hTERT alone immortalizes epithelial cells of renal proximal tubules without changing their functional characteristics

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Wieser M, Stadler G, Jennings P, Streubel B, Pfaller W, Ambros P, Riedl C, Katinger H, Grillari J, Grillari-Voglauer R. hTERT alone immortalizes epithelial cells of renal proximal tubules without changing their functional characteristics. Am J Physiol Renal Physiol 295: F1365–F1375, 2008. First published August 20, 2008; doi:10.1152/ajprenal.90405.2008.—Telomere-dependent replicative senescence is one of the mechanisms that limit the number of population doublings of normal human cells. By overexpression of telomerase, cells of various origins have been successfully immortalized without changing the phenotype. While a limited number of telomerase-immortalized cells of epithelial origin are available, none of renal origin has been reported so far. Here we have established simple and safe conditions that allow serial passaging of renal proximal tubule epithelial cells (RPTECs) until entry into telomere-dependent replicative senescence. As reported for other cells, senescence of RPTECs is characterized by arrest in G1 phase, shortened telomeres, staining for senescence-associated β-galactosidase, and accumulation of γ-H2AX foci. Furthermore, ectopic expression of the catalytic subunit of telomerase (TERT) was sufficient to immortalize these cells. Characterization of immortalized RPTEC/TERT1 cells shows characteristic morphological and functional properties like formation of tight junctions and domes, expression of aminopeptidase N, cAMP induction by parathyroid hormone, sodium-dependent phosphate uptake, and the megalin/cubilin transport system. No genomic instability within up to 90 population doublings has been observed. Therefore, these cells are proposed as a valuable model system not only for cell biology but also for toxicology, drug screening, biogerontology, as well as tissue engineering approaches.

renal proximal tubule cell; renal cell biology; immortalization

CELLULAR SENECESSCE LIMITS the number of population doublings (PDs) of normal human cells in vitro (20). The “classical” stimulus of replicative senescence involves telomere shortening with each cell division (19) due to the end-replication problem (32, 61), resulting in uncappping of telomeres (12) that then resemble DNA double-strand breaks (11, 52). Further senescence stimuli are chronic subtoxic doses of stressors, e.g., reactive oxidative species (57), DNA damage (60), as well as aberrant oncogenic signaling (8). In any case, the terminal growth arrest is executed by either p16/ink4a/pRb or the p53/p21cip1 DNA damage response pathway (1). Stabilization of telomeres prevents activation of p53/p21cip1, and thus stable expression of the catalytic subunit of human telomerase (hTERT) is sufficient to extend the life span of a variety of human cells that retain characteristics of their parental cell strains (2, 4, 13, 23, 63, 65).

In contrast, several cell types cannot be immortalized by telomerase, such as corneal keratinocytes (36) or mammary epithelial cells (23) but also some fibroblast cell strains like IMR90 lung fibroblasts, because of p16/ink4a/pRb activation (42). Accordingly, introduction of viral oncogenes or dominant-negatively acting transgenes either alone or in combination with hTERT expression are necessary for immortalization, at the expense of losing similarity to the parental cell strains (31, 40, 45, 50, 64).

Renal proximal tubule epithelial cells (RPTECs) are involved in blood clearance and resorption of essential metabolites, water, protein, and advanced glycation end-products after glomerular filtration (49). Therefore, they are a widely used model system for basic cell biology but also for various kidney diseases or diabetes (37). Furthermore, they have been discussed for construction of bioartificial tube devices (46, 49). However, only a few continuously growing human kidney cell lines are available, and RPTECs in vitro undergo a very limited number of PDs.

Previous studies on immortalization of RPTECs relied on the use of viral oncogenes, like HK-2 with HPV16 E6/E7 (44) or HKC with a hybrid aden-12-SV40 virus (39). More recently, SV40 with or without hTERT overexpression was used (24, 35).

Here we present cultivation of RPTECs under serum-free conditions without further support by collagen/FCS coating or feeder cells until the cells enter into telomere-dependent replicative senescence. Accordingly, overexpression of hTERT alone was sufficient to immortalize these cells. The resulting RPTEC/TERT1 cell line largely maintained the original differentiation status and functionality without genomic instability for at least 90 PDs. Therefore, RPTEC/TERT1 cells offer a favorable alternative to currently used epithelial kidney cell models like HK-2 cells.

