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Journal of Andrology, Vol. 23, No. 6, November/December 2002
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Immortalized Epididymal Cell Lines From Transgenic Mice Overexpressing Temperature-Sensitive Simian Virus 40 Large T-Antigen Gene

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Received for publication February 28, 2002; accepted for publication June 6, 2002.

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Abstract

Epididymal epithelium is well known as a site of secretion of various proteins present in epididymal luminal fluid. Although there have been many reports of primary cultures of epididymal epithelial cells, their growth is limited over time. We have established immortalized epididymal epithelial cell lines from primary cultures of epididymal cells from transgenic mice harboring temperature-sensitive simian virus 40 large T-antigen gene in order to study the regulatory mechanisms of epididymal function, including specific factor secretion. These cell lines (PC1 from proximal caput; and DC1, DC2, and DC3 from distal caput) have been maintained for more than 1 year and show temperature-dependent growth and expression of cytokeratin, a marker of epithelial cells. These cells express the androgen receptor as well as markers of the murine epididymal epithelium, PEB-like protein (ie, phosphatidye ethanolamine binding protein), E-RABP (ie, epididymal retinoic acid—binding protein), and EP17 (ie, epididymal protein of 17 kd). The androgen-regulated 5-kilobase mE-RABP promoter DNA fragment ligated to the neomycin-resistant gene was used for stable transfection of DC1 cells. Because the mE-RABP gene is specifically expressed in the distal caput, neomycin selection provides a pure population of epithelial cells from that segment. This neomycin-resistant immortalized cell line from the distal caput was cultured for more than 6 months. Such immortalized cell lines should be valuable tools for studying the regulation of tissue-specific gene expression, and may be used to identify one or more epididymal specific transcription factors involved in the expression of epididymal specific proteins.

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Key words: Epididymis, immortalized cell line, transgenic mouse, SV40T-antigen

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The epididymis plays an important role in male fertility because it is the site where spermatozoa develop progressive motility and fertilizing capacity. The epididymis is a long, convoluted tubule surrounded by connective tissue and several layers of smooth muscle. The lumen of the tubule is lined along its length by columnar epithelial cells. Although these principal cells are predominant, other cell types such as apical cells, clear cells, halo cells, and basal cells are also present in the epithelium (Reid and Cleland, 1957). The epididymal epithelium secretes proteins in a highly regulated and regionalized manner, so that maturing spermatozoa encounter discrete micro-environments as they transit through the epididymis (Vreeburg et al, 1990).

Cultured epididymal epithelial cell lines would be useful for gaining information on the function and regulation of these secretory proteins. After the initial reports of primary cultures of rat epididymal epithelial cells (Kierszenbaum et al, 1981; Klinefelter et al, 1982; Olson, 1983), many studies have reported success in establishing primary cultures of epididymal epithelial cells of other species such as mice (Bongso and Trounson, 1996; Carballada and Saling, 1997), hamsters (Moore and Hartman, 1986), rabbits (Orgebin-Crist et al, 1984), rams (Wagley et al, 1984), bulls (Joshi, 1985; Gagnon et al, 2000), dogs (Pera et al, 1996), and humans (Harris and Coleman, 1989; Cooper et al, 1990; Moore et al, 1992; Castellon and Huidobro, 1999). These cells flatten and form mono-layers, but they maintain some of their structural features such as surface microvilli, prominent Golgi apparatus, abundant rough and smooth endoplasmic reticulum, lipid droplets, and multivesicular bodies (Klinefelter et al, 1982; White et al, 1982; Olson et al, 1983). They also maintain some of their functional characteristics such as ion secretion and reabsorption (Wong, 1988; Leung et al, 2001a,b); testosterone metabolism (Klinefelter et al, 1982; Brown et al, 1983); expression of epididymal genes (Kierszenbaum et al, 1981; Pera et al, 1996); secretion of macromolecular components normally found in the lumen such as alkaline and acid phosphatases and glycosidases (Skudlarek and Orgebin-Crist, 1986; Cooper et al, 1989, 1990; Castellon and Huidobro, 1999) and secretory proteins (Carballada and Saling, 1997); and in cocultures, promotion of sperm motility, maturation, and viability (Klinefelter et al, 1982; Moore and Hartman, 1986; Bongso and Trounson, 1996; Akhondi et al, 1997; Gagnon et al, 2000).

Despite demonstration of their usefulness, primary cultures have not been used extensively because their life span is limited. In vivo, the principal cells of the epididymis are slow to renew (Clermont and Flannery, 1970) and they maintain this characteristic in vitro. The mitotic index is low (Brown et al, 1983; Olson et al, 1983; Orgebin-Crist et al, 1984; Byers et al, 1985) unless the cultures are contaminated with fibroblasts or unless they are derived from a fetal or pubertal epididymis (White et al, 1982; Harris and Coleman, 1989; Manin et al, 1992). Recently, the molecular events mediating hormone-responsive gene expression in the epididymis has been studied in HeLa and PC-3 cells (Lareyre et al, 2000). However, these results may not reflect the regulation observed in normal epididymal cells. Research on epididymal function would be greatly facilitated by the establishment of cell lines that would provide a continuous and stable supply of epididymal epithelial cells.

Transformation of primary cell cultures with a plasmid containing a viral oncogene such as simian virus 40 (SV 40) has generated long-term cell lines from several tissues (Ozer, 2000), including fetal human epididymis (Coleman and Harris, 1991) and dog epididymis (Telgmann et al, 2001). A transgenic mouse line has been produced by inserting into its genome a construct consisting of the coding sequence of a temperature-sensitive mutation of SV40 large T-antigen gene (tsA58 mutant).

This transgenic mouse constitutively expresses the mutant gene in every cell of the body (Tegtmeyer, 1975; Yanai et al, 1991). The temperature-sensitive mutant contains a single nucleotide mutation, which results in an oncogene product that is rapidly degraded at the nonpermissive temperature of 39 °C, but functions at the permissive temperature of 33 °C. Therefore, the functional expression of the SV40 large T-antigen gene and cell proliferation can be turned on or off by culturing the cells at 33 ° or 39 °C. These conditionally immortalized cultures of epithelial cells maintained some of the structural and functional characteristics of the normal parent cell populations (Obinata, 1997).

