Regulation of mouse epididymal epithelium in vitro by androgens, temperature and fibroblasts

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The epididymal epithelium provides the microenvironment for sperm maturation. However, the molecular basis of epididymal function is still poorly understood because of the limitations of in vivo systems. For this reason, we have developed an in vitro culture system for mouse epididymal epithelial cells. Cells were purified by enzymatic digestion and centrifugation through a Percoll gradient, and plated on inserts coated with a replacement basement membrane. Cultured cells maintained ultrastructural and immunocytochemical features of epithelia, but did not retain the androgen responsiveness of epididymal cells (as judged by androgen receptor detection and secretion of specific markers) unless cocultured with fibroblasts. The androgen receptor was detected in the nuclei of epididymal epithelial cells only when grown with epididymal fibroblasts in the subjacent chamber. Moreover, specific epididymal secretory proteins were secreted only when epithelial cells were cultured in the presence of both androgens and fibroblasts at 32°C. These results highlight the importance of cell–cell interaction, as well as temperature regulation in the physiology of the epididymis. They also establish the existence of two independent pathways in the differentiation of these cells. The first, leading to the expression of epithelial characteristics, is fibroblast-independent, whereas the second, conferring tissue-specific features, depends upon coculture with fibroblasts.

Introduction

The epididymis is a convoluted tubule embedded in a connective tissue matrix. Embryologically, it is derived from the Wolffian duct and, in adult mammals, reaches considerable complexity from both an anatomical and functional point of view (Bedford, 1975; Hamilton, 1975; Cooper, 1986). The proximal epididymis plays an essential role in sperm maturation which is critical for the ability of the cell to fertilize an egg and is thought to be promoted by the interaction of spermatozoa with epididymal secretions (Orgebin-Crist et al., 1981; Bedford and Hoskins, 1990). In contrast, the main function of the distal epididymis is temperature regulated storage of mature spermatozoa (Bedford, 1978). Study of epididymal physiology may, therefore, provide clues to how spermatozoa achieve and maintain their fertilizing capacity.

Classic studies have shown a role for androgens in the regulation of the epididymis ( Cameo and Blaquier, 1976; Brooks and Higgins, 1980; Tezon et al., 1985); however, it is often difficult to discriminate direct effects on the epithelium from those caused indirectly by an effect on testicular function. In addition, the distal epididymis is temperature regulated (Bedford, 1978; Esponda and Bedford, 1986; Pera et al., 1996), but limitations inherent in the in vivo system have not allowed examination of this feature for the proximal regions. The development of in vitro systems seems essential to improve our knowledge of epididymal physiology. Such an experimental system may also be useful in promoting the in vitro maturation of spermatozoa, which would enable investigation of the molecular mechanisms that underlie epididymal maturation (Moore and Hartman, 1986; Moore et al., 1992; Bongso and Trounson, 1996).

Many attempts have been made to culture epididymal cells, from the initial studies using organ culture (Orgebin-Crist et al., 1987) to the culture of isolated epithelial cells (Kierszenbaum et al., 1981; Cooper et al., 1989; Byers et al., 1992). Rat (Byers et al., 1985; Cooper et al., 1989; Finaz et al., 1991) and human (Cooper et al., 1990; Huang et al., 1992; Moore et al., 1992; Raczek et al., 1995) epididymal epithelial cells grow in culture and form confluent monolayers. The cells keep their structural features, including a well developed Golgi apparatus, and maintain their polarity, endocytotic activity and junctional relationships (Djakiew et al., 1984; Cooper et al., 1989; Byers et al., 1992). The cells secrete several specific products (alkaline and acid phosphatases, N-acetylglucosaminidase and secretory proteins; Kierszenbaum et al., 1981; Smith et al., 1986; Yeung et al., 1989; Cooper et al., 1990) and metabolize testosterone to the active metabolite, dihydrotestosterone (Brown et al., 1983; Amann et al., 1987; Raczek et al., 1994).

