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Award Number: DAMD17-98-1-8296

TITLE: Use of Intraductal Adenovirus Transduction to Assess the Mammary Tumorigenic Potential of a Constitutively Active Prolactin Receptor

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REPORT DATE: September 1999

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	September 1999	Annual (15 Sep 98 - 14 Sep 99)

4. TITLE AND SUBTITLE
Use of Intraductal Adenovirus Transduction to Assess the Mammary Tumorigenic Potential of a Constitutively Active Prolactin Receptor

5. FUNDING NUMBERS
DAMD17-98-1-8296

6. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release
distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 Words)*

We have constructed adenovirus including PRLR and ΔPRLR (the constitutively active form), AKT, kinase dead AKT, the oncogenes fyn and src and ΔAKT (the activated form). In addition a number of control vectors containing green fluorescent protein and β-galactosidase are available. Other constructs relevant to the mammary gland in addition to those listed in the proposal including progesterone receptor (PRA and PRB) have been made. We have found that adenovirus transduction of the mammary epithelium persists up to 3 weeks in vivo and that we obtain transduction of about 20% of the cells. Green fluorescent protein can be visualized *in vivo*.

14. SUBJECT TERMS

15. NUMBER OF PAGES
31

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

FOREWORD

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Margaret P. Wille

PI - Signature Date

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INTRODUCTION

The mammary ductal system, the site of initiation of the majority of breast cancers, is uniquely accessible from the external surface of the body because it terminates with openings on the integument. We have shown that the mouse mammary ducts can be transduced with adenovirus during all developmental stages and that expression of the transduced gene persists some time in the absence of any sign of inflammation. In the first part of this research we will optimize intraductal injection of adenoviral vectors into the mouse mammary gland and determine whither transduction with oncogenes of known tumorigenic potential can increase the proliferative potential of mammary ductal cells. Prolactin has been implicated in mammary tumorigenesis in a number of studies. However, both its role and that of its receptor have been controversial. To evaluate the tumorigenic potential of a constitutively active PRL-R mutant *in vivo* it will be introduced intraductally on an adenovirus vector into the mammary epithelium of normal mice as well as transgenic mice with enhanced susceptibility to mammary tumors. It will be important to determine whether the aPRL-R activates signal transduction *in vivo* as it does *in vitro.* This experiment can be best carried out at the single cell level in sections from tissues where both non-transduced and transduced cells exist side by side. Novel antibodies specific to the phosphorylated intermediate of STAT 5 will be used to develop immunocytochemical methods for evaluating activation of signal transduction pathways in these sections.

BODY

The proposed work was divided into the following tasks.

1. Construction of adenovirus vectors
2. Objective A: Studies of efficacy and persistence of adenovirus transduction
3. Objective B: Transduction with known oncogenes
4. Objective C: Transduction with aPRLR and PRL in adenovirus vectors
5. Objective C: Transduction with activated JAK2 and STAT5
6. Objectives B and C: Evaluation of tumorigenesis
7. Objective C: Evaluation of alterations of signal transduction pathways.

These tasks will be necessary to accomplish the following objectives:

Objective A: *Studies of efficacy and persistence of adenovirus transduction*
Objective B: *Enhancement of oncogenesis.*
Objective C: *Tests of the hypothesis that prolactin promotes mammary tumorigenesis*
The tasks will be divided among the three years of the project as follows:

Progress on these tasks is as follows:

Task 1. Construction of Adenovirus Vectors. The adenovirus vectors planned, completed and tested are listed in Table 1 (Next Page). We have constructed the following vectors described in the proposal including PRLR and ΔPRLR (the constitutively active form), AKT, kinase dead AKT, the oncogenes fyn and src and ΔAKT (the activated form). In addition a number of control vectors containing green fluorescent protein and β-galactosidase are available. Other constructs relevant to the mammary gland in addition to those listed in the proposal including progesterone receptor (PRA and PRB) have been made or are planned to be made

in the next few months.

Table 1. Viruses constructed, to be constructed, or to be grown by the adenovirus core.

virus	plaque purified	PCR	protein	activity
PRA	X	X	X	X
PRA-FLAG				
PRB	X	X	X	X
PRB-FLAG			X	
PRLR-FLAG	X	X	X	X
ΔPRLR-FLAG	X	X	X	X
claudin				
claudin C-tail				
XO pr-luciferase				
Dr. XO				
butyrophilin				
butyrophilin C-tail				
AKT	X			
act AKT	X	X	X	
kin dead AKT	X	X	X	
Fyn	X	X	X	
kin-dead Fyn				
Src	X	X	X	
kin dead Src				
kin dead JNK				
kin dead p38				
WAP-LacZ				
β-casein-LacZ				
β-casein-LacZ mut GRE 1/2 site				
β-casein-LacZ mut STAT site				
various pr. mut. WAP-LacZ				
STAT5A				
GRP				
CBP				
LacZ	X	X	X	X
GFP	X	X	X	X
pTP$^-$ LacZ	X	X	X	X
pTP$^-$ GFP	X	X	X	X

The status of analysis of the various viruses is indicated where "plaque purified" means that virus from the transfection used in the attempted construction have been plaque purified, "PCR" means that PCR analysis of plaque purified stocks has been performed with positive results, "protein" means that western analysis of extracts of transduced cells has yielded positive results, and "activity" means that tests of the expected activity of the protein in transduced cells have yielded positive results. Viruses that have not yet been plaque purified include certain of the viruses expected to be constructed by the adenovirus core.

Task 2. Objective A: Studies of efficacy and persistence of adenovirus transduction. These studies are in progress. More time than anticipated was spent in developing a reproductable method of intraductal injection. However, we have used both GFP and lac-Z reporter systems at this point and appear to have about 20% of the cells transduced with quite high efficiency. Persistance is about 2 weeks maximum. The details of the technique are the subject of a paper accepted for publication (see appendix). In addition to the work specified in the proposal we have found that GFP can be visualized in the live mouse, providing another venue for the eventual experiments on signal transduction intermediates.