In this study, pure cultures of immortalized epithelial cells were obtained by serial cloning of primary cultures from the caput epididymidis of transgenic mice overexpressing the ts SV40 large T-antigen gene. These cells exhibit stable growth; and they express epithelial cytokeratin, the androgen receptor, and the principal cell markers mE-RABP (for murine epididymal retinoic acid binding protein), mEP17 (for murine epididymal protein of 17 kd), and PEB-like protein (for phosphatidylethanolamine binding protein). These permanent cell lines have been used for DNA transfection assays and will be useful for studying the regulation of gene expression in epididymal epithelial cells.

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Cell Isolation and Culture

Adult (10-12 weeks old) male transgenic mice harboring the ts SV40 large T-antigen gene (Yanai et al, 1991) were killed by CO_2 asphyxiation, and the epididymis was freed from the connective tissue and fat tissue. The epididymis was minced into small fragments followed by 3 rinses in Ca^{2+}, Mg^{2+}-free Hanks solution (Life Technologies, Grand Island, NY) to remove spermatozoa. The epithelial cells were transferred to 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA; Life Technologies) solution (5 mg of tissue per milliliter of solution). After incubation at 32 °C for 30 minutes in a thermo-bath shaker (60 cycles/min), the sample was centrifuged at low speed (800 x *g*, for 5 minutes). The supernatant was discarded, then the pellet was suspended with collagenase (1 mg/mL equivalent to 146 U/mg of solid; Sigma, St Louis, Mo) in Hanks solution (5 mg tissue per milliliter of solution). After incubation at 32 °C for 40-60 minutes in a thermo-bath shaker, the cell clumps were almost completely (99%) dissociated. The sample was allowed to settle for 5 minutes and aggregated spontaneously. The supernatant was discarded, and the sediment consisted mainly of epididymal epithelial cells. The cells were suspended into minimum essential medium (MEM) containing D-valine (Life Technologies) supplemented with nonessential amino acids (0.1 mM, Life Technologies), sodium pyruvate (1 mM), L-glutamine (4 mM), 5α-dihydrotestosterone (1 nM), 0.5% fetal calf serum (FCS; Life Technologies), and antibiotics (penicillin G 50 U/mL and streptomycin 50 µg/mL). These cells were cultured using collagen type IV-coated 6-well plastic culture plate (Becton Dickinson Labware, Bedford, Mass) in supplemented MEM at 37 °C for 24 hours in 5% CO_2 in air. The cells were then cultured under the conditions described above at 33 °C to induce SV40 large T-antigen expression. Each single colony was trypsinized using a cloning cylinder (Sigma Chemical Company, St Louis, Mo) and isolated. This cloning procedure was repeated at least twice during the process of cell isolation. The cloned cells were cultured for further studies in Iscove modified Dulbecco medium (IMDM; Life Technologies, Grand Island, NY) supplemented with the same reagents as described above and containing 0.5%-10% FCS.

Cell Growth

The cells (1 x 10^5) were cultured in a 6-well plastic culture plate in the supplemented IMDM containing 10% FCS at 33 °, 37 °, or 39 °C. They were harvested by trypsin-EDTA treatment at given

time points and counted using a hemocytometer.

Immunocytochemistry

For immunostaining, the cells were cultured in a 1-chamber slide (Division Miles Laboratories Inc, Naperville, Ill) at 33 °C, fixed in precooled (-20 °C) 100% ethanol solution for 2 minutes, then air-dried, and stored at -20 °C until used.

The fixed cells were serially hydrated, then treated with phosphate-buffered saline (PBS) pH 7.2 containing 0.3% (v/v) Triton X-100 for 10 minutes at room temperature. The slides were incubated with 1% (w/v) blocking reagent (Roche Molecular Biochemicals, Indianapolis, Ind) in 100 mM maleic acid, 150 mM NaCl pH 7.5 for 2 hours at 4 °C. The fixed cells were incubated with either cytokeratin antibody (68 µg/mL, Z062; DAKO Corporation, Carpinteria, Calif), mouse CRISP1 (22.5 µg/mL), PEB-like protein (20 µg/mL), or mE-RABP (27.5 µg/mL) rabbit polyclonal antibodies (Rankin et al, 1992a) for 12 hours at 4 °C. The secondary antibody employed was Alexa Flora 488-labeled goat anti-rabbit immunoglobulin (Ig) G (20 µg/mL, Molecular Probes, Eugene, Ore). In some cases, the immunoreaction was visualized using an avidin-biotin complex kit according to the manufacturer's instruction (Vectastain ABC kit elite, PK-6101; Vector Laboratories Inc, Burlingame, Calif). When needed, the cells were counterstained with Hoechst 33342 (10 µg/mL, Sigma). Control cells received the same treatment except that the primary antibody was either omitted or replaced with purified nonimmune rabbit IgG.

Immunofluorescence-stained samples were examined with an Axiophot microscope equipped for fluorescence (Carl Zeiss Company Ltd, Thornwood, NY). Images were captured with a CCD camera using SPOT RT software version 3.1 (Diagnostic Instruments Inc, Sterling Heights, Mich) and then edited with Adobe Photoshop 6.0 (Adobe Systems Inc, Mountain View, Calif).

Electron Microscopy

The cells were cultured in a 6-well culture plate coated with collagen type IV at 33 °C and harvested as described above. After centrifugation, the pellet was fixed with 2% glutaraldehyde in phosphate buffer (pH 7.2-7.4) for 12 hours at 4 °C. After thorough washing with phosphate buffer/sucrose solution (pH 7.2-7.4), the pellet was postfixed with 1% OsO_4, dehydrated in ethanol and propylene oxide, and embedded in a mixture of Epon and Araldite. Thin sections (60-nm thickness) were stained with uranyl acetate and lead citrate, then examined for morphology with a Phillips EM-400 electron microscopy (Phillips Electronics Instruments, Mahwah, NJ).