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MATERIALS AND METHODS

Cells and Culture Conditions

Within 24 h after surgery, tissue from the renal cortex was fragmented and incubated at 37°C for 15–20 min in DMEM-Ham’s F-12 (1:1) (Biochrom, Berlin, Germany) containing 1 mg/ml collagenase type IV (PAN-Biotech, Aidenbach, Germany) and 1 mg/ml trypsin inhibitor (Sigma, Vienna, Austria). After being passed through a 105-μm nylon mesh the filtrate was centrifuged, washed twice with phosphate-buffered saline (PBS), resuspended in medium, and dispensed into Roux flasks (Nunc, Wiesbaden, Germany). Twenty-four hours thereafter the medium was changed. The initial passage of confluent cells after 3–5 days was considered population doubling level (PDL) 0. Cells were passaged (1:2 to 1:4) at confluence with 0.25% trypsin-0.02% EDTA, which was inactivated with 1 mg/ml trypsin inhibitor. Cumulative PDL was calculated as a function of passage number and split ratio (4). Medium consisted of DMEM-Ham’s F-12 (1:1) supplemented with 4 mM t-glutamine, 10 mM HEPES buffer, 5 pM triiodothyronine, 10 ng/ml recombinant human EGF, 3.5 μg/ml ascorbic acid, 5 μg/ml transferrin, 5 μg/ml insulin, 25 ng/ml prostaglandin E1, 25 ng/ml hydrocortisone, and 8.65 ng/ml sodium selenite (all from Sigma). For RPTEC/TERT1 cells the medium was supplemented with 100 μg/ml G418 (Sigma). PDLs of RPTEC/TERT1 cells are indicated as PDL posttransfection (PDLpt). HEK-293 cells (American Type Culture Collection) were propagated in DMEM-Ham’s F-12 (1:1) supplemented with 4 mM t-glutamine, 10% fetal bovine serum (FBS; HyClone, Logan, UT), 1 mM NaHCO3, 10 ng/ml EGF, 3.5 μg/ml transferrin, 5 μg/ml insulin, 25 ng/ml prostaglandin E1, 25 ng/ml hydrocortisone, and 8.65 ng/ml sodium selenite (all from Sigma). For RPTEC/TERT1 cells the medium was supplemented with 100 μg/ml G418 (Sigma). PDLs of RPTEC/TERT1 cells are indicated as PDL posttransfection (PDLpt).

For cytogenetic analysis mitotic spreads were stained with Giemsa (kindly provided by Geron, Menlo Park, CA) with EcoR I ligated into the retroviral vector pLXSN (Clontech, Mountain View, CA). For determination of anchorage-independent growth soft agar assay and cytogenetic analysis was performed as described previously (51). HeLa cells served as a positive transfectant. Soft Agar Assay and Cytogenetic Analysis

For determination of anchorage-independent growth soft agar assay was performed as previously described (34). HeLa cells served as a positive control. For cytogenetic analysis mitotic spreads were stained with Giemsa and analyzed as described previously (51).

Bromodeoxyuridine Incorporation (Labeling Index) and Senescence-Associated β-Galactosidase Staining

Determination of cell proliferation was done as described previously (41). After growth in medium supplemented with 50 μM bromodeoxyuridine (BrdU) for 24 h, cells were stained with Hoechst 33258 and analyzed by flow cytometry (FACS Vantage; Becton Dickinson, Franklin Lakes, NJ). The percentage of cells synthesizing DNA within 24 h was calculated.

Senescence-associated β-galactosidase (SA-β-Gal) staining was performed as described previously (14) by incubation of 2% formaldehyde-0.2% glutaraldehyde-fixed cells at 37°C in staining solution (1 mg/ml X-gal, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 2 mM MgCl₂ in 100 mM citric acid/sodium phosphate pH 6.0).

Real-Time Telomeric Repeat Amplification Protocol Assay and Telomere Length Determination

For determination of telomerase activity (TA), a modified real-time telomeric repeat amplification protocol (RT-TRAP) assay was used (59) and TA was calculated relative to HEK293.

Relative telomere lengths were determined with nuclear flow fluorescence in situ hybridization (FISH) assay as recently described in detail (62).

Indirect Immunofluorescent Staining

For staining of megalin, cells grown on eight-well chamber slides (Nunc) were fixed with 4% paraformaldehyde for 10 min at room temperature (RT), permeabilized with 0.1% Triton X-100-0.1% BSA in PBS, and blocked with 0.1% BSA. Rabbit anti-rat megalin antibody generated against an 18-amino acid peptide from the cytoplasmic tail of rat megalin (10) but also reactive to human megalin (30) was used in a 1:1,000 dilution, and cells were stained for 1 h at RT. Secondary antibody was a mouse anti-rabbit FITC antibody (1:100; Sigma); cells were visualized by confocal microscopy with a Leica TCS-SP2.