A major obstacle to the study of purified epithelial cells, however, is their tendency to lose hormone responsiveness (Cunha et al., 1985). Prostatic epithelial cells, immortalized by transfecting them with a plasmid containing SV40 large T antigen, display very low expression of the androgen receptor gene (Rundlett et al., 1992). Moreover, purified prostatic epithelial cells maintained in culture fail to express an
androgen-dependent prostate specific binding protein (Chang and Chung, 1989), yet prostate explants that contain epithelial cells plus fibroblasts respond to androgens by secreting specific markers (Martikainen et al., 1987). Among the studies made thus far on epididymal epithelial cell culture, Raczek et al. (1994) failed to find any androgen dependence of cell height in human epididymal cultures that consisted solely of principal cells. In the few cases in which androgen responsiveness has been shown (Moore et al., 1986; Moore and Hartman, 1986; Smith et al., 1986; Bongso and Trounson, 1996), the authors cultured epididymal epithelia that contained more than one cell layer, so it is very likely that peritubular myoid cells were also present.

We have developed an in vitro culture system for the proximal regions of the mouse epididymis. We have used this system to determine some of the factors that regulate epithelial cell function in vitro as indicative of the mechanisms that operate in vivo. We show that both temperature and androgens regulate protein secretion by epididymal cells and that the androgen responsiveness of the epididymal epithelium depends upon coculture with the epididymal fibroblasts.

Materials and Methods

Purification and culture of epithelial cells

For each experiment, epididymides were obtained from ten 1-month-old Swiss mice (Harlan Sprague–Dawley, Indianapolis, IN). Mice were killed using CO₂ and the epididymal regions to be cultured (Fig. 1) were isolated in medium RPMI-1640 (Gibco BRL, Grand Island, NY). The tissue was incubated at 37°C in a tube rotator for 20 min in PBS containing trypsin (2.5 mg ml⁻¹) and EDTA (1% w/v). After washing the preparation three times with RPMI-1640 supplemented with 5% fetal bovine serum (FBS), tubules (settled by gravity) were added to a dissociation solution consisting of dispase (25 U ml⁻¹; Collaborative Research, Bedford, MA) and collagenase (1.5 U ml⁻¹; Sigma, St Louis, MO) in RPMI-1640 with 5% FBS. After incubation at 37°C in a tube rotator for 30 min, the undigested tissue was allowed to settle and the supernatant containing free cells centrifuged at 200 g for 5 min. The washed cells were resuspended in 1 ml RPMI containing 5% FBS and fractionated in a discontinuous Percoll gradient prepared as described by Finaz et al. (1991). For each fraction, density was estimated using density marker beads (Pharmacia, Uppsala) and cell viability assessed by Trypan blue exclusion. The cell types present were estimated by the use of specific fluorochrome conjugated lectins. The lectins used were Ulex europaeus agglutinin conjugated to rhodamine (UEA-1-TRITC) and Bandeiraea simplicifolia conjugated to fluorescein (BS-1-FITC) (Sigma). These lectins have been used previously to distinguish the different cell types in the epididymis (Burkett et al., 1987a, b). The layers enriched in principal cells were pooled and plated in culture medium. The medium (referred to as s-RPMI) was RPMI-1640 medium in which d-valine had been replaced l-valine to discourage fibroblast growth (Orgebin-Crist et al., 1984; Cooper et al., 1989) supplemented as described by Moore et al. (1986). When indicated, the medium also contained 200 nmol testosterone 1⁻¹ and 1 µmol dihydrotestosterone 1⁻¹. Cultures were maintained in 0.3 cm² inserts (Collaborative Research, Bedford, MA), freshly coated with a thin layer of Matrigel (Collaborative Research) diluted 1:7 (Byers et al., 1992) in 24-well tissue culture plates. A volume of 0.25 ml was used in the upper cell-containing chamber, and 0.5 ml in the lower chamber. In experiments examining the effect of coculture with fibroblasts, the latter were confined to the lower chamber. Epithelial cells were plated at 0.3~0.6×10⁶ cells cm⁻² (low density) or 0.6~1.0×10⁶ cells cm⁻² (high density). The plates were cultured in 5% CO₂ at 37°C or at 32°C. After 2 days, a serum-free defined medium was used (SFDM: s-RPMI in which FBS was replaced by 100 nmol epidermal growth factor (EGF) 1⁻¹). Thereafter, the medium was changed every 2 days. In experiments involving the recovery of proteins from supernatants, SFDM was supplemented with 1 mg BSA ml⁻¹.

