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A Morphological and Immunohistochemical Comparison of Mammary Tissues from the Short-Tailed Fruit Bat (*Carollia perspicillata*) and the Mouse[1]

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ABSTRACT

In the present study, mammary tissues from the fruit bat (*Carollia perspicillata*) and mouse (*Mus musculus*) were compared using histological and immunohistochemical methods. Because the female bat exhibits greater reproductive similarities to humans, it might provide a useful animal model for studying mammary physiology and disease with relevance to our own species. In lactating and recently lactating specimens, bat tissue had significantly fewer adipocytes and more collagenous connective tissue compared to the mouse. The proteins Stat5a, keratin 5, Npt2b, and E-cadherin were all similarly localized in mouse and bat mammary tissues taken from lactating animals. The present study demonstrates that whereas the epithelial compartment and the presence of differentiation markers are conserved between the mouse and bat, differences exist in the stromal compartment.

developmental biology, mammary glands

INTRODUCTION

Studies of mammary gland development and physiology are mainly performed in mice and rats. The short generation time, available genetic mutants, and methods for gene modification make these species model organisms for investigations. For economic reasons, a large body of work has also been published regarding bovine milk and lactation. As yet, much less research has been done on lactation in bats and the development of bat mammary glands.

Bats are interesting and potentially informative animals in which to study mammary gland physiology, because they are such an extraordinarily diverse order of mammals. No group, for example, exhibits greater species variability in feeding habits. Foods taken by different bats include insects (both volant and nonvolant), scorpions, spiders, flower parts, nectar and pollen, fruits, smaller vertebrates, and blood (both mammalian and avian). These foods vary not only in nutritional composition but often also differ significantly in seasonal abundance and ease of procurement. This, in turn, creates a variety of problems for the bats with respect to the timing of reproductive activities (e.g., particularly lactation, a time of peak nutritional demand), maternal provision for the nutritional requirements of their suckling young, and transition from milk to an adult diet. It is perhaps not surprising, for example, that the vampire bats, which feed only on blood as adults, exhibit unusually long periods of lactation [1]. During this period the young must learn how to successfully stalk and feed on their prey. Bats also exhibit substantial species differences in the relative development of their young at birth, the extent to which they carry their young during lactation, and the microhabitats within which suckling young spend much of their early lives. Presumably, these factors influence suckling duration and frequency, nutritional characteristics of the milk, and mammary gland function. Finally, most bats bear and nurse only one young, but some members of one family (the Vespertilionidae) produce from two to as many as four young in a litter. For these reasons, it is impossible to generalize very much about lactation in bats.

Until now, much of the research into bat lactation has focused on milk composition and nursing behavior [2]. Several of these studies have explored changes in milk composition across the different phases of the lactation period within species [3–7]. Others have focused on differences across species in an attempt to find correlations between milk composition, size, diet, and/or foraging strategy [3, 5, 8–10]. These studies have only begun to scratch the surface of bat lactation, because few of the more than 1000 bat species have been investigated.

One area that has been particularly neglected is the structure and physiology of the bat mammary gland itself. The few studies that have investigated this gland have focused on the presence of leukocytes in the mammary epithelial tissue [11], temporal differentiation of the epithelial cells during lactation [12], and the unusual phenomenon of male lactation [13]. To our knowledge, no published work has characterized the morphology of any bat mammary gland either histologically or biochemically and compared it with that of other mammals. The mammary gland of the short-tailed fruit bat (*Carollia perspicillata*) would seem to be particularly deserving of study, because this species exhibits many reproductive similarities with humans. These include being monovular and a spontaneous ovulator with a functional luteal phase to its reproductive cycle, having a moderately long cycle terminated by true menstruation, giving birth to a single infant after a relatively long gestation period (normally 113–119 days), and having two mammary glands [14–16]. Furthermore, under natural conditions, fe-

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male *Carollia* apparently spend much of their adult lives pregnant and/or lactating. In captivity, however, this can easily be prevented. Finally, *Carollia* can be conveniently and inexpensively maintained in a research setting [17]. For these reasons, this species might be developed as a useful model for the study of mammary gland development, lactation, and possibly cancer with relevance to humans.