For staining of occludin and E-cadherin, confluent cells on glass coverslips were fixed in ice-cold 99% methanol and blocked in 5% BSA-1% Triton X-100 in PBS. Coverslips were incubated with primary antibody at 1:100 dilution in 1% BSA-0.2% Triton X-100 for 1 h, washed, and incubated with Texas red-conjugated secondary antibody for 1 h. Fluorescent images were obtained with a Zeiss Axioskop fluorescent microscope. Antibodies against occludin, E-cadherin, and URO-5 were purchased from Zymed Laboratories (San Francisco, CA). Transduction Laboratories (Lexington, KY), and Abcam (Cambridge, UK).

For detection of aminopeptidase N (APN), cells were harvested by trypsinization. After blocking for 20 min in 10% FBS in PBS, cells were resuspended in a 1:100 dilution of the primary antibody (Southern Biotech, Birmingham, AL) and, after washing, incubated with a 1:100 dilution of goat anti-mouse FITC antibody (Sigma). Analysis was performed with a FACSCalibur (Becton Dickinson) and CellQuest Pro software.

Transmission Electron Microscopy

RPTECs were cultured to confluence on Permanox petri dishes. Monolayers were fixed for 15 min in 1% glutaraldehyde in PBS, washed in PBS, postfixed in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer, dehydrated in graded series of isopropanol, and embedded in Durcupane ACM (Fluka, Buchs, Switzerland). Sectioning for both light (0.5 μm) and electron (0.1 μm) microscopy was performed perpendicular to the cell layer(s). Sections were stained by toluidine blue (light microscopy) or by uranyl acetate and lead citrate (electron microscopy).

Scanning Electron Microscopy

Cells were cultured to confluence on Thermaplast coverslips (Nunc). Monolayers were fixed for 45 min with Karnovsky’s fixative (2% formaldehyde, 0.5% glutaraldehyde) and postfixed for 30 min with 1% osmium tetroxide in 0.1 M cacodylate buffer, dehydrated in graded series of isopropanol, and embedded in Durcupane ACM (Fluka, Buchs, Switzerland). Sections were stained by toluidine blue (light microscopy) or by uranyl acetate and lead citrate (electron microscopy).
Fig. 1. Growth and immortalization of renal proximal tubular epithelial cells (RPTECs). A: representative growth curve of parental cell strain (RPTEC) and catalytic subunit of human telomerase (hTERT)-immortalized (RPTEC/TERT1) cells. B: bromodeoxyuridine (BrdU)-Hoechst labeling index of early-passage RPTECs as a control (left) and senescent RPTECs (right) showing G1 growth arrest in senescent cells. C: morphology and senescence-associated (SA)-ß-galactosidase staining of early-passage (left), senescent (center), and immortalized (right) RPTECs. D: relative telomerase activity determined by real-time telomeric repeat amplification protocol (RT-TRAP) assay of parental RPTECs, neo (vector control)-transfected cells, and immortalized RPTECs. BDL, below detection limit; PDLpT, population doubling level posttransfection. E: telomere erosion of parental RPTECs and telomere stabilization after hTERT expression measured by nuclear flow fluorescence in situ hybridization (FISH). MESF, molecules of equivalent soluble fluorochromes. *P < 0.05, **P < 0.01.
45 min with 1% OsO4 in 0.1 M sodium cacodylate buffer (pH 7.4). Specimens were dehydrated with methanol following a critical point drying and finally sputter-coated with a 30- to 50-nm gold-palladium layer for observation with a scanning electron microscope (JEOL jsm-25s).

**Functional Assays**

**Transepithelial electrical resistance.** Cells were seeded on 0.5-cm diameter, 0.2-μm pore size, aluminum oxide filter inserts (Nunc). Transepithelial electrical resistance (TEER) of monolayers was measured with the Endohm and EVOM systems from World Precision Instruments (Berlin, Germany). TEER of blank filters was subtracted from all samples. TEER values are expressed as ohms times square centimeter.

**Hormonal stimulation.** Cells were cultured to confluence on 12-well culture plates and treated overnight with 0.1 mM IBMX in culture medium. Thereafter, cells were treated with varying concentrations of parathyroid hormone (PTH) and vasopressin for 15 min in culture medium also containing 0.1 mM IBMX. Medium was removed, and 0.5% trichloroacetic acid cell extracts were prepared. cAMP was assayed in the neutralized extracts with a competitive immunosay (Cayman Chemicals, Loerach, Germany).

**pH-dependent ammonia genesis.** Cells were cultured to confluence on 24-well culture plates. Cells were incubated in DMEM-Ham’s F-12 with 2 mM sodium bicarbonate-20 mM HEPES from pH 6.6 to pH 8.6 in a humidified 37°C incubator without CO2 for 4 h. Supernatant was collected, and ammonia was assayed with a commercially available assay (Sigma).

