

Isolation and culture of epithelial cells from rat ductuli efferentes and initial segment epididymidis

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Abstract. To improve the study of epithelial function in rat ductuli efferentes (efferent ductules) and initial segment epididymis, we developed a primary cell culture system with modifications of the Klinefelter method (1992). The cultured efferent ductal epithelium was grown to confluence and the cells maintained many of the organelles characteristic of these cells *in vivo*, including dense-staining granules, indented nuclei and apical cilia. Ciliary beat was observed for up to 10 days in culture. Cultured initial segment epithelial cells were elongated and characterized by apical branched microvilli. Electron microscopy revealed intact cell junctions, an endocytotic apparatus and lysosomal granules. Ultrastructurally, the initial segment epithelium contained a well developed Golgi apparatus. For both epithelia, cell characteristics were also confirmed by indirect immunofluorescent staining for cytokeratins 8, 18. Endocytotic activity was detected by the uptake of cationic ferritin at the apical surface and within vesicles. Estrogen receptor and clusterin mRNAs were expressed in the cultured epithelia and no difference was found in their expressions when cultured with or without 10⁻⁹ M 17- β estradiol. Indirect immunofluorescent staining for clusterin further indicated that this protein was present in the cultures. In conclusion, these *in vitro* methods will be useful for the investigation of epithelial function in the head of the epididymis.

Keywords: Ductuli efferentes, epididymis, cell culture, estrogen receptor, clusterin,

Introduction

Three *in vitro* approaches have been used to study the function of epididymal epithelium. These include the culture of whole organs, individual cells, and epithelial cell aggregates. Organ cultures have been useful in demonstrating that sperm maturation in the caput epididymis is androgen-dependent (Orgebin-Crist et al., 1976; Orgebin-Crist & Menezo, 1980; Klinefelter & Hamilton, 1984). However, a disadvantage of whole tubule or organ culture is the inability to collect bioelectrical response data for ion fluxes. Another problem is the inability to separate epithelial from

connective tissue responses. To circumvent these problems, others have isolated individual epididymal cells through repeated digestions of microdissected tissues. Although isolated cells attached to glass and plastic surfaces, they tended to become rounded after isolation and showed less physiological appearance than desired (Killian et al., 1977; Klinefelter & Amann, 1980a; Killian, 1981; Olson et al., 1982). Improvement in the yield of principal cells was achieved by using Percoll gradient centrifugation (Robaire & Hermo, 1988) and cell elutriation (Wagley et al., 1984). Another improvement, was the growth of isolated cells on an extracellular matrix (Klinefelter & Amann, 1980b; Klinefelter et al., 1982; Wagley et al., 1984). This method has been used to culture epithelial cells of efferent ductules, initial segment and cauda epididymides regions in the human (Wong, 1988; Chan et al., 1995). Although the human cells reached confluence after 4 days in culture, and bioelectrical data was collected, cellular morphology and epithelial ultrastructure were not documented in those studies.

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Recent culture methods have emphasized the isolation of tubular aggregates or fragments of epithelium, rather than individual cells. Using these new procedures, primary epithelial cultures of efferent ductules have been established using tissues from rats, boars and humans (Byers et al., 1985b; Raczek et al., 1992; Raczek et al., 1994; Heimuer et al., 1996). However, the methods presented in these studies did not work well in our laboratory, as epithelial appearance of the ductules was compromised, and the population of ciliated cells was greatly diminished. Klinefelter (1992) used a similar but more detailed method to culture epithelium from caput epididymidis. His method involved two controlled enzymatic digestions of dissected tubules, repeated pipetting, attachment of cell plaques to culture filter inserts coated with defined extracellular matrix and then draining of fluid from the upper into the lower chamber. This method resulted in epithelial cells displaying excellent morphology and expressing normal patterns of protein secretion (Klinefelter et al., 1992). Therefore, the goal of the present study was to adapt these successful methods for the culture of epithelium from efferent ductules and initial segment regions. Based upon the establishment of confluent layers with this culture system, it will be possible to investigate steroid regulation of bioelectrical functions and ion transport in the head of the epididymis.

Materials and methods

Animal management

Adult (90- to 120-day-old) male Sprague-Dawley rats were purchased from Harlan Sprague-Dawley Inc. (Charles River Breeding Laboratories Inc., Kingston, NY, USA) and were maintained in a constant temperature (25°C) and humidity (50%) vivarium on a 12L:12D cycle. Purina Laboratory Chow (Purina, St Louis, MO, USA) and water were available ad libitum.

Preparation of buffers, culture medium, and basement membrane

Medium 199, and Hank's balanced salt solution (HBSS) were obtained from Sigma (St. Louis, MO) and prepared according to the protocols established by Klinefelter (1992). Millicell®-CM inserts (Millipore Corporation, Bedford, MA, USA) were coated with rat tail collagen, collagen IV and laminin (Collaborative Research, Lexington, MA, USA) as described by Klinefelter (1992), and overlaid with 50 µl of Matrigel® (Collaborative Research, Lexington, MA, USA) diluted 1:10 in sterile phosphate-buffered saline (PBS), and air-dried in the incubator. Sterile technique was maintained throughout these preparations.

Isolation and culture of epithelial cells

Adult male rats were euthanized with sodium pentobarbital. Both the efferent ductules and epididymis were isolated and microdissected under sterile conditions to remove fat and connective tissue. Tubules of the efferent ductules and

initial segment epididymidis (Herms et al., 1991a) were uncoiled and cut into several small segments. The tissue fragments were transferred to a 50 ml centrifuge tube containing 10 ml of HBSS and 1 mg/ml collagenase (Boehringer Mannheim Biochemistry, Indianapolis, IN, USA) and incubated in a shaking water bath at 34°C for 30 min. The tissue fragments were disrupted by repeated pipetting with a 10 ml pipette. To stop enzymatic digestion, 20 ml of HBSS was added and the tubule fragments were allowed to settle for 10 min. The floating connective tissue fragments and sperm were removed by aspirating the supernatant until 1.5-2 ml of fluid was left in the centrifuge tube. The remaining fluid containing free released tubule fragments was transferred to a 15 ml centrifuge tube and subjected to repeated pipetting with a Pasteur pipette until the size of tubule fragments was smaller than 1 mm³. Ten milliliters of HBSS was added to the tube and the tubule fragments were allowed to settle. The supernatant was removed leaving 0.5 ml of fluid in the centrifuge tube.

Medium 199 containing 10% fetal calf serum (FCS) and 1 mg/ml of collagenase was added to a final volume of 2 ml, and the tubule fragments were further digested in a shaker water bath at 34°C for 5 min. To stop enzyme digestion, 10 ml of medium 199 was added and the tubule fragments were allowed to settle for 10 min. The supernatant was removed, the pellet was subjected to repeated pipetting, washing with medium 199 and settling three times to form small cell plaques. The diameter of these smaller cell plaques was between 100 and 300 µm with each plaque containing 50-250 cells.