In the present study, we have compared the mammary glands of *Carollia* and the mouse histologically. We have also used immunohistochemistry to evaluate the expression and location of several proteins indicative of secretory mammary epithelium.

MATERIALS AND METHODS

Sources of Animals

The captive animal used in the present study was obtained from a breeding colony maintained at the Weill Medical College of Cornell, New York, NY, and then the Memorial Sloan-Kettering Cancer Center, New York, NY, with the approval of the respective institutional animal care and use committees. Care was provided in accordance with guidelines set forth in the Principles of Laboratory Animal Care (NIH publication no. 86-23).

The remaining animals were collected in the wild with the permission of the Wildlife Section, Forestry Division, Ministry of Agriculture, Land, and Marine Resources of the Republic of Trinidad and Tobago. The collection methods used were approved by the institutional animal care and use committees at Cornell and the SUNY Downstate Medical Center. The bats were captured with hand nets in their diurnal roosts and transported in specially designed, darkened cages. These cages were constructed of wood and wire mesh and were well-ventilated to prevent overheating of the animals. The bats were then held briefly (for up to 12 h) in an air-conditioned laboratory of the Department of Life Sciences at the University of the West Indies (St. Augustine, Trinidad) until processed. Using the same capture and field-transport methods, large numbers of *Carollia* have been introduced into long-term captivity with negligible mortalities from their time of original capture in the wild [15, 18].

Specimens Examined

Most of the bats utilized in this study were collected during May 2002 from a wild population living on the West Indian island of Trinidad. The virgin female was captive-reared, had attained adult body size, but still retained the darker pelage of a juvenile.

Staging of Wild-Caught Specimens

Most adult females in the wild population appear to carry two pregnancies per year and exhibit substantial reproductive synchronization. For many of these females, the first pregnancy appears to be established between September and early November, includes a period of postimplantational developmental delay at the primitive streak stage, and is completed in March or April [15, 19]. Most of these females then conceive again at a postpartum estrus (unpublished observations). In captive animals, this estrus usually occurs between 3 and 6 days after parturition but occasionally may be up to several days later [17]. The second pregnancy in the wild population does not appear to include a significant period of delay. When parous females were collected and examined during late May in four successive years (2000–2003), many carried conceptuses that had progressed to the somite stage or beyond, as would be expected in normal (nondelayed) pregnancies (unpublished observations). Mammary tissues were collected from three early pregnant, wild-caught *Carollia* in late May. Based on the appearance of their mammary glands, these animals had recently given birth and lactated. The probable times at which they had given birth and then conceived again were estimated from data on the timing of the postpartum estrus and conceptus development during normal pregnancies in captive-bred animals [15, 17, 19; unpublished observations]. Mammary tissues were also collected in late May from two *Carollia* carrying newborn young. These animals were unusual in being somewhat out of reproductive synchrony with most of the population. The ages of their babies were estimated using weight data for captive-born animals in two colonies of Trinidadian origin [20, 21; unpublished observations].

Mouse Tissue Samples

Mammary tissue was harvested from primiparous mice on the morning following delivery of pups, fixed for 4 h in Tellyesnizky fixative (70% ethyl alcohol, 5% glacial acetic acid, 5% formaldehyde), and embedded in paraffin.

Tissue Fixation and Immunohistochemistry

The bat mammary tissues were fixed in two different solutions: One gland was fixed in Bouin solution overnight; the other gland was placed in 4% formaldehyde (methanol-free) overnight at 4°C. Both were then stored in 70% ethanol until paraffin embedded. Sections (thickness, 5 μm) of these glands were stained with hematoxylin and eosin or with the Masson trichrome procedure.