Task 3. Transduction with known oncogenes. Controls for this experiment been started. We have mice over-expressing the *neu* oncogene on hand and are currently determining the tumor incidence in virgin mice and mice that have undergone a single pregnancy and lactation.

Task 4. Transduction with aPRLR and PRL in adenovirus vectors. These vectors have currently been constructed and have been shown to be active in *in vitro* cell culture systems. They will be used in mice shortly.

Task 5. Transduction with activated JAK2 and STAT5. To be completed

Task 6. Evaluation of tumorigenesis. This task has been started in *neu* mice.

Task 7. Evaluation of alterations of signal transduction pathways. To be completed.

KEY RESEARCH ACCOMPLISHMENTS

- Construction of adenoviral vectors
- Studies of efficiency and persistence using GFP and Lac-Z reporters
- Ability to visual fluorescent probes in the live mouse.

REPORTABLE OUTCOMES

Manuscript:
Nguyen, D.A.D., Beeman, N., Lewis, M.T., Schaack, J., Neville, M.C. Intraductal injection in the mouse. J. Mammary Gland Biology and Neoplasia. Supplement on Methods, In Press.

Abstract:
Neal Beeman and Margaret C. Neville. Injection of adenovirus vectors into the mouse mammary gland achieves efficient, long term transduction of mammary epithelial cells without inflammation. American Society for Cell Biology, December, 1999.

Presentations:
M.C. Neville. Intraductal Injection in the Mouse. Bar Harbor Conference "Modelling Human Mammary Cancer in Mice". Brief Platform Presentation

CONCLUSIONS:

Thus we have made a good start on the project and continue to follow the track laid out in the proposal.

REFERENCES: See Reportable outcomes, above.

APPENDICES. Manuscript: Intraductal Injection in the Mouse

Abstract

Abstract for ASCB, Washington, D.C. December, 1999

Injection of adenovirus vectors into the mouse mammary gland achieves efficient, long term transduction of mammary epithelial cells without inflammation. Neal Beeman and Margaret C. Neville. American Society for Cell Biology, December, 1999.

A non-invasive, non-inflammatory, gene delivery system is needed for controlled temporal and spatial expression of introduced genes in the mammary epithelium. Adenovirus vectors encoding either LacZ or GFP reporter genes were injected into the mammary lumen through the teat canal. LacZ transduced glands were processed for histochemistry. GFP transduced glands were surgically reflected, maintaining blood flow, and observed in living mice. Transduction was patchy and much of the gland was negative; however, up to 100% of cells were positive for reporter activity in transduced areas. Only luminal cells were transduced. Lactation appeared to be unaffected. Transduced alveoli were distended with milk and milk fat globules. Milk fat globules were observed within transduced cells and immunofluorescence demonstrated the presence of milk proteins in morphologically normal transduced cells. Transduced glands did not show lymphocytic invasion and transduction persisted for over 60 days without pathology. A population of transduced cells has been shown to survive the normal involution of the mammary gland at the end of lactation. These findings suggest that adenovirus vectors provide an effective means to alter the genetics of the mammary epithelium in a spatially and temporarily controlled manner.
Supported by DOD Grant BC971759.

Intraductal injection into the mouse mammary gland

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ABSTRACT

The mammary epithelium is continuous with the skin through a teat canal leading to a single primary duct in the mouse. Using fire-polished micropipettes 60 to 75 μm in diameter it is possible to inject any desired substance directly through the teat into the lumen of the mammary gland. If the primary duct of the gland is exposed surgically hypodermic needles can also be used for injection. Both techniques can be used to investigate the state of tight junctions in the mammary gland by examining transepithelial movement of radioactive sugars or fluorescent-labeled proteins. The intraductal or up-the-teat injection of adenoviral and plasmid vectors provides a convenient means of altering gene expression in the luminal epithelium. Finally, injected fluorescent probes as well as adenovirus-transduced green fluorescent protein can be directly visualized in the mammary gland in the living mouse using confocal microscopy.

Key Words: adenovirus, intraductal injection, up-the-teat injection, mouse mammary gland

Abbreviations: GFP, green fluorescent protein; pfu, plaque forming units

The mammary epithelium is accessible from the exterior of the animal through the teat canal. This unusual characteristic is technically advantageous and was exploited nearly 30 years ago by James Linzell and his colleagues for assessing the transepithelial permeability of the mammary gland to a variety of substances in the pregnant and lactating goat (1-3). Falconer injected ^{125}I-prolactin intraductally in rabbits to assay its distribution. More recently intraductal injection of the mammary gland has been used in the rat for transduction of the epithelium with retroviruses (4) and adenovirus (5,6). Intraductal injection of antitumor agents has been proposed for breast cancer therapy in women but reports of its efficacy do not seem to be available in the literature. This powerful technique has not been much exploited in the mouse, because of the small size of the teats and major mammary duct. This difficulty can be overcome with the use of appropriately sized micropipettes inserted directly into the teat canal through the nipple (up-the-teat injection) or with the use of large gauge needles inserted into the primary duct of the surgically exposed mammary gland. In this laboratory the technique has been used to assess the status of the tight junctions between epithelial cells by measuring the permeability of the mammary epithelium to radioactive sucrose and to fluorescently labeled proteins. It has also been used for adenoviral transduction and transfection with plasmids and for visualization of the mammary gland *in vivo.*

In this article we describe fabrication of the micropipettes used for up-the-teat injection. For the injection we utilize a dissection microscope and a drawn micropipette mounted on a micromanipulator to enter the teat canal. An alternative technique uses surgical exposure of the 3rd or 4th mammary gland and injection with a Hamilton syringe directly into the primary duct. We illustrate the use of up-the-teat injection to measure transepithelial permeability using both [^{14}C]-sucrose and fluorescently-labeled proteins. Transduction of the luminal epithelium with an

adenoviral vector and visualization of the mammary lumen with the confocal microscope in the living mouse are also described briefly.

