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Immortalization and characterization of proximal tubule cells derived from kidneys of spontaneously hypertensive and normotensive rats

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Immortalization and characterization of proximal tubule cells derived from kidneys of spontaneously hypertensive and normotensive rats. Epithelial cell lines from the proximal tubule of SHR and WKY rats were generated by microdissection, cell growth on 3T3 cell feeder layers, and transduction of the SV40 large T-antigen gene. The cell lines that formed confluent, electrically-resistive monolayers (basal conductance 1 to 20 mS/cm²) were selected for further study. Of these, cell lines generated from one rat did not show evidence of T-antigen expression or integration, and apparently immortalized spontaneously. Cell lines from three other rats expressed high levels of T-antigen, and showed evidence of integration of one or more copies of T-antigen. All cell lines formed polarized monolayers with apical microvilli, tight junctional complexes, and convolutions of the basolateral plasma membrane. Most cell lines grew in the absence of extracellular glucose indicating a capacity for gluconeogenesis. Sodium succinate cotransport and P₂-purinergic receptor mediated signaling were demonstrated in all lines tested. The cell lines also showed that Na/H exchanger activity is regulated by angiotensin II. The results indicate that these cell lines express a proximal tubular phenotype, and are morphologically and functionally similar to primary cultures. These rat cell lines represent a new, potentially useful cell model for elucidating the cellular and molecular mechanisms of genetic differences in proximal tubule Na⁺ reabsorption.

Essential hypertension is a major cause of cardiovascular morbidity and mortality throughout the world. Its pathogenesis and pathophysiology are complex and influenced by many inter-related genetic, environmental, and ethnic factors [reviewed in 1]. The accumulated data suggest a polygenetic disease manifesting a variety of cellular and molecular changes that are poorly understood in terms of physiological importance.

Salt-sensitive hypertension is a subtype of essential hypertension in which blood pressure can be controlled by restrictions in salt intake or diuretics. The kidney is central to the pathogenesis of high blood pressure in salt-sensitive individuals, due to its dominant role in metabolic regulation of Na⁺ homeostasis. Pathophysiological considerations and experimental evidence suggest that a decrease in the renal set point for Na⁺ reabsorption, that is an increase in Na⁺ reabsorption, leads to a hereditary form

of hypertension, for example in Liddle's syndrome [2, 3]. Although the renal tubular segments involved in essential hypertension have not been established, three lines of evidence point to a more proximal site for altered Na⁺ reabsorption: (1) hypertension due to increases in distal Na⁺ reabsorption, but not essential hypertension, can be treated with mineralocorticoid antagonism and/or distal diuretics (amiloride); (2) increased distal Na⁺ reabsorption due to genetic abnormalities in a Na⁺ channel is associated with a relatively severe, early onset type of hypertension (Liddle's syndrome), which is different from the later onset in essential hypertension (weeks in rat models; decades in humans); and (3) hyperaldosteronism is a frequent cause of increased distal Na⁺ reabsorption, while aldosterone levels are not generally elevated in essential hypertension. The late onset of high blood pressure in essential hypertension is consistent with an increase in the activity of one of the proximal tubular Na⁺ transporters as this change could be compensated by other Na⁺ transporters in the same segment or in more distal segments [discussed further in 4]. To test the hypothesis of a proximal tubular origin in essential hypertension, it would be a great advantage to have an *in vitro* model system that is genetically homogeneous in terms of expressing the phenotypic characteristics of normo- and hypertensive cells. Presently, there is not an established, characterized cell model system to serve in this role.

Viral oncogene immortalization of primary cells offers the possibility of cell line generation by the introduction of a single gene without full tumorigenic transformation and with preservation of differentiated phenotype [reviewed in 5]. Some of the newer engineered vectors coding for immortalization genes provide a longer life span for cell lines without compromising major features of the differentiated epithelia, such as contact inhibition, tight junctional formation, cell polarity, and segmental-specific solute transporters [reviewed in 5].

We report here the immortalization of primary cultures of epithelial cells from the proximal tubule of spontaneously hypertensive (SHR) and control Wistar-Kyoto (WKY) rats, using the oncogene SV40 large T-antigen. These cell lines were characterized by electrophysiology, electron microscopy, and microscopic fluorescent imaging, demonstrating that cells retain the major differentiated, phenotypic characteristics observed in primary cultures of proximal tubule epithelial cells.

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Preliminary reports on the immortalization of these cells have been published previously [6–8].

Methods

Primary cell culture of rat proximal tubules

Male, 4- to 8-weeks-old, spontaneously hypertensive SHR and control WKY rats (Charles River Laboratories, Wilmington, MA, USA) were anesthetized with ether, and their kidneys were perfused through the renal artery with rat renal tubular epithelium (RTE) medium composed of D-MEM:F-12, 1:1, supplemented with 15 mM HEPES, 1.2 mg/ml NaHCO₃, 5 µg/ml insulin, 5 µg/ml transferrin, 10 ng/ml epidermal growth factor, 4 µg/ml dexamethasone, 100 U/ml penicillin G, and 100 µg/ml streptomycin sulfate. Following complete blood washout, the kidneys were excised and superficial cortical slices were incubated in rat RTE medium containing 1 mg/ml collagenase type IV for 10 to 20 minutes at 37°C. S1 segments of the proximal convoluted tubule were microdissected from the surrounding tissue and placed on Ethicon collagen-coated 12 mm Millicell-CM culture plate inserts (Millipore, Bedford, MA, USA). Collagen-coating of Millicell-CM culture plate inserts was accomplished by wetting the inserts with a 20% Ethicon collagen dispersion in 60% ethanol, allowing collagen to air dry in a biological safety cabinet, and irradiating with a UV light to ensure sterility. S1 segments were maintained in a minimum volume of rat RTE medium supplemented with 10% FBS for 12 to 24 hours. After attachment to the culture plate insert, proximal tubule segments were co-cultured with lethally irradiated (5,000 to 6,000 rads) NIH 3T3 fibroblasts on both the collagen-coated insert and the underlying plastic well, and the cultures were continued in rat RTE medium plus 10% FBS at 37°C in a 5% CO₂ humidified atmosphere.

Immortalization, selection, and cloning of proximal tubule cells

Approximately 7 to 10 days after microdissection, primary cell outgrowths were dissociated with trypsin-EDTA (0.25% trypsin, 1 mM EDTA-4Na⁺ in Hanks' balanced salt solution without Ca²⁺ or Mg²⁺) and then re-seeded onto new collagen-coated culture plate inserts with a fresh complement of lethally irradiated fibroblasts. Within 24 hours, the culture medium was replaced with an equal volume of virus-containing supernate from ψ -CRE cells [9] generated by transfection with a replication-defective retrovirus encoding wild-type SV40 large T-antigen and neomycin phosphotransferase under the control of the Moloney Murine Leukemia Virus promoter [10, 11]. Primary cultures were sequentially infected over the course of six to eight hours with the addition at approximately two hour intervals of fresh virus-containing medium supplemented with 4 µg/ml polybrene [12]. Since the initial doubling time of these cells is about 7 to 10 days, they were maintained in culture for 10 to 14 days before passage. In some cases, following the second and third passages, the infection procedure was repeated. Cells were expanded in the presence of lethally irradiated NIH 3T3 fibroblasts over the course of 6 to 10 weeks or until approximately passage ten. Without fibroblast co-culture or viral infection, primary cultures of rat proximal tubule cells die within two weeks (unpublished observations).