Isolation and culture of fibroblasts

Epididymal tubules were subjected to a 30 min digestion in 1.2 U collagenase ml⁻¹ and 10 U dispase ml⁻¹. Tissue fragments were allowed to settle by gravity, washed and plated in

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**Fig. 1.** Schematic representation of the mouse epididymis, with the shaded area indicating the regions used for cell culture. Cp: caput; Co: corpus; Cd: cauda; Vd: vas deferens.

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**Fig. 2.** Ultrastructure of cultured epididymal epithelial cells. Cells were cultured for 4 days prior to fixation. (a) General view of the epithelial monolayer. The cell density at plating was 10⁶ cells cm⁻². There are numerous apical microvilli in the luminal border of the cells and several organelles and vacuoli in the cytoplasm. Nuclei (N) are rounded and mainly located in the basal pole. Scale bar represents 2 µm. (b) Detail of a cell showing a well developed Golgi apparatus (G) close to the nucleus (N) and a lysosome granule (L). Scale bar represents 0.5 µm. (c) Detail of the apical border of the cultured cells. Below the microvilli (mv) there are some vesicles (arrowheads). Accumulation of mitochondria (M) can also be seen. Scale bar represents 1 µm. (d) Basal pole of the cultured cells. The filter can be seen in the right corner at the bottom. Beneath the nucleus there are mitochondria (M) and some vesicles (arrowheads). Scale bar represents 1 µm. (e) General view of the epithelial monolayer. The cell density at plating was 0.5×10⁶ cells cm⁻². The cells are flattened and display scarce and shorter microvilli when compared with those plated at higher densities (see (a)). N, nucleus. Scale bar represents 2 µm.
Fig. 2.
culture medium (s-RPMI in which d-valine was substituted for l-valine). Culture was performed using 24-well Costar tissue culture plates, in 5% CO₂ at 37°C. Under such conditions, a mixed population of cells plated and grew. On day 2, most of the cells were epithelial, but as the culture progressed, they were replaced by fibroblast-like cells, which had completely overgrown the epithelial cells by day 10. After this, they could be maintained in culture for at least a further 1–2 weeks in a serum-free defined medium (f-SFDM). f-SFDM is f-RPMI in which FBS has been substituted by 3 nmol bFGF l⁻¹. For coculture experiments, the epithelial cells were isolated as described above and plated in inserts that were placed in the wells in which the fibroblast-like cells were growing. In some experiments, rabbit endothelial vascular cells (REVVC) were used as controls, and cultured similarly to epididymal fibroblasts.

Electron microscopy

Inserts containing the cells were washed in PBS and fixed for 1 h with 1% glutaraldehyde in 0.125 mol cacodylate buffer l⁻¹ containing 1% (w/v) tannic acid. Cells were then washed in cacodylate buffer, and the filter bearing the fixed cells was cut out and washed in distilled water. After dehydration through graded alcohols and infiltration with Spurr resin (Polysciences, Warrington, PA), the filter was cut into strips and embedded. Ultrathin silver–gold sections were stained with lead citrate and uranyl acetate, and examined in a Phillips 301 electron microscope.