Materials:

General

Anesthetic: Pentobarbital, I.P. A 1:10 dilution of a 50 mg/ml solution is injected in sufficient volume to give 60 μg/gm body weight. A 10 to 20 μg/gm as follow-up dose is used if the mouse must be maintained under anesthesia for some time.

Dissecting microscope

Fiber Optic Light Source (Fisher Scientific)

Fabrication of micropipettes for up-the-teat injection.

Bunsen burner with flame spreader

Fine forceps (old ones that can be put in the flame)

10μl or 25μl glass micropipettes (Drummund Precision Disposable micropipettes, 10 or 25 μl or Wiretrol Calibrated Micropipettes with stainless steel plunger, up to 100 μl, Fisher Scientific)

Compound microscope with calibrated ocular reticle and adjustable stage

Micromanipulator (small, Prior, U.K.)

Platinum wire for making electrode for Microforge (26 gauge, Fisher)

Other supplies for constructing microforge (homemade) include variable power source and various electrode holders and clamps to fit the microscope stage available

Up-the-teat injection:

Prepared micropipette with 60 to 75 μm tip (see above)

Drummond digital micropipettor (10 or 100µl; Fisher Scientific)

Micromanipulator

Blunt-tipped forceps

Electric light to keep mouse warm

Intraductal injection:

Surgical:

Dissecting microscope and fiberoptic light source

Cork board (4" x 6")

Straight pins

Surgical tape

70% Ethanol

Opening scissors

Forceps - Mouse tooth and smooth

Cotton-tipped wooden applicators

Wound clips or suturing supplies

Recovery chamber, warm

Black "Sharpie" or magic marker (optional)

Experimental:

Injectable substance (viruses, plasmids, tracer dyes etc.)

301/2G needle or higher (Becton Dickinson & Co. Franklin Lakes, NJ 07417-1884)

Hamilton syringe with hub for removable needles (50µl) or similar

Tracer dye (optional) - *e.g.*, 0.1-0.5% trypan blue or Evan's blue in 1X PBS or 0.9% saline

Measurement of transepithelial permeability

^{14}C-sucrose (2 x 10^6 cpm/ 8 µl

Drummond digital microdispenser set to 8.8 µl

Injection apparatus as above

Observation of the mammary alveolus in a living mouse

Inverted confocal microscope with removable stage diaphragm.

Heating pad

Large coverslips

Duct or surgical tape

Fine scissors

Forceps

Blunt probe

Gauze pads

Ringer's solution

METHODS

Fabrication of micropipettes for up-the-teat injection.

The goal is to pull a calibrated micropipette (Drummund Precision Disposable micropipettes, 10 or 25 µl) so that it has a strong, tapered and polished tip 60 to 75 µm in diameter. The trick in achieving proper tip size and conformation is to pull each pipette in two stages. The first pull is slow and reduces the diameter by a factor of two; the second pull is rapid and results in a gradual taper to a diameter less than 75 µm. The pipette is then broken under the microscope and fire polished in a microforge.

Details: Attach a flame spreader to a large Bunsen burner or fashion a flame spreader from heavy

foil. Adjust the flame to one inch. One of the tines of the forceps is inserted into the lumen of the dispensing end of the pipette and the glass is grasped gently. The plunger end is held with the fingertips. The dispensing end of the pipette is held at the top of the flame with the pipette level and perpendicular to the band of flame. Light tension only is maintained so that the wall thickness is unchanged and the glass melts to form an hour glass shape (Figure 1A, pipette 1). When the dispensing end begins to move, the pipette is removed from the flame as rapidly as possible keeping the glass tube as straight as possible. Dip the dispensing end in water to cool. Water will be drawn up into the pipette. The external diameter at the waist of the pulled section is about one half the original diameter of the tube (Figure 1A, pipette 1).

For the second pull the pipette is held as before, but tension is exerted horizontally throughout the pull. The pipette is exposed to the flame as above with the band of flame at the waist of the hour glass. The water in the lumen may boil but this will not affect the correct pull. As the pipette becomes plastic, both hands begin to move apart rapidly. At this point the pipette is immediately removed from the flame while horizontal tension is maintained and the dispensing end dipped in water to cool. Water should be drawn into the pipette past the waist of the elongated hour glass. The waist of the hour glass should be 75 μm or less in external diameter at its narrowest point; and the wall of the pipette at this point is very thin; it is often flexible (Figure 1A, pipette 2). If water is not drawn into the pipette it has been sealed. The end of the pipette is often pulled off accidentally during this step. These and the sealed pipettes should be examined under the microscope to determine whether the lumen is patent at the waist. If not, they are discarded.

The pulled tubes are broken with fine forceps under a dissecting microscope to obtain the a tip of proper diameter. The ocular reticle of a *compound* microscope is used to locate the

region of the pipette with an external diameter between 60 μm and 75. The shape of this target region is carefully noted and the pipette is placed flat on the stage of a *dissecting* microscope. The pipette is grasped just below the target diameter with fine forceps held flat against the stage. The forceps are rotated gently to force the dispensing end upwards and snap it off. If the forceps are maintained at the level of the stage and perpendicular to the pipette, the dispensing end will snap off at the desired diameter and a reasonably smooth break will be obtained (Figure 1A, pipette 3). Once a pipette of ideal tip diameter is obtained it can be used as a guide in breaking other tips correctly under the dissecting microscope.