At about passage ten, the typical protocol involved selection of infected cells by treatment with 0.8 mg/ml G418. Surviving cells were cloned by microscopically picking colonies from a sparsely

populated 100 mm collagen-coated plastic culture plates after trypsinization in 1% methyl cellulose. Using this method, cells were detached in place, that is, immobilized by the viscosity of the solution, and then isolated and removed from the plate using an inverted microscope and micropipette. Colonies were cultured on collagen-coated 12 mm culture plate inserts and only those colonies that formed confluent, electrically resistive, polarized monolayers were selected for long-term maintenance in culture.

Culture of cell lines

Cell lines were maintained in rat RTE medium supplemented with 5% FBS (without antibiotics) on Ethicon collagen-coated 100 mm plastic culture plates and were passaged at 80 to 95% confluence or approximately 5 to 7 days in culture using a split ratio of 1:20 to 1:40. Optimum cell growth occurred when plastic culture plates, filter inserts, or glass coverslips were coated with Ethicon collagen. Although cells did grow in the absence of collagen on plastic culture plates or Transwell-clear (polyester) filter inserts (Corning Costar, Cambridge, MA, USA), cells only poorly attached to the uncoated surfaces, and cells could easily be dislodged, such as during the changing of nutrient medium. Cells did not grow in the absence of collagen on Millipore-CM inserts.

Nomenclature

The first four upper case letters represent the species, tissue, and cell type. Thus, "S" and "W" denote SHR and WKY rats, respectively, "K" refers to kidney, and "PT" represents proximal tubule cells. The next four numbers refer to the date of microdissection, or equivalently the date when cells were first placed into culture, thereby giving a reference to the cell line's approximate age. "Cl." and the accompanying number identify the clone or subclone. As an example, then, SKPT-0193 Cl.1 refers to proximal tubule cells (colony 1) that were microdissected from the kidney of an SHR rat and placed into culture in January 1993.

Gene transfer validation

Flow cytometry was used to simultaneously measure T-antigen (Tag) expression and DNA levels following fluorescent staining, as previously described [12, 13]. T-antigen expression was measured after indirect immunofluorescence staining with a T-antigen-specific monoclonal antibody (PAb416; Oncogene Science, Manhasset, NY, USA) and fluorescein-conjugated secondary antibody (fluorescein-conjugated goat anti-mouse immunoglobulin G F(ab')₂ fragments; Boehringer Mannheim Biochemicals, Indianapolis, IN, USA). DNA levels were simultaneously determined using propidium iodide, a DNA-specific fluorochrome.

Clonality of the cell lines was assessed as follows: high molecular weight genomic DNA was extracted from each cell line [14], dialyzed, and 3 µg from each line was digested with the restriction enzyme *Stu*I, which was demonstrated to make one cut in the retroviral genome outside of the Tag region. After overnight electrophoresis [15] on a 0.8% agarose gel with a buffer composed of 40 mM Tris-Cl, 18 mM NaCl, 20 mM Na-acetate, 20 mM EDTA, pH 8.0, the DNA was transferred by capillary blotting (Southern blot) [16] onto Zeta-Probe membrane (Bio-Rad, Hercules, CA, USA) and probed with a biotinylated cDNA for Tag (Random octamer labeling kit, Tropix, Bedford, MA, USA). Hybridization, stringency washes, and chemiluminescent probe detection using CDP-Star substrate (Tropix) were according to manufacturer's instructions.

Electron microscopy

SKPT and WKPT cells on culture plate inserts were washed twice in PBS and then fixed with glutaraldehyde in the following sequential manner: (a) 0.75% glutaraldehyde in PBS for 15 minutes at 37°C; (b) 2.5% glutaraldehyde for 15 minutes at room temperature; and (c) 2.5% glutaraldehyde overnight at room temperature. Fixed samples were washed twice with PBS and then stored at 4°C. In preparation for electron microscopy, samples were post-fixed with OsO₄, dehydrated, embedded in Polybed 812, and allowed to polymerize at 65°C. After sectioning normal to the culture plate inserts, samples were examined with a Jeol 100CX transmission electron microscope.

Electrical conductance and electrophysiology

Electrical conductance measurements and detailed electrophysiology studies were conducted as previously described [17]. For electrophysiology experiments of intact monolayers, luminal and basal compartments were continuously perfused with a HCO₃⁻ buffered Ringer solution composed of 114 mM NaCl, 4 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, 28 mM NaHCO₃, 25 mM D-glucose, and 0.1% (wt/vol) bovine serum albumin, and measurements were determined at 37°C in a controlled atmosphere of 5% CO₂.

Na,K-ATPase activity was also measured in monolayers by electrophysiology, after permeabilization of the apical membrane with 10 μM amphotericin B and 0.1% DMSO [18]. The cell monolayers were continuously perfused both apically and basolaterally with a HCO₃⁻-free solution composed of 20 mM Na-gluconate, 120 mM NMG-acetate, 4.7 mM K-gluconate, 1.5 mM CaCl₂, 1.5 mM MgCl₂, 10 mM HEPES pH 7.4 (with acetic acid), 25 mM D-glucose, and 0.1% (wt/vol) bovine serum albumin. Measurements were made at 37°C.

SBFI-AM loading and intracellular Na⁺ measurements

SKPT and WKPT cells were grown on Ethicon collagen-coated coverslips and incubated with D-MEM:F-12, 1:1, that was supplemented with 15 mM HEPES, 1.2 mg/ml NaHCO₃, 7 μM SBFI-AM, 2 mM probenecid, 0.25% pluronic F-127, and 0.1% FBS for three hours at 37°C. Following the incubation, cells were placed on ice and washed twice with an excess of D-MEM:F-12 immediately prior to use.

Cells were placed in a thermostated chamber (maintained at 37°C) and constantly perfused with buffer composed of 114 mM NaCl, 4 mM KCl, 1 mM MgCl₂, 1.5 mM CaCl₂, 23 mM HEPES pH 7.4 (with NaOH), 10 mM D-glucose, 5 mM L-glutamine and 0.01% (wt/vol) BSA. Under the microscope, cells were alternately illuminated through epifluorescence optics with light at 340 and 380 nm, and light emitted above 435 nm was captured by an intensified CCD camera. The video images were analyzed and digitized with Image-1/FL software (Universal Imaging, Media, PA, USA) and saved on a hard disk as a function of time. In addition, the software generated the ratio image from the captured fluorescence at 340 and 380 nm at the approximate rate of one image every eight seconds. The 340/380 ratio image normalized the fluorescence for different amounts of dye in the optical section and was calibrated in terms of Na⁺ concentration. Intracellular Na⁺ calibration was performed *in situ* using three different Na⁺ concentrations, 10 μg/ml gramicidin D, and 25 μM verapamil (to inhibit the multidrug resistance gene product).

