteins are secreted by both constitutive and regulated pathways. The primary function of the constitutive pathway is the delivery of new membrane proteins, however in some cases it functions to deliver proteins for secretion.[307] Conversely, the regulated pathway specifically targets proteins to the plasma membrane for secretion in response to physiological signals. This pathway is the primary mechanism by which proteins are secreted in the pancreas, salivary, and adrenal glands. Elements of regulated secretory pathway have been identified by proteomic analysis of Golgi preparations from lactating rat mammary glands,[306] and evidence of regulated secretion of casein has been detected in isolated milk secreting cells.[308] However, this mechanism appears to account for only a small percentage of total casein secretion.

LACTOSE AND OLIGOSACCHARIDES   s0190

p0420 Lactose is synthesized within the Golgi complex by the transfer of galactose from UDP-galactose to glucose

in a reaction that is catalyzed by a complex between β-4-galactosyltransferase-1 and alpha-lactalbumin.[309] Biochemical studies showing that lactose is enriched in preparations of casein-containing secretory vesicles from mammary glands of lactating rats suggest that it may be secreted along with milk proteins.[310] Milk oligosaccharides are synthesized by addition of glycosyl groups, including *N*-acetylglucosamine, fucose, and *N*-acetylneuraminic acid to the galactose residue of lactose. This basic mechanism accounts for the synthesis of both linear and branched-chain oligosaccharides.[311] Relatively few details are known about the regulation of milk oligosaccharide synthesis, what determines oligosaccharide structural diversity, or how oligosaccharides are packaged into secretory vesicles for secretion during lactation. The various glycosyltransferase reactions responsible for generating specific oligosaccharide structures localize to different Golgi compartments,[312,313] suggesting the existence of specific mechanisms for detecting and regulating oligosaccharide abundance, as well as for transporting oligosaccharides within the Golgi to achieve specific structural characteristics. Specific domains within the C-terminal regions of glycosyltransferases control their localization within Golgi cisternae,[314] however, the mechanisms controlling glycosyltransferase targeting to a particular Golgi compartment have not been identified.

CALCIUM, PHOSPHATE AND CITRATE

s0195

p0425   Milk is rich in calcium, $PO_4^=$, and citrate, which exist in a variety of chemical forms including free ions, as $Ca^{++} PO_4^=$ or Ca^{++}citrate complexes, or bound to casein for Ca^{++} and $PO_4^=$ ions. Citrate is synthesized de novo by MECs, whereas milk Ca^{++} and $PO_4^=$ are derived from the maternal circulation. Biochemical, physiological, and kinetic evidence indicates that all three molecules are secreted into milk by exocytosis of Golgi-derived secretory vesicles.[315] Golgi membranes contain Ca^{++} pumps and citrate transporter activities that presumably mediate the transport of these substances into Golgi-derived vesicles.[316] Although it is likely that $PO_4^=$ enters the Golgi system by transport, in some species Golgi $PO_4^=$ may also originate by UDP hydrolysis during lactose synthesis. Although some of the Ca^{++} in milk must be transported from the exocytotic pathway, clear evidence for a powerful apical membrane transporter, PCMA2, that transports the majority of Ca^{++} in rodent and bovine milk, has been obtained from several laboratories.[207,317,318]

Lipid Secretion (Pathway II)

s0200

p0430   The mechanism of milk lipid secretion is distinct from the exocytic pathway used to secrete proteins, sugars, and water into milk and from exocytic pathways used by hepatocytes or enterocytes to secrete lipids into the circulatory system.[319] Milk lipids originate from CLD,[320–322] which are secreted into milk as membrane-bilayer coated structures, known as MFG, through an apocrine mechanism.[322,323] Proteomic analyses,[324] which documented that the overall protein composition of CLD isolated from milk secreting cells is similar to that of the secreted MLG, suggest that CLD are secreted into milk in toto, without significant modification of their compositions. Such studies also demonstrated that the protein signature of CLD isolated from milk-secreting cells is distinct from that of CLD isolated from hepatocytes, which are not secreted, suggesting that protein composition of mammary CLD may be specialized for transport and secretion. The reason why milk-secreting cells have evolved a specialized mechanism(s) of lipid secretion that bypasses the endoplasmic reticulum and Golgi packaging machinery used in the secretion of other substances is unclear. However, the presence of a "direct lipid secretion" pathway may provide means of delivering large amounts of lipid to neonates during critical growth periods.[319] In addition, the membrane surrounding MFG protects milk lipids from emulsification and may possess additional biological functions.[325–327]

p0435   Based on ultrastructural evidence showing CLD contacting and being surrounded by the apical plasma membrane,[320] it has been proposed that CLD are secreted by a "membrane envelopment" mechanism.[322] Although most evidence favors this mechanism,[322,328] an alternative mechanism involving interactions between secretory vesicles, CLD, and the plasma membrane has been proposed.[329] In support of this alternative pathway, the SNARE protein SNAP-23, which has been implicated in the secretion of casein vesicles, has also been detected on CLD by immunofluorescence microscopy.[305] The possibility that CLD may be secreted by more than one mechanism is further suggested by observations that lipid-containing structures with a diverse range of sizes and lipid compositions have been detected in milk.[330]

p0440   Details about the mechanisms by which CLD form contacts with the apical plasma are limited. However, CLD are known to accumulate near the apical plasma during lactation,[289] and ultrastructural analyses have shown that they are separated from the plasma membrane by a 10–20-nm-thick layer of electron dense material.[290] Material of similar appearance has been shown to separate the surface of the lipid droplet from the surrounding membrane in secreted MFG.[290,331] Proteomic analyses of human, bovine, caprine, and mouse MFG membranes have shown that they are uniquely enriched in three proteins: the apical plasma membrane protein butyrophilin (BTN); the cytoplasmic enzyme, xanthine oxidoreductase (XOR); and the CLD-associated protein, adipophilin (ADPH/ADRP/perilipin 2).[332] Biochemical and immunocytochemical analyses indicate that these proteins exist as a stable complex in MFG,[333] and loss of function studies suggest that each protein contributes to normal milk lipid secretion.[334–337]

p0445 How BTN, XOR, and ADPH interact to mediate lipid secretion is not well understood at the molecular level, but interactions between these proteins have been documented and critical functional domains are beginning to be identified. ADPH on the CLD surface is proposed to form a complex with BTN and XOR that allows CLD to dock at the apical plasma membrane.[332] This mechanism is supported by experiments showing that ADPH exists as a complex with BTN and XOR on MFG membranes and co-localizes with these proteins on the apical plasma membrane at sites of CLD secretion.[107] Binding studies have further shown that XOR binds tightly to a cytoplasmically oriented domain, B30.2, of mouse BTN.[338,339] The C-terminal region of ADPH, which forms a four-helix bundle structure,[337] has also been implicated in CLD secretion by experiments in which mutant ADPH lacking the four-helix bundle domain was shown to disrupt CLD secretion in lactating mice.[337] Four-helix bundle motifs bind to phospholipid membranes and have been implicated in the induction of membrane curvature[340,341] and the recruitment of other proteins to the curvature site.[341] It is an intriguing possibility that interactions between the C-terminal four-helix bundle domain of ADPH and the apical plasma membrane of milk-secreting cells may initiate changes that lead to BTN and XOR recruitment to CLD contact sites and ultimately to the envelopment and secretion of CLD.[342]

s0205 *Transcytosis (Pathway III)*

p0450 Transcytosis provides a pathway for proteins and other macromolecules from the maternal circulation to be transported into milk. Substances transported by this pathway are taken up either by clathrin-dependent or clathrin-independent endocytosis at the basal plasma membrane of milk secreting cells and enter into a basolateral early endosome (BEE) compartment. Substances within this compartment are sorted into a rapid recycling pathway, to late endosomes/lysosomes, to the trans-Golgi network, or to a common endosome recycling (CER) compartment.[343,344] There is further sorting of substances within the CER to the apical membrane or back to the basolateral membrane.[344] Thus, transcytosis involves a series of complex sorting events that may or may not intersect with the exocytotic pathway used for secreting endogenously synthesized proteins, lactose, and oligosaccharides. The extent to which the exocytotic and transcytosis pathways intersect in the secretion of milk substances derived by endocytosis is unknown. However the demonstration that concanavalin A-ferritin complexes taken by endocytosis were found in secretory vesicles containing casein[345] provides experimental evidence of endocytic and exocytotic pathway intersection in the milk secreting cells of lactating animals.

IMMUNOGLOBULINS s0210

p0455 Of the substances transported into milk by transcytosis, immunoglobulin A transport (IgA) is the best understood. Immunoglobulin A exists as a dimer (dIgA) and is by far the predominant immunoglobulin class in human milk.[346] At the onset of lactation, plasma cells home to the mammary gland where they lodge in the interstitial spaces, producing much of the IgA secreted into milk. Dimeric IgA synthesized by plasma cells or elsewhere in the body binds to polymeric immunoglobulin receptors (pIgR) on the basolateral surface of milk-secreting cells.[347] The entire IgA–pIgR complex is internalized by clathrin-dependent endocytosis and transferred to the apical membrane. In other cell types, dIgA–pIgR transcytosis has been shown to involve the BEE and CER compartments and an apical recycling endosome compartment.[348] Fusion of endocytic vesicles with the apical plasma membrane delivers the dIgA–pIgR complex to the apical plasma membrane, where the receptor undergoes proteolytic cleavage at arginine 585 to release dIgA bound to a portion of the pIgR extracellular domain called secretory component.[347] The mechanism of fusion of dIgA–pIgR vesicles with the plasma membrane in kidney cells involves *N*-ethylmaleimide sensitive factor (NSF) and components of the SNARE machinery.[350,351] Presumably a similar mechanism operates in milk-secreting cells, but the process has not been studied. Transgenic pIgR expression in mouse mammary glands demonstrated that pIgR levels limit dIgA transport into milk.[351] Dimerization of pIgR, induced by dIgA binding, stimulates the transcytosis of the complex in cultured immune cells,[352] possibly by activating PKC/PI3 kinase signaling pathways.[344] It is unknown if similar processes regulate dIgA transfer into milk.