For immunostaining, tissue sections were first deparaffinized, rehydrated, and then incubated for 20 min in 0.3% hydrogen peroxide in 10% methanol to block endogenous peroxidase activity. Next, antigen retrieval was performed by microwave treatment in antigen unmasking solution (Vector Laboratories, Burlingame, CA). The sections were blocked with 3% horse serum for 30 min before application of the primary antibody. The primary antibodies used were rabbit polyclonal anti-mouse keratin 5 (1:200; catalog no. PRB-160B; Covance, Richmond, CA), rabbit polyclonal anti-mouse Stat5a (1:200) [22], a rabbit antibody against the Npt2b protein (diluted 1:100; a kind gift from Jürg Biber), and a mouse antibody against E-cadherin (1:200; catalog no. C20820; Transduction Laboratories, Palo Alto, CA). Primary antibodies were applied at 37°C for 1 h except for Stat5a, which was incubated at 4°C overnight. For immunoperoxidase staining, anti-rabbit secondary antibodies (Vector Laboratories) were applied for 30 min at room temperature. The Vectastain Elite ABC Kit (Vector Laboratories) was used for detection according to the manufacturer's protocol. The sections were counterstained with hematoxylin and mounted using Permount (Fisher Scientific, Pittsburgh, PA).

For immunofluorescence staining, the sections were subjected to the unmasking procedure described above and washed with PBS. After a 30-min incubation with 3% horse serum, sections were incubated for 1 h with the primary antibody at 37°C. After washing with PBS, the sections were subjected to a 30-min incubation with fluorescent goat anti-rabbit and goat anti-mouse secondary antibodies (Molecular Probes, Eugene, OR), washed again with PBS, and then mounted using Vectashield (Vector Laboratories). Images were captured with a Zeiss Axioskope and Sony DKC-5000 digital camera and were manipulated using Adobe Photoshop software. Four sections from each animal were used for staining.

RESULTS

Stages Examined

All the bat tissues came from wild-caught animals with the exception of those from one captive-reared virgin animal. One of these females carried a fully furred newborn that had open eyes, weighed 4.5 g, and still had much of its umbilical cord attached. This newborn was estimated to be less than 24 h old and is hereafter referred to as the Lactational Day 1 (L1) specimen. Another female carried an 8.1-g infant. This infant was estimated to be between 6 and 8 days old based on data collected from a captive colony. Weights for newborn animals in this colony were as follows: on the day of birth, 12 infants weighed 4.66–6.39 g (mean ± SD, 5.69 ± 0.54 g); on Day 5 postpartum, 3 infants weighed 6.79–7.72 g (mean ± SD, 7.11 ± 0.31 g); and on Day 10 postpartum, 3 infants weighed 9.20–10.03 g (mean ± SD, 9.72 ± 0.37 g) [19; unpublished observations]. Using data from another captive colony [21], the 8.1-g infant would have been estimated to be between 12 and 20 days old. The reason for the size difference between infants reared in the two colonies is not known.

Three wild-caught animals carried embryos that were estimated to be 44–45, 46, and 48 days postcoitum by comparison with embryos removed at carefully timed intervals during normal (nondelayed) pregnancies in captive-bred *Carollia* [19; unpublished observations]. These animals were assessed to be parous and postlactational because of