Electrophoresis and Western Blot Analysis

An equal amount of cell lysates was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions. The molecular mass of the antigen bound by the antibodies was determined by enzyme immunostaining of the protein after blotting to a polyvinylidene difluoride (PVDF) membrane (Immobilon-P, Millipore Corp, Bedford, Mass) from the SDS-PAGE gel according to standard methods described previously (Yu et al, 2000).

Transfection

Preparation of the Construct— A chimeric plasmid containing a 5 kilobase (kb) mE-RABP promoter fragment (Lareyre et al, 1998b, 1999) upstream of the neomycin phosphotransferase (*neo*) gene was constructed as follows: the coding region of the *neo* gene (*Hin*dIII-*Bam*HI fragment) was excised from the pCI-*neo* vector (Promega, Madison, Wis) and subcloned into the *Hin*dIII-*Bam*HI site of pBluescriptII SK(+) (Stratagene, La Jolla, Calif). To generate the *Hin*dIII site at the 3' end of the mE-RABP promoter, p*Hind*III was amplified by polymerase chain reaction (PCR) using promoter-specific

primers SM18 (5'-TGCTGGTCATACAGC-3' corresponding to -1268 to -1254 of the mE-RABP gene sense sequence) and SM23H (5'-CCAAGCTTTGGGTTCAGCTCCCCACCAGA-3' corresponding to +6 to +26 of the mE-RABP gene antisense sequence) (Lareyre et al, 1998) plus the *Hin*dIII digestive site at the 5' end of the primer (underlined). The PCR fragment was purified with Wizard PCR preps (Promega) and subcloned into pGEM-T Easy vector (Promega). The nucleotide sequence of the clone containing the PCR fragment was confirmed on both DNA strands and named pGT23HS. The partial fragment of the mE-RABP promoter (4.5 kb, *Sal*I-*Sma*I fragment) was digested from p*Hind*III and subcloned into the *Sal*I-*Sma*I site of pGT23HS to generate a 5 kb mE-RABP promoter fragment containing *Hin*dIII sites on both ends. The 5 kb mE-RABP promoter fragment was subcloned into the *Hin*dIII site in front of the *neo* gene and used for transfection.

Transfection— Cells (2 x 10^5) were suspended in 2 mL of supplemented Dulbecco modified Eagle medium (DMEM; Life Technologies) with 10% FCS, then seeded onto a 6-well culture plate (Becton Dickinson Labware). After 24 hours, 5 µL of PLUS reagent (Life Technologies) and 0.5 µg of plasmid construct was diluted in 100 µL of DMEM and incubated for 15 minutes at room temperature. Four or 8 µL of lipofectin reagent (Life Technologies) was diluted in 100 µL of DMEM and incubated for 15 minutes at room temperature. The 2 solutions were combined, gently mixed, and incubated for 15 minutes at room temperature as the transfection mixture. The medium was replaced on the cells with 800 µL of fresh DMEM, then the transfection mixtures were added to the wells and incubated for 4 hours at 37 °C in 5% CO_2. The medium was replaced with 2 mL of supplemented IMDM with 10% FCS and cultured for 24 hours at 33 °C in 5% CO_2. Transfected cells were cultured in the presence of G418 (200 µg/L) for 2 weeks. G418-resistant colonies were isolated using cloning cylinders as described above, and stable transformant clones were established within 8 weeks after the transfection.

Reverse Transcriptase-Polymerase Chain Reaction

Total RNA was isolated from 1-5 x 10^7 cells using an RNeasy midi kit (Qiagen Inc, Valencia, Calif). One microgram of total RNA was used to synthesize complementary DNA (cDNA) using a Thermoscript reverse transcriptase (RT)-PCR System (Life Technologies). After cDNA synthesis, 2 µL (10% of the total volume) of cDNA was subjected to 40 cycles of PCR, denaturing at 94 °C for 15 seconds, annealing at 55 °C for 30 seconds, extension at 72 °C for 30 seconds, and a final extension at 72 °C for 5 minutes using Platinum*Taq* DNA polymerase (Life Technologies). To amplify the message of specific molecules, SV40 large T-antigen—specific primers (5'-GAAAATGGAAGATGGAGTAAA-3' and 5'-AATAGCAAAGCAAGCAAGAGT-3'), *neo*-specific primers (5'-CCGGTGCCCTGAATGAACTGCAGG-3' and 5'-CCAACGCTATGTCCTGATAGCGGT-3'), or PEB-like protein primers (5'-TTAGCAGTGGCACTGTCCTC-3' and 5'-GGTTATACTTCTTGCGGAAGG-3', GenBank accession number BC008169) were used. For mE-RABP RT-PCR, primary primers (5'-TCAGCGAAGTACAAGGTCACC-3' and 5'-CATTGTTGTCCAAGCTCCG-3') were used first. PCR reactions were then diluted with water (1:100) and 1 µL of that solution was used for nested PCR with mE-RABP-specific primers (5'-AGAGAACCTTCTGGCTCTGACC-3' and 5'-TGCTTCAAGGCTATCTTCTGG-3'). Specific primers for ß-actin (Life Technologies) were used as a control.

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Establishment of Epididymal Cell Lines From Transgenic Mice

To establish epididymal cell lines, tissues were obtained from 10- to 12-week-old transgenic mice and dissociated cells were cultured on a collagen type IV—coated plastic plate in supplemented modified essential medium (MEM) with 0.5% FCS. Cells were cloned by colony formation during 4 to 6 weeks, then cultured in supplemented MEM containing 2.5% of FCS. After recloning, cells were maintained in

supplemented IMDM containing 2.5% FCS for 2 weeks, 5% for 2-5 weeks, 7.5% for 4-6 weeks, and up to 10% after 6 weeks. Within 15 weeks, 4 cell lines (PC1 from proximal caput; and DC1, DC2, and DC3 from distal caput) were established and they were maintained on a collagen type IV—coated plastic plate and passaged by trypsin treatment each week. All cell lines established have been through more than 50 passages in continuous culture without reaching a marked crisis. These cell lines grow on either plastic plates (with or without collagen coat) or glass slides. However, the population doubling time of the cells on glass slides is significantly lower than that observed on plastic plates or collagen-coated plastic plates (data not shown).