The broken pipettes are polished on the stage of the *compound* microscope with a platinum wire electrode (or microforge) held in a micromanipulator (Figure 1B). The broken pipette is placed flat on the microscope stage so the tip is just visible with a 10X objective. Using the micromanipulator, the tip of the microforge is advanced into the other side of the field of view (M, Figure 1B). The variable power source is used to adjust the power gradually upward until the wire just begins to glow in the microscope field. The wire loop will extend forward slightly as it is heated. When the pipette tip and the tip of the microforge are brought within 3 to 4 micrometers of each other, a smoothly broken tip will be polished rapidly and should be removed as soon as the tip appears smooth. More jaggedly broken tips can often be polished smoothly if they are positioned carefully near the electrode so jagged projections are closer to the heat source. It is often necessary to reposition such a tip several times during polishing. Polished tips do not have to be perfectly regular. Two finished tips are shown in Figure 1C.

Before attempting an injection, always test the pipette to make certain that fluid can be drawn up and expelled freely. A pipette can be used until it is broken or clogged. For cleaning the pipette can be attached to a vacuum apparatus and large volumes of fluid drawn rapidly

through the pipette. Seventy % ethanol followed by sterile water is recommended for cleaning pipettes.

Up-the-teat injection.

Animal preparation. For injection into the lumen of the mouse mammary gland through the teat canal (up-the-teat injection), the mouse is positioned on a platform beneath a dissecting microscope (Figure 1E, A). A micropipette (E) inserted into a Drummond digital microdispensor (B) held in a micromanupulator (C) is positioned above the 2nd, 3rd, or 4th nipple that has been prepared as described below. A fiber optics light (D) is convenient for visualizing the field. The tip of the micropipette is inserted into the lumen of the teat canal and 8 to 25 µl of solution injected. For larger volumes a Wiretrol micropipette (5 to 100 µl) can be held in the micromanipulator using a length of narrow bore rubber tubing to fit the pipette to the micromanipulator and the solution can be dispensed with the accompanying stainless steel plunger.

The first step is to prepare the teat for injection. The removal of the hair around the teat to obtain a clear unobstructed work area can facilitate the injection procedure. For lactating mice, the opening for the teat canal is readily apparent at the center of the teat and the capillary tip will readily slip in when pushed against the teat at this location. Staining the teat with trypan blue will make the opening more visible in the beginning. In nonlactating mice, the opening to the teat canal is often covered with a layer of dead skin which must be removed by repeated gentle grabbing and pulling with a pair of microscissors.. Although the layer of dead skin can also be removed by cutting off a thin layer of skin at the tip of the nipple, excessive cutting can deform the nipple and makes the task of locating the opening more difficult.

Injection. To insert the tip of the capillary tube into the teat canal, it is first positioned

immediately adjacent to the base of the nipple using the micromanipulator. The center of the nipple is then positioned under the tip of the capillary by manipulating the skin around the nipple with a pair of blunt forceps. Direct manipulation of the nipple is possible, but can result in damage. Next, the center of the teat is pushed upward against the tip of the capillary using the natural elasticity of the nipple or a light upward motion of the forceps. If correctly performed the tip of the capillary should enter the teat canal easily. If it does not, then the tip may not be positioned properly at the opening of the teat canal or the opening is still covered by skin. A delicate touch is critical to these procedures.

Finally, the material in the capillary tube is injected through the teat into the lumen of the mammary gland. Since the tip of the capillary was initially positioned at the level of the base of the nipple, the nipple will be under compression and exert an upward force against the capillary tip. This upward push helps to prevent the capillary tip from slipping out of the teat canal during slight movements of the mouse, such as breathing. The nipple and the inserted capillary tip should be observed carefully throughout the injection as even slight movement can dislodge the tip from the teat canal.

Intraductal injection.

For younger or nulliparous virgin mice, injection through the teat canal is difficult. In this case the gland can be exposed surgically and the primary duct just below the teat injected with a Hamilton syringe.

Animal preparation: The mouse is anesthetized and affixed on its back to a cork board using either surgical tape or straight pins through the paw webbing. The ventral fur is wetted with 70% ethanol to reduce fur entry into the wound. If the animal has a light coat color (e.g. Balb/C, CD1) the nipple region may be "painted" using a black magic marker to provide better visualization of

the nipple and primary duct.

A 1.5-2 cm mid-ventral incision is made beginning just above the pubic area. Two additional incisions are made from the base of the incision at an angle toward the midpoint of the hind legs. The angled cuts should be well behind the nipple for the #4 mammary gland. The triangular patch of skin together with the attached #4 mammary gland are peeled back gently using mouse tooth forceps and a cotton-tipped wooden applicator and pinned to the cork board such that the skin is tight and the gland is roughly flat.

Injection: Locate the nipple under the dissecting microscope - it will appear as a greyish circular spot against a lighter background of the skin. The nipple should be near the corner created by the two incisions but not so close that the pin will interfere with the injection.

Next, locate the primary duct of the mammary gland. The primary duct will extend from the nipple back toward the #4 fat pad. If necessary, use the smooth forceps to wiggle the fat pad gently. The location of the primary duct should become apparent since it is attached to the nipple and will move with the fat pad.

Load the syringe with approximately 25-30µl of the injectable substance; there will be some dead air space in the syringe. Using an oblique angle of attack in the same orientation as the length of the primary duct, carefully insert the tip of the needle into the lumen of the primary duct near the nipple. It should be inserted a few millimeters. The skin may need to be held with forceps to facilitate needle penetration. Take care not to penetrate the back wall of the duct. Slowly inject 10-20µl of the substance into the gland. If done correctly, the entire mammary ductal tree will fill with the injected fluid. The exact volume tolerated by the gland before the terminal end buds or duct termini burst varies with the age of the animal and the extent of gland development and should be determined empirically using a tracer dye on a few test animals.