The cell plaques were resuspended in medium 199 containing 10% FCS. A suspension (400 µl) containing 200-300 cell plaques for the efferent ductules, or 300-400 cell plaques for the initial segment epididymidis, was added to the apical chamber of a Millicell®-CM insert (12 mm diameter and 0.4 µm pores), and 200 µl of medium 199 containing 10% FCS was added to the well in a 24-well culture plate. Cultures were incubated at 34°C in a humidified atmosphere of 95% air and 5% CO₂. In one experiment, efferent ductal cells were plated on a smaller chamber (7 min) with a filter composed of Type I collagen and Matrigel®.

Culture conditions

The cell plaques were maintained in medium 199 containing 10% FCS for the first 18 hours. For the following 5 days, the cells were cultured in a serum-free medium containing 0.16 mg/ml bovine lipoprotein and 4×10^{-10} M dihydrotestosterone (DHT) or 2×10^{-7} M DHT plus 10^{-9} M 17β -estradiol (E₂).

Light and electron microscopy

The hematoxylin-stained cell monolayers were observed and photographed by bright field microscopy. To prepare the electron micrographs, 5-day-old cell monolayers of the efferent ductules and initial segment epididymidis were fixed in a solution containing 5% glutaraldehyde, 0.05 M

Collidine (Tousimis, Rockville, MD), 0.1 M sucrose (pH 7.4) at 4°C for 1 h. After glutaraldehyde fixation, the cells were washed 3 times in the buffer, and post fixed in a solution containing 1.5% potassium ferrocyanide and 2% osmium tetroxide (pH 7.4) at 4°C for 1 h. The samples were washed three times with deionized water, dehydrated through a graded series of ethanol solutions and propylene oxide, and embedded in Epon resin and polymerized at 90°C for 2 h. Sections 1 µm thin were stained with 1% toluidine blue in 1% sodium tetraborate and observed by bright field microscopy. Ultrathin sections of selected areas were stained with uranyl acetate and lead citrate and viewed in a JEOL IOOCX electron microscope at 60 KV.

Endocytosis

Five-day-old cell monolayers were rinsed with fresh medium 199 twice and incubated with 1 mg/ml of cationic ferritin (Sigma, St Louis, MO, USA) at 34°C for 10 min. Samples were then processed according to the protocol for electron microscopy.

Immunocytochemistry

Indirect immunofluorescent staining of cytokeratin was performed by a modification of the procedure of Mostov et al. (1986) and Dinges et al. (1991). After 5 days of culture on the collagen- and Matrigel-coated Millicell®-CM insert, the cell monolayers of efferent ductules and initial segment epididymidis were washed twice with PBS solution, and fixed in a PBS (pH 7.4) solution containing 1.5% paraformaldehyde and 0.2% Triton X-100 at 4°C for 1 h. After two PBS rinses, the cultured cells were blocked with a PBS solution containing 0.2% gelatin (PBS-G). The cells were incubated with mouse anti-cytokeratins 8 and 18 antibody (Sigma, St Louis, MO, USA), diluted 50-100X in PBS-G at 44°C for 30 min. Another set of cell cultures was incubated with a mouse preimmune ascites fluid to block specific binding sites for the cytokeratins of the cells. After two PBS-G rinses, both groups of samples were labeled with a fluorescein isothiocyanate (FITC)-conjugated goat antimouse IgG (Sigma, St Louis, MO, USA), diluted 100X in PBS-G, at room temperature for 30 min. The cell monolayers were washed twice with PBS-G, and mounted in a Slow Fade (Molecular Probes Inc, Eugene, OR, USA) solution on a slide. The samples were photographed on Kodak T-400 film (Eastman Kodak, Rochester, NY, USA) at 400 ASA, using Zeiss Fluorescent Microscope.

Indirect immunofluorescent staining of Clusterin was detected by a modification of the method of Sylvester et al. (1984). The cell monolayers were fixed with 50% picric acid, 4% paraformaldehyde, and 0.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) at 4°C for 1 h, and then washed twice in PBS. The cultured cells were incubated in ice-cold PBS containing 0.1% Tween 20 and 0.1% BSA for 10 min and then blocked with a blocking buffer containing 0.1% BSA and 5% goat serum in PBS for 15 min. The cells were incubated with a rabbit anti-rat clusterin antibody, which was obtained from Drs S.R. Sylvester and M.D.

Griswold (University of Washington, Seattle, WA, USA), diluted 50-100X in PBS containing 0.1% bovine serum albumin (PBS-B), at 4°C for 1 h. Another set of samples were treated with rat preimmune serum (Sigma, St. Louis, MO) to block the specific binding of the clusterin. After several rinses with PBS-B, the samples were labeled with FITC-conjugated goat anti-rabbit IgG (Sigma, St Louis, MO, USA), diluted 10X in a PBS-B, at room temperature for 30 min.

Both fibroblasts and smooth muscle cell were determined in the primary cell cultures according to the method of Skalli et al. (1986) and Travo et al. (1982). The cell monolayers of efferent ductules and initial segment epididymidis were fixed in methanol at -10°C for 10 min and washed twice in PBS. The cell cultures were incubated with a 20X diluted mouse anti-desmin antibody (Sigma, St Louis, MO, USA) for 30 min at 25°C to detect fibroblasts. After two rinses of PBS, the samples were labeled with a 100X diluted FITC-conjugated anti-mouse IgG (Sigma, St Louis, MO, USA) at 25°C for 30 min. The presence of smooth muscle cells in the cell cultures was detected on the basis of direct immunofluorescent staining using 200X diluted FITC-conjugated anti-smooth muscle actin IgG (Sigma, St Louis, MO, USA).

RNA isolation

The cell cultures from 12 rats were pooled together. The total cellular RNA was isolated from the efferent ductules and initial segment epididymidis according to the method described by Sambrook et al. (1989). The RNA samples were treated with 10 units of DNase I at 37°C for 30 min to digest chromosome DNA according to the method of Message-Clean Kit (GenHunter, Nashville, TN, USA) and subjected to spectrophotometric quantitation at A260 and stored at -70°C until further use.

Reverse transcription polymerase chain reaction

The gene specific primers (GSP) for estrogen receptors, including GSP-1: primer 1351 (5'-TTCACCTTCTGGAGTGTGAA-3') and GSP-2: primer 1804 (5'-TTGTAGAGATGCTCC-ATGCC-3') were selected to amplify the conserved regions of the estrogen receptor, according to the sequence in the GeneBank (Koike et al., 1987). The GSPs for clusterin, including GSP-1: primer 343 (5'-TGCCTGAAGCACA CCTGCAT-3') and GSP-2: primer 686 (5'-ATGAGGCT GCGACCAAGCG-3'), were used to amplify a conserved region of clusterin, according to the sequence suggested by Collard and Griswold (1987).