Using the software, SPLMOD, v. 2DP (April 1986) [19, 20], Na⁺ transport data were fitted to two models: (1) two exponentials plus a linear component and (2) three exponentials plus a linear component. The estimated parameters from the better of the two fits, as determined by the random distribution of the residuals, were used to calculate the maximum rate of Na⁺ influx.

BCECF-AM loading and intracellular pH measurements

SKPT and WKPT cells were grown on Ethicon collagen-coated coverslips and incubated with 10 μM BCECF-AM in Ringer's medium at 23°C for 25 minutes. Following the incubation, cells were washed with excess Ringers, and place on ice until use.

Cells were placed in a thermostated chamber (maintained at 37°C) and constantly perfused with buffer composed of 120 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, 20 mM HEPES pH 7.4 (with NaOH), 5 mM D-glucose, 5 mM L-glutamine. Imaging of the cells was carried out as described for SBFI, except that cells were illuminated at 495 and 450 nm, and fluorescence was collected between 500 and 560 nm.

Intracellular pH was calibrated *in situ* by using the method described by James-Kracke [21]. Briefly, at the end of an experiment, 10 μM nigericin was added followed by changing the experimental medium to a high K⁺ salt-solution (K⁺ replacing Na⁺ in experimental buffer) which was first at pH 5.5 then at pH 8.5. These two pH extremes gave the minimum (R_{min}) and maximum (R_{max}) 495/450 ratio, respectively, thus allowing intracellular pH to be estimated through following expression

$$pH_i = pK_a - \log \frac{R_{max} - R}{R - R_{min}}$$

where pK_a is the logarithm of the association constant for BCECF [21].

The software, SPLMOD [19, 20], was used to determine the rate of change of intracellular pH, dpH_i/dt. The rate of change of intracellular H⁺ concentration, d[H⁺]_i/dt, was determined by the product of dpH_i/dt and buffer capacity for the respective cell line, obtained in separate experiments performed essentially as described in Boyarsky et al [22].

Chemicals

Collagenase type IV, D-MEM:F-12, FBS, G418 (geneticin), goat serum, Hanks' balanced salt solution, L-glutamine, penicillin-streptomycin, and trypsin-EDTA were purchased from Gibco-BRL Life Technologies, Inc. (Grand Island, NY, USA). Amphotericin B, BSA, dexamethasone, gramicidin D, HEPES, insulin, leupeptin, pepstatin, phlorizin, PMSF, probenecid, propidium iodide, transferrin, and verapamil were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Glutaraldehyde and Polybed 812 were purchased from Polysciences (Warrington, PA, USA). BCECF-AM, EIPA, Pluronic F-127, and SBFI-AM were obtained from Molecular Probes, Inc. (Eugene, OR, USA). Collagen dispersion was from Ethicon, Inc. (Princeton, NJ, USA). Polybrene (hexadimethrine bromide) was from Aldrich Chemical Co. (Milwaukee, WI, USA). Epidermal growth factor was obtained from Upstate Biotechnology, Inc. (Lake Placid, NY, USA). Ang II was purchased from United States Biochemical Corp. (Cleveland, OH, USA). All other chemicals were of the highest quality commercially available.

Table 1. T-antigen and electrophysiological properties of rat proximal tubule cells

Cell line	Relative tag	% Cells Tag +	Conductance mS/cm^2	Δ Isc ($\mu A/cm^2$)		Growth without D-glucose?
				w/4 mM Na-succinate	w/2 mM ATP	
SKPT-1092 Cl.1	0	0	1.2 \pm 0.5 (3)	0.9 \pm 0.5 (3)	7.4 \pm 0.5 (3)	+++
SKPT-1092 Cl.2	0	0	1.8 \pm 0.7 (3)	1.5 \pm 0.3 (3)	9 \pm 2 (3)	ND
SKPT-0193 Cl.1	0.60	100	1.0 \pm 0.6 (3)	0.9 \pm 0.4 (5)	2.2 \pm 0.5 (3)	ND
SKPT-0193 Cl.2	1.0	100	0.40 \pm 0.04 (3)	2.3 \pm 1.6 (2)	6 \pm 1 (2)	+++
SKPT-0193 Cl.6	1.1	100	0.70 \pm 0.10 (2)	0.9 \pm 0.4 (3)	6.0	ND
WKPT-1292 Cl.3	1.0	100	1.3 \pm 0.2 (2)	5.2	14	0
WKPT-1292 Cl.4	0.60	100	1.9 \pm 0.2 (2)	5 \pm 4 (2)	12 \pm 2 (2)	ND
WKPT-1292 Cl.8	0.60	97	1.0 \pm 0.1 (3)	5 \pm 2 (3)	32 \pm 7 (3)	0
WKPT-1292 Cl.10	0.90	100	0.80 \pm 0.10 (3)	3 \pm 2 (3)	20 \pm 3 (3)	ND
WKPT-1292 Cl.16	1.5	100	3.6 \pm 0.5 (3)	ND	ND	+++
WKPT-0293 Cl.2	1.5	100	23 \pm 5 (4)	ND	ND	+++
WKPT-0293 Cl.3	0.80	100	19 \pm 9 (3)	31 \pm 16 (3)	37 \pm 17 (3)	ND

Cells were immortalized with a retroviral construct encoding wild-type SV40 large T-antigen (Tag) as described in **Methods**. T-antigen expression, measured in cells in the G1 phase of the cell cycle, was measured in arbitrary fluorescence units by flow cytometry, and Tag levels were normalized to the SKPT-0193 Cl.2 cell line. Percentage cells expressing Tag (% cells Tag+) was determined by comparing cells stained in the presence and absence of antibody to Tag. Electrical conductances of visually confluent monolayers on culture plate inserts were measured as previously described [17] or, alternatively, by estimating the basal conductance levels from electrophysiology experiments. Electrophysiology experiments were performed on confluent cell monolayers on culture plate inserts in a modified Ussing chamber that includes a four electrode system for measuring transepithelial Isc, conductance, and potential. Changes in Isc (Δ Isc) were measured on-line with a voltage clamp module. Maximal Δ Isc after apical addition of substrate (succinate) or agonist (ATP) are reported. Luminal and basolateral compartments were continuously perfused with a HCO_3^- buffered Ringer solution containing 0.1% BSA. Sodium succinate and ATP were added to the luminal compartment. Conductance and Isc measurements are the mean \pm standard deviation, or where $N = 2$, the average \pm difference between the measured value and the average. The number of experiments are indicated in parentheses. Cell growth in the absence of D-glucose was determined visually, where +++ indicates positive growth. ND = not determined.