After injection, wait about 5 seconds for the back pressure to reduce and, using the smooth forceps, gently squeeze the fat pad around the needle and slowly pull the needle out of the duct. Pinch the duct with the forceps as the needle passes the tip and hold to prevent leakage of the injected substance. Close the animal using either wound clips or sutures and allow recovery in a warm chamber or fresh cage. Repeat for the contralateral gland.

Injection of adenovirus.

Adenovirus Preparation. Adenovirus preparation has been described in detail in Li *et. al* (7) in this issue. For these experiments adenovirus containing either green fluorescent protein (GFP) or Lac Z under control of the cytomegalovirus major immediate early promoter (CMV promoter) was used. Adenoviral E1A and E1B genes within the left end of the adenovirus chromosome were deleted so that the virus was replication defective, although replication may not be completely eliminated (8).

Transduction of the mammary epithelium *in vivo*: We have found that adenovirus transduction is more efficient when the virus is injected into the gland of the pregnant animal. We have had no experience with the virgin animal, although we have found it possible to inject intraductal into a non-pregnant animal, if she has had a litter of pups. Presumably the presence of large amounts of milk in the lumen interfere with viral infectivity in the lactating animal. For observations during lactation we carry out the injections on day 19 of pregnancy, injecting 5-8 x 10^{10} pfu (plaque forming units) of virus particles diluted with Ringer's containing 10% calf serum and 25 mM polybrene (hexmethrine bromide, Sigma). In general we inject a volume of 50 µl. Although we often assay the mouse at 3 days of lactation, marker expression has been found to persist at least a week or two longer.

Observation of the mammary alveolus in a living mouse

For certain types of studies of milk secretion it may be iimportant to visualize the mammary alveolus in a living mouse. For this purpose fluorescent probes can be injected up the teat or intraductally to visualize the mammary lumen. Adenovirally encoded fusion proteins containing GFP can also be injected prior to the experiment for visualization *in vivo*. The fourth mammary gland is the easiest to visualize with the anesthetized mouse placed on the stage of an inverted microscope equipped for laser or digital confocal fluorescence microscopy. Some preliminary results are given below.

Preparation of the animal: The microscope stage is warmed with a stage heater (ideal) or heating lamp. If a heat lamp is used it must be turned off while taking micrographs. The stage insert is removed from the microscope and placed with a 50 ml centrifuge tube of Ringer's solution on a heating pad to warm. A coverslip is taped over the aperture in the insert.

The mouse, anesthetized as described above, is placed on its back on a warm heating pad. A 2 cm mid-ventral incision is made beginning midway between the 4^{th} and 5^{th} nipples. An angled incision is made from the base of the incision at an angle toward the midpoint of the hind legs passing over the junction of the 4th and 5th mammary glands. A third incision is made at the anterior end of the midline incision angled laterally between the third and fourth mammary glands, leaving a wide margin of skin around the fourth mammary gland. A blunt probe or cotton-tipped applicator is worked along the length of the three incisions and is used to lift the ventral two-thirds of the fourth mammary gland entirely free of the body wall while preserving mammary circulation.

The mouse is now placed prone on the warmed stage insert and the deflected flap of skin is arranged on the secured coverslip over the aperture. The mammary gland should be visible through the aperture. In general most fluorescent probes can also be seen with the naked eye, an

aid in positioning the animal. The flap of skin is taped lightly to the insert taking care not to compress the gland. A flaccid fold of skin between the mouse and the taped area helps to reduce the transmission of breathing movements to the visualized tissue. Place a gauze pad dampened with warm Ringer's beneath the mouse, making sure that the gauze contacts all exposed tissues. The edge of the gauze pad should extend over the edge of the coverslip, but not across the aperture. A film of saline will spread between the coverslip and the visualized tissue and prevent drying.

Visualization of the mammary gland. The stage insert bearing the mouse is placed on the stage of the inverted microscope and the mammary gland is visualized through the objective. To prolong visualization of a living gland the gauze pad should be kept well moistened with Ringer solution and body temperature of the mouse maintained. The stage should remain warm to the touch but not hot. A dark redness of the ears, feet and tail indicates over-heating. Etiolation or whitening of these areas indicates under-heating. If the anesthetic begins to wear off, small doses may be injected intra-peritoneally through the ventral body wall by simply rolling the mouse towards the tape.

Throughout the experiment red blood cells should be seen coursing through the vasculature of the gland indicating adequate circulation. While it is occasionally possible to obtain usable images with a 100X oil emersion objective, because most structures lie more deeply in the tissue, a long working distance 60X objective is generally more satisfactory. In addition, best results are obtained in a mouse that has been lactating for 10 days or more because the thickness of the adipose layer that generally surrounds the parenchyma is reduced at this time. More detailed discussion of the use of digital or laser confocal microscopy is beyond the scope of his article.

COMMENTS

In this section we give examples of the application of up-the-teat injection to studies of mammary function.

Measurement of transepithelial permeability.

The teat canal injection procedure can be applied to the assessment of permeability of tight junctions between mammary epithelial cells *in vivo*. Tracer injected into the lumen of the mammary gland will remain there if the tight junctions are closed. However, if they are open the injected tracer will leak out of the lumen and into the interstitial space. Since small molecules such as sucrose can readily transfer across the endothelium of the capillaries and enter the bloodstream, the peak level in the blood mirrors the leakage rate of such molecules. In our study, we injected the tracer [^{14}C]-sucrose into the lumen of the mammary glands during pregnancy and lactation. During pregnancy, [^{14} C]- sucrose appeared in the bloodstream almost immediately after injection, as the tracer leaked across the permeable tight junctions (Figure 2). The level in the blood stream then fell with a half-time of about 20 minutes as the non-metabolizable sugar was cleared by the kidneys. During lactation, injected [^{14}C]-sucrose was not detectable in blood samples, because it was confined to the lumen by the impermeable tight junctions of lactation.