The first strand cDNA was synthesized by annealing 1 µg RNA sample, 9.5 µl of DEPC-H₂O and 1 µl of 10 µM Adapter Primer (Gibco BRL, Gaithersburg, MD, USA), while heating to 65°C in a water bath for 10 min, immediately chilling in an ice bath for 2 min, and quickly spinning on a bench centrifuge. The samples were mixed with 4 µl of 5x reverse transcription (RT) buffer, 1 µl of 10 mM deoxyribonucleoside triphosphates (dNTPs), 2 µl of 0.1 M dithiothreitol (DTT), 0.5 µl RNase inhibitor, and incubated in a

42°C water bath for 2 min. One microliter of Super Script 11 reverse transcriptase (200 unit/ μ l, Gibco BRL) was added to the reaction mixtures, which were incubated at 42°C for 30 min. The reaction was terminated by incubating at 70°C for 15 min and chilling in ice for 5 min. After reverse transcription, the DNA samples were incubated with 1 μ l of RNase H (Gibco BRL) at 42°C for 10 min to digest excess RNAs, and purified using a GlassMax DNA Clean Cartridge system (Gibco BRL) to remove the excess primers and small RNA products.

To amplify the DNA of estrogen receptor, 2 μ l of first strand cDNA, 5 μ l of 10X PCR buffer, 37 μ l of DEPC-H₂O, 2.5 μ l of 50 mM MgCl₂, 1 μ l of 10 mM dNTPs, 1 μ l of 10 μ M Universal Amplification Primer, and 1 μ l of GSP-1 (i.e. primers 1351) were mixed together and overlaid with 75 μ l of mineral oil. After the addition of 0.5 μ l of *Taq* DNA polymerase (5 unit/ μ l), the reaction mixtures were subjected to a thermal cycle as follows: 35 thermal cycles of 94°C for 45 s, 57°C for 25 s and 72°C for 2 min, followed by 75°C for 15 min and storage at 4°C. The PCR products were purified using a GlassMax DNA Clean Cartridge to remove the excess primers. The PCR products of estrogen receptor were diluted 1:20 with distilled water, and 1 μ l of diluted PCR products was subjected to nested amplification with 5 μ l of 10X PCR buffer, 37 μ l of DEPC-H₂O, 2.5 μ l of 50 mM MgCl₂, 1 μ l of 10 mM dNTPs, 1 μ l of 10 μ M GSP2 (i.e. primer 1804), and 1 μ l of 10 μ M GSP-1 overlaid with 75 μ l of mineral oil. After the addition of 0.5 μ l of *Taq* DNA polymerase (5 units/ μ l), the reaction mixtures were subjected to thermal cycle as follows: 35 thermal cycles of 94°C for 45 s, 57°C for 25 s and 72°C for 2 min, followed by 72°C for 15 min and storage at 4°C. The estrogen receptor cDNA samples were subjected to electrophoresis in a 1.5% agarose gel and stained with ethidium bromide. The DNA bands were visualized and photographed with Polaroid film under UV light.

To amplify the DNA of clusterin, 2 μ l of first strand cDNA, 5 μ l of 10X PCR buffer, 37 μ l of DEPC-H₂O, 2.5 μ l of 50 mM MgCl₂, 1 μ l of 10 mM dNTPs, 1 μ l of 10 μ M Universal Amplification Primer (Gibco BRL, Gaithersburg,

MD, USA), and 1 μ l of GSP-1 (i.e. primer 343) were mixed together and overlaid with 75 μ l of mineral oil. After a preheating period of 5 min at 94°C, 0.5 μ l of *Taq* DNA polymerase (5 units/ μ l, Gibco BRL, Gaithersburg, MD, USA) were added to each tube. The reaction mixtures were subjected to a thermal cycle as follows: 35 thermal cycles of 94°C for 45 s, 57°C for 25 s and 72°C for 2 min, followed by 72°C for 15 min and storage at 4°C. The PCR products were purified using a GlassMax DNA Clean Cartridge to remove the excess primers. The PCR products of clusterin were diluted 1:20 with distilled water, and 1 μ l of diluted PCR products was subjected to nested amplification with 5 μ l of 10X PCR buffer, 37 μ l of DEPC-H₂O, 2.5 μ l of 50 mM MgCl₂, 1 μ l of 10 mM dNTPs, 1 μ l of 10 μ M GSP-2 (i.e. primer 667), and 1 μ l of 10 μ M GSP-1 overlaid with 75 μ l of mineral oil. After the addition of 0.5 μ l of *Taq* DNA polymerase (5 units/ μ l), the reaction mixtures were subjected to thermal cycle as follows: 35 thermal cycles of 94°C for 45 s, 57°C for 25 s and 72°C for 2 min, followed by 72°C for 15 min and storage at 4°C. The amplified PCR product of clustering was electrophoresed in a 1.5% agarose gel and stained with ethidium bromide. The DNA bands were visualized and photographed with Polaroid film under ultraviolet (UV) light.

Results

Cultured efferent ductule epithelium

Epithelial plaques of efferent ductules (Fig. 1) contained both ciliated and nonciliated cells. The surface of the cell plaque was covered with bundles of cilia, and active ciliary beat caused the cell plaques to swim and rotate in the β medium before seeding. To remedy this problem, during the first 18 h, it was necessary to drain fluid from the apical chamber for 1-2 h by removing fluid from basal chamber. If this procedure was performed, the plaques would begin to attach during the next 24 h and began to spread (Fig. 2). By day 4-5, the cells covered 70-80% surface area of the insert. Ciliary beat was observed for up to 10 days in

Fig. 1 Phase contrast micrography of an epithelial cell plaque from the efferent ductules. The surface of the cell plaque contained numerous cilia, and active ciliary movement was readily observed before plating. x 230