SERUM ALBUMIN s0215

p0460 In addition to transporting dIgA, elements of the dIgA–pIgR pathway also appear to be responsible for transcytosis of serum albumin into milk.[233] Fluorescently labeled serum albumin is found in close proximity to dIgA on the basolateral membrane of milk-secreting cells of lactating mice, and it co-localizes with dIgA in endosomal vesicles within these cells.[233] However, the identity of the vesicular compartment containing dIgA and serum albumin was not defined, and it remains unclear if dIgA and serum albumin undergo co-transcytosis at each step of the pathway. Nevertheless, co-localization analyses of serum albumin and casein demonstrated that the albumin transcytosis pathway does not intersect with the post-Golgi compartment mediating milk protein secretion.[233] Receptor mediated uptake has been implicated in the transport of albumin across various epithelial barriers. The presence of approximately 30 times more serum albumin than dIgA in mouse milk suggests the existence of mechanisms for concentrating albumin, or the presence of albumin receptors, for

transport into milk that differ from those for dIgA. In contrast to mice, where the serum albumin concentration in milk is approximately equal to that in serum,[233] the serum albumin concentration in human milk is significantly lower than that in serum.[353] Thus there appear to be species differences in aspects of serum albumin transcytosis into milk.

s0220 **TRACE ELEMENTS**

p0465   Transport of trace elements, such as iron, copper, and zinc, combines elements of transcytotic and exocytic pathways. Distinct mechanisms exist for trace element uptake into milk-secreting cells. Like IgA and albumin, iron is internalized by a receptor-mediated endocytotic process. Iron bound to transferrin (TFN) binds to the transferrin receptor (TFNR) on the basolateral surface of the mammary gland. The TFN–TFNR complex is internalized by clathrin-dependent endocytosis and transported into the BEE of the mammary epithelium, where the acidic environment releases iron from TFN. It is thought that released "free" iron is transported out of endocytic vesicles into the cytoplasm by the divalent metal ion transporter identified in hepatic cells and placental tissue,[354,355] but direct evidence that this process occurs in milk-secreting cells is lacking. In the cytoplasm, iron complexes with iron-binding proteins, such as ferritin, or it is transported into the secretory machinery by ferroportin where it complexes with transferrin or lactoferrin and is packaged into secretory vesicles carrying milk proteins.[356] While significant amounts of iron are transported into rodent milk to meet the nutritional demands of the growing offspring, human milk iron is very low as the full-term infant stores enough iron from the breakdown of fetal hemoglobin to meet infant demand for four to six months postnatally.[357]

p0470   Copper is transferred from the circulatory system into milk-secreting cells by basolateral located transporters. Three copper transporters have been identified in the mammary gland: Copper transporter 1 (CTR1), ATPase7A (ATP7A), and ATPase7B (ATP7B). CTR1, an essential regulator of copper import in most tissues,[358] including the mammary gland,[359] is found at the plasma membrane and forms a multimeric complex having a central copper channel with high affinity for copper.[358] ATP7A and B are polytopic transmembrane cation-transporting P-type ATPases that are closely related to each other.[360] Both proteins are found in the secretory pathway and function in the transfer of copper to copper-binding proteins or copper-dependent enzymes in this compartment.[361] Copper complexed to ceruloplasmin or metalothionine is packaged into secretory vesicles and secreted by the exocytotic pathway. Mutations in ATP7A and B are responsible for copper excretion defects observed in livers of Menke and Wilson disease patients. In each case, the defects are associated with impaired

trafficking of these proteins from the Golgi to secretory vesicles.[362] ATP7A and 7B have been implicated in the secretion of copper into milk.[356] However, only impaired ATP7B function has been shown to influence milk copper levels in mice.[363] Transgenic studies in fact suggest that ATP7A mediates copper secretion from the basolateral membrane of the mouse.[364] Although ATP7A and 7B are expressed in human MECs,[364,365] their roles in milk copper secretion are not fully established. Milk copper levels are reported to be lower in patients with Wilson's disease,[366] however Wilson's disease does not appear to interfere with lactation.[367] Whether ATP7A is able to compensate for ATP7B dysfunction in the milk copper secretion in Wilson's disease patients is unknown. However, ATP7B has been shown to rescue copper accumulation defects observed in cells derived from mice with defective ATP7A,[360] thus overlap in the functions ATP7A and B may account for the limited effect of ATP7B loss on mammary gland function and milk copper levels.

p0475   The organization of the zinc secretory system is broadly similar to the copper secretory system in that specific transporters mediate zinc uptake from the maternal circulation and its subsequent transport from the cytoplasm into the secretory system. Twenty-four different zinc transporters (ZnT) have been identified in mammals; they fall into two categories, 10 ZnT and 14 zinc import proteins (ZIP).[368] ZnT family members transport zinc from the cytoplasm across the plasma membrane or into the secretory or endosomal pathways.[369] ZIP family members mediate transfer of zinc into the cytoplasm from the circulation or intracellular compartments.[370] ZnT family members are known to be critical regulators of zinc transport into milk of humans and rodents. In humans, a missense mutation in ZnT2 has been identified that is associated with low milk zinc levels,[371] and in mice a spontaneous ZnT4 mutation appears to be responsible for decreased milk zinc levels associated with the lethal milk phenotype. Specific roles for ZIP family members in zinc secretion into milk have not been described.

Membrane Transport (Pathway IV)   s0225

p0480 Na^+, K^+, and Cl^- ions are the most prevalent minerals in most milks, comprising about 10% of the osmolality of human milk, 25% of the osmolality of bovine milk, and about 65% of the osmolality of rabbit milk,[372] with lactose making up most of the rest. Because the total concentrations of Na^+ and K^+ in milk are equivalent whether determined by ion selective electrodes or flame photometry,[373,374] these ions are considered to be free, e.g., not complexed with other molecules. The same assumption is usually made for Cl^-.[375] The important questions are: What are the concentrations of these ions within the various compartments of the mammary tissue? What are the mechanisms by which the

t0010 **TABLE 31.1**   Monovalent Ion Concentrations in the Guinea Pig Mammary Gland

Component	Interstitial, mEq/L	Cell, mEq/L		Milk, mEq/L	
		Guinea Pig; Ruminant	Mouse	Guinea Pig; Ruminant	Mouse
Sodium (Na$^+$)	150	41.7	47	8	27
Potassium (K$^+$)	4.5	122 (free); 143 (total)	129	24	47
Chloride (Cl$^-$)	116	66.5		12	N/A

Data from Ref. 5.

concentration gradients between interstitial fluid, cell, and milk are maintained in the lactating epithelium? The first question was answered at least for goats and guinea pigs in a 1971 publication by Linzell and Peaker.[5] Table 31.1 shows the concentrations of Na$^+$, K$^+$, and Cl$^-$ in the interstitial space, the cell water, and the milk. Similar results were reported for goats, cows, and sheep.[5] Before dealing with potential mechanisms for monovalent ion transport, it is useful to reiterate the basic principles proposed by Linzell and Peaker in 1971:[5]

o0010 **1.** All mammary membranes are freely permeable to water so that the concentrations of osmolytes essentially determine the volume of milk.

o0015 **2.** The ducts have the same ionic permeability properties as the alveolar cells, and milk composition is unchanged as it passes through the mammary ducts.

o0020 **3.** The ionic concentration in the cytoplasm is maintained by pumps and exchangers present on the basolateral membranes of the cells.

o0025 **4.** The apical membrane is permeable to Na$^+$, K$^+$, and Cl$^-$.

o0030 **5.** Na$^+$ and K$^+$ are at electrochemical equilibrium across the apical membrane, whereas the concentration of Cl$^-$ in the milk is lower than predicted by passive distribution.

p0510   These concepts have not changed since Linzell and Peaker first proposed them;[5] they apply during full lactation when the paracellular pathway is closed.

s0230 **WATER PERMEABILITY OF MAMMARY TISSUES**

p0515   The major evidence for the free permeability of the mammary membranes, both epithelial and endothelial, to water is that milk is iso-osmotic with plasma under any circumstances in which it has been measured.[376,377] An interesting example is that during Ramadan, when women fast, their blood becomes slightly hypertonic and their milk follows suit.[378] Water channels called aquaporins are thought to be responsible for the water permeability of cell membranes. Aquaporin 1 (AQP1) was clearly shown to be present in myoepithelial cells in the bovine mammary gland as well as the capillary endothelium.[379] Aquaporin 3 (AQP3) was found on the

basolateral membranes of secretory epithelial and ductal cells in the rodent mammary gland[380] and more selectively in the bovine gland.[379] Aquaporin 5 (AQP5) was present on the apical membranes of the mammary ducts of virgin animals[381] and possibly intracellularly during lactation. Aquaporin 7(AQP7), a water and glycerol channel, has been shown to be expressed in the murine mammary gland at lactation (Ramanathan and Neville, unpublished) but its localization is unknown. It can be assumed that these channels are at least partly responsible for water permeation across mammary membranes, but final answers are not yet in.