The movement of large tracer molecules, such as proteins that might not cross the capillary endothelium, can be followed using fluorescently labeled proteins and fluorescent microscopy. Figures 3A and B show the results of an experiment in which FITC-BSA was injected into the lumen of the mammary glands of deeply anesthetized mice during pregnancy (A) and lactation (B). The probe was followed immediately by injection up-the-teat of 4% paraformaldehyde in in PBS up-the-teat to fix the tissue. After dissection the gland was embedded

in polymethacrylate embedding medium (JB4), sectioned, and viewed with the digital confocal microscope. Subsequent fluorescent microscopy showed that during lactation, the FITC-BSA was present exclusively within the lumen (Figure 3B), while during pregnancy, the FITC-BSA was found throughout the interstitial space of the mammary gland as well as in the lumen (Figure 3A). Although data obtained using protein probes are less quantitative than those using radiolabeled tracer, such probes do allow the leakage path of the tracer to be followed morphologically. In this case the tracer allowed us to ascertain that the probe leaked around the cells rather than being transcytosed, since the injected FITC-albumin was seen in the paracellular space, but was not detectable within the mammary epithelial cells.

Adenoviral transduction of luminal epithelium

Figure 3C shows a low power view of a mammary gland from a day 3 lactating mouse transduced with adenovirus containing the GFP gene on day 19 of pregnancy. At this magnification, which does not distinguish individual cells, a large proportion of the gland appears to be transduced. In order to better estimate the proportion of cells transduced, a mouse was similarly injected with a virus containing the lacZ gene. The gland was dissected, reacted with the lacZ chromagen, bluo-gal (Sigma), to obtain the characteristic blue color and processed for frozen section microscopy. The reaction is carried out at pH 8 to inhibit endogenous galactosidase. A representative section is shown in Figure 3D. Many cells in the section stain a very dark blue, indicating a high degree of expression. In some alveoli all the cells appear to be stained. These alveoli are expanded and contain evidence of milk in the lumen, indicating that neither the adenovirus transduction nor the presence of the foreign gene, lacZ, interfere with the normal function of the gland. Further, no signs of inflammation have ever been seen. However, the proportion of cells transduced does not exceed 25%. This degree of transduction needs to be

kept in mind when experiments are planned, particularly if morphological assays are not to be used. If the criteria for the effect of a particular gene substitution are morphological then the presence of adjacent transduced and untransduced cells and alveoli provides a control for the effect of the transgene.

Visualization of the mammary lumen and epithelium in vivo.

We have found that the lumen of the mammary gland can be visualized with injection up the teat of fluorescent dyes. Figure 3E shows an *in vivo* view of the mammary gland of a mouse that received an injection of 150 μl of Cy3-labeled IgG (0.375 mg/ml) with 40 μg/ml DAPI, to visualize the nuclei. Scattered light was removed from the image by digital deconvolution to make an optical section in the tissue (Slidebook software). The milk fat globules are clearly visible in the lumen of many of the alveoli and the DAPI has rendered the nuclei visible. We hope to use this technique, perhaps in conjunction with lipophilic dyes, to visualize milk fat globule secretion. The possibility that calcium binding dyes can be used to quantitate the level of intracellular calcium in various stages of lactation is intriguing. We have just begun to explore this area and are still working out the possibilities and limitations of the technique.

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LEGENDS

Figure 1. Fabricating and using micropipettes for up-the-teat injection. A. Stages in pulling micropipettes. After a slow pull the heated portion of the pipette has an hour-glass shape and the wall has not appreciably thinned (bottom pipette, 1). When the pipette is pulled a second time the waist of the indentation narrows to less than 75 μM (middle pipette, 2) or breaks entirely (upper pipette, 3). Bar, 5 μm B. Microforge (M) positioned over movable stage (S) of a compound microscope with wires to a variable power source (P). The platinum heating wire can be seen above the objective in the opening on the movable stage. For fire-polishing, a freshly broken micropipette is placed on the left-hand side of the stage with the tip about 4 μm from the platinum wire. C. Tips of two fire polished pipettes. Bar is 100 μm. D. A pasteur pipette attached to a piece of flexible tubing and a micropipette for cleaning. (The micropipette was taped down for the purposes of the photograph). E. Set up for teat canal injection using a Drummond microdispensor (B) mounted on a micromanipulator (C) beneath a dissecting microscope (A). The micropipette (E) is inserted into the teat canal by manipulating the tissue surrounding the nipple with a pair of fine forceps.

Figure 2. Appearance of [^{14}C]-sucrose in the blood stream after microinjection of 10^6 cpm into the lumen of the mammary gland in pregnant and lactating mice. After injection, the [^{14}C]sucrose level in the bloodstream rapidly increased then declined in the 18 day pregnant mouse as the isotope was cleared by the kidney (Open squares). The level of [^{14}C]-sucrose in the blood remained at background in the 2 day lactating mouse (Open circles).

Figure 3. Views of injected mammary gland. A. Distribution of FITC-albumin in the mammary glands of a pregnant mouse. Several alveoli with FITC in the lumina are shown. Nuclei are stained with DAPI (blue). Note the leakage of the FITC-albumin (green) from the lumen into the basolateral and interstitial space of the mammary alveoli (100X magnification). B. Distribution of FITC-albumin in sections of mammary glands of a lactating mouse. The FITC-albumin was confined to the lumen. 400X. C. Adenovirus transduction of mammary epithelium. Low power view (40X) on the FITC channel of GFP transduction of fourth mammary gland visualized in an anesthetized lactating mouse prepared as described in text. D. Section of mammary gland transduced with virus containing gene for Lac Z. Section stained with Bluo-gal (Sigma) as described by Sanes *et al.* (9) . E. Visualization of the mammary lumen and nuclei of mammary alveoli in the live mouse. The 4th mammary gland of a 10-day lactating mouse was injected with Cy3-labeled IgG and the vital dye DAPI. The nuclei of the alveolar cells are shown in blue. Dark lines crossing the alveoli are capillaries. Blood cells can be seen to move in these vessels under transmitted light (not shown).