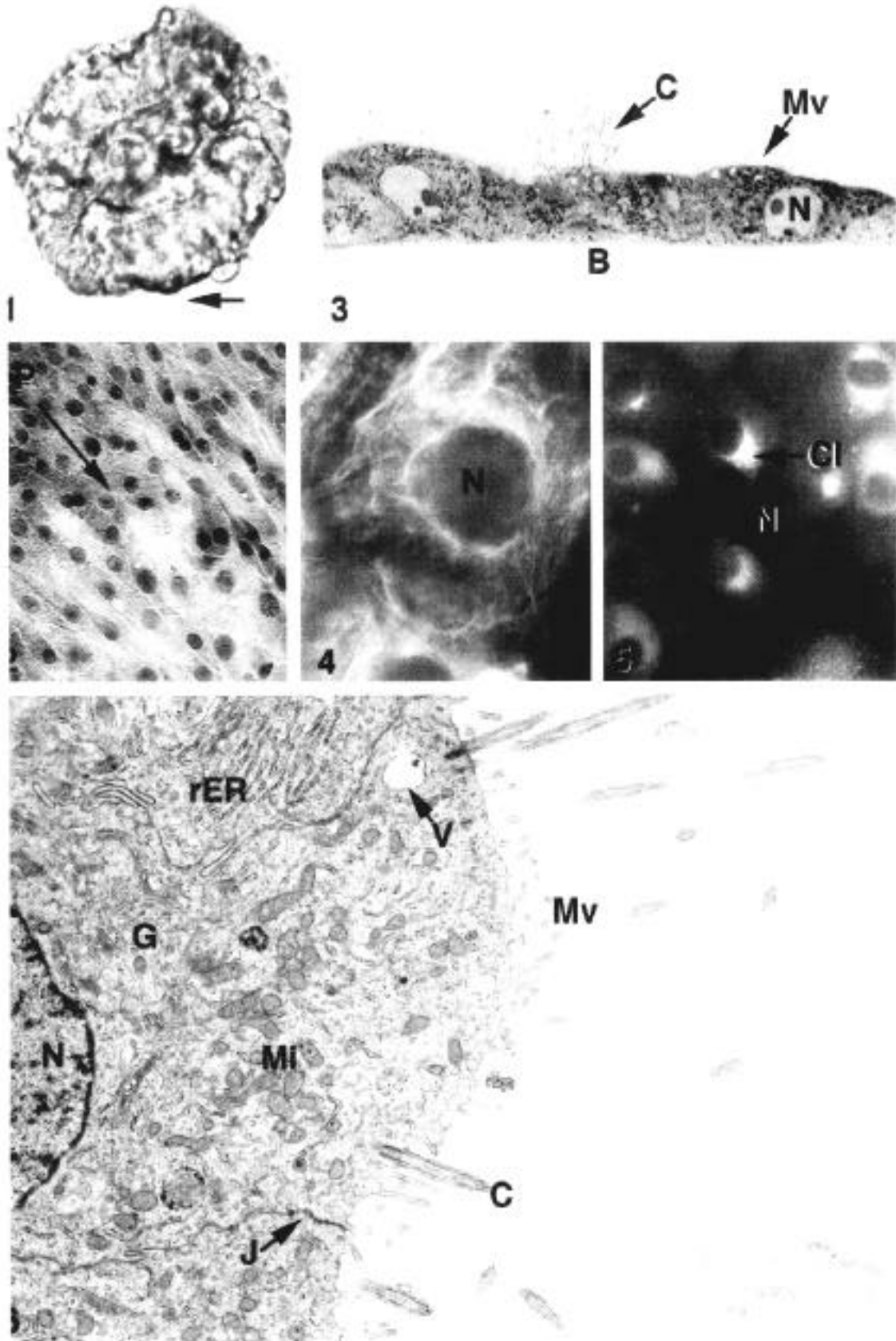
Fig. 2 A surface view of a 5-day-old cell monolayer of the efferent ductules. The polygonal epithelial cells have grown in the direction of the arrow away from the original cell plaque (P). Hematoxylin staining. X 400

Fig. 3 A perpendicular section of a 5-day-old cultured efferent ductule epithelium. This monolayer was composed of ciliated and nonciliated cells. Polarity of this cell monolayer was discerned by the apical cilia (C), microvilli (Mv) and basement membrane (B) domains. The cells exhibited a cuboidal appearance and contained vesicles and numerous densely stained granules. (N) = Nucleus. X 1130

Fig. 4 Indirect immunofluorescent staining for cytokeratins 8, 18 in cultured efferent ductule cells. The cytokeratin was detected as a filament network in the cytoplasm surrounding the nucleus (N) of efferent ductule epithelium. x 1380

Fig. 5 Indirect immunofluorescent staining for clusterin (Cl) indicates that clusterin was present in the perinuclear region of cultured efferent ductule epithelia. (N) = Nucleus. x 630

Fig. 6 Electron micrograph of a 5-day-old cultured epithelium from the efferent ductules. Cilia (C) and microvilli (Mv) were observed on the apical surface. Numerous mitochondria (Mi) were found beneath the ciliary basal bodies, A few vesicles (V), lysosomal bodies (L) and Golgi apparatus (G) were present in the apical cytoplasm. A well-developed rough endoplasmic reticulum (rER) was seen in an adjacent nonciliated cell. An apical tight junctional Complex (J) revealed the structural integrity that was maintained in these cultured cells. x 9300



Culture. The monolayer of efferent ductule cells displayed a polarized appearance, noted by their asymmetric structures of apical cilia and microvilli, and basement membrane domains (Fig. 3). In culture wells having a smaller diameter (7 mm), the monolayer reached confluence by day 5.

The epithelial nature of the cultured cells was detected by indirect immunofluorescent staining for cytokeratins 8 and 18, which are expressed abundantly in the epithelial cells of the efferent ductules and epididymis *in vivo* (Achstatter et al., 1985). Positive immunofluorescent staining for cytokeratin was observed in the perinuclear region of the cultured cells of the efferent ductules (Fig. 4). Contamination by fibroblasts or smooth muscle cells in the cell cultures was determined by immunocytochemical staining for desmin or α -smooth muscle actin, respectively (not shown). Neither fibroblasts nor smooth muscle cells were detected in the cell monolayers after 4 days in culture. Clusterin, an epididymal protein, is synthesized and secreted by the epithelial cells lining the efferent ductules and epididymis (Sylvester et al., 1991; Igouda et al., 1994). Therefore it was of interest to know whether cultured efferent ductule cells retained clusterin *in vitro*. In this study, a positive immunofluorescent stain for clusterin was detected in the cytoplasm of the cultured cells (Fig. 5).

Ultrastructurally the cells contained apical cilia, an indented nucleus, dense-staining cytoplasmic granules, numerous mitochondria and endocytotic vesicles. A well developed rough endoplasmic reticulum was present in the nonciliated cells (Fig. 6). Apical tight junctional complexes appeared normal (Fig. 7) and interdigitating folds of the lateral plasmalemma were observed in the efferent ductal epithelium (Fig. 8).

The endocytotic activity of cultured efferent ductule cells was determined by the uptake of cationic ferritin. After 10 min incubation with cationic ferritin, the cultured epithelia exhibited endocytotic activity, with the presence of ferritin particles in coated pits, apical tubes, and endosomes (Fig. 9).

Cultured initial segment epithelium

Epithelial plaques of initial segment epididymidis possessed microvilli on their apical surface (Fig. 10). The cell plaques began to attach to the substrate and spread after 18 h in culture. The cells covered approximately 90% of the surface area of an insert within 5-6 days and formed a centrifugal growth peripheral to the original cell plaques and displayed epithelial arrangement (Fig. 11). Figure 12 shows a perpendicular section of the initial segment monolayer, which exhibited a well developed polarized appearance. Many dense-staining granules and vesicles were present in the perinuclear region of the epithelial cells.

Epithelial characteristics of the cultured cells were detected by immunocytochemical staining for cytokeratins (Fig. 13). Contamination with fibroblasts or smooth muscle cells was not detected in the cell monolayers (not shown). Indirect immunofluorescent staining for clusterin indicated that clusterin was present in the initial segment epithelial cells (Fig. 14).

Ultrastructurally, the cultured cells of initial segment epididymidis contained numerous mitochondria, a prominent Golgi and rough endoplasmic reticulum (ER) in the perinuclear region (Fig. 15). At higher magnification, the Golgi apparatus was composed of abundant small vesicles and sacculi (Fig. 16).