Detection of cytokeratin and uvomorulin

Cells were fixed on the inserts with 2% (w/v) p-formaldehyde in PBS (pH 7.5) for 10 min and incubated for 30 min in 20 mmol glycine l⁻¹ in PBS (pH 7.5) to block free aldehyde groups. Cells were permeabilized for 30 min in a solution of PBS containing 0.5% (v/v) Triton X-100 and processed for indirect immunofluorescence following standard procedures. The primary antibody was mouse anti-pan cytokeratin (1:20) or rat anti-uvomorulin (1:100) in PBS–1% (w/v) BSA. The second antibody solutions contained anti-mouse IgG conjugated to TRITC and anti-rat IgG conjugated to fluorescein isothiocyanate (FITC), respectively, diluted in 1% BSA in PBS. These antibodies were purchased from Sigma. Stained cells were mounted on slides with Gelvatol (Monsanto, St Louis, MO) and examined using a Zeiss microscope equipped with epifluorescence and appropriate filters. Controls, including irrelevant monoclonal antibodies as primary antibody, were also conducted.

[³H]methyl thymidine labelling experiments

[³H]methyl thymidine (2 μCi; ICN, Costa Mesa, CA) was added to the lower culture chamber at plating (day 0) or on days 1, 2, 3, 4, 5 and 6. After 24 h, epithelial cells on the inserts were rinsed in PBS, fixed for 1 min in methanol-acetic acid (3:1; v:v), and air-dried. Cells were coated with a photographic emulsion (NTB-2, Kodak, Rochester, NY) and exposed for 48 h at 4°C. The emulsion was developed in D-19 (1:2 in distilled water) and fixed in rapid fix (Kodak). After staining with toluidine blue, the mitotic index (percentage of labelled nuclei) was estimated by counting more than 500 cells per slide for each time interval and for each of the conditions tested.

Localization of androgen receptor

Cells were fixed and permeabilized as described and then incubated for 3 h in a blocking solution consisting of PBS with 0.05% (w/v) sodium azide, 5% (w/v) BSA and 0.4% (w/v) Triton X-100. After washing in PBS with 1% BSA, a solution of rabbit anti-human androgen receptor (1:100; Affinity Bioreagents, Mechanic Station, NJ) in PBS–1% BSA–0.4% Triton X-100 was added and incubated overnight at 4°C. Reactivity was detected using biotinylated anti-rabbit IgG (1:200 in PBS–1% BSA; Sigma, St Louis, MO) and anti-biotin antibody conjugated to horseradish peroxidase (HRP) (1:200 in PBS–1% BSA; Sigma), followed by incubation in a substrate solution containing 0.75 mg diaminobenzidine ml⁻¹, 0.09 μl H₂O₂ (30%) ml⁻¹ and 0.03% (w/v) NiCl₂ in Tris buffer (50 mmol l⁻¹; pH 7.5).
Supernatants from cultured cells were collected every 2 days, clarified by centrifugation (10,000 g for 5 min), and the proteins precipitated with trichloroacetic acid (TCA) and recovered in sample buffer. For SDS-PAGE, 50 μg of protein (corresponding to half of the total protein recovered) were used (Laemmli, 1970) and the proteins were transferred to 0.2 μm pore nitrocellulose filters (Schleicher and Schuell, Keene, NH) following standard procedures. The filters were blocked with 5% non-fat dried milk in washing buffer (50 mmol Tris $1^{-1}, 150$ mmol NaCl $1^{-1}, 0.2\%$ (w/v) gelatin, 0.05% (w/v) Tween-20) overnight at 4°C and then used for western blots. The primary antibody used was a guinea-pig serum that recognizes the protein CP 27 (Flickinger et al., 1988), diluted 1:1000 in washing buffer. Filters were incubated sequentially with biotinylated anti-guinea-pig IgG (1:4000 in washing buffer) and anti-biotin-HRP (1:4000 in washing buffer) and positive staining was detected using an enhanced chemiluminiscent substrate (ECL, Amersham, Little Chalfont), WGA-binding sites were detected with commercial WGA conjugated to biotin (Genzyme, Cambridge, MA). Briefly, blocking of non-specific sites was carried out for 1 h in a solution containing 0.5 mol NaCl $1^{-1}, 20$ mmol Tris $1^{-1}, 10$ mmol CaCl$_2$ $1^{-1}, 10$ mmol MgCl$_2$ $1^{-1}, 10$ mmol MnCl$_2$ $1^{-1}$ and 2% (w/v) gelatin (pH 7.4) and then 4 μg of biotinylated lectin ml $^{-1}$ was added and incubation progressed for 2 h. The proteins recognized were revealed by use of an anti-biotin-HRP antibody (1:5000 in washing buffer), followed by ECL.