ROLE OF THE DUCTAL EPITHELIUM IN THE CONCENTRATION OF IONS IN MILK
s0235

p0520   Although the concentration of many secretions such as saliva is altered as the fluid passes through the ducts, the mammary gland appears to be an exception. The best evidence is that the concentration of ions is the same in milk obtained early in milking, which presumably has lingered in the ducts, as in milk obtained at the end of milking as long as high levels of OT are not used to obtain milk letdown.[382]
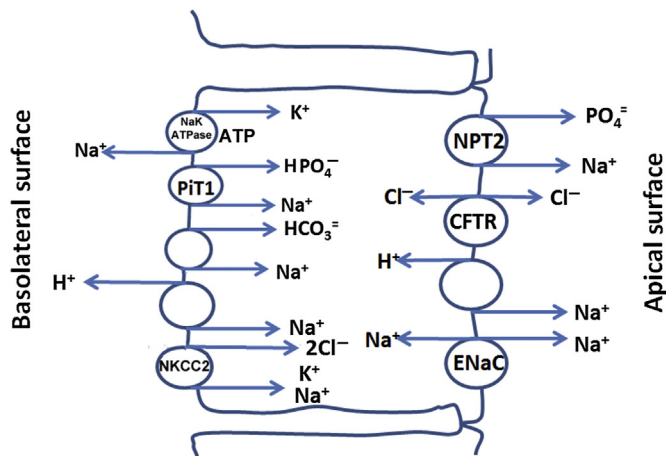
BASOLATERAL MEMBRANE TRANSPORTERS
s0240

p0525   There is abundant evidence for a number of pumps and exchangers in the basolateral membrane. The functional and immunohistochemical evidence for a ouabain-sensitive Na/KATPase in the basolateral membranes of the lactating mammary alveolar cells was first reported in the early 1970s[229,383] and has since been obtained in many laboratories (reviewed in Ref. 376); it is widely accepted that the Na$^+$ and K$^+$ levels in the mammary cell are mostly maintained by this enzyme. However, Na$^+$ levels in the cell (43 mM in the guinea pig, for example, 26 mM in the mouse[384]) are higher than in many other cells. The mechanism is not clear, but many Na$^+$-dependent exchangers are thought to be present on the basolateral membrane,[376] which could account for excess entry of this ion into the cell (Figure 31.15). Evidence for an amiloride-sensitive Na$^+$/H$^+$ exchanger as well as a DIDS-sensitive Na/HCO$_3^-$ cotransporter were obtained in the 31EG4 cell line,[385] a nontransformed mouse mammary cell line derived from the IM-2 cell line.[386] Shennan

f0080

FIGURE 31.15 **Transporters and channels in the basal and apical membranes of the mammary alveolar cell.** This figure shows the membrane transporter for monovalent ions for which there is evidence from studies in the lactating mammary gland and tissue culture models. PiT-1 is the product of the Slc20a1 gene; the $Na^+/PO_4^=$ transporter Npt2, also known as NaPi-IIb, is the product of the Slc34a2b gene; the $Na^+K^+2Cl^-$ transporter NKCC2 is the product of the Slc12a2 gene; CFTR is the cystic fibrosis Cl transporter encoded by *Cftr* in the mouse: and ENaC is the nonvoltage sensitive amiloride sensitive Na^+ channel encoded by the murine Scnn1b gene. While Na^+ hydrogen exchangers have been proposed for both membranes, their molecular identity is not yet clear. (For a color version of this figure, the reader is referred to the online version of this book.)

and colleagues[387] obtained physiologic evidence for electroneutral $Na^+/K^+/2Cl^-$ exchange in mammary explants inhibitable by furosemide. Clear immunohistochemical evidence supports the presence of this transporter at the basolateral membrane.[122,381,388] Levels were highest in the ducts of the virgin gland, but the protein was detected throughout pregnancy and lactation generally localized to basolateral membranes. Mice with a null mutation of this transporter showed no obvious defects in mammary morphology during lactation; however, the pups failed to thrive.[388] What could not be determined from the experiments was whether the lactation failure was due to a defect endogenous to the mammary gland or some other deficiency, since the mice have defects in a number of tissues.

APICAL MEMBRANE TRANSPORT

Apical membrane transport has also been characterized in a number of laboratories. The earliest experimental evidence was obtained in goats in 1974. Linzell and Peaker found that NaCl and KCl solutions infused up the teat were absorbed into the blood stream, whereas isotonic sucrose solutions infused in the same way drew ions into the milk space.[230] In addition, measurement of the effect of changes in the Na^+, K^+, and Cl^- concentration in the milk space of the goat mammary gland on the transepithelial potential difference

provided evidence for the presence of a nonselective cation channel and a Cl^- channel in the apical membrane.[389] Additional evidence for K^+ and Cl^- channels comes from studies of ion transport across membrane vesicles isolated from MFG membranes.[390,391] In the guinea pig (Table 31.1), rat, and rabbit the ratio of the concentrations of Na^+ and K^+ in milk and mammary gland was approximately equal at 3, and both were at approximately electrochemical equilibrium across the apical membrane, suggesting that they are passively distributed.[372] This was also the case in the mouse where careful measurements of the transepithelial and basolateral potential in vivo, gave a transepithelial potential of $-35\,mV$ and a basolateral potential of $-49\,mV$. By subtraction the apical potential should be in the range of $-14\,mV$, not too different from the equilibrium potentials of Na^+ and K^+ across the apical membrane of -26 and $-15\,mV$, respectively.[232,384]

The Cl^- concentration is much higher in the cell than in the milk of all species where it has been measured,[372] and the ion is obviously out of electrochemical equilibrium. The mechanism by which milk Cl^- is maintained at a low level is not clear, but experiments in tissue culture models are beginning to show how Cl^- transporters may function. The cell line, 31EG4, mentioned before, is a reasonably satisfactory model as it forms a tight monolayer when grown on a Transwell support. The resistance of this monolayer is substantially increased by glucocorticoid in a manner reminiscent of the in vivo mammary gland.[392] 31EG4 cells were found to possess both the amiloride sensitive epithelial Na^+ channel (ENaC) and the cystic fibrosis transmembrane conductance regulator (CFTR), a Cl^- channel that is stimulated by cyclic AMP and inhibited by diphenylamine-2-carboxylate.[392] Both were localized to the apical border of 31EG4 cells by immunostaining, a location that was confirmed by electrophysiological experiments. CFTR was also identified in sections of human mammary gland at the apical border of the epithelium.[392] This transporter could be responsible for Cl^- movement between the milk and the cell in the lactating mammary gland: however, because it is a passive channel, it cannot account for the movement of Cl^- down its electrochemical gradient from the lumen into the cell. A further difficulty with an important role for CFTR in the ionic composition of milk is that women with cystic fibrosis, a disease with defective CFTR, have no difficulty with lactation.[393] While considerable physiologic evidence indicates the presence of a K^+ channel in the apical membrane,[376] the molecular identity of this channel remains unknown, unless it is the Ca^{++}-activated $K_{Ca}3.1$channel described following. There appears to be a good deal to be learned about the molecular nature of the channels in the apical membrane of the mammary epithelium.

s0250 **PHOSPHATE TRANSPORT IN THE MAMMARY GLAND**

p0540 Na$^+$ PO$_4^=$ cotransport in the mammary gland of the lactating rat was identified physiologically in 1996[394] when it was thought to mediate basal transport of both ions into the mammary cell in vivo. It is hypothesized that this transport is mediated by a Type III neutral Na$^+$ HPO$_4^-$ transporter known as *PIT* encoded by Slc20 genes.[395] *Slc20a1* is upregulated at lactation in the mouse (Ramanathan and Neville, unpublished), suggesting that PIT-1 is the basal Na$^+$ HPO$_4^-$ carrier in the mammary epithelium. A specific Na$^+$ PO$_4^=$ transporter (NPT2B or NaPi-IIB; gene name *Slc34a2*) was shown to be highly expressed at the apical membrane of the lactating mammary gland in the mouse[381] and goat[396] and can be either electrogenic, transporting Na$^+$ PO$_4^=$, or electroneutral, transporting Na$^+$ HPO$_4^-$.[395] These findings suggest that Na$^+$ and PO$_4^=$ are transported across the basolateral membrane into the cell by PIT-1 and across the apical membrane to the milk by NPT2B. However, detailed proof of this hypothesis is lacking.

s0255 **A POTENTIAL ROLE FOR PURINERGIC RECEPTORS IN REGULATION OF MAMMARY GLAND ION TRANSPORT**

p0545 Mammary tumor cells were found to secrete the nucleotides UTP and ADP after mechanical stimulation; these compounds in turn increased intracellular Ca^{++} in the tumor cells.[397] The receptor responsible for these nucleotide effects was found to be the metabotropic P2 purinergic receptor, P2Y. Blaug and colleagues[249] using 31EG4 cells showed that ATP and UTP stimulated apical Cl$^-$ movement by acting on P2Y to increase Ca^{2+} release from the endoplasmic reticulum. Studies of purinergic receptor activation in primary human mammary epithelial cell cultures produced evidence for K$_{Ca}$3.1channels. K$^+$ intermediate/ small conductance Ca^{++}-activated channel, subfamily N, member 4, also known as KCNN4, is a human gene encoding the K$_{Ca}$3.1 protein, which is responsive to P2Y receptors in the basolateral membrane of these MECs. Increased cellular Ca^{++} also activates ENaC in the basal membrane to alter Na$^+$ transport.[250] The physiological meaning of these findings is not entirely clear, but they indicate that Na$^+$ and K$^+$ secretion into milk is subject to a higher level of regulation than previously thought.

p0550 In conclusion, the major outlines of monovalent transport into milk have not changed since they were first proposed in 1971 by Linzell and Peaker.[5] However, considerably more molecular information about the mechanism of transport across the basolateral membrane is available. Many questions remain about the molecular mechanisms of apical transport.