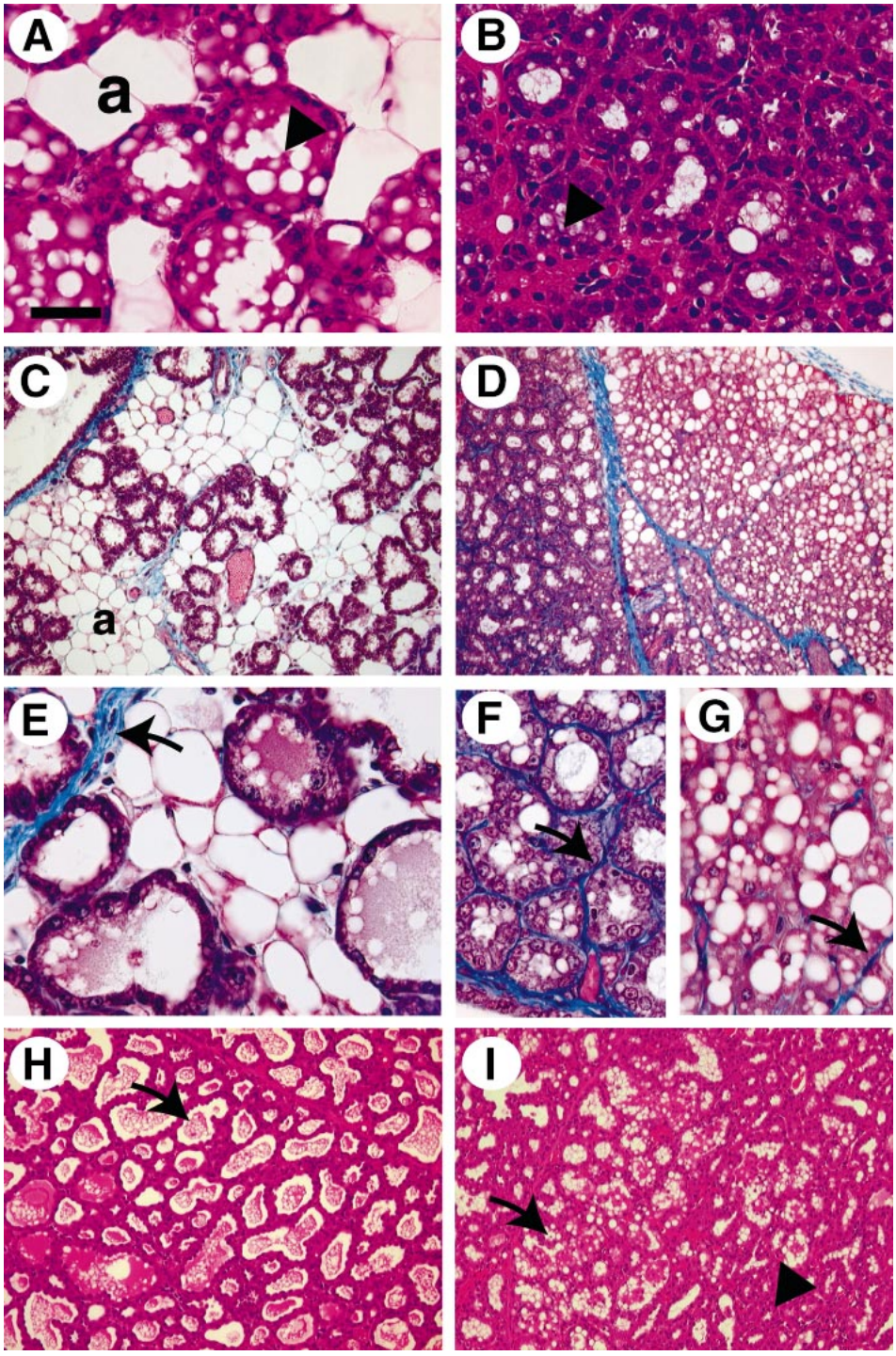

FIG. 1. Comparison of lactating and parturient mammary tissues. Hematoxylin-and-eosin staining reveals similarly structured, round alveoli (arrowhead) that contain lipid droplets and milk in glands from mouse (**A**) and bat (**B**). In the mouse gland (**A**), the alveoli are surrounded by adipocytes (a). In the bat, secretory portions of the gland contain a higher proportion of epithelial elements (**B**, **D**, and **F**), and multilocular adipocytes are segregated on the periphery (**D** and **G**). The Masson trichrome-stained sections show a higher collagen content (stained blue) in bat tissue (**D**) compared to mouse tissue (**C**). Prominent collagen bundles (arrow) are adjacent to large ducts, but not the alveolar tissue, in the mouse gland (**E**). In the bat, each alveolar unit is surrounded by a robust sheath of collagen (arrow; **F**). Collagen bundles (arrow) are also found in the adipose portion of the bat gland (**G**). Hematoxylin and eosin-stained sections from an early pregnant bat that had recently lactated have large alveoli that are lined by cuboidal cells lacking lipid droplet inclusions. Secreted material is present in the lumina (arrow; **H**). Tissue from a bat with an older pup contains areas that resemble the gland from an animal that had just delivered and is actively nursing (arrow) as well as areas that are devoid of lipid droplets (arrowhead; **I**). Bar = 25 μm (**A**, **B**, **E**, and **F**) and 75 μm (**C**, **D**, **H**, and **I**).

the time of year when they were removed from the reproductively synchronized population and the condition of their mammary glands. As is typical of recently postlactational females, their mammary glands exhibited an early to moderate regrowth of hair, enlarged but significantly regressed nipples, and only a low content of retained milk.