Figure 1 represents the growth of the epididymal cell lines at permissive temperature (33 °C) and nonpermissive temperature (39 °C). The growth rate was markedly decreased from 2 to 4 days of culture at 39 °C in all established cell lines. Prolonged culture at nonpermissive temperature caused significant cell death, especially in DC3 cells. At 33 °C, these cell lines have been maintained for more than 1 year. A partial inhibition of cell growth was seen at 37 °C (data not shown). RT-PCR analysis using specific primers for SV40 large T-antigen demonstrated that the message was expressed in all established cell lines (Figure 2).

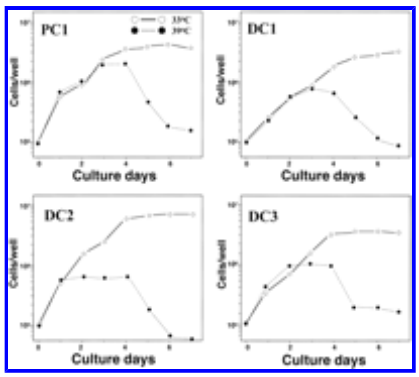

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Figure 1. Cell growth of the cell lines under different temperature conditions. Epididymal cell lines (PC1, DC1, DC2, and DC3) were cultured on 6-well plate coated with collagen type IV in supplemented IMDM with 10% FCS, and harvested with trypsin for cell counting. Open and solid circles indicate cell numbers cultured at permissive (33°C) and nonpermissive (39°C) temperatures, respectively. All cell numbers are the average of triplicate experiments. Values of the triplicates are so close that standard errors are too small to be seen with the y-axis in log phase.

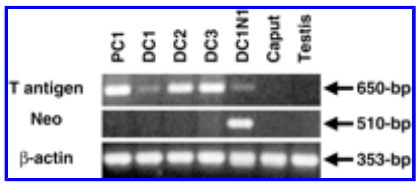

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Figure 2. Transgene expression in the cell lines. T-antigen transcripts or *neo* transcripts were determined by RT-PCR using gene-specific primers (see "Materials and Methods"). Beta-actin was used as a control to monitor RNA quality and template amount.

Morphology of Epididymal Cell Lines

Confluent cells were polygonal in shape with large nuclei (Figure 3, PC1 and DC2). Before reaching confluency cells were more elongated, but they displayed large nuclei (Figure 3, DC3). One cell line (DC1) had consistently smaller nuclei than the other 3 cell lines.

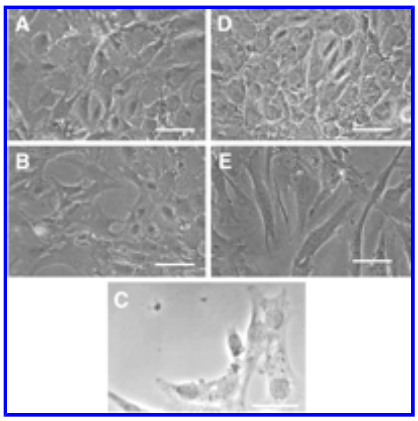

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Figure 3. Photomicrographs of established cell lines PC1 **(A)**, DC1 **(B)**, DC1N1 **(C)**, DC2 **(D)**, and DC3 **(E)**. Cells were cultured for 3 days after approximately 30 passages on collagen type IV—coated plastic plate in supplemented IMDM with 10% FCS. Bar = 10 µm in **A, B, D**, and **E**. Bar = 15 µm in **C**.

Transmission electron microscopy revealed that cultured cells retained many of the cytoplasmic features that characterize epithelial cells in the murine epididymis (Soranzo et al, 1982; Abe et al, 1983), including a prominent Golgi apparatus, abundant rough and smooth endoplasmic reticulum, lipid droplets, and dense granules (Figure 4). Depending on the plane of the section, some cells display a polarity similar to that seen in vivo (Figure 4; PC1, DC2, and DC3). A large highly lobulated nucleus is seen at one pole with rough endoplasmic reticulum located between the nucleus and the smooth plasma membrane. The Golgi apparatus, interspersed with lipid droplets and dense granules, is localized in the supranuclear region, and the plasma membrane at that pole displays blebs, microvilli, and interdigitation, which are suggestive of the stereocilia border seen at the apex of epididymal cells in vivo.

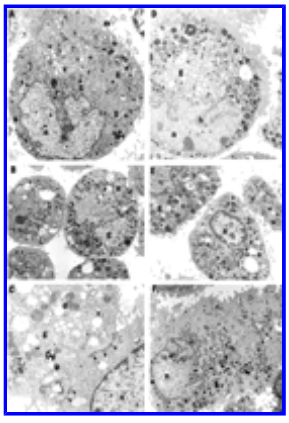

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Figure 4. Transmission electron micrographs showing PC1 **(A)**, DC1 **(B** and **C)**, DC2 **(D)**, DC1N1 **(E)**, and DC3 **(F)** cultured for 4 days after approximately 20 passages on collagen type IV—coated plastic plates in supplemented IMDM with 10% FCS and fixed with 2% glutaraldehyde. Note the polarized distribution of organelles in PC1 **(A)**, DC2 **(D)**, and DC3 **(F)** cells: the infranuclear surface of the cell shows a sparse distribution of microvilli (m), whereas the supranuclear surface show blebs and interdigitated microvilli. DC1 **(B)** and DC1N1 **(E)** cells are smaller than PC1, DC2, and DC3 cells. The cells possess an abundant Golgi apparatus (g), vacuoles (v), and dense granules (d). Nuclei (n) are highly lobulated and junctional complexes (arrowheads) can be seen between adjacent cells. Magnification: **A**, x6382; **B**, x7500; **C**, x10260; **D**, x5750; **E**, x5750; **F**, x3167.

DC1 cells and DC1N1 cells, derived from DC1, are smaller than PC1, DC2, and DC3 cells, but they contain the same organelles. Tight junctions between neighboring cells were frequently seen and are illustrated in Figure 4 between 2 DC1 cells. In vivo lateral desmosomes and gap junctions are a prominent feature of the epididymal epithelium (Soranzo et al, 1982).