MEETING THE ENERGY REQUIREMENTS OF LACTATION

s0260

p0555 The energy demands of lactation are significant and require a major shift in energy homeostasis. Some animals can meet this energy demand wholly by mobilizing endogenous energy stores.[398] Such animals (some bears, seals, baleen whales) usually have a large body size and the storage capacity that allows them to remain fasted throughout lactation. For most animals, however, the energy consumed in the diet is needed to support the demands of this expensive process. One adaptation of maternal physiology is the growth and increased absorptive surface area of the alimentary tract to extract the available nutrients from dietary intake.[399,400] This adaptation, in isolation, however, would be insufficient. To meet these demands, multiple signals converge to increase maternal caloric intake, minimize the energy requirements in nonessential tissues, and direct nutrients that will be used for milk production to the mammary gland.

Exogenous Nutrients

s0265

p0560 Much of the research on the role of exogenous nutrients in providing substrate for milk synthesis has been carried out in rodents, animals with large litters that require an enormous increase in energy transfer to support their high rate of growth. While the general adaptive mechanisms to meet these demands are thought to be present in most lactating animals, including humans, there are certainly species-specific adaptations. Here we will deal mainly with the extensive information available from studies in rodents as well as earlier studies in dairy animals.

Increasing Energy Intake

s0270

p0565 Two primary hormonal signals are thought to be involved in promoting hyperphagia during lactation: (1) PRL release associated with suckling; and (2) a decline in the adiposity-associated hormone, leptin (Figure 31.16).

p0570 Suckling can stimulate energy intake in the absence of milk production,[401–403] even when peripheral signals of negative energy imbalance are not present.[404] Suckling is a major stimulus to PRL secretion,[405] and PRL is thought to promote feeding in order to meet the high energy demands of lactation. PRL induces hyperphagia in nonlactating dams in a dose-dependent manner[406–408] and rescues the inhibitory effects of bromocriptine on the appetite of galactophore-cut dams (dams who are still being suckled but not producing milk).[409] This effect on appetite is thought to involve key areas of the hypothalamus that regulate energy balance. Both PRL and suckling can increase the expression of orexigenic (e.g., appetite promoting) neuropeptide Y in the dorsomedial hypothalamus.[410,411] In addition, these areas of the brain

FIGURE 31.16 **The regulation of energy intake during lactation.** PRL secretion reduces the sensitivity of the brain to leptin. Suppressed leptin levels and central leptin resistance converge to promote feeding. Other signals from the suckling response may also contribute to this interplay between the mammary gland, adipose tissue, and the brain. The neural pathway linking suckling to energy intake is currently uclear as indicated by "?". The increased energy intake helps meet the energetic demands of lactation. PRL = prolactin; NEFA = nonesterified fatty acids. (For a color version of this figure, the reader is referred to the online version of this book.)
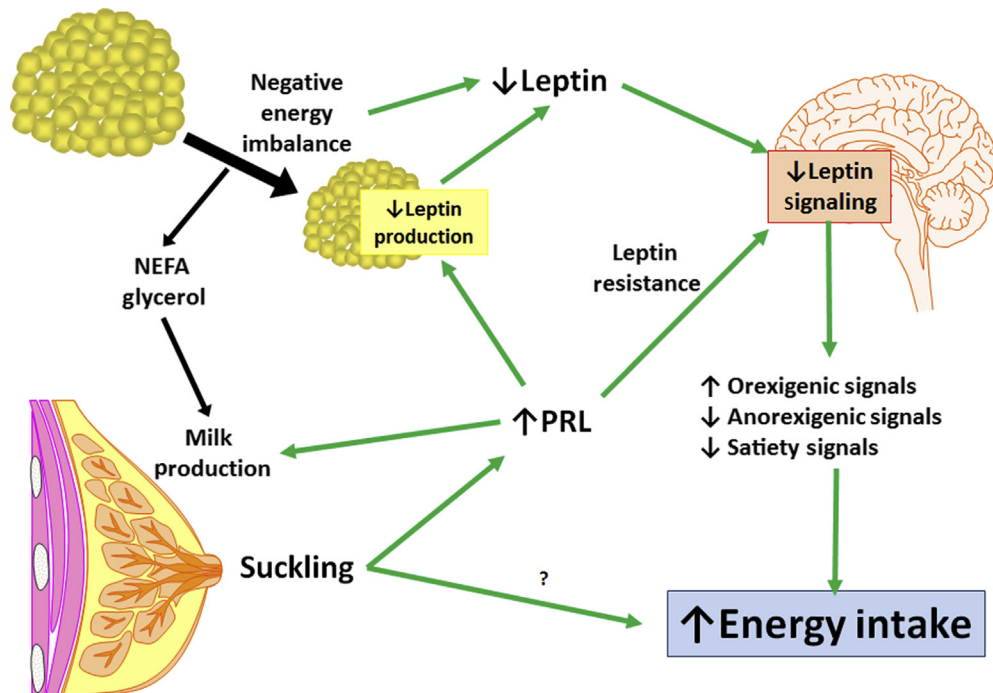
f0085

become resistant to the anorexigenic (anti-appetite promoting) actions of leptin. PL signals through the PRL receptor and promotes hypothalamic leptin resistance by mid-pregnancy in the rat.[412,413] Central leptin resistance is maintained throughout lactation,[414] likely by PRL,[415] and appears to be essential for the maintenance of hyperphagia.[416–419]

p0575   Leptin signaling during lactation is also suppressed by decreased levels of the hormone.[403,420] Circulating levels become less responsive to meals and more responsive to maternal energy balance, while displaying a diminished diurnal fluctuation throughout the day.[403,420] The decline in leptin levels may result from the depletion of maternal adipose stores during lactation, as endogenous lipid stores are trafficked to the mammary gland.[403,404,418] Alternatively, PRL may directly inhibit the secretion of leptin.[421] Regardless of the mechanism, with a brain that is resistant to leptin, and leptin levels falling, the inhibition of orexigenic signals is diminished and the stimulation of anorexigenic signals is minimized, both of which result in increased appetite.[422]

s0275 *Adaptations in Expended Energy*

p0580   Lactation is an expensive process, particularly in rodents who increase their metabolic requirements up to threefold to nourish a rapidly growing litter of pups. This increased requirement is founded not only on the energetic value of the milk constituents but also in the maternal energy expended to produce and secrete the milk. In women, the efficiency of milk production is predicted to be between 80% and 95%,[423] suggesting that during lactation basal metabolic rate should increase anywhere from 5% to 20% due to the energy cost of milk production.[424,425] Butte et al. elegantly showed in women that there is a direct correlation between milk production and total energy expenditure, consistent with the idea that metabolic rate is affected by the costs of milk production.[426] However, this effect on metabolic rate has not been observed in all studies, suggesting that the energetic efficiency of milk production may *vary* substantially between individuals or that peripheral tissues become more metabolically efficient to compensate for the increased energetic demand of milk production.

p0585   The efficiency of milk production is inherently linked to the type of macronutrient used to make milk lipid (Figure 31.17). Thus, it is energetically more expensive to produce milk lipid from carbohydrate or protein because as much as 25% of the energy must be expended to convert them to a free fatty acid precursor. On the other hand the energetic cost of producing milk lipid with preformed fatty acids derived from the diet or from endogenous stores is substantially less (<1–2%). For this reason the composition of the diet and the availability of preformed precursors can have a significant effect on the energetic requirements of milk production.

p0590   In addition, evidence suggests that the body can adapt in other ways to conserve energy for milk production (Figure 31.17). Three of the proposed adaptations are: (1) decreased physical activity[427–429]; (2) suppressed anabolic activity of adipose and muscle[430–432]; and (3) reduced nonshivering thermogenesis by brown adipose tissue (BAT).[427,433–437] With respect to BAT, falling leptin levels may coordinately explain the inhibition of BAT and the suppression of nonshivering thermogenesis during lactation.[404,427,438]
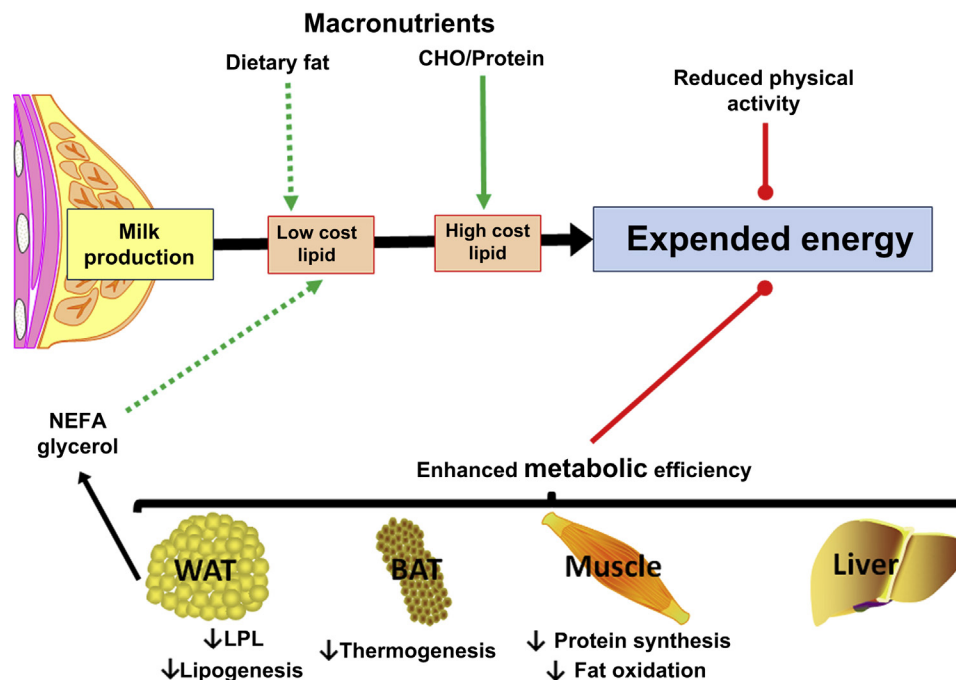
f0090