Results

Strategy

This study was undertaken to develop immortalized epithelial cell lines from the S1 segment of kidney proximal tubule of both hypertensive (SHR) and normotensive (WKY) rats, which would continue to remain differentiated in terms of morphology and cell function. An important functional criterion of differentiation was the formation of electrically-resistive cell monolayers. The strategy employed was the same as that successfully used for the immortalization of rabbit S1 proximal tubule cells [17]. Briefly, this strategy involves the microdissection of the tissue of interest so that the origin is well defined (S1 proximal tubule segment), expansion of the primary cells under conditions that allow the cells to remain differentiated (growth on collagen-coated porous filter supports in the presence of lethally-irradiated NIH-3T3 fibroblast feeder layer), infection with a retrovirus construct to maximize the probability of incorporating an immortalization gene (SV40 large T-antigen), and continued maintenance of immortalized cells on collagen-coated porous filter supports (such that cells retain their differentiated features), as well as selecting for cells that form confluent, electrically-resistive monolayers (a major epithelial characteristic).

Generation of WKPT and SKPT cell lines

Following this strategy, we have developed five SKPT and seven WKPT cell lines from four different isolates obtained from two SHR and two WKY rats, respectively. As defined by the selection protocol, these cell lines form visually confluent, electrically-resistive monolayers when grown on collagen-coated porous supports. All cell lines were actively maintained through 50 passages, while several lines have continued to be maintained

through greater than 130 passages. The cell lines do not behave as transformed cells in that cells are contact inhibited upon confluence of the cell monolayer.

The high passage numbers obtained thus far suggest that the cells have been immortalized. Even if not truly immortal, sufficient numbers of cells have already been generated such that numerous experiments can be performed on cryopreserved stocks. Because T-antigen (Tag) bypasses the cellular controls that negatively regulate cell cycle and confer senescence, expression of this immortalization gene was determined by staining with a Tag-specific antibody and flow cytometry (Table 1). Two cell lines expressed no Tag and probably represent spontaneous immortalization whereas the remaining ten cell lines expressed Tag (Table 1). Among the latter, Tag expression levels varied by approximately threefold. This variation is consistent with expression levels observed with astrocytes immortalized with the same or similar vectors, with Tag expression under the control of the Moloney Murine Leukemia Virus LTR [23].

To determine if the lines are clonal or variants (subclones) of the same clone, Southern blot analysis was performed on the genomic DNA obtained from all of the cell lines (Fig. 1). Confirming the flow cytometry measurements, the Tag gene was not detected in the SKPT-1092 cell lines, thus supporting that immortality of the SKPT-1092 lines is not dependent on Tag expression. Of the SKPT-0193 cell lines, Cl.1 and Cl.2 each have single integration site, and are apparently descendants of the same parent, that is, variants of the same clone, whereas Cl.6 appears to have either two Tag integration sites or, as is less likely, is composed of two stem lines with single site integrations. Similarly, all five clones of the WKPT-1292 cell lines appear related, as all contained three Tag integration sites. Both WKPT-0293 cell lines have apparently one identical integration site.

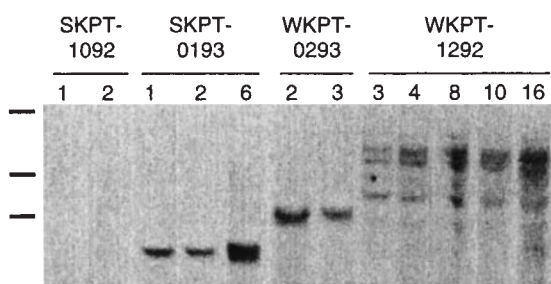


Fig. 1. Southern analysis of SKPT and WKPT cells. Genome DNA was isolated, digested with a restriction enzyme that makes one cut outside the T-antigen region, electrophoresed, transferred to Zeta-Probe membrane, then probed with a T-antigen fragment as described in the **Methods** section. Lanes represent cell lines designed by the name of the cell line, and the clone number. Molecular weight markers from the top are 23, 9.4, and 6.6 kb.

Morphology of WKPT and SKPT cells

To characterize the ultrastructural morphology of SKPT and WKPT cells, cell monolayers were grown to confluence on collagen-coated culture plate inserts, fixed, and processed for electron microscopy. Figure 2 shows two SKPT and two WKPT cell lines, each from different isolates, and demonstrates that each cell line forms a polarized monolayer of cells typical of simple epithelia. The length of the microvilli was comparable to other established cell lines [17, 24] and to primary cultured cells [25–27], although diminished compared to proximal tubules *in vivo* [28].

Prominent ultrastructural characteristics that are common to both SKPT and WKPT cells are shown in a representative electron micrograph of an SKPT cell line (Fig. 3). These include tight junctional complexes, that is, tight junction and desmosomes, convolutions of the basolateral plasma membrane forming intracellular spaces, numerous mitochondria, rough and smooth endoplasmic reticulum, Golgi apparatus, clathrin-coated vesicles, and elements of the vacuolar apparatus. Taken together, these ultrastructural features are consistent with the active metabolic and transport processes of proximal tubular epithelium.

Epithelial nature and proximal tubule origin of WKPT and SKPT cells

A characteristic typical of simple epithelia is the formation of “occluding junctions” between cells. Confluent WKPT or SKPT cells exhibited basal conductances between 1 and 20 mS/cm² (Table 1), demonstrating their epithelial origin and their ability to homogeneously organize as tissue over a large area, such as a confluent monolayer on a 4.2 cm² filter.

A further characteristic of epithelial cells is vectorial transport of ions across the cell monolayer. Frequently, the nature of this vectorial transport is a defining feature of the epithelia [29]. One such example found in the proximal tubule is electrogenic, Na⁺-dependent absorption of di- and tricarboxylic acids, primarily Krebs cycle intermediates such as succinate and citrate [30–32]. Confluent SKPT or WKPT cell monolayers actively absorb succinate via an apical Na-dicarboxylate cotransporter (3 Na⁺ to 1 dicarboxylate coupling ratio), as demonstrated by an increase in I_{sc} on apical addition of Na-succinate (Table 1). Methylsuccinate, a non-metabolic analog of succinate, gave similar increases in I_{sc} (data not shown), supporting the conclusion that the increases in I_{sc} on Na-succinate addition are related to an electrogenic

transport, and not to metabolism. Retention of the electrogenic Na-succinate co-transporter in SKPT and WKPT cells is consistent with, that is, a marker of, the proximal tubular origin of these cell lines.

A further characteristic of proximal tubule cells *in vivo* is their large capacity for gluconeogenesis compared with other nephron segments [33]. Cells derived from each isolate continued to thrive when maintained in a glucose-free nutrient medium (Table 1). This growth behavior suggests that the metabolic machinery necessary for gluconeogenesis was retained. Note, however, that there are two cell lines from the WKPT-1292 isolate that do not grow in the absence of glucose. The reason for the lack of gluconeogenesis observed in the WKPT-1292 Cl.3 and Cl.8 cell lines remains to be investigated.