Location of the Mammary Glands

Carollia has paired mammary glands, with the thickest portions located on the front of the chest, to either side of the midline. These become progressively thinner as they extend deep into the axillary regions. Sections for the histological and immunocytochemical studies were taken from the thickest portion of the glands.

Carollia has two nipples, located on the lateral sides of the chest. Their locations seem to facilitate carrying of the very large babies during early postnatal life. These bats do not have additional, false nipples. Because the mothers roost hanging by their feet (upside down), the babies are usually positioned head down when suckling. During early postnatal life, each baby adheres to its mother, both at rest in their roosts and in flight, by holding onto one of her nipples with its mouth and her ventral body surface with its feet. As the babies grow, they generally take to adhering diagonally to the females, in ''bandolier'' fashion. When

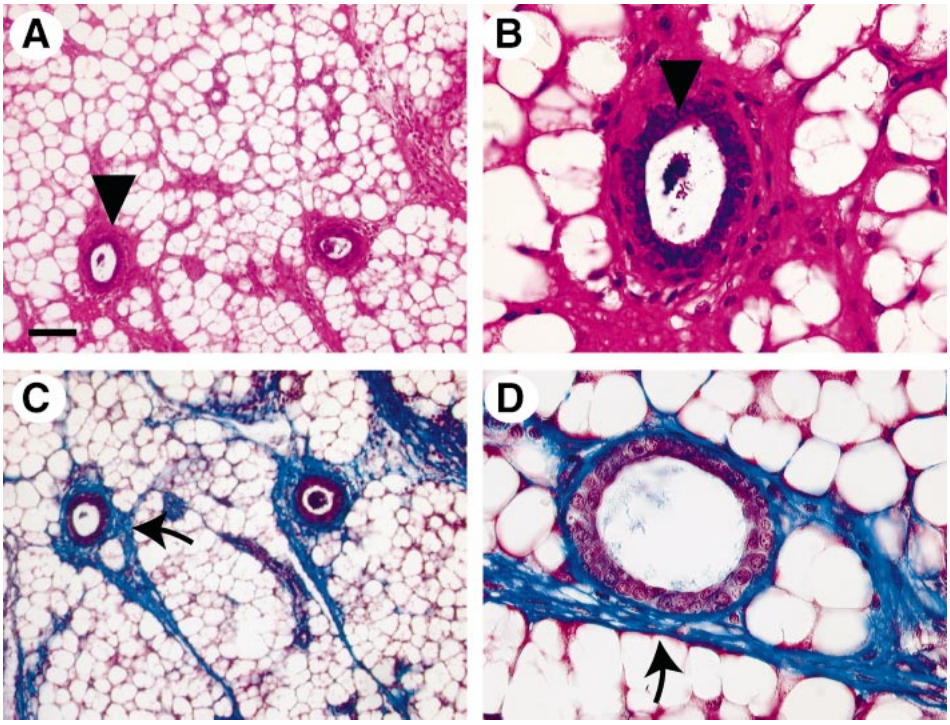

FIG. 2. Virgin bat mammary tissues. **A** and **B**) Hematoxylin and eosin-stained sections at low (**A**) and high (**B**) magnifications. **C** and **D**) Sections stained with Masson trichrome. In the virgin state, bat glands are filled with sparse ducts (arrowhead), and the collagen (arrow) distribution is very similar to the lactating sample. Bar = 60 μm (**A** and **C**) and 20 μm (**B** and **D**).

the babies become still larger, they hang separately by their own feet in the roost as they suckle.