Endocytotic activity of cultured initial segment epithelium was determined by the uptake of cationic ferritin. The ferritin particles were adsorbed on the apical cell membrane and translocated into endocytotic vesicles (Fig. 17).

Expression of estrogen receptor- α and clusterin mRNAs

Since estrogen acts through a nuclear receptor, it is important to know whether the epithelial cells retain estrogen receptor mRNA *in vitro*. Total cellular RNA was isolated from 5-day-old cell monolayers and subjected to RT-PCR analysis. PCR primers selected for amplifying the conserved regions of the estrogen receptor- gene were detected by the presence of a 453-bp DNA fragment (Fig. 18). Estrogen receptor mRNAs were detected in the cell cultures of the efferent ductules (Fig. 18: lanes A and B) and initial segment epididymidis (Fig. 18: lanes C and D). No difference in the expression of estrogen receptor- mRNA was found in cultured cells treated with DHT alone (Fig. 18: lanes B and D), or DHT plus E2 (Fig. 18: lanes A and C).

The expression of clusterin mRNA in culture was determined by amplifying a 343-bp cDNA fragment from the conserved region of the clusterin gene. Clusterin mRNA was present in the tissue homogenates of efferent ductules, and initial segment epididymidis (Fig. 19: lanes E and F), as well as in the tissue cultures of the efferent ductules and initial segment epididymidis (Fig. 19: lanes A-D). No difference in the expression of clusterin mRNA was found in the cell cultures treated with DHT (Fig. 19: lanes A and C), or DHT plus E2 (Fig. 19: lanes B and D).

DISCUSSION

This study demonstrated the successful growth of epithelium from efferent ductules and initial segment epididymidis *in vitro*. Excellent cellular morphology in both regions was

Fig. 7 Electron micrograph of the tight junction (Ti) between two nonciliated efferent ductule cells cultured for 5 days. x 30 400

Fig. 8 Electron micrograph of the lateral plasmalemma between two nonciliated efferent ductule cells cultured for 5 days. The interdigitating folds of the cell membranes (arrow) were similar to those seen *in vivo*. The cytoplasm also contains mitochondria (Mi) and lysosomes (L). x 34 200

Fig. 9 Electron micrograph of a nonciliated epithelium in a 5-day-old monolayer of the efferent ductules. After a 10 min incubation with cationic ferritin at 34°C, the ferritin particles were detected in coated pits (CP) between microvilli (Mv) and in apical tubes, (At) and endocytotic vesicles (V). x 35 400

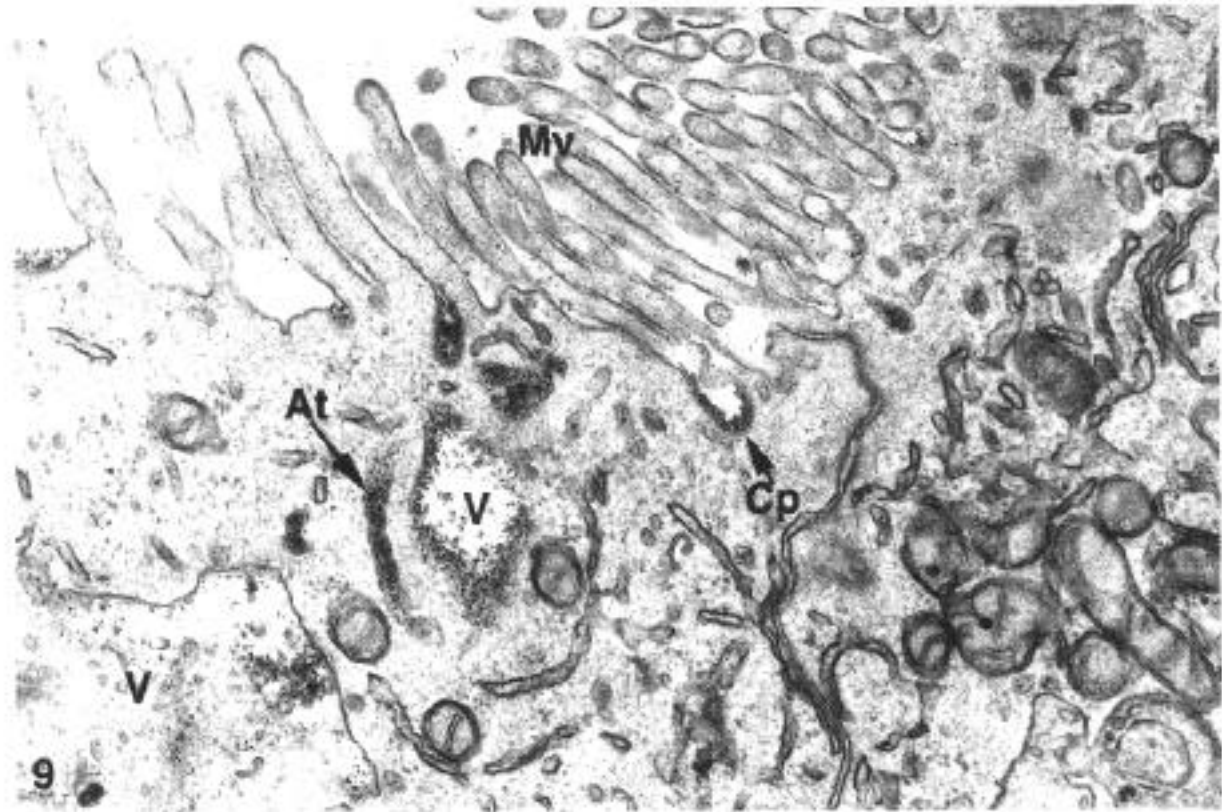
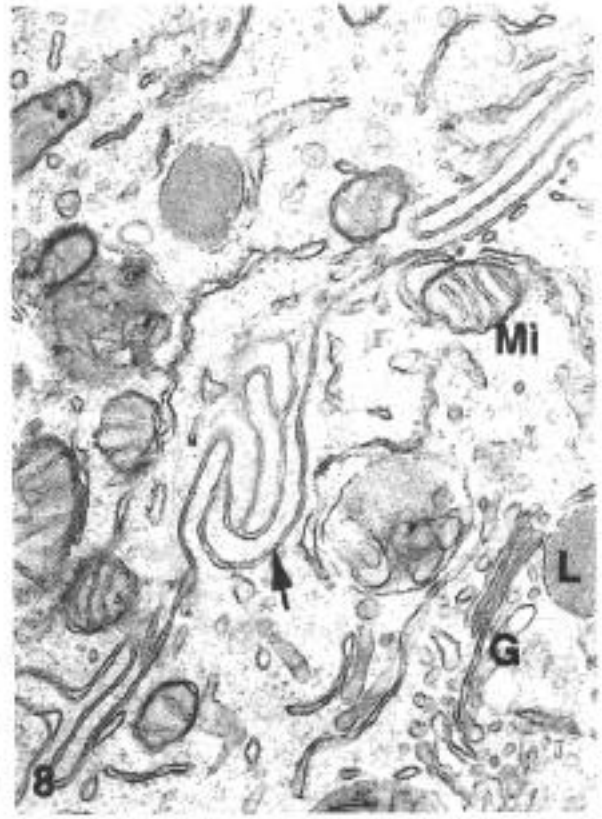
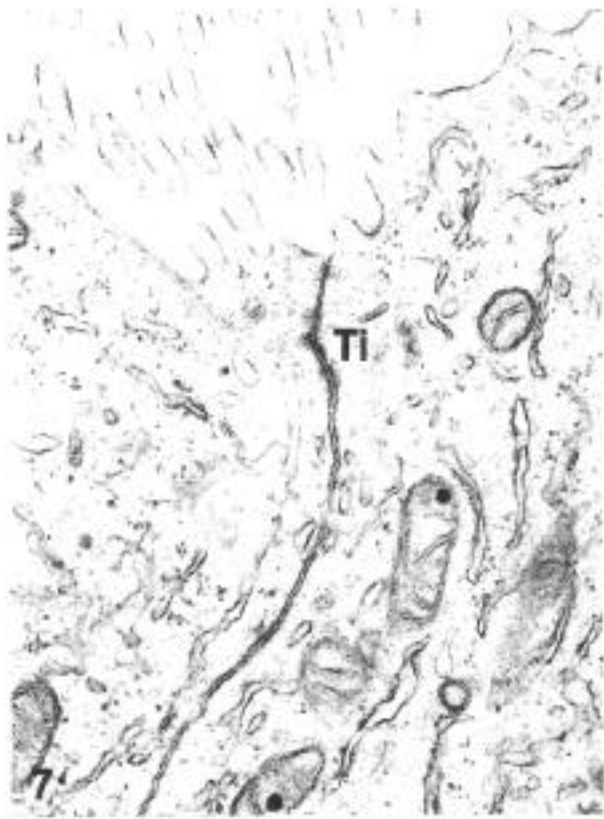