Expression of Molecular Markers in Epididymal Cell Lines

In order to demonstrate the epithelial origin of the established cell lines, a cytokeratin antibody was used for immunofluorescence staining. As shown in Figure 5, positive immunostaining of cytokeratin was observed in the cytoplasm of the principal cells of the epididymal epithelium and the epididymal cell lines. The immunoreaction observed in immortalized cells indicates that they are derived from the epithelium of the epididymis. The absence of nonreactive cells in all cell lines indicated that the subcloning procedure had been successful and that we had established pure populations of epithelial cells.

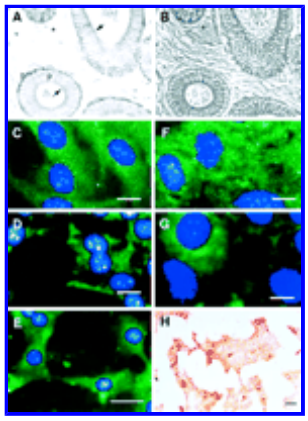

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Figure 5. **(A)** Photomicrograph of epididymis fixed in Bouins fluid, stained for immunocytochemistry after incubation with a cytokeratin antibody (272 µg/mL). **(B)** Phase contrast image of the same section shown in **(A)**. Note the immunostaining of the principal cells (p), the stronger staining of the basal cells (b), and absence of staining in the peritubular tissue (star). **(C-G)** Immunofluorescence of the cell lines cultured for 1-2 weeks after approximately 30 passages on glass slides with supplemented IMDM with 10% FCS, stained with a cytokeratin antibody (68 µg/mL). Cytokeratin immunofluorescent staining is green and Hoechst 33342-stained nuclei are blue. **C**, PC1; **D**, DC1; **E**, DC1N1; **F**, DC2; **G**, DC3. **(H)** Photomicrograph of DC1 cells cultured for 1 week on a glass slide with supplemented IMDM with 10% FCS, stained for immunocytochemistry with a cytokeratin antibody (68 µg/mL), and counterstained with hematoxylin. Note that at this low magnification, all cells are immunopositive. Magnification: **A** and **B**, x270. Bar in **C-G** = 10 µm. Bar in **H** = 40 µm.

Normal epididymal epithelial cells secrete several proteins that can be used as molecular markers of cell line function (CRISP1, PEB-like protein, mE-RABP, and mEP17 [Araki et al, 1992; Rankin et al, 1992a; Vierula et al, 1992; Lareyre et al, 2001]). As shown in Figure 6, all the cell lines examined after 3-10 weeks in culture retained positive staining with the PEB-like protein antibody; however, there was no significant positive immunoreaction with preimmune rabbit IgG or with the CRISP1 or mE-RABP antibodies (data not shown).

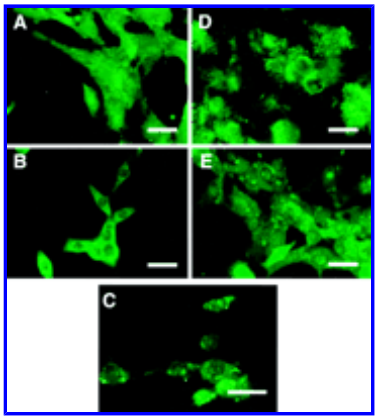

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Figure 6. Immunofluorescence of the epididymal cell lines cultured for 1 week after approximately 30 passages on a glass slide with supplemented IMDM with 10% FCS and stained with PEB-like protein antibody (20 µg/mL). **A**, PC1; **B**, DC1; **C**, DC1N1; **D**, DC2; **E**, DC3. Bar = 10 µm in **A, B, D**, and **E**. Bar = 15 µm in **C**.

A Western blot analysis of a gel probed with the PEB-like protein antibody revealed a distinct band at an apparent molecular mass of 25 kd in all cell lines after approximately 30 passages after isolation, and in epididymal tissue extract obtained from control adult mice (Figure 7). A positive band with an apparent molecular mass of ~24 kd probed with the mE-RABP antibody was clearly observed in PC1, DC1, DC2, and DC3 cells (Figure 7), whereas in native epididymal tissue extract, the mE-RABP antibody mainly reacts with 4 bands at an apparent molecular mass of 18, 21, 24, and 26 kd (Figure 7). An immunopositive band was also observed in all cell lines, except HeLa cells probed with an androgen receptor (AR)-specific antibody against the N-terminal peptide of the AR (Figure 8A). The reactivity was suppressed when the AR antibody was pretreated with the blocking peptide sc-816P (Figure 8B). No immunoreactive band was observed in any of the cell lines with the CRISP1 or the mEP17 antibodies (Figure 7).

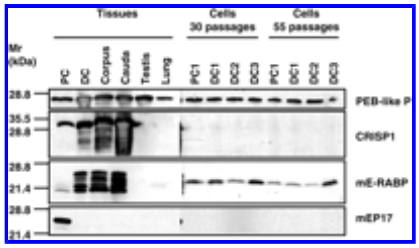

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Figure 7. Western blotting analysis of the epididymal tissues and lysates of epididymal cell lines from different passage periods. Aliquots containing proteins (20 µg from epididymal extracts, 50 µg from testis or lung extracts, or 100 µg from packed cell extracts) were resolved in 12% SDS-PAGE under reducing conditions. After electrophoresis, the proteins were transblotted to a PVDF membrane and detected as described by either PEB-like protein (PEB-like P, 5 µg/mL), CRISP 1 (5 µg/mL), mE-RABP (5 µg/mL), or mEP17 (3 µg/mL) antibodies. The cells were cultured for approximately 30 passages or approximately 55 passages after isolation, and cultured on a plastic plate coated with collagen type IV with supplemented IMDM and 10% FCS. PC indicates proximal caput; DC, distal caput.

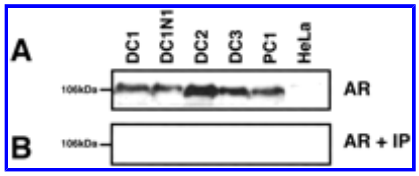

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Figure 8. Immunodetection in the cell lysates probed by the androgen receptor antibody sc-816 (1 µg/mL) (**A**) and the same reaction in the presence of the blocking peptide for the antiandrogen antibody (**B**).