Histological Comparison of Mammary Glands from Postparturient Bats and Mice

Mammary tissues collected on Day 1 of lactation from both species contained abundant, round alveoli composed of epithelial cells surrounding lumina filled with milk secretion and large lipid droplets (Fig. 1, A–D). The epithelial component of the bat tissue had a denser appearance in comparison to the mouse tissue. A paucity of adipocytes was found within the parenchyma (secretory portion) of the L1 bat mammary tissue relative to the mouse. The alveoli were surrounded by a prominent layer of collagen fibers (Fig. 1, D and F). Adipocytes were found only at the periphery of the gland and were separated from the epithelial tissue by bundles of collagen (Fig. 1, D and G). In addition, the L1 bat stroma contained fibroblasts.

Generally, the mouse alveoli were larger than the bat alveoli (Fig. 1, A and B) and were interspersed with large adipocytes in the stroma (Fig. 1, A and C). Collagen was also found within the mouse mammary tissue; however, it was relatively inconspicuous immediately around the alveoli, and much was instead packaged into large bundles scattered throughout the tissue (Fig. 1E). Compared with the bat gland, the mouse stroma had few to no fibroblasts interspersed with the adipocytes (Fig. 1, E and G).

Bats Parturient and Pregnant

Mammary tissue collected from three pregnant bats (between 44 and 48 days of gestation) contained alveoli of similar density with no stromal tissue being apparent. However, the epithelial cells were cuboidal and lacked the lipid droplets seen in the L1 bat. The lumina were larger, and the intralumina material was lighter (Fig. 1H). Based on our observation of the annual reproductive cycle of this bat population, these bats likely had nursed recently and conceived within a few days of delivery. Sections from tissues of the bat with the 8.1-g pup displayed a heterogeneous appearance, with some areas containing alveoli with lipid-containing secretory cells and areas that resembled the less active tissue of the pregnant, postlactational animals (Fig. 1I). This heterogeneity is observed in mice at the end of lactation, when pups are nursed infrequently.

Virgin Bat Tissues

The stromal component of mammary tissue from virgin bats contained few fibroblasts and many adipocytes that were similar in appearance to those of the fat pads of mouse mammary glands (Fig. 2, A and B). The mammary ducts within virgin bat tissue were lined by epithelial cells and surrounded by collagen fibers, similar to those seen in the L1 bat tissue (Fig. 2, C and D).

Expression of Differentiation Markers

In an attempt to further characterize the cell types and epithelial cell differentiation of bat mammary tissue, we used antibodies that are able to discern the differentiation status of secretory mammary epithelial cells in the mouse [23]. Keratin 5, signal transducer and activator of transcription Stat5a, Npt2b, and E-cadherin are apparently well-conserved across species, because all four proteins were easily stained in the bat tissue. Keratin 5 was observed in the myoepithelial cells of the alveoli, immediately surrounding the secretory epithelial cells, in Lactational Day 1 tissue from both species (Fig. 3, A and B). Stat5a, a protein obligatory for the prolactin signal-transduction pathway, is located in the nuclei of mammary epithelium of lactating mice [23] (Fig. 3C). Stat5a was also found to be nuclear in the secretory epithelial cells of the bat (Fig. 3D). Npt2b, a sodium-potassium cotransporter in secretory epithelial cells, was detected on the apical membrane of the alveolar cells of both the mouse and the bat mammary tissue (Fig. 3, E and F). Finally, E-cadherin, an epithelial cell adhesion