Attempts were made to view the electrogenic activity of the apical Na-glucose cotransporter (1:1 Na⁺ to glucose transport coupling ratio), which is another functional marker of proximal tubule origin [34]. Measurements of I_{sc} on confluent SKPT and WKPT cells did not provide any evidence for glucose-dependent, phlorizin-inhibitable apical-to-basal fluxes, even with cultures grown in glucose-free medium for up to four passages. These results indicate that levels of Na-glucose co-transport are below the electrical detection limit of approximately 15 pmol/(second · cm²) [34], but not necessarily that the cells completely lack this cotransporter. In the case of the proximal tubule specific H⁺-peptide cotransporter, PEPT-2, direct measurements of radiolabeled peptide uptake were necessary to demonstrate the presence of this transporter in the SKPT-0193 Cl.2 cell line, because the maximal velocity was equivalent to an electrical flux of 1 μA/cm² [8], and therefore within the noise level of electrophysiological measurements.

Signal transduction in WKPT and SKPT cells

The functional expression of signaling pathways is important in assessing the state of differentiation of cells in culture. The purinergic, receptor-mediated signal transduction pathway is well established in epithelial cells [reviewed in 35]. Since purinergic stimulation has been shown to increase membrane Cl⁻ current [36], changes in I_{sc} can be used as a functional measure of expression of purinergic signal transduction machinery. Luminal addition of ATP to confluent SKPT and WKPT cells produced an increase in I_{sc} representative of anion secretion (Table 1). Thus, SKPT and WKPT cells express a complete set of functional P₂-purinergic receptors, associated intracellular signaling system(s), and affected membrane transporter(s) to give a measurable electrical response.

Sodium transport in WKPT and SKPT cells

One of the primary reasons for the generation of these proximal tubule cell lines from SHR and WKY rats was to investigate their capacity for Na⁺ reabsorption. There are three major transporters involved in Na⁺ reabsorption in the early proximal tubule: apical Na/H exchanger, and basolateral Na,K-ATPase and Na-bicarbonate cotransporter [reviewed in 37]. The transport activity of Na,K-ATPase and the Na/H exchanger can easily be quantified and is discussed below. Quantification of the transport activity of the Na-bicarbonate cotransporter is more complex, but has recently been accomplished in the SKPT-0193 Cl.2 cell line and is reported elsewhere [38].

Na,K-ATPase activity was measured as short-circuit current

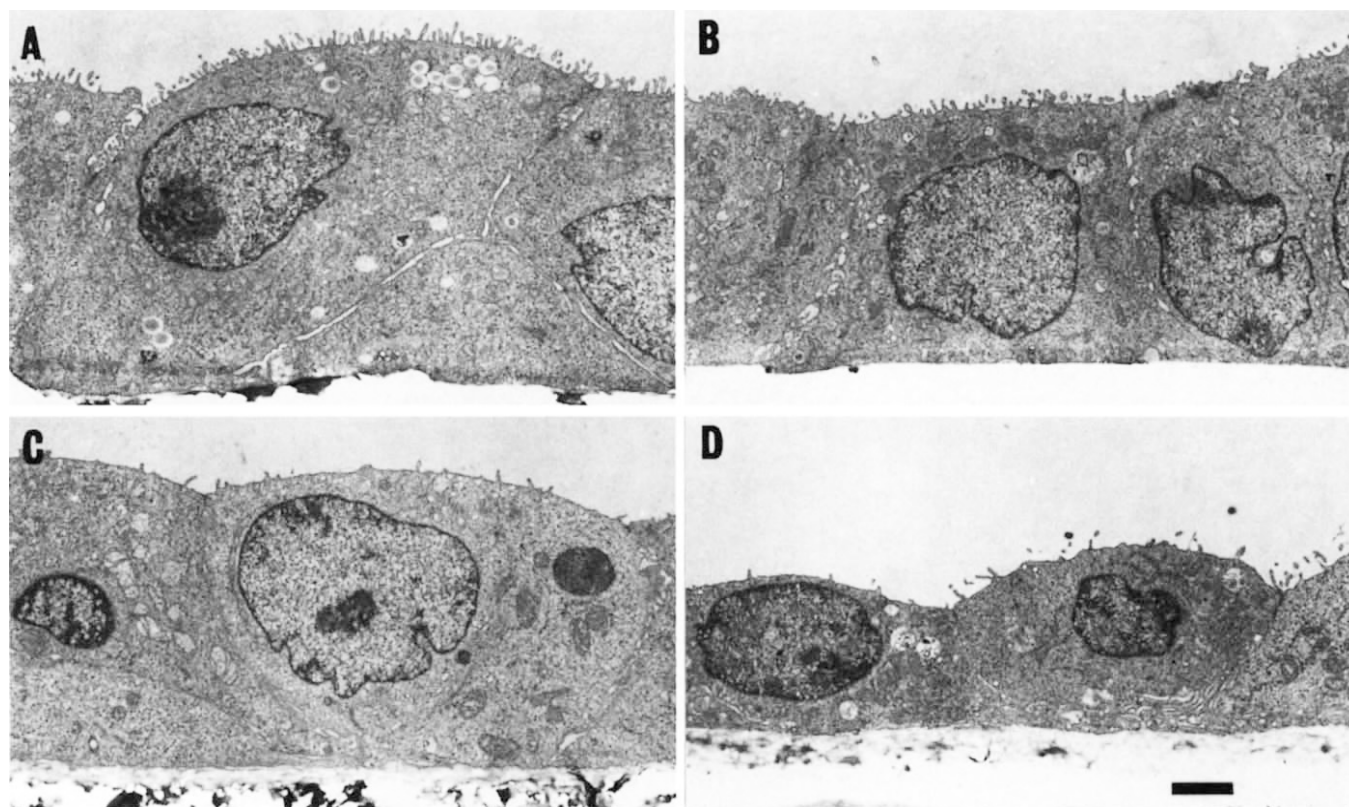


Fig. 2. Electron microscopy of SKPT and WKPT cells. Proximal tubule cells (passages 30 to 40) were grown to confluence on collagen-coated Millicell-CM culture plate inserts and then processed for electron microscopy. A, B, C, D: Cross-sections of SKPT-1092 Cl.1, SKPT-0193 Cl.2, WKPT-1292 Cl.8, and WKPT-0293 Cl.2, respectively. Calibration bar is 2 μm .

(Isc) in intact monolayers after permeabilization of the apical plasma membrane with amphotericin B [18] (Fig. 4). The procedure involves making the membrane selectively permeable to monovalent ions without destroying the permeability barrier to divalents and larger organic ions. Since the apical and basal compartments were bathed in symmetrical solutions, the Isc measured represents primary active transport (ATPase), Ca^{2+} currents, or secondary active transport dependent on the Ca^{2+} gradient between the extracellular solution and the cytosol. The baseline Isc was completely dependent on Na^+ and K^+ , and inhibited about 80% by 1 mM by basolateral ouabain (Fig. 4), consistent with the presence of the $\alpha 1$ -isotype Na,K-ATPase in rat kidney which is poorly sensitive to ouabain [39, 40]. The ATPase-generated current ranged between 5 to 14 $\mu\text{A}/\text{cm}^2$, and was stable over many passages (Table 2). The current density to which this current corresponds is about 150 to 420 pmol of Na^+ transported per second per cm^2 (normalized to filter support area). This current density represents 6 to 17% of that seen in the proximal tubule *in vivo* [reviewed in 41], but is high compared with other cells, for example, 2 to 5 times higher than in freshly isolated myocytes [42].