RT-PCR analysis using total RNA from the cell lines (after approximately 30 passages) with PEB-like protein, mE-RABP, and mEP17 specific primers is shown in Figure 9. Primary RT-PCR detected PEB-like protein messenger RNA (mRNA) in all cell lines, mEP17 mRNA in DC2 and DC1N1 cells, but it failed to detect mE-RABP in the cell lines, although nested PCR detected a positive band in all cell lines (Figure 9). All RT-PCR fragments were confirmed as mE-RABP, PEB-like protein, and mEP17 cDNA by sequencing (data not shown). HeLa cells did not express PEB-like protein, mEP17, and mE-RABP mRNA. RT-PCR reactions done without cDNA (Figure 9) or with RNA but without reverse transcriptase (data not shown) were negative. Data obtained from a qualitative analysis of the various molecular markers in established cell lines are summarized in the Table.

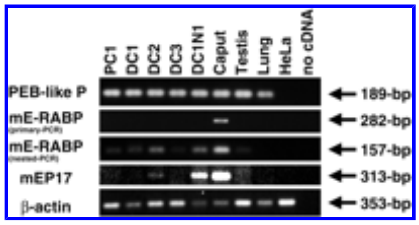

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Figure 9. RT-PCR analysis of PEB-like protein, mE-RABP, and mEP17 in the cell lines. Cells were cultured in IMDM supplemented with 10% FCS in a plastic culture bottle until they were confluent, and then harvested for RNA isolation. The cells were 30 weeks old (passaged approximately 30 times) after isolation. PEB-like protein, mE-RABP, or mEP17 mRNA expression was confirmed by RT-PCR using gene-specific primers (see "Materials and Methods"). Beta-actin was used as a control.

Neomycin-Resistant Stable Transfectants Derived from DC1 Cells Using a 5-Kilobase DNA Fragment of mE-RABP Promoter

Previously, we showed that the gene encoding mE-RABP is specifically expressed in the epithelium of mid and distal caput epididymidis under androgen control (Lareyre et al, 1998a,b), and that the 5 kb promoter fragment of the mE-RABP gene contains all the information required for hormonal regulation and spatial and temporal expression of the mE-RABP gene in the epididymis (Lareyre et al, 1999). Therefore, we attempted to establish a stable transfectant cell line using the androgen-regulated mE-RABP promoter to direct the *neo*-resistant gene in DC1 cells.

The construct for transfection, composed of the 5 kb mE-RABP promoter and *neo* resistant gene, was transfected into 1.2 x 10^6 DC1 cells. One day after the transfection, cells were cultured in IMDM with 10% FCS in the presence of G418 (200 µg/mL). After 8 weeks, 6 colonies showed G418 resistance and were recloned by colony formation. Each clone was then transferred into 24-well culture plates coated with collagen type IV for expansion. The concentration of G418 in the culture medium was increased to 400 µg/mL and the cells were maintained. Using this procedure, 5 independent neomycin-resistant transfectants from DC1 cells, termed DC1N1 to DC1N5, were established. Their morphology and size are similar to those of the original DC1 cell line (Figures 3 and 4). RT-PCR analysis demonstrated *neo* gene expression in the cells (Figure 2). In the neomycin-resistant cell line, expression of the mE-RABP protein and the PEB-like protein was similar compared to that of the parent cell line, DC1 (data not shown).

Discussion

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The epididymis, a tubular organ exhibiting vectorial functions of sperm concentration, transport, maturation, and storage is a unique model for studying tissue- and region-specific gene expression (Orgebin-Crist, 1996; Cornwall et al, 2002). In order to study the molecular mechanisms regulating this tissue and region-specific gene expression, pure epididymal epithelial cell lines that express epididymis-specific genes are useful.

Although several reports have described primary cultures of epididymal epithelial cells, a common problem of these cultures is their short life span. Primary cultures from mature epididymis do not proliferate for more than approximately 1 week after seeding. However, a common problem is the variability between cultures due to the varying degree of contamination with nonepithelial cell types (Klinefelter et al, 1982). Cultures from vas deferens epithelial cells from immature mice maintain some proliferating activity and can be used for up to 1 month (Manin et al, 1992). Long-

term cell lines generated from human fetal epididymal primary cultures followed by transformation with an origin-defective SV40 gene have been reported (Coleman and Harris, 1991). These lines maintain expression of the cystic fibrosis gene normally present in the epididymal epithelium. More recently, primary cultures of canine epididymal epithelium were immortalized by retroviral transfection with SV40 large T-antigen. These cells maintain expression of molecular markers abundantly expressed throughout the epididymis, but they do not express markers with a region-specific pattern of expression (Telgmann et al, 2001). This may occur either because region-specific expression is lost in these immortalized cells or because the cells were derived from an epididymal segment not expressing these markers.

We have established immortalized cell lines from the epididymis of mature transgenic mice overexpressing the ts SV40 large T-antigen (Yanai et al, 1991). The same transgenic mice previously have provided various immortalized epithelial cell lines from several organs (Obinata, 1997). The properties of these established lines suggested that their growth was dependent on large T-antigen expression, and that they retained some of the differentiated functions of each particular tissue (Obinata, 1997). The immortalized epididymal cell lines we developed are the first described for mice, the most common mammalian species used for molecular genetics and fertilization studies. Moreover, these cell lines were derived from 4 defined segments of the caput epididymidis. We have also established a stable transformant of the DC1 cell line expressing the neomycin-resistant gene under the control of a 5-kb promoter fragment of the mE-RABP gene. This cell line has been cultured for more than 6 months in the presence of neomycin. It should be noted that transfection may disrupt its gene expression pattern. This appears not to be the case in the DC1N1 cell line because the expression of endogenous mE-RABP is similar to the parent cell line DC1.