FIGURE 31.17   **The regulation of energy expenditure during lactation.** Milk production is energetically expensive. This cost includes not only the energy found in the milk constituents but also the energy that must be expended to produce and secrete the final product. Preformed fatty acids from the diet (exogenous) or mobilized from adipose tissues (endogenous) require relatively little expended energy to produce milk lipid. If milk lipid is made from CHO or amino acid precursors, these substrates must be converted to fatty acids via de novo lipogenesis, increasing the amount of energy needed to produce milk lipid. To conserve energy during lactation and maintain thermoneutrality, peripheral tissues become more metabolically efficient and physical activity declines. WAT = white adipose tissue; BAT = brown adipose tissue; NEFA = nonesterified fatty acids; LPL = lipoprotein lipase. (For a color version of this figure, the reader is referred to the online version of this book.)

s0280 ### Dietary Fat versus De novo Derived Lipid: Effects on Milk Lipid Content

p0595   The positive energy imbalance resulting from the combined effects of hyperphagia and enhanced metabolic efficiency can provide the extra energy needed for milk production. The extra energy makes its way to the mammary gland in the form of carbohydrate, lipid, or protein, and may be trafficked through other tissues prior to being utilized by the mammary gland for milk production (Figure 31.18).

p0600   Breakdown products from hydrolysis of dietary lipids are taken up by the intestinal cells, re-esterified into triglycerides (TAG), and released in a lipoprotein called a chylomicron. Chylomicra are released into the lymphatic circulatory system and eventually enter the portal vein from which they are distributed through the blood stream to be utilized by lipoprotein lipase (LPL) containing tissues for oxidation or storage or synthesis by MECs into milk TAG. If there is very little fat in the diet, a larger portion of the lipid component of the milk must be produced from the available carbohydrate and/or protein, via a process called de novo lipogenesis. If this process occurs in the mammary gland, the fatty acids synthesized are limited to 8- to 16-chain saturated fatty acids, while in the liver and adipose tissues fatty acids of 16 carbons and longer are produced (Figure 31.18). Increased dietary fat has been shown to downregulate de novo lipogenesis in the mammary gland[439–445] and in turn reduce the proportion of medium chain fatty acids in the milk fat. As such, milk TAG composition reflects dietary fat composition.[446]

Endogenous Nutrients s0285

p0605   In addition to the positive energy imbalance, endogenous nutrients are mobilized and directed to the mammary gland to meet the demands for lactation. PRL and β-adrenergic stimulation from the sympathetic nervous system provide critical signals for this shift in fuel trafficking. However, these signals have tissue-specific effects in the periphery. Fat stores in adipose tissue and liver (made via de novo lipogenesis or transported to these tissues from dietary sources[446]) are mobilized during lactation to provide milk fat substrates and energy for milk production.
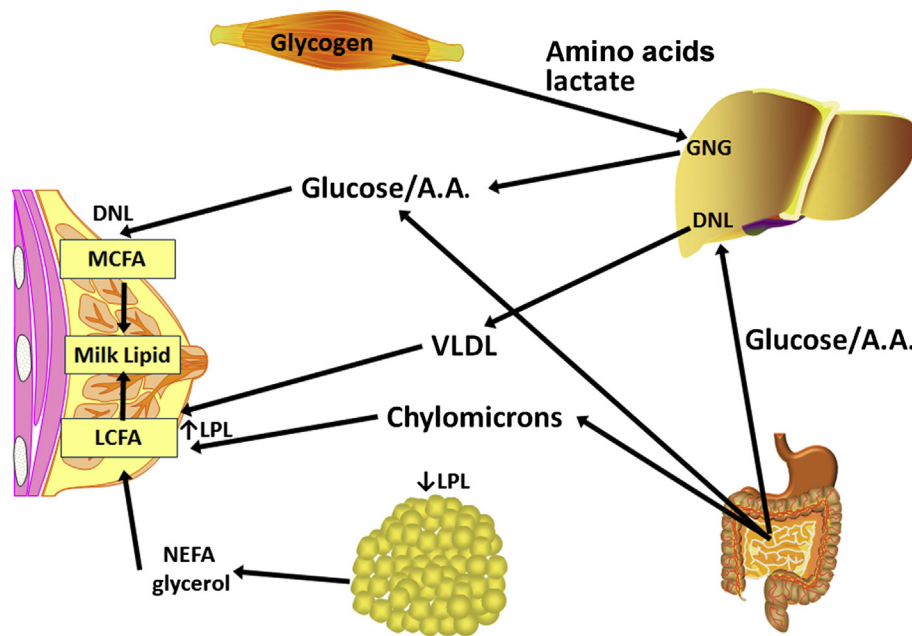
Adipose Tissues: The Primary Source of Endogenous Energy s0290

p0610   During pregnancy, ingested energy gradually accumulates in white adipose tissues (WAT) in preparation for lactation, stored as TAG in lipid droplets. During

f0095

FIGURE 31.18 **Exogenous and endogenous nutrients affect the composition of milk lipid.** Dietary fats primarily enter circulation through the lymphatic system as triacylglycerol in chylomicrons, while the liver releases stored lipid in the form of VLDL. The differential expression of LPL in the mammary gland and adipose tissues leads to the trafficking of these neutral lipids toward milk production. Adipose tissue lipid is mobilized and trafficked to the mammary gland in the form of NEFAs. Glucose and amino acids, mobilized from endogenous stores or absorbed from the diet, are directed to the mammary gland as precursors for milk carbohydrate and protein. When in excess, the mammary gland and liver convert these precursors to fatty acids via de novo lipogenesis. The product in the mammary gland is primarily MCFA, while in the liver it is long-chain fatty acids (LCFA) and subsequently triacylglycerol. The types of fats that end up in milk lipid are a function of dietary fat (usually LCFA), the amount that is mobilized from endogenous stores (usually LCFA), and the location of DNL (MCFA, mammary gland; LCFA, liver). LPL = lipoprotein lipase; NEFA = nonesterified fatty acid; VLDL = very low density lipoprotein; DNL = de novo lipogenesis; A.A. = amino acid; MCFA = medium chain fatty acid; LCFA = long chain fatty acid. (For a color version of this figure, the reader is referred to the online version of this book.)

lactation, this stored energy is then mobilized to provide fatty acid substrates for energy production and milk lipid synthesis.[77,431,446] Lipolytic enzymes, such as hormone-sensitive lipase and adipose triglyceride lipase, hydrolyze the lipid droplets to release the stored TAG. The released fatty acids and mono-acylglycerol are transported out of the cells into the blood. They bind to albumin and are circulated throughout the body.

p0615   This shift of WAT from an anabolic state during pregnancy to a catabolic state during lactation is in part due to the reversal of the low sympathetic tone that develops in this tissue during pregnancy. Shortly after parturition, the sympathetic tone in adipose tissues increases. The concentration of norepinephrine in WAT increases, stimulating β-adrenergic receptors on adipocytes, which become more responsive to the activation of lipolysis by cyclic AMP.[430,447–450] At the same time, insulin suppression of lipolysis in WAT and its ability to promote lipogenesis becomes impaired.[451,452] Under normal postprandial conditions or hyperphagia, elevated insulin will activate lipogenic pathways and inhibit lipolysis,[453] resulting in a net deposition of energy in WAT.[454,455] During lactation, impaired insulin sensitivity resulting from decreased expression[456,457] and responsiveness of IR[452,458,459] in WAT blunts the uptake and deposition of

energy and diverts ingested energy to the mammary gland.[432,456,460] Evidence from both humans[461] and animals suggests that the PRL secreted after secretory activation may have direct effects on adipose tissues that suppress adiponectin secretion and impair insulin suppression of lipolysis.

p0620   In addition to mobilizing the stored lipid, the impairment in insulin signaling decreases the deposition of ingested carbohydrate and protein[430,449,462,463] so that these nutrients can be directed to the mammary gland. Glucose uptake and oxidation decline,[432,451,464,465] making the available glucose carbons for the production of milk.