Na/H exchange activity was measured in these cell lines by either Na^+ influx or H^+ efflux after an acid-load protocol. The acid-load protocol utilized involved bathing of the cells with an ammonium salt solution, followed by removal of ammonia which results in acidification of the cytosol [43, 44]. Mathematical

simulations of this acid-load protocol in an early proximal tubule model [4] indicate that the initial rate of recovery of intracellular pH or the initial change in the intracellular Na^+ concentration exclusively reflect exchanger activity rather than a combination of other transporters involved in Na^+ or pH homeostasis. Figure 5 depicts representative experiments of the acid-load protocol (ammonia pulse) on SKPT-0193 Cl.2 cells loaded with either the Na^+ -sensitive fluorescent dye, SBFI (Fig. 5A) or the pH-sensitive fluorescent dye, BCECF (Fig. 5B). The observed rate of Na^+ uptake was 13 $\text{mm Na}^+/\text{min}$, while the acid efflux corresponded to 9 $\text{mm H}^+/\text{min}$. Experimental evidence for Na/H exchanger activity was obtained by complete dependence of the recovery on apical Na^+ and inhibition of recovery by the amiloride analog EIPA when Na^+ was present in the apical solution (data not shown). EIPA is a relatively specific inhibitor of the Na/H exchanger [45]. Similar Na^+ measurements in SKPT-1092 (passage 59) and WKPT-1292 Cl.8 (passage 45) yielded rates of 53 ± 6 ($N = 14$) and 37 ± 4 ($N = 24$) $\text{mm Na}^+/\text{min}$. All these fluxes are relatively high compared to a steady-state Na^+ concentration of about 20 mm , and suggest a highly absorbing epithelium with intracellular Na^+ turnover times of approximately one minute or less.

Angiotensin II signal transduction in WKPT and SKPT cells

Angiotensin II (Ang II) signaling is an important feature of proximal tubules cells *in vivo*, and is involved, among other roles, in the regulation of salt reabsorption [reviewed in 46, 47]. To

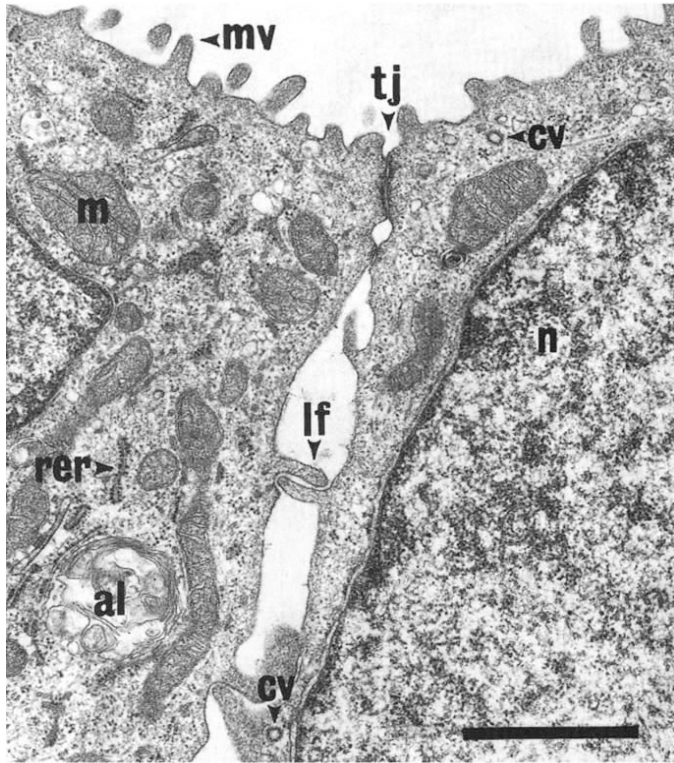


Fig. 3. Ultrastructure of SKPT-0193 Cl.2 cells. SKPT cells were grown to confluence on collagen-coated Millicell-CM culture plate inserts, and adjacent cells were viewed in cross-section by electron microscopy. Ultrastructural features include microvilli (mv), tight junction (tj), clathrin-coated vesicle (cv), autolysosome (al), mitochondria (m), rough endoplasmic reticulum (rer), nucleus (n), lateral cytoplasmic folds (lf). Calibration bar is 1 μm .

demonstrate that Ang II regulation of Na^+ metabolism can be studied in these cell lines, cells were treated with 1 μM Ang II immediately after intracellular acidification. As shown in Figure 5A, bottom panel, Ang II stimulated the rate of Na^+ influx from 13 to 54 $\text{mm Na}^+/\text{min}$ (compare to Fig. 5A, top panel). Further, the intracellular Na^+ concentration overshoot the basal level by 30 mm Na^+ , followed by a gradual Na^+ efflux to the basal level, suggesting the possible regulation of a Na^+ efflux pathway, such as Na,K-ATPase or Na-bicarbonate cotransporter, by Ang II. Similar effects of Ang II on the rate of Na^+ metabolism were observed in the SKPT-1092, WKPT-1292 Cl.8, and WKPT-0293 Cl.2 cell lines (data not shown).

Since the biological effects of Ang II on the kidney are known to be concentration dependent and biphasic [reviewed in 46, 47], the experimental protocol was repeated for several Ang II concentrations. Figure 6 summarizes the dose-dependent effect of Ang II on the rate of Na^+ influx in SKPT-0193 Cl.2 and WKPT-1292 Cl.8 cell. Each data point is the mean response of a population of cells from a single experiment \pm SEM. Interestingly, the SKPT and WKPT cells displayed very different dose-response characteristics, with SKPT cells being stimulated by Ang II at lower concentrations than in WKPT cells. Further experiments are necessary to determine if this difference is related to a genetic difference between SHR and WKY rat strains, or is specific for the selected clones or isolates.