Fig.10 Phase contrast micrograph of a cell plaque from initial segment epididymidis. Microvilli (arrow) of the epithelial cells are discerned on the surface of the plaque. X 250

Fig.11 A surface view of a 5-day-old cell monolayer from initial segment epididymidis. The polygonal epithelial cells have grown in the direction of the arrow away from the original cell plaque (P). Hematoxylin staining. x 320

Fig. 12 A Perpendicular section of a monolayer of initial segment epithelium. This monolayer contained principal cells that were polarized, as noted by the apical microvilli (Mv) and basement membrane (B) domains. The cells became elongated in appearance. (N) = Nucleus. x 1130

Fig. 13 Indirect immunofluorescent staining for cytokeratins 8,18 in cultured initial segment cells. The cytokeratin was detected as a filament network surrounding the perinuclear region of the cultured initial segment cells, (N) = Nucleus. x12 10

Fig. 14 Indirect immunofluorescent staining for clusterin (Cl) indicates that clusterin was present in the perinuclear region of cultured initial segment epithelial cells. (N) = Nucleus. x 580

Fig. 15 Electron micrograph of a 5-day-old cultured epithelial cell from initial segment epididymidis. A prominent Golgi apparatus (G) was noted near the nucleus (N). Many of the apical microvilli were shortened due to the flattening of this epithelial cell in culture. x13 250

preserved and epithelial characteristics were observed throughout the monolayers. The cells displayed several functional traits of *in vivo* epithelia, including endocytosis and the expression of mRNA for estrogen receptors and the protein clusterin.

Efferent ductule morphology

It was more difficult to culture epithelial cells from the efferent ductules than from initial segment epididymidis. The difficulty stemmed from the ability of efferent ductal plaques to swim and rotate in the culture medium, due to the active beat of cilia. As the plaques rotated rapidly, there was no opportunity for the cells to interact with the basement membrane substrate on the semipermeable supports. If the cell plaques did not attach, their survival was relatively short compared to those that formed monolayers. Three strategies were used to improve the attachment of cell plaques in efferent ductule cultures. First, the tissue were subjected to a shorter period of enzymatic digestion, compared to other published methods (Byers et al., 1985b; Raczek et al., 1992). Second, the total volume of fluid in the apical and basal chamber was reduced to create a surface tension, which helped to hold the cell plaques near the substrate and facilitated their attachment. Third, the composition of the substrate played an important role in cell attachment. In this study, a significant improvement was noted when the semipermeable supports were coated with collagens Type I (rat tail collagen extract), and Type IV, and laminin, in addition to Matrigel®, compared to the use of collagens or Matrigel® alone.

The cultured efferent ductule cells had a cuboidal appearance and the cell height ranged from 5 to 20 µm, which was shorter than that reported *in vivo* (Hermo et al., 1992b). However, the cells appeared active in fluid reabsorption, as they contained an active endocytotic apparatus and lateral interdigitating folds of the plasmalemma, which were consistent with *in vivo* characteristics of adult rat efferent ductules (Hermo & Morales, 1984; Ilio & Hess, 1994).

Both the ciliated and nonciliated cells of the efferent ductules contained several clear vesicles and lysosomes in the cytoplasm, which were associated with the uptake of fluid or macromolecules *in vitro*. However, the number of

these organelles was reduced dramatically in cultured cells, compared to those in histologic sections of the efferent ductules (Morales & Hermo, 1983; Hermo & Morales, 1984; Hermo et al., 1988; Hermo et al., 1992a). Since cultured efferent ductule cells were maintained in a defined medium, these cells were no longer exposed to rete testis fluid macromolecules which would lead to reduced endocytosis and fewer lysosomes in the cultured cells. This result is consistent with observations in cultured efferent ductule cells of rats (Byers et al., 1985b) and boar (Heiniger et al., 1996), but differs from results of human efferent ductule, which contained numerous clear vesicles in the supranuclear region (Raczek et al., 1992). Both Golgi apparatus and rough endoplasmic reticulum (RER) were extensive in the nonciliated cells but less in the ciliated cells of this study.

In the present study, ciliated cells were maintained for the duration of the study (10 days). However, previous reports have indicated that the ciliated cells are less frequent in culture (Byers et al., 1985b; Raczek et al., 1992). The difference between laboratories may be due to variations in the preparation of cell plaques, the composition of the media, and the preparation of basement membrane for the culture well inserts. Alternatively, the cells may have resorbed their cilia, as noted by Byers et al. (1985b).

Initial segment morphology

The cultured initial segment epithelium was elongated, and cellular height showed wide variation, ranging from 10 to 40 µm, which was shorter than observations *in vivo* (Hermo et al., 1992b; Hermo et al., 1995). The cultured cells were polarized, with apical microvilli and a distinct basement membrane domain. However, the number of microvilli was decreased in the cultured initial segment cells compared to *in vivo* (Robaire & Hermo, 1988; Hermo et al., 1992b). The cultured cells appeared active in fluid reabsorption on the basis of interdigitating folds in the plasmalemma and large intercellular spaces. These features have been reported by others in cultures of epididymal epithelium (Moore et al., 1986; Byers et al., 1985a; Cooper et al., 1989; Cooper et al., 1990; Klinefelter, 1992). It is likely that the cultured initial segment cells were also involved in protein synthesis and secretion, as a prominent Golgi apparatus was well preserved *in vitro*.