The cells divide rapidly and, until they reach confluency, are elongated, but they do not display the spindly characteristic of proliferating fibroblasts. At confluency they form a monolayer of flattened polygonal cells typical of epithelial cells in culture. In vivo, the epididymal epithelium consists of tall highly polarized columnar cells joined by tight junctional complexes forming a permeability barrier between the systemic circulation and the lumen, where spermatozoa mature and are stored. The morphology of the principal cells in primary cultures is best preserved when cells are cultured at high density on matrix-impregnated permeable support (Byers et al, 1986). When these cells are placed in a dual compartment chamber they maintain some of their polarized function such as impermeability to tracers and electrical resistance across the monolayer (Byers et al, 1992). Our immortalized cell lines were cultured on plastic dishes coated with collagen type IV. They retain the cellular organelles that are characteristic of epithelial cells in intact epididymis such as large lobulated nuclei, abundant Golgi apparatus, numerous lipid droplets, and dense bodies. Although the cells were harvested by trypsin-EDTA treatment and centrifuged before fixation, they retain the type of junctional complexes between cells seen in epididymal principal cells. Our data indicate that immortalized epididymal epithelial cells form de novo junctional complexes on contact. Although they do not retain the typical cell shape seen in vivo, they maintain a similar polarity in the distribution of cellular organelles.

Previous studies have shown that keratin-containing intermediate filaments are found only in epithelial cells and not in connective tissue or muscle cells of various tissues (Franke et al, 1978; Sun et al, 1979), including the epididymis (Olson et al, 1983). Our results show that the subcloning procedure generated cell lines that show some morphological differences (Figures 3 and 4), but were 100% positive when a cytokeratin antibody was used for immunostaining (Figure 5), strongly suggesting that we have obtained pure epithelial cell lines derived from the epididymis. These cell lines isolated from the caput epididymidis (PC1 from proximal caput; and DC1, DC2, and DC3 from distal caput) have been maintained for more than 1 year and show temperature-dependent cell

growth (Figure 1).

Immunofluorescence studies as well as Western blotting analysis revealed that all 4 cell lines are immunopositive for PEB-like protein (Figures 6 and 7). PEBP is a 21- to 25-kilodalton protein, originally purified from bovine brain (Bernier and Jolles, 1984) and is now recognized as belonging to a family of proteins widely expressed and evolutionarily conserved from plants to mammals (Schoentgen and Jolles, 1995). It has been reported that PEBP functions as either a serine protease inhibitor (Hengst et al, 2001) or a protein kinase inhibitor (Yeung et al, 1999). In the murine male reproductive tract, a PEB-like protein is present in the cytoplasm of testicular germ cells, and the cytoplasmic droplet of epididymal spermatozoa, but it is also secreted into the lumen by the principal cells of the caput epididymidis (Rankin et al, 1992a). The epididymal form differs from the reported sequence of brain PEBP by 1 amino acid (Chaurand, personal communication). The epididymal origin of the luminal PEB-like protein was confirmed by the normal distribution of immunoreactive sites in the epididymal epithelium after unilateral castration or severance of the efferent ducts, and in the epididymis of immature mice and XXS*xr* mice with no spermatozoa in the epididymis (Vierula et al, 1992). Although the secretion of PEB-like protein has been questioned based on the lack of secreted signal in its sequence and the absence of PEB-like protein in sperm-free epididymal fluid from castrated, androgen-treated rats (Perry et al, 1994; Frayne et al, 1998), other studies have shown that PEB-like protein is secreted by rat germ cells in the testis (Onoda and Djakiew, 1993; Saunders et al, 1995) and in conditioned medium of PEBP-expressing cells (Hengst et al, 2001). The presence of PEB-like protein in immortalized cell lines isolated from the proximal and distal caput epididymidis (Figures 5,6,7) indicates that these cells have retained expression of the PEB-like protein gene and confirms their epithelial origin because the PEB-like protein is not expressed in other cell types in the epididymis (Rankin et al, 1992a; Vierula et al, 1992). Although radiolabeling studies of synthesis and secretion showed that protein with a molecular weight similar to PEB-like protein is synthesized in the proximal and the distal caput (Holland et al, 1992), immunolocalization studies indicated that PEB-like protein is first detected in mid caput (Rankin et al, 1992a; Vierula et al, 1992). It appears there is no difference in immunoreactivity between the cell lines derived from the proximal or the distal caput. This could suggest that sectioning of the proximal caput has included a more distal segment; however, this is unlikely because the sectioning was performed conservatively in order to obtain cells from the initial segment. It may be that in vivo expression of PEB-like protein is negatively regulated in the proximal caput.

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Detection of molecular marker proteins in epididymal cell lines

CRISP1 is an abundant protein, under androgen control, secreted by the distal caput-corpus and cauda of mouse epididymis (Rankin et al, 1992a; Vierula et al, 1995). Although metabolic studies have showed that CRISP1 is first synthesized in the distal caput (Cornwall et al, 1990; Vreeburg et al, 1990), immunolocalization studies first detected the protein at the junction of the distal caput-corpus (Rankin et al, 1992a; Vierula et al, 1995). This may explain the absence of immunoreactivity in the proximal caput and the distal caput cell lines, especially if the subcloning procedure selected for cell clones did not express CRISP1.

mE-RABP is an androgen-regulated lipocalin of 18.5 kd synthesized by the principal cells of the mid and distal caput epididymidis and secreted in the lumen (Cornwall et al, 1990; Vreeburg et al,

1990). It belongs to the superfamily of lipocalins and, in vitro, binds retinoic acid (Rankin et al, 1992b). mEP17 is a testicular factor—regulated 17-kd lipocalin synthesized by the principal cells of the initial segment of the caput epididymidis (Lareyre et al, 2001). The mE-RABP and mE17 genes evolved by gene duplication from an ancestral gene (Lareyre et al, 2001). mEP17 mRNA was expressed in DC2 and DC1N1 cell lines. Expression in DC1N1 cells is not surprising because the mE-RABP promoter used to transfect the neomycin-resistant gene contains the mE17 gene (Lareyre et al, 2001). However, it is not clear why mEP17 expression would occur in the DC2 cell line rather than the PC1 cell line derived from the epididymal segment, where mEP17 is specifically expressed in vivo. Because mEP17 is regulated by one or more yet-unidentified testicular factors, one may surmise that the medium did not provide the necessary factor or factors for mEP17 expression in PC1 cells, but expression in DC2 cells may suggest a negative regulation of mEP17 in the distal caput. Western blot analysis failed to detect immunoreactive mEP17 in the cell lines. In vivo, mEP17 is secreted and found in the lumen of the initial segment and segment 2, but, unlike mE-RABP, it disappears from the lumen more distally. mEP17 may be unstable or more rapidly degraded than mE-RABP, and this may explain the absence of immunoreactivity in the cell lines.