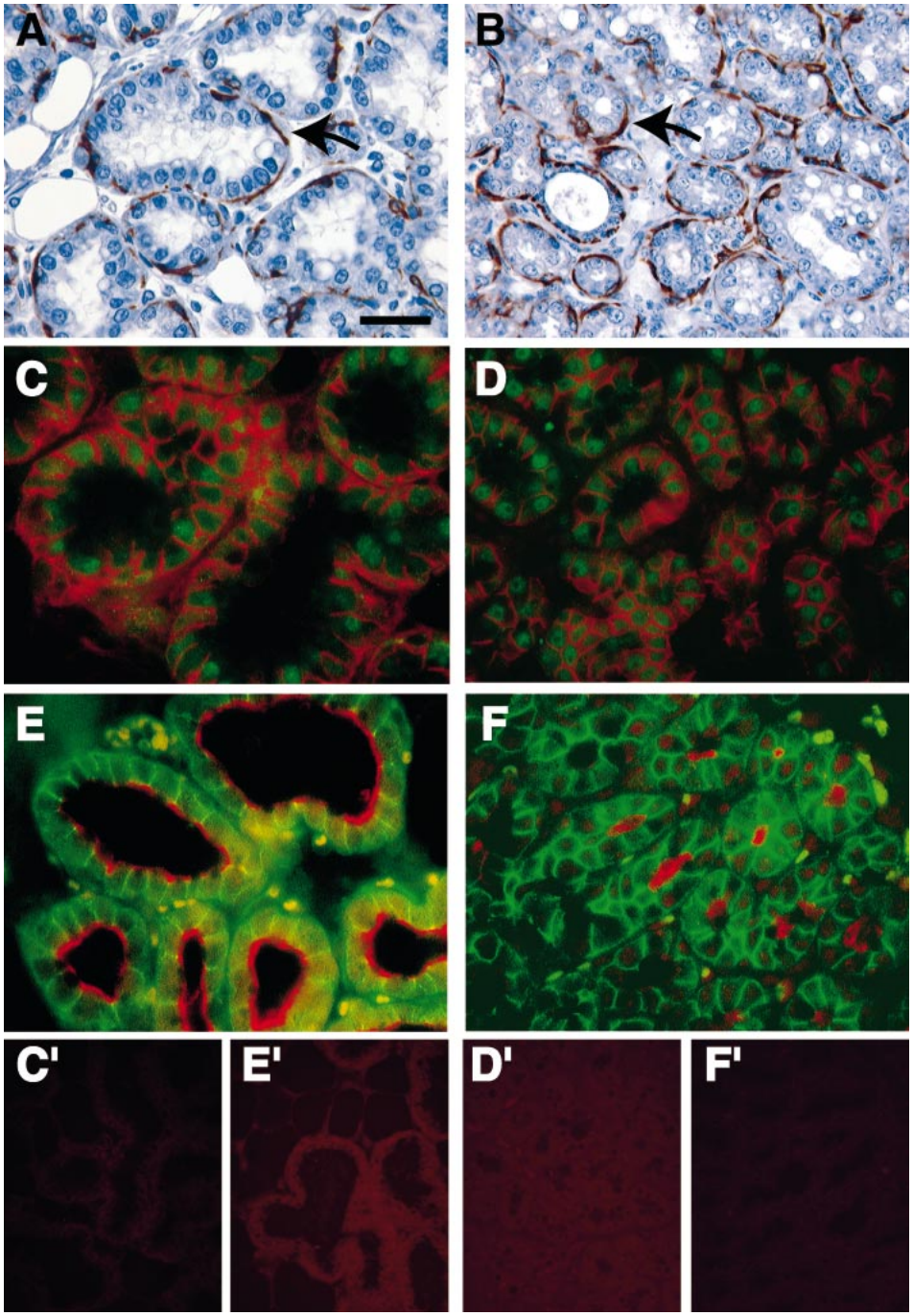

FIG. 3. Immunohistochemical characterization of epithelial cells. Alveoli in mouse (**A**) and bat (**B**) glands are surrounded by a layer of myoepithelial cells (arrow) that express keratin 5 (brown stain). Nuclear localization of Stat5a (green) can be seen in both mouse (**C**) and bat (**D**) epithelial cells. Npt2b, a transporter protein, is localized on the apical membrane (red) of both mouse (**E**) and bat (**F**) secretory epithelial cells. E-cadherin is visible in the plasma membrane of mouse (red in **C** and green in **E**) and bat (red in **D** and green in **F**) epithelial cells. **C′** through **F′** are negative controls stained with omission of the primary antibodies to demonstrate staining specificity. Bar = 20 μm (**A** and **B**) and 16 μm (**C**–**F**).

protein, was present on the lateral borders of the secretory epithelial cells in the mammary glands of both species (Fig. 3, C–F). These observations demonstrate that despite differences in stromal composition, the epithelial organization and the prolactin-signaling pathway in the mammary glands of bats and mice are similar. Furthermore, both species utilize the same membrane transporter in lactating tissue.