Liver: An Alternate Source for Carbohydrate, Lipid, and Protein

s0295

p0625   During lactation, the liver serves as both a way station for endogenous nutrients and a metabolite interconverter to meet the nutrient needs of the mammary gland. Under postprandial conditions, excess nutrients accumulate in the liver and are stored until endogenous sources become less available. Unlike adipose tissues, hepatic lipid is released into the circulation in the form of very low density lipoproteins (VLDL). As such, this lipid becomes available to the mammary gland in much the same form as dietary fat (but via VLDL).

p0630   In addition to providing lipid, the liver converts metabolic intermediates into glucose in order to help to provide a regular supply of carbohydrate to the mammary gland. Skeletal muscle provides the precursors in the form of lactate[466] and amino acids[465] to support hepatic gluconeogenesis,[447,467] which increases during lactation.[424] Increased gluconeogenesis has been explained as a consequence of liver hypertrophy, a lower insulin-to-glucagon ratio in the portal vein, and intermittent hypoglycemia.[468] Hepatic glycogen also becomes a source for hepatic glucose production.[469] In humans, glycogenolysis may become the primary pathway for hepatic glucose production under fasting conditions.[470] However, there is evidence that the relative contributions of glycogenolysis and gluconeogenesis vary with species and metabolic state. Regardless, the end result serves to divert glucose to the mammary gland to meet the immense demands of milk production.

Improved Metabolic Control
s0300

p0635   Despite the fact that studies in animal models suggest that the insulin resistance of peripheral tissues helps to direct nutrients to the mammary gland, studies in humans indicate that this extension of pregnancy-associated insulin resistance resolves as lactation proceeds.[471] This improvement in metabolic control may lead to levels of insulin sensitivity that surpass that of nonlactating controls.[472] The mechanism of this improvement is not well understood, but it likely reflects the depletion of endogenous energy stores.

Trafficking Exogenous and Endogenous Nutrients to the Mammary Gland
s0305

p0640   Controlled in large part by the rate of milk secretion, active blood flow to the mammary gland increases the delivery of glucose, amino acids, fatty acids, and lipoproteins.[446,473,474] PRL also inhibits the expression of LPL in WAT,[475–477] while increasing LPL expression in mammary gland tissue.[477] This tissue-specific effect of PRL traffics triglyceride carried by chylomicra and VLDL away from WAT and to the mammary gland.

p0645   Glucose is the major substrate for synthesis of lactose and glycerol in the mammary gland during lactation. Glucose uptake in the mammary gland significantly increases during lactation, and although blood glucose may be somewhat elevated from lactation-induced hyperphagia, glucose uptake in the mammary gland is elevated independent of dietary supply.[465,478]

p0650   In rodents the proportion of long-chain fatty acids in milk is high during early lactation reflecting the mobilization of fat stores.[432] The increased availability of substrate for milk fat synthesis decreases the need for the mammary gland to produce its own lipid via de novo lipogenesis.[446] Endogenous fatty acids from WAT contribute 10–20% of the lipid used for milk fat production,[446] and

mice mobilize up to 70% of their adipose reserves during lactation.[432] However, most of these endogenous stores are used up during the first several days of lactation.

Evidence for Impaired Lactation in Maternal Diet-Induced Obesity
s0310

Animal Models of High Fat Feeding and Maternal Diet-Induced Obesity
s0315

p0655   Rodent models of diet-induced obesity have historically been the most common models used to study the effects of obesity on pregnancy and lactation. Typically, obesity is induced by feeding a HF diet using either a cafeteria-style diet where rodent chow is supplemented with high calorie human snacks[439,443,479–481] or a semi-purified diet using lard or vegetable oil as the primary fat source.[445,482,483] There are benefits and downfalls of both HF-feeding paradigms. Cafeteria-style diets are more representative of the human condition; however, the specific source of calories is not as well controlled as with semipurified HF diets. Additionally, when a rodent is left to choose its own food there is the potential for deficiencies in essential nutrients, which can have adverse effects on lactation independent of HF feeding.[484,485] Finally, it is important to consider the composition of the fatty acids used to induce obesity, as different types of fat substantially affect milk composition.[22,480,486]

p0660   Further, the conclusions that can be drawn from studies with diet-induced obesity models are confounded by the problem that HF diets themselves may have effects on lactation separate from effects of obesity. Typically, short-term exposures to HF diets are employed in lean animals to dissect these two effectors.[445,487–489] This approach is particularly useful in examining how HF feeding affects the various stages of mammary gland development. Separating the effects of chronic HF feeding from obesity is more challenging. To achieve this objective, some researchers have switched HF-fed, obese animals to a low fat diet during pregnancy, lactation, or both.[490] Such an approach, however, reflects an acute dietary change, rather than dissecting chronic HF feeding from the effects of obesity.

Diet-Induced Obesity Impairs Mammary Gland Development and Function
s0320

p0665   Obesity in dairy cows has long been known to impair milk production,[491] and rodent studies show similar effects.[443,492] When an HF diet was employed for 16 days prior to puberty in the mouse, one group found that the branching frequency and the width of mammary ducts were reduced along with the presence of abnormal myoepithelial cells in virgin mice.[492] In another study, lobuloalveolar development of the mammary gland was impaired when HF feeding was begun after puberty.[443] Morphologically, these authors reported abnormal side

branching and alveolar development by day 14 of pregnancy. In addition they showed a significant difference in mammary gland weight due to the increased size of the fat pad. Decreased pup weight, depressed milk synthesis genes, and the retention of large CLDs in the epithelium by day one of lactation suggested an inherent impairment in milk synthesis.[443] Together, these studies support the concept that diet-induced obesity can interfere with mammary gland function at different stages of development, but they fail to differentiate the effects of chronic HF feeding and obesity.

p0670   Unlike its effects on mammary gland development, the impact of HF feeding and obesity on milk production and composition has been relatively well characterized in rodents. The overall consensus is that diet-induced obesity impairs milk production early in lactation.[443,490,493] Observations of delayed pup growth and sometimes even death[479] within the first few days after parturition were an indication of decreased milk production and/or secretion. Additionally, milk composition is altered by HF feeding and obesity.[22,488] As mentioned previously, milk lipid composition is reflective of dietary lipids. However, it has also been shown that HF feeding can increase milk lipid content[480,490,493] as well as decrease milk protein.[443,480] However, in one study HF feeding during lactation actually led to decreased milk fat production.[445] Additionally, feeding conjugated linoleic acid or transfatty acids during lactation has resulted in suppressed milk fat production.[22,486,494] Therefore, the type of dietary fat may prove to be a determining factor in how milk composition is affected.

p0675   The effects of high-fat feeding and obesity during lactation on neonatal growth have also been inconsistent[441,443,444,495,496] with some studies reporting impaired neonatal growth linked to lactation defects[443,444] and others reporting increased neonatal growth correlating with an obese phenotype.[495,496] These inconsistencies suggest that the impact of HF feeding and obesity may vary with the stage of mammary development.

s0325 ### Evidence for Impaired Lactation in Humans with Diet-Induced Obesity

p0680   There is clear data indicating an increased risk for a failure of breastfeeding in obese women (BMI≥30).[497–500] Based on findings that obese white and Hispanic women are more prone to fail in initiating breastfeeding than normal weight women,[501,502] it has been proposed that obesity delays secretory activation[503,504] possibly by blunting PRL release in response to suckling.[505] Obesity is also associated with a shorter duration of breastfeeding.[497,506–509] Taken together, these studies indicate that obesity is an important risk factor for dysfunctional lactation. However, the biological basis for this increased risk has not been elucidated, and because of the current epidemic of obesity, it is an important area for further research.

MILK EJECTION   s0330

p0685   Secreted milk is stored in the alveoli and, to a limited extent, in the udder of ruminants. Milk ejection, necessary for milk delivery to the suckling, is effected by the contraction of myoepithelial cells whose processes form a basket-like network around the alveoli (Figure 31.19). Stimulation of nerve terminals in the nipple or teat produces impulses in spinal afferent nerves that reach the magnocellular neurons in the supraoptic (SON) and paraventricular (PVN) nuclei of the hypothalamus. The axons of these neurons run through the pituitary stalk terminating in the posterior pituitary[510] and releasing the nonapeptide, OT, when stimulated. Released OT is carried through the circulation to the lactating mammary gland where it interacts with specific receptors on myoepithelial cells, initiating a coordinated contraction that expels milk from the alveoli into the ducts and subareolar sinuses or udder. Milk ejection is essential for lactation, as shown by the observation that mice genetically deficient in OT have a severe lactation deficit.[511,512] Although milk ejection was the first recognized action of OT, the hormone and its receptors are found in many organs including the brain, where its release is regulated by a wide variety of peptides and hormones.[513] Methods are becoming increasingly available for assessing the effects of targeted mutations on this process. A particularly useful video illustrating techniques for
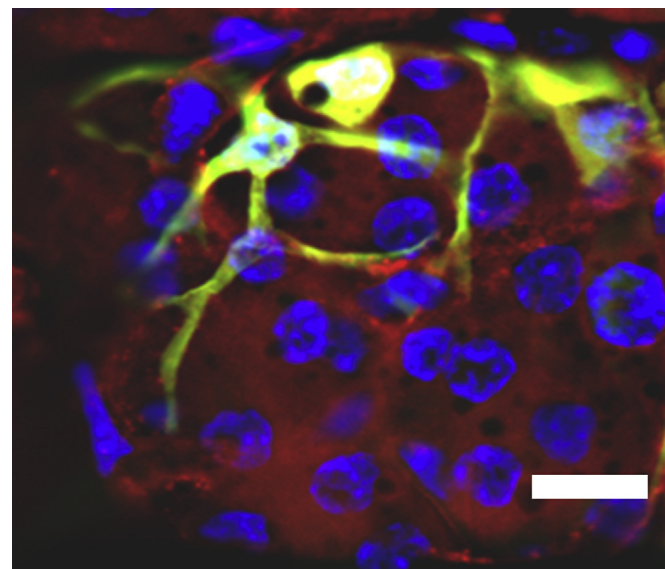
f0100