**Dot blot and densitometry**

Supernatants recovered at 2 day intervals were clarified as described above and blotted onto nitrocellulose filters using a Bio-Dot Microfiltration Apparatus (Bio-Rad, Richmond, CA). Filters were then used for immunoblot with anti-CP 27 as described above. The exposed films were scanned using a OneScanner (Macintosh, Apple Computer Inc, Cupertino, CA) and analysed using NIH-Image software. Analysis of variance (ANOVA) followed by Duncan's test was used to examine statistical differences among the different treatments.

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**Fig. 4.** Detection of cytokeratin and uvomorulin in epididymal cells, fixed 3 days after plating. Cytokeratin forms a network in the cytoplasm of the epithelial cells (a), while uvomorulin is restricted to cell borders (arrows), being part of the junctional complexes between cells (b). Controls, including an irrelevant monoclonal as primary antibody, were negative for both cytokeratin (c) and uvomorulin. Fibroblasts were also negative for both markers. Fibroblasts stained with the anti-uvomorulin antibody are shown in (d). Scale bar represents 20 μm.
Results

Characterization of the cultured epithelial and fibroblast-like cells from the epididymis

The epithelial cells, regardless of the presence or absence of fibroblasts or androgens, plated and grew, reaching confluence by day 3–5. Electron microscopy confirmed that the cells formed a monolayer and displayed a marked polarity with apical microvilli and basal nuclei (Fig. 2a). Junctional complexes were observed, especially in the apical pole. The endoplasmic reticulum and Golgi apparatus were well developed (Fig. 2b) and there were vesicles of several sizes that may have been multivesicular bodies, secretory granules or endocytotic vesicles. These vesicles were especially abundant in the apical pole, in which accumulations of mitochondria could also be found (Fig. 2c). At the basal pole, some vesicles and mitochondria could also be distinguished (Fig. 2d). Polarity was dependent on cell density, with a flattened shape and fewer microvilli at lower density (Fig. 2e). Fibroblast-like cells presented a markedly different morphology. Electron micrographs confirmed that they neither formed monolayers nor displayed signs of polarity (Fig. 3a). They had elongated central nuclei, abundant rough endoplasmic reticulum and relatively few organelles. Cytoplasmic fibrils could be seen in some areas (Fig. 3b). For further characterization of these populations, the epithelial cell markers cytokeratin and uvomorulin were used. Epididymal epithelial cell preparations stained positively (Fig. 4a, b) and specifically (Fig. 4c) for both markers, whereas fibroblast-like cells were negative (uvomorulin is shown in Fig. 4d; data for cytokeratin are not shown).

Dynamics of epithelial cell growth in culture

The evolution of mitotic indices was examined by labelling with [3H]methyl thymidine at different time points and analysing the parameters of cell density, androgen dependence, fibroblast coculture and temperature for effects on the dynamics of cell growth. Temperature was the only factor that displayed a significant effect (Fig. 5). The mitotic indices at 37°C peaked on day 3 while, at 32°C, the peak was not reached until day 4 (Fig. 5a). Temperature also had an effect on cell viability. At 37°C, the peak of growth occurred rapidly and, once proliferation stopped, the cells did not survive. At 32°C, however, although growth occurred at a slower rate, cells survived even when they were no longer proliferating (Fig. 5, compare (a) with (b)). Aside from temperature, the other factors examined (cell density, androgens and coculture with fibroblasts) did not detectably influence cell proliferation.

Localization of androgen receptor

Under our culture conditions, androgen receptors were detected in the nuclei of fibroblasts, but only in those cultured in the presence of androgens (Fig. 6a, b). They were also present in the nuclei of epithelial cells cocultured with fibroblasts in the presence of androgens, regardless of the temperature (Fig. 6c, d). Although present initially, the androgen receptor was undetectable by day 3 when the epithelial cells were cultured with fibroblasts but in the absence of androgens (Fig. 6e), or with androgens in the absence of fibroblasts (Fig. 6f).