DISCUSSION

Carollia has considerable potential for development as a relevant model in mammary gland research. We have demonstrated that the mammary epithelium of this bat expresses some of the same protein markers as that of the mouse, currently the most commonly utilized model. Moreover, the reproductive biology of the fruit bat is much more like that of humans. They are monovular, spontaneous ovulators with a functional luteal phase to their nonpregnant cycles. These cycles are moderately long and are terminated by true menstruation, apparently induced by involution of the most recent corpus luteum [14, 16]. Exact cycle length has not been determined. However, it appears to be between 24 and 30 days, because breeding activity is spread throughout the first month after housing mature, nonpregnant females with stud males [17]. *Carollia* eventually gives birth to a single infant after a relatively long gestation period (normally 113–119 days) [15, 19, 20]. Finally, *Carollia* is small and relatively inexpensive to maintain and breed in large numbers within modest research facilities.

The most remarkable difference between mammary tissue in the mouse and the bat *Carollia* at parturition is the

paucity of white adipose tissue in the bat. Although both mouse and bat demonstrate round alveoli with secretory epithelial cells surrounded by a layer of myoepithelial cells, the mouse gland contains a large amount of white adipose tissue and comparatively little fibrous connective tissue within the fat pad. Cows, sheep, and humans also have much more of a fibrous connective tissue component within their stroma (for review, see [24]). Collagenous connective tissue may be prominent in mammary glands of the fruit bat for structural reasons. *Carollia* gives birth to large and precocious young. These young are extensively furred, with open eyes, and weigh approximately 30% of the mother's mass on the day of birth. After giving birth, the females continue to carry their growing young, both when resting within the diurnal roost and during foraging flights. This apparently continues for much of the next couple of weeks [21]. In this respect, *Carollia* is different from a number of other bat species that leave their young behind in their diurnal roosts while foraging [2, 25]. In the case of *Carollia*, which generally prefer to hang from the ceilings of their roosts, firm adherence to the mother early in life is extremely important. Accidental separation could be directly injurious to the young or subject them to a greater risk of predation. The young attach themselves primarily by firmly grasping one of the mother's nipples with the mouth and teeth and her fur with the feet. Abundant collagenous tissue within the mammary glands no doubt strengthens them to withstand the weight of the infants, both when at rest and during vigorous muscular movements while in flight. The importance of this is indicated by the fact that some bats have also evolved ''false'' or ''pubic'' nipples on the ventral body surfaces closer to the genitalia. Although these function primarily as ''holdfasts'' for the infants, in some species they have a lactiferous function as well [2, 26].

In mice, much of the adipose tissue within the mammary gland is replaced during the epithelial proliferation and differentiation phases of late pregnancy. By midlactation, white adipose tissue is virtually nonexistent within the gland. The stroma is then reorganized during the involution stages and once again accumulates an abundance of lipid-rich adipocytes. The fibroblasts also become more numerous as the epithelial cells die [27]. In *Carollia*, on the other hand, very few adipocytes are evident within the mammary glands at the onset of lactation. The stroma is instead characterized by fibroblasts and prominent collagenous bundles, with accumulations of adipose tissue being disposed toward the periphery of the gland. Because the mammary gland in virgin *Carollia* is mostly comprised of white adipose tissue, the onset of pregnancy must initiate a large amount of epithelial proliferation and development, which then replaces the adipose tissue.

Prolactin is present in bats and is thought to stimulate mammary gland development and the production of secretions during lactation [28]. In several bat species, the number of prolactin-expressing cells in the anterior pituitary gland increases during midpregnancy and plateaus during late pregnancy and lactation [29, 30]. Our demonstration of nuclear Stat5a in the lactating bat tissue supports the presence of a similar Jak2/Stat5 signaling pathway as in the mouse.

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