mE-RABP could not be detected in the cytoplasm of the cell lines by immunostaining. In vivo, a faint immunostaining is seen in the supranuclear area of the principal cells of the mid and distal caput by light microscopy (Rankin et al, 1992a). However, in contrast to CRISP1, no specific labeling of the organelles involved in the secretory pathway could be detected by electron microscopy (Vierula et al, 1995). The transport kinetics of newly made secretory proteins from the endoplasmic reticulum is a highly regulated event that may be affected by differential folding rates, posttranslational modification of the proteins (Mellman and Simons, 1992), or both. It was suggested that, in vivo, the rate of transport and secretion of mE-RABP may be more rapid than that of CRISP1, and that the amount of mE-RABP in the organelles involved in the secretory pathway of the principal cells may be below the limit of immunohistochemical detection (Vierula et al, 1995). This may also be the case in our cell lines, especially if the endoplasmic reticulum and Golgi apparatus are not as well developed as in the principal cells in situ. RT-PCR did not detect mE-RABP mRNA in any cell lines, but nested PCR did detect it in PC1 cells and testicular tissue. However, in both, the levels were lower than that seen in DC1 and DC2 cells, and caput tissue. In vivo mE-RABP expression in proximal caput was not detected by in situ hybridization (Lareyre et al, 1998a), suggesting a negative regulation of mE-RABP in the proximal caput. Despite low levels of mE-RABP mRNA, Western blot analysis clearly showed that mE-RABP was expressed in the cell lines (Figures 6,7,8), including PC1, even after 55 passages. In Western blots stained with diaminobenzidine, mE-RABP resolves as a doublet of 18.5 kd (Rankin et al, 1992a). With enhanced chemiluminescence, 4 immunoreactive bands of 18, 21, 24, and 26 kd were observed. The immunoreactive band in the cell lines has a molecular weight of 24 kd. Because the sequence of the PCR product corresponds to mE-RABP, it suggests that posttranslational modifications occurring in the cultured cells are different from that occurring in the tissue. The relatively high level of protein compared to its mRNA may suggest that mE-RABP is very stable, or it is not secreted as readily as in in vivo and it accumulates in the cell lines, or both.

Our results demonstrate that immortalized cell lines have maintained the spatial expression pattern seen in vivo for the androgen-regulated mE-RABP gene. In contrast, epididymal cells from immature mice cultured on matrix-impregnated permeable support in a dual compartment chamber in serum-free medium did not maintain androgen receptor expression and secretion of an androgen regulated marker unless fibroblasts were cultured in the basal compartment of the dual culture chamber (Carballada and Saling, 1997). Likewise, epithelial cells from the vas deferens of immature mice, amplified by subculturing over a 3T3 feeder layer in a serum-free medium and transferred to a plastic dish coated with collagen type 1, proliferate without androgens in the medium, but do not retain their androgen

responsiveness, as judged by secretion of a specific marker of the vas deferens, mouse vas deferens protein (Manin et al, 1992). Only when cells are seeded at confluency onto matrix microporous membranes in a dual compartment chamber do they become polarized and become able to respond to androgen stimulation by expressing the *mvdp* gene (Manin et al, 1992; Dassouli et al, 1994, 1995; Darne et al, 2000; Manin et al, 2000). In contrast, the growth of our immortalized cell lines on collagen type IV—coated plate appears to be dependent on androgen and fetal calf serum, as has been reported for other cell lines (Tajana et al, 1984), and they express both the androgen receptor and mE-RABP, a highly sensitive marker of androgen stimulation (Holland et al, 1992). The level of expression of mE-RABP, as determined by nested RT-PCR, is low and it is likely that, as was demonstrated for primary cultures of vas deferens epithelial cells from immature mice, maximum androgenic stimulation may be achieved only after full polarization on extracellular matrix coated microporous membranes.

In summary, we have established for the first time immortalized cell lines from the caput of the murine epididymis. The functional status of the cell lines from the proximal and distal caput was evaluated by both Western blotting and RT-PCR analysis. Results show that these cells express both the androgen receptor gene and the mE-RABP gene, which are known to be androgen-regulated in vivo. Furthermore, these cells have been successfully transfected with plasmids containing the mE-RABP promoter ligated to the neomycin-resistant gene. The transfection of expression vectors with a reporter gene into cultured cells has been extensively used to characterize *cis*-regulatory elements such as promoters and enhancers. This study constitutes a primary characterization of epididymal immortalized cell lines and further analysis will be carried out to identify more epididymal markers expressed in these cell lines. They should be valuable tools for studying the regulation of tissue-specific expression and may be used to identify one or more transcription factors involved in the expression of epididymis specific genes.

Acknowledgments

We are indebted to Drs Kotaro Ishibashi (Daiichi Pharmaceutical Co Ltd), Shane Cutler, Pierre Chaurand, Naoya Masumori, Robert Whitehead, and the Electron Microscopy Core of the Department of Pathology (Vanderbilt University) for their technical support and helpful discussions. The excellent secretarial assistance of Mrs Loreita Little is gratefully acknowledged.

Footnotes

Supported in part by the Rockefeller/Ernst Schering Foundations and grant HD36900 from the National Institutes of Health.

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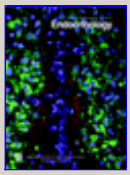

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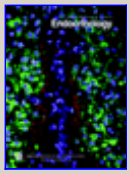

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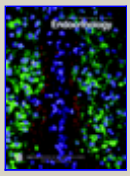

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