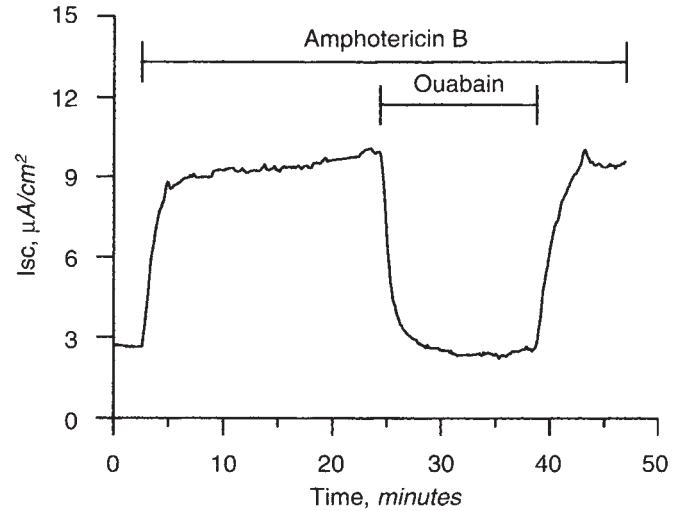


Fig. 4. Na,K-ATPase-mediated short-circuit current. Na,K-ATPase activity was measured as short-circuit current (Isc) in intact monolayers of SKPT-0193 Cl.1 cells (passage 25) after permeabilization of the apical plasma membrane to monovalent ions with 10 μM amphotericin B in 0.1% DMSO. Apical and basolateral compartments were perfused symmetrically with a bicarbonate-free solution composed of 20 mm Na^+ , 4.7 mm K^+ , and low Cl^- as described in the *Methods* section. Isc under these conditions is completely dependent on Na^+ and K^+ and was taken as I_{max} for the Na,K-ATPase (Table 2). Ouabain (1 mM) was added by basolateral perfusion.

Table 2. Na,K-ATPase activity in rat proximal tubule cells

Cell line	Passage numbers(s)	I_{max} for Na,K-ATPase $\mu\text{A}/\text{cm}^2$
SKPT-0193 Cl.1	25–26	7.3 ± 0.6 (4)
SKPT-0193 Cl.2	27–110	11.0 ± 0.7 (7)
SKPT-0193 Cl.6	22	6.5 ± 1.3 (2)
WKPT-1292 Cl.8	35–44	6.8 ± 0.4 (8)

Na,K-ATPase activity was measured as short-circuit current (Isc) as described in the legend to Fig. 4. Passage number(s) indicates the range of passages of cell monolayers from which Isc was measured. Isc measurements are the mean \pm standard error of the mean (SEM), or where $N = 2$, the average \pm difference between the measured value and the average. The number of experiments are indicated in parentheses.

Discussion

Our results with the cells derived from the microdissected proximal tubules indicate that cell lines were successfully established from only two SHR and two WKY rats. The characterization so far, although incomplete, has relied on metabolism, electrical activity, growth, morphology, and expression of specific proximal tubular transporters as criteria. The results show retention of a proximal tubular phenotype, with characteristics similar to what has been observed in primary cultures. The state of differentiation of our cell lines is consistent with previous observations using the same immortalization vector with other cell types, namely, that the immortalization gene does not abolish the differentiated state at the time of immortalization [reviewed in 5]. Based on Na,K-ATPase levels, the transport capacity has not been diminished through passage numbers of greater than 100.

Immortalized cell lines have considerable advantages over primary cells in that expansion of the cell lines can result in

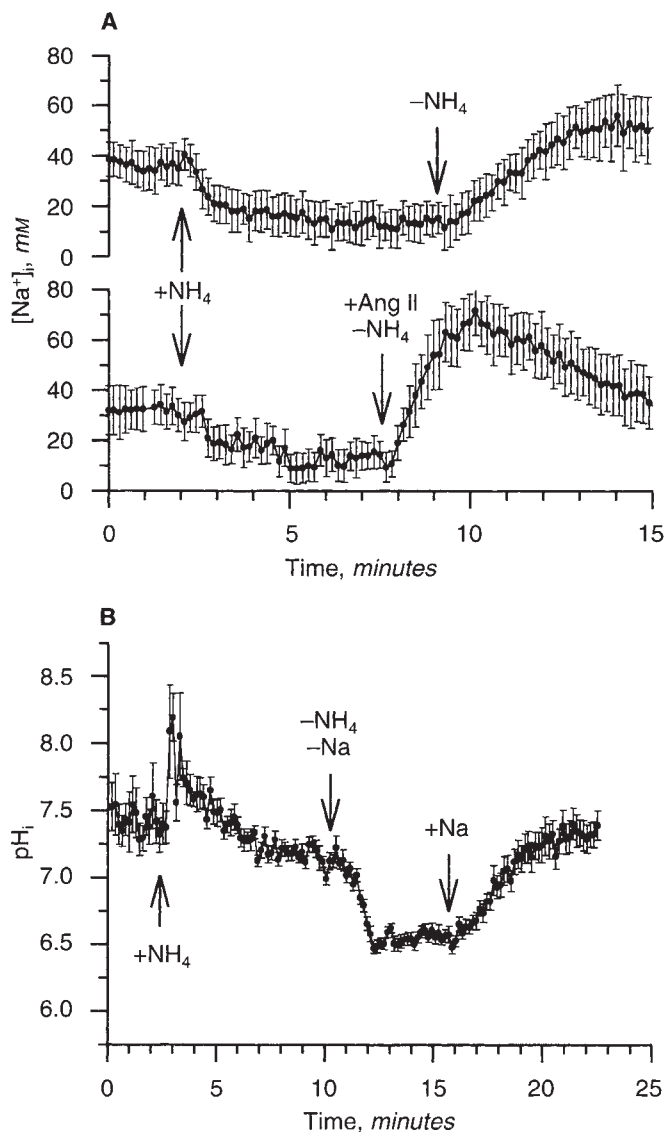


Fig. 5. Sodium and proton transport following an acid-load. **A.** Top panel. Sodium is transported into the cell after an acid-load. Intracellular Na^+ concentration ($[\text{Na}^+]_i$) in SBF1-loaded SKPT-0193 Cl.2 cells (passage 37) was monitored with a fluorescent imaging microscope as described in the **Methods** section. Cells were continuously perfused with a Ringer's solution, and at the indicated times the buffer was supplemented with 20 mM NH_4Cl ($\pm \text{NH}_4$). Bottom panel. 1 pM Ang II stimulated Na^+ transport following ammonia pulse in SKPT-0193 Cl.2. The experimental protocol was the same as in the top panel, except the perfusate was supplemented with 1 pM Ang II (+Ang II) immediately following NH_4Cl treatment. In both top and bottom panels, each record illustrates the fluorescence (mean \pm SD) of twelve cells as a function of time. The maximum Na^+ influx rate after an acid-load was 13 ± 2 mM Na^+/min (control, top panel) and 54 ± 5 mM Na^+/min (+1 pM Ang II, bottom panel) (mean \pm SEM). **B.** Recovery of pH_i from acid-load depends on extracellular Na^+ . Intracellular pH (pH_i) in BCECF-loaded SKPT-0193 Cl.2 cells (passage 64) was monitored with a fluorescent imaging microscope as described in the **Methods**. Cells were continuously perfused with Ringer's solution, and at the indicated times the buffer was supplemented with 20 mM NH_4Cl or Na-free ($-\text{Na}$) (Na^+ replaced by NMG^+). Each data point illustrates the fluorescence (mean \pm SD) of eighteen cells as a function of time. The initial rate of H^+ transport during the recovery of pH_i from the acid-load on the addition of Na^+ was 9 ± 2 mM H^+/min (mean \pm SEM).