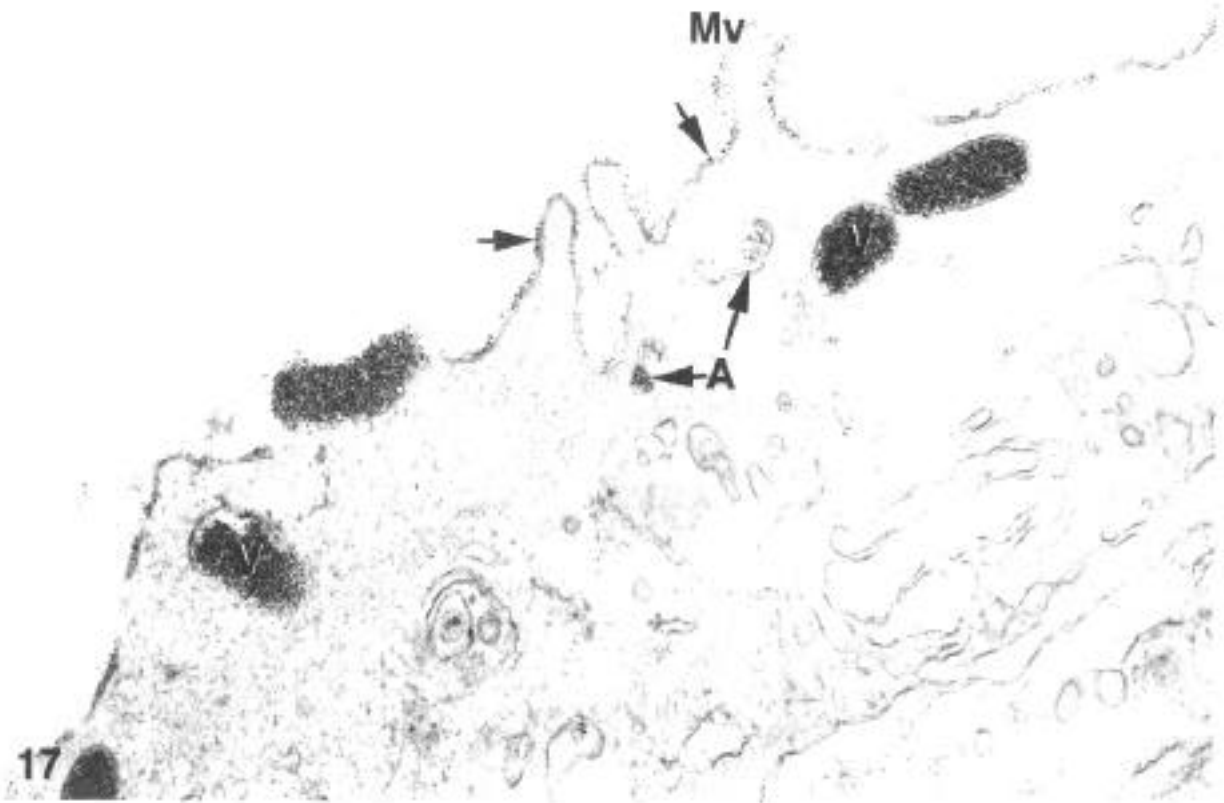
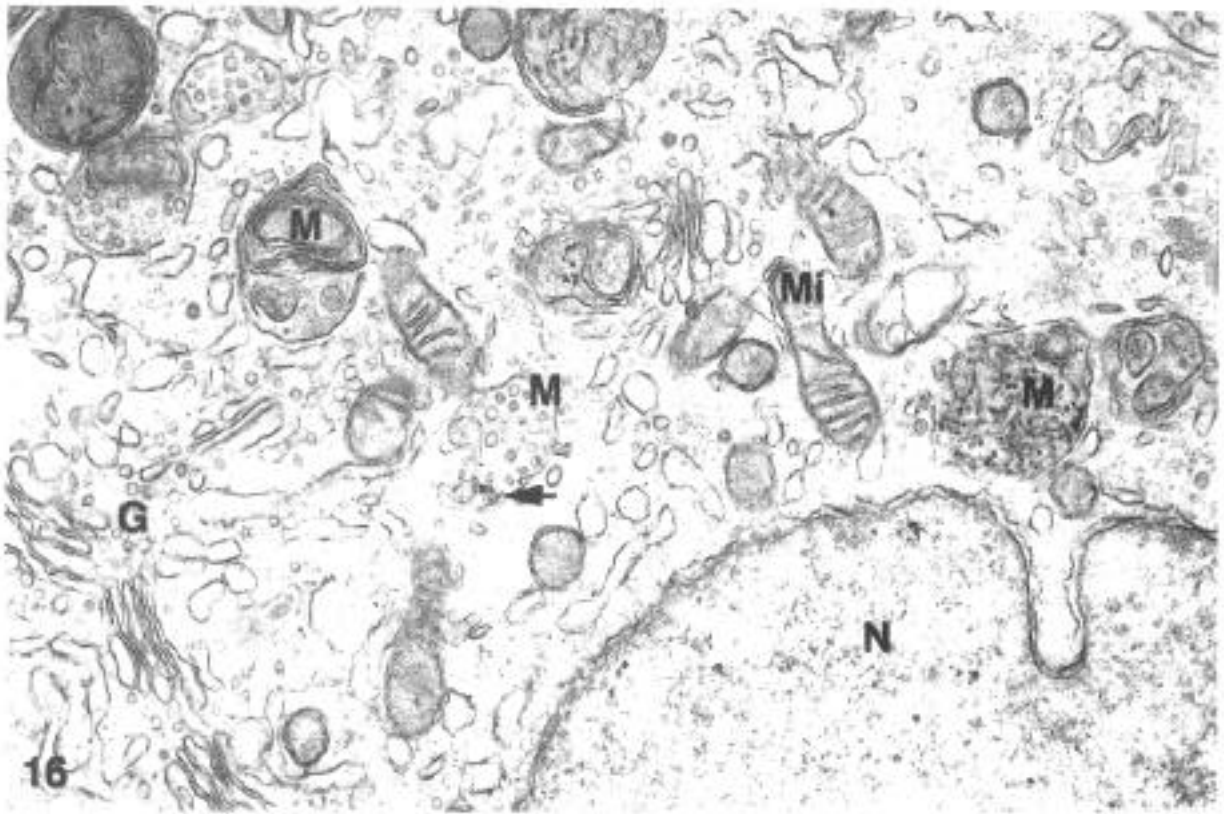


Fig. 16 Electron micrograph of a 5-day-old cultured initial segment epithelium. The conspicuous Golgi apparatus (G) was adjacent to the nucleus (N) along with numerous mitochondria (Mi) and multivesicular bodies (M). x 28 200