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Figure 1

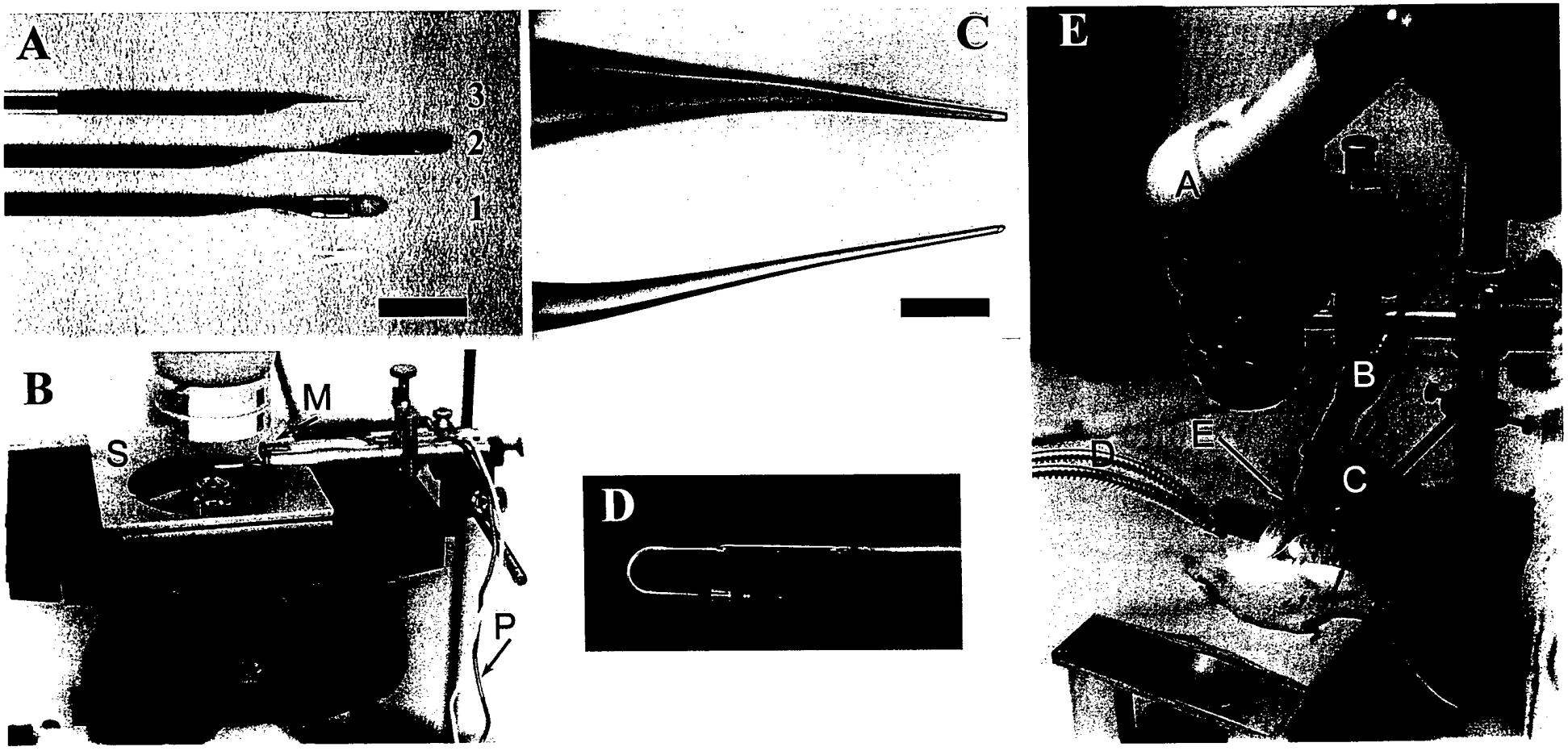

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