Protein secretion by the cultured epithelial cells

The expression of a specific protein marker such as CP 27 was analysed (Figs 7 and 8). CP 27 was detected readily in supernatants recovered after incubation of whole epididymal tissue (Fig. 7, lane f) and in supernatants from cells that had been cultured in the presence of androgens and fibroblasts (Fig. 7, lanes d and e). When the epithelial cells were not cocultured with fibroblasts (Fig. 7, lane b) or when coculture occurred in the absence of androgens (Fig. 7, lane c), CP 27 was substantially reduced. CP 27 was also undetectable in supernatants from cultured fibroblasts (Fig. 7, lane a). The secretion of CP 27 by cultured cells varied with time (Fig. 8). During the first 2 days, CP 27 was present in all cultures regardless of temperature, androgen or fibroblast culture conditions. Since the CP 27 signal was maximal in supernatants collected after the first 2 days of culture, this was considered to represent 100% secretion and the amounts of CP 27 present in the supernatants on successive days were normalized as a percentage of this value. This analysis revealed that CP 27 production and secretion, or both, decreased abruptly on days 3–4 unless the cells were cultured at 32°C with fibroblasts and androgens. In this case,
Fig. 6. Localization of the androgen receptor in cultured epididymal cells. (a, b) Cultured fibroblasts, fixed 12 days after plating. The androgen receptor was detected in the nuclei of fibroblasts cultured in the presence (a), but not in the absence (b), of androgens. (c–f) Epithelial cells, fixed 4 days after plating. Epithelial cells, stained positively when cultured in the presence of androgens and fibroblasts, regardless of the temperature of the culture, (c: 37°C; d: 32°C). The intensity of staining varied, being more intense in some nuclei (arrows) than in others (arrowheads). However, androgen receptors were not detected in cells cocultured with fibroblasts at 32°C but in absence of androgens (e) or cells cultured at 32°C with androgens but in absence of fibroblasts (f). The speckled pattern seen in all figures is due to the pores of the filter that supported the cells. Scale bar represents 20 μm.

the amount of CP 27 present in the supernatants was about 70% of the maximum and differences between androgen and non-androgen conditions were clear (Figs 7 and 8). It is noteworthy that coculture with epididymal fibroblast-like cells produced this effect on CP 27 secretion and a mesenchymal cell line, REVC, was ineffective (Fig. 8). After 5–6 days, the amount of CP 27 decreased in all cases and could not be detected in supernatants collected on day 8.

Lectin blots were used to determine whether cultured epithelial cells secreted other tissue-specific markers such as the two WGA binding proteins GP-49 and GP-83 (Fig. 9). The results showed that the protein expression pattern of epididymal fluid in vivo (lane a) was preserved in vitro (lane d). Resembling the expression of CP 27, the expression of GP 49 and GP 83 required all three factors (32°C, androgens and fibroblasts); culture at 37°C (lanes e, f) or at 32°C without androgens (lane c) or without fibroblasts (lane b) prevented detection of these proteins.