Fig. 17 Higher magnification electron micrograph of a 5-day-old cultured epithelial cell from initial segment epididymis. After a 10 min incubation a 34°C cationic ferritin particles were seen adhering to the apical plasmalemma (arrows), over microvilli (Mv) and within apical tube (A) and endocytotic vesicles (V), suggesting an active endocytotic apparatus was preserved in the cell cultures. x 39 400

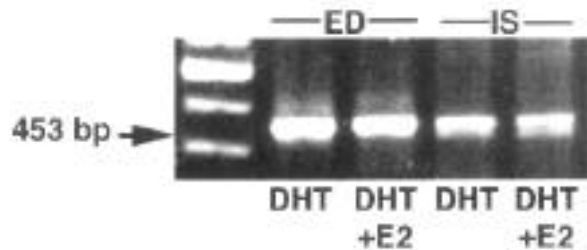


Fig. 18 RT-PCR analysis of estrogen receptor mRNA in 5-day-old cell monolayers of the efferent ductules and initial segment epididymis. Expression of estrogen receptor mRNA, a 453-bp fragment, was present in the cell cultures of Lane A and B: efferent ductules, and Lane C and D: initial segment epididymis, which were incubated in 10⁻⁷ M DHT (Lane A and C), and 10⁻⁷ M DHT +10⁻⁹ M E2 (Lane B and D) for 5 consecutive days.

Previously, a detailed immunohistochemical study in the male excurrent ducts revealed that the expression of cytokeratins 8 and 18 was predominant in epithelial cells lining the efferent ductules and epididymis but was not found in the connective tissue cells (Achtstatter et al., 1985; Kasper and Stosiek, 1989; Dinges et al., 1991 Wakui et al., 1994). In the present study, cytokeratins 8 and 18 were also found in the cultured epithelial cells. Furthermore, immunocytochemical staining for desmin (Lazarides, 1980) or -smooth muscle actin (Skalli et al., 1986) failed to detect contamination with fibroblasts or smooth muscle cells in the cultures. Therefore, we concluded that the monolayers consisted of epithelial cells and that contamination by fibroblasts or smooth muscle cells was negligible.

Evaluation of functional characteristics

Since the epithelial cells of efferent ductules and initial segment were cultured in a defined medium, it was necessary to determine whether the cells exhibited functional characteristics of cells in vivo. The internalization of proteins, ions and water in the efferent ductules and head of the epididymis has been documented as one of the major functions of epithelial cells lining these regions (Robaire & Hermo, 1988; Clulow et al., 1994). The endocytotic activity of the efferent ductules and epididymis (Goyal et al., 1980; Hermo & Morales, 1984, Djakiew et al., 1985; Hermo et al., 1985; Djakiew et al., 1986; Gerard et al., 1988; Goyal & Williams, 1988; Veeramachaneni & Amann, 1990, Veeramachaneni & Amann, 1991) has been demonstrated by the presence of electron-dense tracers in the endocytotic apparatus of the epithelial cells lining these regions (i.e. coated pits, apical tubules, endocytotic vesicles, and lysosomes). In this study, cationic ferritin was taken up by the cultured cells of the efferent ductules and initial segment epididymis. The ferritin particles were localized in the coated pits, apical tubes and endocytotic vesicles of the cultured cells in this study. This observation is in agreement with previous reports from cultured cells of the efferent ductules (Heiniger et al., 1996) and epididymis (Cooper et al., 1990). However, cultured human efferent ductule cells exhibited lower endocytotic activity (Raczek et al., 1992) compared to that of cultured epididymal cells from humans (Cooper et al., 1990).

Another major function of the efferent ductules and initial segment epididymis is synthesis and secretion of proteins, which contribute to the maturational process of sperm as they transit through these regions (Cooper, 1992; Hermo et al., 1994; Hinton & Palladino, 1995). If the



Fig. 19 RT-PCR analysis of clusterin mRNA in the tissues and cultured epithelia of the efferent duct and initial segment epididymis. Expression of clusterin mRNA, a 343-bp fragment, was present in the epithelial cell cultures of Lane A and B: efferent ductules, and Lane C and D: initial segment epididymis, which were treated with 10⁻⁷ M DHT (Lane A and C), and 10⁻⁷ M DHT + 10⁻⁹ M E2 (Lane B and D) for 5 consecutive days. Clusterin mRNA was also expressed in the tissue homogenates of Lane F: efferent ductules and Lane F: initial segment epididymis.

cultured epithelia of the efferent ductules and initial segment epididymidis exhibit functional similarity to the native cells *in vivo*, they should be able to maintain mRNAs for synthesized proteins *in vitro*. In this Study, we used clusterin, a major secretory protein in the head of the epididymis (Sylvester et al., 1984; Sylvester et al., 1991; Hermo et al., 1991b), as a biomarker to determine whether the cultured cells of either region retained this capability. It was demonstrated for the first time that clusterin mRNA can be maintained in cultured epithelium from efferent ductules and initial segment epididymidis. This result is consistent with previous reports of the expression of clusterin mRNA in efferent ductules and different segments of the epididymis using Northern blot analysis (Cyr & Robaire, 1992) and *in situ* hybridization (Garrett et al., 1991, Hermo et al., 1991b). The cells did not lose their capacity to synthesize clusterin *in vitro*, which supports the previous report that the epithelial cells of efferent ductules contained an endogenous form of clusterin (Igdoura et al., 1994). Clusterin synthesis by efferent ductal epithelium is unresponsive to testosterone (Igdoura et al., 1994), and based upon the present study is also not regulated by estrogen, as clusterin mRNA was present in the cultured cells with or without the presence of estrogen.

Finally, the efferent ductules have been shown to contain high concentrations of estrogen receptors and in some species to be the only region of the male reproductive tract containing estrogen receptors (Schleicher et al., 1984; West & Brenner, 1990; Cooke et al., 1991; Iguchi et al., 1991; Fisher et al., 1997; Goyal et al., 1997; Hess et al., 1997). In fact, estrogen receptor mRNA expression in the rat efferent ductules is 3.5-fold higher than in uterine tissues (Hess et al., 1997). Thus it appears that the efferent ductules are a major target for estrogen in the male. In some species, estrogen receptor was not found in the epididymis (West & Brenner, 1990; Goyal et al., 1997), but in the rat there is evidence both for and against the presence of the receptor in initial segment epididymidis and other regions of the tract (Fisher et al., 1997; Hess et al., 1997). Therefore it was of interest to determine whether estrogen receptors could be maintained in cultured cells of the efferent ductules and whether the initial segment epithelium contained receptor. In the present study, estrogen receptor mRNA was expressed in cultured cells from both regions, regardless of the presence or absence of estrogen in the medium. This data further supports the immunohistochemical data showing receptor in the epididymis of the rat (Hess et al., 1997).

These results demonstrated that cultured epithelial cells of the efferent ductules and initial segment epididymidis can retain considerable morphologic and functional characteristics of native cells *in vivo*. The establishment of this epithelial culture system provides a basis for the investigation of steroid hormone regulated functions in the head of the epididymis.

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