FIGURE 31.19   **Myoepithelial cell in the mammary gland of a lactating mouse.** The cell has been transduced with adenoviral GFP (yellow) showing processes embracing the luminal epithelial cells (red). Nuclei stained with DAPI (blue). Scale bar 10 μm. *Source: From Russell T, Fischer A, Beeman N, Freed E, Neville MC, Schaack J. Transduction of the mouse mammary epithelium with adenoviral vectors in vivo.* J Virol 2003;**77**(10):5801–09. http://dx.doi.org/10.1128/JVI.77.10.5801–5809.2003, © 2003, American Society for Microbiology. (For interpretation of the references to color in this figure legend, the reader is referred to the online version of this book.)

assessing mammary development from whole mounts of tissue as well as an ex vivo technique for assessing myoepithelial contraction has recently been published.[514]

s0335 The Letdown Reflex

p0690 Figure 31.20 shows neurophysiological correlates of OT release in rats, humans, and cows. These processes are the subject of an excellent review by Armstrong.[516] The rat nurses her litter for about 30 min each hour. Letdown is delayed for at least 15 min after the attachment of the pups.[510] Thereafter, increases in mammary pressure corresponding to OT-induced milk ejection can be measured every 5–12 min (Figure 31.20(A) and (B)). In cows a more sustained release of OT has been measured (Figure 31.20(D)). In women, Cobo and colleagues[517] showed that ejection can be measured as a rise in pressure sensed with a small catheter placed in a mammary duct or noted subjectively by the mother as a "tingling sensation" in the breast prior to or shortly after the start of suckling. When milk was continuously pumped from the breast, these contractions lasted about 1 min and occurred with a frequency of 4–10 contractions pe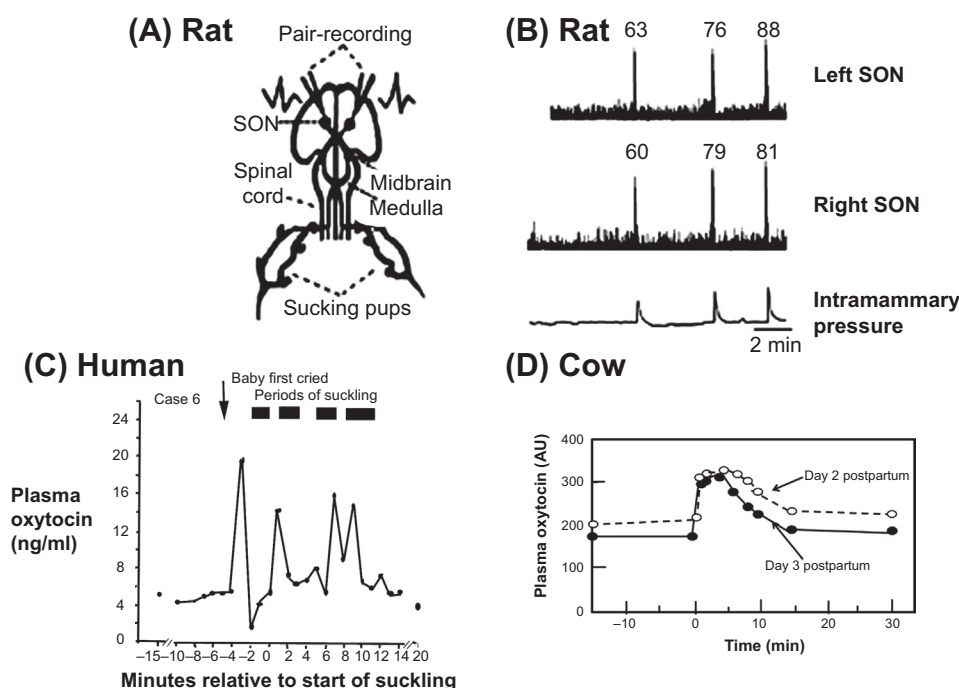r 10 minutes. When measured from blood samples drawn at 2 min intervals, the pulses of OT in the blood stream correspond to these contractile episodes. Unlike OT release in the rat, in women OT release begins prior to suckling in response to the cry of the infant or the mother preparing for the feed[518] (Figure 31.6(C)), indicating a psychological input to the magnocellular OT neurons.

The Neuroendocrinology of OT Synthesis and Release   s0340

p0695 OT holds the distinction of being the first naturally occurring peptide hormone to be synthesized,[519] a feat for which Vincent du Vigneaud received the Nobel Prize in Chemistry in 1955. OT release involves exocytosis of secretory granules stored in the posterior pituitary following a burst of impulses carried from the OT neurons in the SON or PVN.[510] Immunostaining techniques were used to show that OT is synthesized mainly in magnocellular neurons in these nuclei of the hypothalamus, separate from the vasopressin-synthesizing neurons.[520] Pulse chase experiments showed that OT is synthesized as part of a 30 kDa prohormone in the hypothalamus.[521] After cleavage to smaller subunits, the prohormone is packaged into secretory granules and transported down the axonal

(A) Rat Pair-recording
SON
Spinal cord
Midbrain Medulla
Sucking pups

(B) Rat 63 76 88
Left SON
60 79 81
Right SON
Intramammary pressure
2 min

(C) Human Case 6 Baby first cried Periods of suckling
Plasma oxytocin (ng/ml)
Minutes relative to start of suckling

(D) Cow
Plasma oxytocin (AU)
Day 2 postpartum
Day 3 postpartum
Time (min)

f0105 **FIGURE 31.20 Oxytocin (OT) secretion.** (A, B) Recordings from OT-releasing neurons in the anesthetized rat made simultaneously with recordings of intramammary pressure. Bursts of neuronal activity with firing rates indicated by the numbers above each peak are spaced at 5–12 min intervals. Neurons from both sides of the brain fire simultaneously leading to a pulse of OT release from nerve terminals in the posterior pituitary and a rise in the plasma OT level, followed shortly by a rise in intramammary pressure. (C) OT release in the woman during suckling. Plasma OT rises when the woman first hears the infant cry. Pulses of OT continue during suckling intervals. (D) OT release in the cow showing the prolonged pulse of OT in the plasma during milking on days 2 and 3 postpartum. *Source: (A, B) Used with permission from Wang YF, Negoro H, Higuchi T. Lesions of hypothalamic mammillary body desynchronise milk-ejection bursts of rat bilateral supraoptic OT neurones.* J Neuroendocrinol 2013;**25**(1):67–75. (C) Used by permission from Ref. 515. (D) Used by permission from Akers RM, Lefcourt AM. Milking- and suckling-induced secretion of OT and prolactin in parturient dairy cows. Horm Behav 1982;**16**:87–93.

processes of the SON and PVN neurons to the posterior pituitary. There it is further cleaved to OT and its binding protein, a 10 kDa neurophysin. The complex between OT and neurophysin is stable at pH 5.5, but dissociates at pH 7.4, freeing OT as the complex is released to plasma.[510,522]

p0700   The neurophysiology of OT release is complex and subject to regulation by steroid hormones and many other agents.[515,523] Critical to the appropriate release of OT during lactation is conditioning of the OT neurons during pregnancy.[523] OT release requires an increase in cytoplasmic Ca^{++}, both in magnocellular neurons and in nerve terminals; however, the Ca^{++} homeostatic mechanisms differ at the two locations with involvement of the endoplasmic reticulum in the dendrites, but not in the axonal terminals.[524] OT release is modulated by many agents including the sex steroids P4 and E2 as well as norepinephrine;[515,523] the mechanisms involved remain the object of intense study by neurophysiologists.[525] Of importance for lactation, endogenous opioid peptide systems inhibit release of OT at the magnocellular neurons in the hypothalamus and at their neurosecretory terminals in the posterior pituitary. Opioids likely also have an inhibitory effect on the cell bodies of afferent input cells that stimulate magnocellular neuron activity.[526]

s0345 Myoepithelial Cell Contraction

p0705   The processes of the myoepithelial cells lie within the basement membrane of the mammary alveolus and along the interlobular ducts. Autoradiographic studies showed that OT binding sites are similarly localized[527] and that there is 10-fold increase in the concentration of OT receptors in the rat mammary gland during pregnancy. The gradual increase in mammary OT receptor concentration contrasted sharply with the sudden increase in these receptors in the uterus on the day of parturition. The contractile response depends on interaction of smooth muscle alpha-actin (ACTA2) with myosin, and it has been found that lack of ACTA2 expression in null mice leads to severely impaired pup growth.[528] However, it appears that other pathways are involved in relaxation following this contractile response. Raymond et al.[529] found that conditional deletion of a laminin receptor, α3β1 integrin, from myoepithelial cells also led to low rates of milk ejection with impaired phosphorylation of focal adhesion kinase, an altered balance of elements of the Rac/Rho pathway and sustained phosphorylation of myosin light chain. These authors found that the lack of relaxation after OT-induced contraction in vitro could be rescued with constitutively active Rac or treatment with an inhibitor of myosin light chain kinase (MLCK). The authors suggest that α3β1 integrin stimulates Rac signaling that in turn inhibits MLCK activity, bringing about completion of the contraction relaxation cycle associated with milk letdown.

p0710   OT also appears to stimulate the growth of myoepithelial cells both in vitro and in vivo. When administered with E2 and P4 via implantable pellets into a virgin mouse mammary gland, it also enhanced myoepithelial differentiation of the cap cells surrounding the terminal end bud.[530] The potential role of OT in stimulating myoepithelial cell proliferation has recently been reviewed.[531]