Discussion

The isolation and culture of epididymal epithelial cells allows detailed studies on the physiology of the epididymis. The
epithelial cells used in these studies were purified from the distal caput and proximal corpus of the epididymis since these regions play an important role in sperm maturation (Orgebin-Crist et al., 1981; Bedford and Hoskins, 1990; Soler et al., 1994) and are easily digested using collagenase and dispase. One-month-old mice were used because, in young animals, fewer spermatozoa are present in the tubules and the connective tissue is more easily digested, enabling consistent recovery of larger numbers of cells than from older mice. The cultured epithelial cells exhibited < 5% contamination with other cell types, as judged by staining with lectins and cytokeratin. Regardless of the coculture with fibroblasts, these epithelial cells behaved as predicted; they formed confluent monolayers and retained ultrastructural and cytochemical features of epithelial cells. The most typical feature, marked polarity (mainly measured by cell height and development of apical structures, such as microvilli) was maintained and, importantly, it was correlated with cell density. Cells at low densities were flattened and had scarce microvilli, whereas at higher density they varied from cuboidal to columnar, although they never reached the height of the epithelium in vivo. They also displayed longer and more abundant microvilli. This effect on cell morphology has been reported by others (Byers et al. 1986) and suggests that intraepithelial paracrine factors play a role in maintaining a differentiated epithelial cell pattern. Finally, the cultured cells displayed positive staining for cytokeratin and uromorulin, recognized markers of epithelial cells (Byers et al., 1985; 1992; Finaz et al., 1991). These markers were present in the cells during the entire period of culture, regardless of the presence of androgens or fibroblasts.

Epididymal fibroblasts were isolated by taking advantage of the fact that these cells grow very well in vitro in a serum-rich medium. They overgrew the short-lived epithelial cells and, by day 10, were present at 100% purity. However, it is important to state that in these studies the broadest definition of the term “fibroblast” is used. In fact, it is likely that the fibroblast-like cells observed represent a mixed population since the epididymis contains peritubular myoid cells in addition to stromal cells. This fibroblast-like cell population showed a pattern of growth and appearance that is characteristic of all mesenchymal-derived cells when grown in culture. Their ultrastructural characteristics were also those of fibroblast-like cells (no signs of polarity, fibrils in the cytoplasm) and they secreted extracellular matrix. Neither of the epithelial markers examined (cytokeratin and uromorulin) showed a positive reaction with this population.

The cultured epithelial cells were able to undergo mitosis in the culture conditions used. There was no effect of either fibroblasts or androgens in promoting cell growth, in contrast to the situation described in prostatic cells (Cunha et al., 1985; Chang and Chung, 1989; Rundlett et al., 1992). However, unlike the situation in the prostate, the mitotic index in the epididymal epithelium decreases during postnatal development and is virtually zero in the adult (Sun and Flickinger, 1982). In fact, the cultured cells in the present study stopped proliferating spontaneously. Of the parameters examined, only temperature affected the dynamics of epididymal epithelial cell growth.

The localization of the androgen receptor in the cultured cells was studied since this is one of the most conspicuous features of differentiated epididymal epithelium in vivo. Androgen receptors are localized in the nuclei of epithelial cells and also in the subjacent stromal cells (Sar et al., 1990); during embryonic development of the organ, androgen receptors are also detected in mesenchymal cells before their localization in epithelial cell nuclei (Cooke et al., 1991). The androgen receptor was detected in the nuclei of epididymal fibroblasts cultured in the presence of androgens. In epithelial cells, nuclear localization was lost after 3 days of culture unless the cells were cocultured with fibroblasts in the presence of androgens. Temperature did not affect this pattern, which is in agreement with previous reports (Regalado et al., 1993; Pera et al., 1996).

For examination of protein secretion, the first marker studied was CP 27. This protein is the most abundant protein in fluid from the mouse cauda epididymides and is produced and secreted in the distal caput and proximal corpus epididymides (Flickinger et al., 1988). CP 27 was detected in supernatants of epithelial cells regardless of the culture conditions during the first 2 days. This represents a lag period in which the cells still reflect in vivo conditions. From day 3, the content of CP 27 in the supernatants decreased unless the cells were cocultured with fibroblasts at 32°C in presence of androgens. Importantly, the effect of coculture was somehow specific to epididymal fibroblasts, since coculture of epididymal epithelial cells with other mesenchyme-derived cells (REVC) failed to restore androgen sensitivity. It is noteworthy that the secretion of CP 27 cannot be maintained for long periods; after a week of culture, it was undetectable even with optimal conditions.
WGA staining was also used to evaluate protein secretion. Although not as specific as antibody detection, this probe has been used to define two glycoproteins, GP-49 and GP-83, which are androgen-dependent proteins secreted by the mouse corpus and cauda epididymides (Liu et al., 1992). GP-49 and GP-83 secretion paralleled the situation described above; only those epididymal epithelial cells cocultured with fibroblasts at 32°C in the presence of androgens displayed a pattern of secretion similar to that of the organ in vivo.