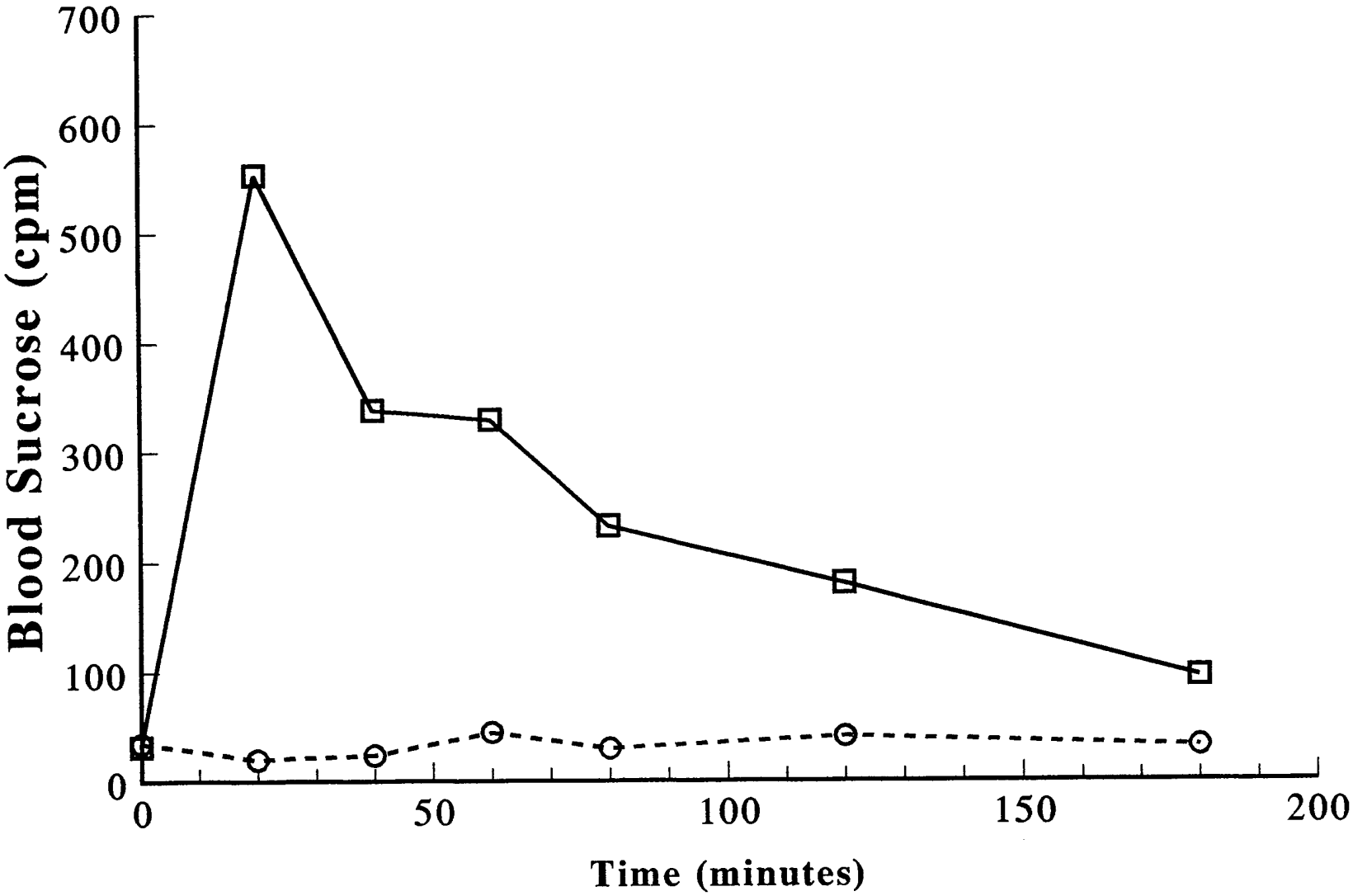
Figure 2



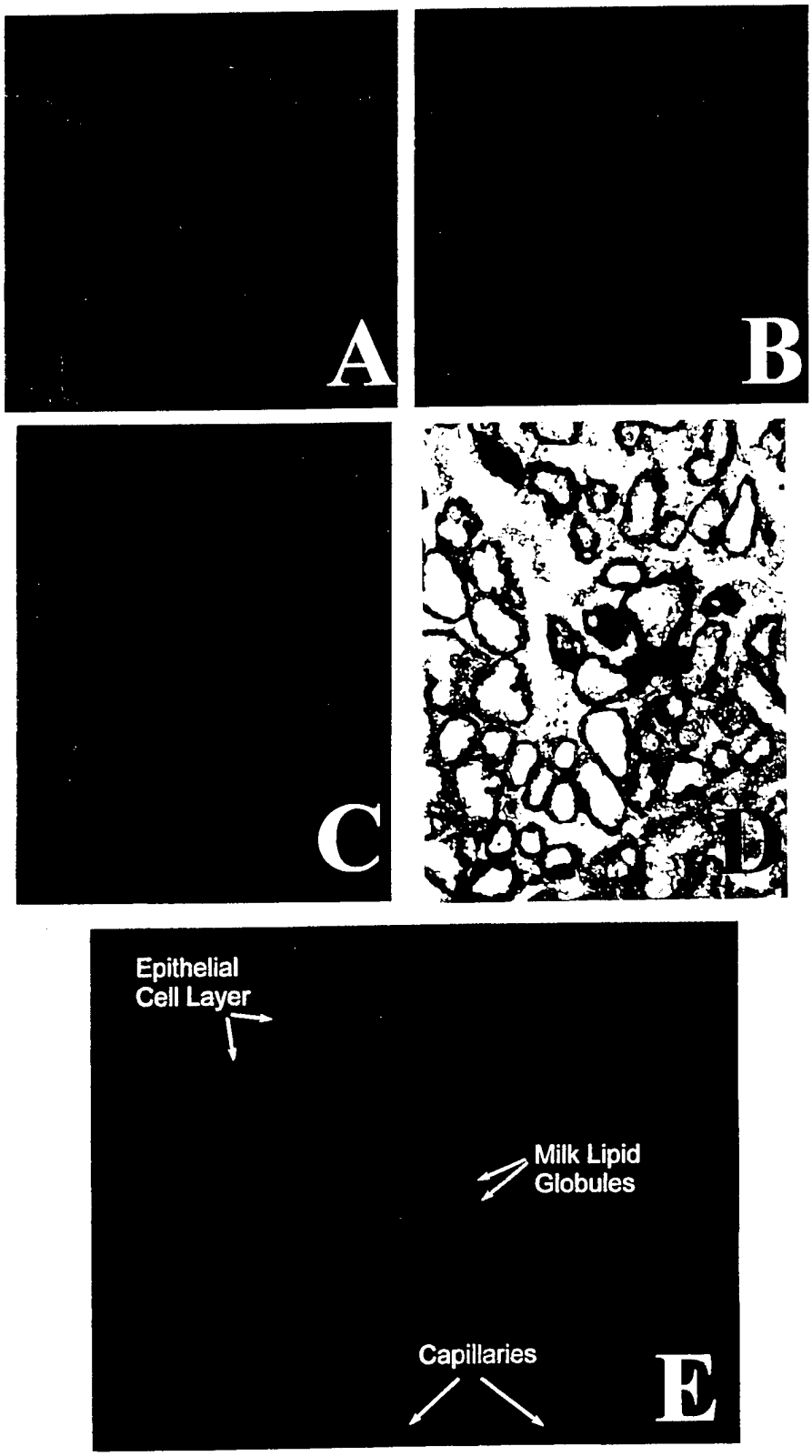


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Figure 3