The OT Receptor   s0350

p0715   The OT receptor is one of a family of nonapeptide hormone receptors that belong to the seven membrane spaning Rhodopsin-like Class I G-protein coupled receptors.[532] The human OT receptor has 389 amino acids and a core molecular mass of ~40–45 kDa that can be increased by glycosylation. The OT receptor gene has been cloned from a number of species[533,534] and a number of regulatory elements identified in the promoter including a palindromic estrogen response element and a half-steroid response element[534] that may be responsible for the E2-induced stimulation of OT receptor mRNA. In an elegant study, Olins and Bremel[535] found that OT stimulated the influx of extracellular Ca^{++} ions leading to phosphorylation of the myosin light chain in rat mammary myoepithelial cells. Influx of extracellular Ca^{++} ions regulated the duration of the response. There is also evidence that intracellular Ca^{++} stores are involved. Because the OT receptor is found in many locations, both in the brain and in organs such as the myometrium, heart, and peripheral nervous system, it is considered as a prime candidate for pharmacotherapeutic interventions.[536]

p0720   Cholesterol is an essential allosteric modulator of OT receptor function as is Mg^{2+},[533] although whether either plays a role in modulation of the activity of this receptor in the mammary gland is unknown. P4 has little effect on mRNA expression but exerts a powerful inhibitory action on receptor activity at a nongenomic level.[537] Whether this effect is mediated by interaction of P4 directly with the OT receptor as reported by Grazzini et al.[538] or by in some way altering the essential interaction of cholesterol with the receptor[539] remains to be elucidated. An interesting question is whether this interaction of cholesterol with the OT receptor plays a role in the elusive mechanism by which secretory activity is downregulated by P4 in late pregnancy.

OT and Maternal Behavior   s0355

p0725   OT has been implicated in a host of physiological and pathological functions such as stress management, maternal behavior, appetite, and social recognition. In the late 1970s, Pederson and Prange and others noted that intraventricular administration of

OT induced maternal behavior in virgin rats.[540] This observation led to extensive studies of the role of OT in maternal behavior (summarized in Chapter 52 and in Ref. [533]). It has been found that OT neurons release the hormone not only from their axons but also from their dendrites in the hypothalamus where it acts on OT receptors that are widely distributed in the brain to decrease anxiety and appetite and facilitate social recognition.[541] It seems likely that these mechanisms are involved in the positive response to nursing observed by many mothers; in rats NMR images showed increased activity associated with OT in regions of the brain associated with mother–pup bonding.[542] The extensive literature relating OT and mood is beyond the scope of this chapter; a number of recent review articles are available for the interested reader.[536,541,543]

CONCLUSIONS

s0360

p0730 We are at a strategic junction in research on mammary development and function. Starting from a clear understanding of the systems physiology and biochemistry of milk secretion developed in the 1970s, in the succeeding decades the stages of mammary development as they coordinate with reproductive maturation have been well defined. We know the structures of the genes, mRNAs, and proteins that are involved in both development and the synthesis and secretion of milk components. More recently new technologies have allowed a finer structural definition of the minor components of milk, and we are on the cusp of understanding the role of oligosaccharides, cytokines, and glycosylated proteins in infant development. We have visualized the cell biology of the mammary cell at the level of both the light and electron microscope and now can marry structure and function using advanced immunocytochemical techniques to understand where proteins are located in the cell and how that localization varies with developmental stage and functional activity. Although the role of nutrition in milk secretion has occupied dairy scientists for decades, with the now clear effects of obesity on lactational competence in women, the whole body flux of nutrients and the dietary and hormonal regulation of these processes are coming to prominence. PRL and OT are no longer just hormones that promote milk synthesis and ejection, respectively; it is now clear that they have actions in the central nervous system that regulate nutrient intake as well as maternal-infant bonding. Our next steps are to delve more deeply into the protein–protein interactions that mediate both regulation of mammary development and milk secretion and to come to an improved understanding of how the nutrient and emotional demands of lactation fit into systemic metabolism.

p0735 A few of the many interesting questions that we must address in the next decades are:

1. Molecular regulation of mammary development o0035
   a. Ductal development: What regulates the temporal and special pattern of expression of the receptors for the hormones, particularly estrogens, and growth factors that regulate development? Is this pattern of expression established within the mammary stem cell compartment, and does it respond to environmental factors? o0040
   b. Alveolar proliferation and differentiation in pregnancy: While we know that changes in circulating P4, PRL, and PL drive these processes, what are the molecular pathways that lie between the receptors for these hormones and the genes whose expression is changed? o0045
   c. What are the roles of microRNAs, gap junction proteins, insulin, and other factors in secretory differentiation in pregnancy? o0050
   d. Could the inhibitory effects of P4 on milk secretion in late pregnancy be mediated by P4 interaction with the OT receptor in myoepithelial cells? o0055
   e. What are the membrane channels that mediate transfer of monovalent ions at both the apical and basolateral membrane? How do they change with development and what are the mechanisms by which they are regulated? o0060
2. Involution o0065
   a. How do the many signaling molecules that have been shown to affect involution interact at the molecular level? o0070
   b. What is it that macrophages do to facilitate the involution process? o0075
   c. Do the tight junctions play a role in the increased Na^+ and Cl^- in milk during involution and mastitis, or does increased stress on the alveolar cells set in motion purinergic pathways that alter secretion and transport in alveolar cells? Could ATP and UTP be the elusive "feedback inhibitors of lactation" that have been shown to be present in both ruminant and human milk?[544,545] o0080
   d. In addition to macrophages, are there other immune cells and factors that interact with the involution process? o0085
3. Nutrition, metabolism, and mammary development o0090
   a. Are SREBP and STAT5 the only transcription factors that drive alveolar development in pregnancy, and how is this process modulated by diet, obesity, or malnutrition? o0095

o0100 **b.** What is it that macrophages do to facilitate the involution process?

o0105 **c.** What are the mechanisms by which nutrients are directed toward the lactating mammary gland for milk synthesis?

o0110 **d.** How are the activities of organs such as the liver, digestive tract, and heart stimulated in lactating animals? Are hormones involved? If so, which ones, and by what mechanisms?

o0115 **e.** What are the mechanisms that regulate appetite so that food intake is upregulated to meet the demands of lactation?

o0120 **4.** Systemic factors regulating milk secretion and ejection in lactation

o0125 **a.** How do obesity and food intake interact with mammary gland development and milk secretion to alter lactation?

o0130 **b.** Can we utilize the mRNA from the MFG to analyze the molecular basis of effects of obesity, emotional state, immune disorders, etc., on lactation in women?

o0135 **c.** What are the mechanisms by which infant gender and maternal emotional states alter milk secretion and composition?

o0140 **5.** Interactions between the immune system and mammary development and function

o0145 **a.** What are the details of the interaction of dIgA with its receptor and the cleavage event that releases secretory IgA into the milk?

o0150 **b.** More recent evidence indicates that immune cells such as macrophages and eosinophils are necessary for both ductal development[546] and involution.[183] What other immune interactions promote or impede mammary development, particularly during pregnancy?

o0155 **c.** Are there specific immune reactions that link maternal diseases, obesity, and metabolic disorders to lactation?

o0160 **6.** The maternal and infant microbiome

o0165 **a.** What are the critical nonnutrient components that support normal neonatal development, and how do they interact with the infant gut and immune system?

o0170 **b.** What components of milk foster development of the infant microbiome, and how is their secretion into breast milk regulated?

o0175 **c.** If microbes from the mother are actually transferred to the infant through the milk, how do they get across the mammary epithelium? Is the process selective?

o0180 **7.** Lactation and breast cancer

o0185 **a.** How does breast-feeding before the age of 30 suppress breast cancer risk?

o0190 **b.** What are the mechanisms by which pregnancy promotes breast cancer risk?

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Non-Print Items

Abstract

The mammary gland undergoes its final development in the adult animal, filling the mammary fat pad with a ductal tree and rudimentary alveoli during puberty, and expanding and fully differentiating during pregnancy. The hormones of pregnancy, prolactin (PRL), placental lactogen, growth hormone, and progesterone (P4) cooperate to produce a gland that is fully developed but nonfunctional. Secretory activity commences around parturition, with the withdrawal of progesterone and maintained levels of PRL and glucocorticoid. During lactation PRL provides a comprehensive signal that fosters synthesis and secretion of milk components and the survival of the alveolar cell. The lactating gland produces milk of a composition defined for the species using several specialized pathways including: (1) exocytosis for the secretion of milk proteins, lactose and divalent ions; (2) a unique lipid secretion pathway that produces membrane-bound milk fat globules; (3) transport systems for monovalent ions, glucose, and amino acids; and (d) transcytosis for the secretion of immunoglobulins and other milk components. Tight junctions form a gasket around the apical surface of the epithelial cells that is open to traffic of large and small molecules in pregnancy but tightly closed in lactation. The volume of milk produced is determined by milk removal from the gland, a function dependent on oxytocin secretion by the posterior pituitary and contraction of myoepithelial cells to force milk out of the alveoli. With the termination of milk removal an orderly involution process involving interactions with immune cells returns the gland to its resting state. In short, we provide a summary of our present understanding of the cellular and molecular biology of mammary development and milk secretion in the context of the whole body mechanisms that ensure adequate flux of nutrients to the gland to provide sufficient milk to meet the needs of the neonate.