The results of the present study suggest that mesenchymederived cells in the epididymis play a key role in the maintenance of androgen responsiveness in the postnatal epithelium, supporting the suggestion of Cunha et al. (1985). This is not a characteristic of androgen regulation alone, but also occurs for oestrogen (Cooke et al., 1986). It has also been demonstrated that the culture of epithelial cells on feeder layers composed of stromal cells has beneficial effects regarding epithelial cell growth (Manin et al., 1992) and differentiation (Kedinger et al., 1987). With regard to a putative mechanism of action, the detection of larger amounts of secretory markers in epithelial cell supernatants after coculture cannot be explained on the basis of increased cell numbers, since the fibroblasts did not promote cell growth. Alternatively, it seems that fibroblasts may provide some signal to epithelial cells, enabling them to retain a differentiated pattern. This signal must be a soluble factor, since it can pass from the lower to the upper compartment. Swinnen et al. (1990) propose that soluble factors secreted by different cell types of mesenchymal origin modulate the androgen responsiveness of Sertoli cells in culture. Coculture of Sertoli cells with stromal or peritubular cells increases the secretion of some specific markers from the Sertoli cells, such as androgen-binding protein and transferrin (Verhoeven et al., 1992; Janecki and Steinberger, 1987; Thompson et al., 1995). Moreover, prostatic stromal cells and testicular peritubular myoid cells are effective in promoting such an effect, while footsole fibroblasts are not (Swinnen et al., 1990). The results of the present study are consistent with a similar mechanism of action.

Temperature was found to be a major factor controlling protein synthesis and secretion in the cultured epithelium, indicating that temperature regulates protein synthesis not only in the testis and distal portions of the epididymis (Esponda and Bedford, 1986; Bedford, 1991), but also in the proximal epididymis. Temperature effects do not appear to be mediated by androgens, and are probably also independent of fibroblast-secreted factors, since in the present study

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Fig. 8. Secretion of protein CP 27 as a function of time and culture conditions. CP 27 was quantified in the supernatants from the different cultures by densitometry. The quantities detected on day 2 were maximal for all samples; these values were considered 100% and used to normalize the other values measured. Data represent the mean ± so (n = 5). T, temperature; A, androgens; F, fibroblasts; *rabbit endothelial vascular cells (REVC) were used instead of epididymal fibroblasts. **Significantly different (P < 0.05).
temperature affected protein secretion but not androgen receptor localization. This is in agreement with previous reports in rats (Regalado et al., 1993) and dogs (Pera et al., 1996).

In summary, three factors appear to be essential to maintain a differentiated state in cultured epididymal epithelial cells. Temperature and androgens appear to operate via independent pathways. Fibroblasts, however, appear to facilitate the androgen response of the epithelium. Collectively, the results of the present study suggest that fibroblasts do not play a role in the differentiation of these cells as epithelial, since they retain their epithelial pattern in the absence of fibroblasts. Indeed, some epithelial features, such as marked polarity, may be self-regulated. Fibroblast factors may act to refine the epithelial pattern by inducing tissue-specific protein secretion. The system described here may be a useful tool with which to undertake molecular studies on differential gene expression and the secretory activity of the epididymis, as well as to promote sperm maturation in vitro.

The authors are grateful to C. J. Flickinger, J. C. Herr and K. L. Klots from the University of Virginia for providing the antisera against protein CP 27. They also thank J. M. Bedford (Cornell University, NY), T. G. Cooper (Institute of Reproductive Medicine, Münster, Germany), C. Kirchhoff (iHF, Hamburg, Germany) and R. Ivell (iHF, Hamburg, Germany) for helpful suggestions that allowed them to improve the manuscript. This work was supported by NIH grant HD 18201. R. Carballada is also grateful for support from the Andrew W. Mellon Foundation.

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