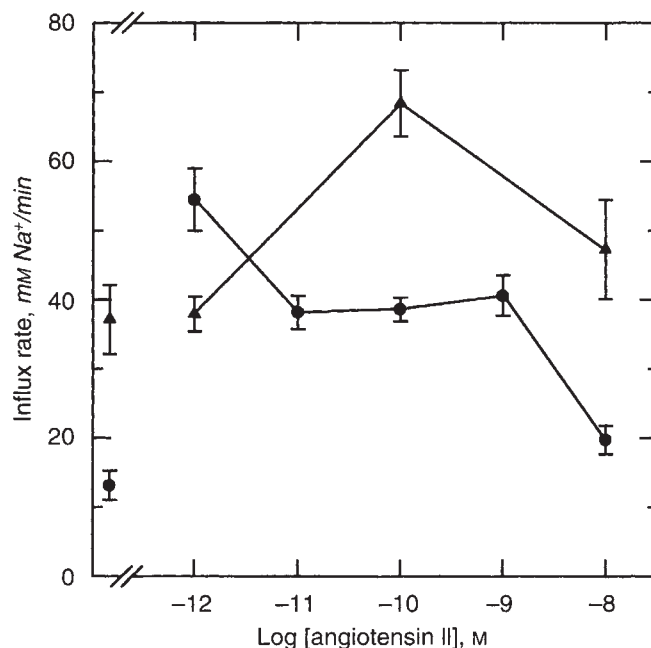


Fig. 6. Maximum rate of Na^+ influx as a function of Ang II concentration in SKPT and WKPT cells. Sodium transport data as a function of Ang II concentration was collected from SBF1-loaded SKPT-0193 Cl.2 (passage 37) (\bullet) and WKPT-1292 Cl.8 (passage 45) (\blacktriangle) cells by light microscopic fluorescence imaging as described in the **Methods** section and Figure 5A. Transport data were fit to two models: (a) two exponentials plus a linear component and (b) three exponentials plus a linear component. The calculated parameters from the better of the two fits, as determined by the random distribution of the residuals, were used to calculate the maximum rate of Na^+ influx. Each data point represents Na^+ influx rates for the population of cells analyzed in a single experiment \pm SEM.

sufficient number of cells for specific investigations, while the cells can also be distributed to other investigators. This property already proved advantageous for demonstrating the presence of the proximal tubule specific PEPT-2 transporter in the SKPT-0193 Cl.2 cell line [8]. Detailed studies can now be undertaken into the properties and consequence of the PEPT-2 transporter in the proximal tubule, such as in aminoglycoside transport using the same transporter.

The cell lines described in the article were derived from microdissected S1 proximal tubule segments, such that the site of origin is known. The use of retroviral constructs for incorporation of the immortalization gene into proximal tubule cells should be applicable to cells from other segments, as long as the cells from the segment of interest grow in primary culture. We have had success in immortalizing proximal tubular cells from human kidney specimens [48, and unpublished data]. In most immortalization protocols for epithelial cells, the major problems are either immortalization of undesirable epithelial cells or of mesenchymal cells. Manual microdissection avoids the first problem, while the use of fibroblast feeder layers and epithelial-specific selection criteria, such as the ability of epithelial cells to form confluent, resistive monolayers, avoids the latter. The use of glucose-free medium to select for proximal tubule cells may provide an even easier selection procedure to avoid both problems in the future.

It is important to emphasize that our every attempt to develop cell lines from primary cultures have proved successful. Our

success probably reflects that sufficiently high virus titers were used for the immortalization. Interestingly, an apparent spontaneous immortalization was observed. This is similar to a previous observation with rabbit proximal tubule cells [17]. Spontaneous immortalization was likely to be the result of the increased lifespan of cultured proximal tubule cells in the presence of fibroblast feeder cells, providing a greater opportunity for spontaneous mutations. Our experience would indicate that spontaneous immortalization occurs sufficiently frequently in rat and rabbit tissue, such that it would be possible to use the property as a basis for cell line generation. It is unlikely, however, that such a strategy based on spontaneous immortalization could be successfully applied to human specimens, since human cells have not been observed to immortalize spontaneously despite much effort [reviewed in 5]. An immortalization protocol involving introduction of retroviral constructs encoding for immortalization gene(s) will therefore be crucial for achieving success with human cell lines. Virus-dependent immortalization of human proximal tubule cells have already proved to be as efficient as that of rats [48, and unpublished data].

The spontaneously immortalized cell lines did, however, provide a convenient control for the effect of T-antigen expression on the measured cellular properties. We did not find any correlation of the properties investigated and reported thus far with T-antigen expression levels. These properties include basal conductance, Na,K-ATPase levels, or presence of purinergic or angiotensin receptors and associated signaling and effector systems. Thus, either T-antigen does not appear to affect these transport systems, or the mutations in the spontaneously immortalized cell lines were equivalent to T-antigen with respect to these transport systems. In general, it appears that as long as the effect of T-antigen is small enough such that contact inhibition is retained, the characteristic epithelial phenotype will continue to be expressed.

One important observation of these cell lines is that they have apparently retained the appropriate proximal tubule phenotype for Na⁺ reabsorption. Since cell lines were developed from both SHR and WKY rats, we are in a position to investigate, in detail, the molecular or cellular changes that might explain genetically determined *in vivo* differences in Na⁺ reabsorption. A change in the set-point of proximal tubular Na⁺ reabsorption could potentially be caused by a change in one of many different regulatory or structural elements involved in Na⁺ reabsorption. In other words, a large number of different mutations could result in the same phenotype. The advantage is that cell line differences observed *in vitro* cannot be ascribed to neuronal or hormonal influences that may be different *in vivo*. The WKPT and SKPT cell lines have already proved useful in elucidating a difference in dopaminergic response of SHR and WKY rats [49].

While the detailed investigation of animal models via cell lines may prove to be valuable and time effective, the application of this technology to humans should be invaluable, since substitute technologies, such as transgenic or knockout methodologies, required to understand the cellular and molecular basis of altered renal Na⁺ transport are not applicable.

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Appendix

Abbreviations are: Ang II, angiotensin II; ATP, adenosine 5'-triphosphate; BCECF-AM, 2',7'-biscarboxyethyl-5 [6]-carboxyfluorescein-acetoxymethyl ester; BSA, bovine serum albumin; D-MEM, Dulbecco's Modified Eagle's Medium; DMSO, dimethyl sulfoxide, EDTA, ethylenediaminetetraacetate; EGTA, ethylene glycol-bis(β -aminoethyl ether) N,N,N',N'-tetraacetate; EIPA, ethylisopropylamiloride; F-12, Nutrient Mixture F-12 (Ham); FBS, fetal bovine serum; G_m, monolayer conductance; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; I_{sc}, short circuit current; NMG, N-methyl-D-glucamine; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; RTE, renal tubular epithelium; SBF1-AM, Na⁺-binding benzofuran isophthalate-acetoxymethyl ester; Tag, SV40 large T-antigen.

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