**γ-Glutamyl transferase activity.** γ-Glutamyl transferase (GGT) activity was determined as described previously (27). Cells were grown in 12-well culture plates and incubated with substrate solution containing 1 mM γ-glutamyl-para-nitroanilide (GPNA), 60 mM Tris·HCl pH 8.0, and 20 mM glycy-glycine for 20 min at room temperature. Enzymatic cleavage of GPNA was stopped by adding 10% acetic acid (1:2). Para-nitroanilide (pNA) release was determined by spectrophotometry at 405 nm in a Hitachi U 2001 photometer. GGT activity is expressed as nanomoles of pNA per minute and milligram of protein.

**Sodium-dependent phosphate uptake.** Sodium-dependent phosphate uptake assay was performed as described previously (25) with minor modifications. In brief, cells were grown to confluence in 48-well plates. Before assay cells were rinsed three times with uptake buffer consisting of (mM) 137 NaCl, 5.4 KCl, 2.8 CaCl2, 1.2 MgSO4, 48-well plates. Before assay cells were rinsed three times with uptake buffer consisting of (mM) 137 NaCl, 5.4 KCl, 2.8 CaCl2, 1.2 MgSO4, and 1 KH2PO4 or a buffer in which sodium chloride was replaced by 137 mM choline chloride. For uptake, sodium chloride- or choline chloride-containing buffer was supplemented with 1 μCi of [13P]phosphoric acid (Hartmann Analytic, Braunschweig, Germany) per well. Uptake was allowed for 1 or 10 min at RT. Thereafter, cells were washed three times with ice-cold choline chloride buffer and lysed for 30 min with 0.5 M NaOH. Two hundred microliters of lysate per well was analyzed for total radioactivity on a Packard 1900CA TRI-CARB (Packard Instrument, Sterling, VA) liquid scintillation analyzer.

**Protein uptake.** Cell culture-grade bovine aprotinin (Sigma) was labeled with Alexa Fluor 633 succinimidyl ester (Molecular Probes, Invitrogen, Eugene, OR) according to manufacturer’s instructions. Unincorporated dye was removed with a 1-kDa-cutoff minidialysis kit (Amersham Biosciences, Piscataway, NJ) according to manufacturer’s instructions at 4°C overnight. Uptake was analyzed similarly to the analysis in Ref. 18. In brief, confluent cells were starved for 24 h in DMEM-Ham’s F-12-4 mM l-glutamine. Labeled aprotinin was diluted to final concentrations in PBS containing Ca2+/Mg2+, and cells were incubated for 30 min at 37°C or 4°C. Thereafter, cells were put on ice and rinsed seven times with ice-cold PBS, and protein uptake was determined on a FACSCalibur.

**Semiquantitative PCR.** cDNA for semiquantitative PCR was isolated as described previously (17). Primers for detection of SLC34A3 (NM_0080877) were CCGAAGCGCCCGACATCC (forward) and ACCGTGTTGGCCGACGGAGCTCA (reverse); for human cubilin (NM_001081) GCCATCCAGGGCTCCGACTCTAC (forward) and TGAAGCCTGCCCTGTTAACCT (reverse); and for human β-actin (NM_001101) CTGGAACGGTGAAAGTGCA (forward) and AAGGACTTCTCTGTAACATGCA (reverse).

**Statistics**

Data are presented as means ± SD. Student’s t-test was used to compare data unless otherwise indicated. P values for statistical significance are as indicated.
RESULTS

RPTECs Undergo Telomere-Dependent Senescence That Is Circumvented by Introduction of hTERT Alone

To establish RPTECs as a novel model system for kidney function and aging, we serially passaged them under (optimized) serum-free culture conditions and retrovirally transduced them with pLXSN-hTERTneo (59). RPTEC/TERT1 cells were propagated as a mass culture because of poor cloning efficiency. They showed growth rates of 0.25 PD/day and a population doubling time of ~96 h, comparable to the normal counterpart, and have now accomplished ~90 PDs, corresponding to more than threefold life span extension since normal RPTECs enter growth arrest after 24 PDLs (Fig. 1A). In contrast, cells transduced with an empty control vector ceased to grow almost immediately after selection (data not shown).

To confirm replicative senescence, we analyzed the cell cycle distribution and labeling index by BrdU-Hoechst 33258 staining. While RPTECs at PDL 16 showed a labeling index of 24.3% (SD ± 1.1%) (Fig. 1B, left), cells at PDL 24 were arrested in G1 phase with as few as 2% (SD ± 0.9%) of the cells performing DNA synthesis (Fig. 1B, right). In addition, RPTECs at late PDLs displayed morphology changes reminiscent of senescence in other cell models and homogeneous staining for SA-β-Gal (Fig. 1C). In contrast, RPTEC/TERT1 cells retained morphology very similar to early-passage cells as well as few SA-β-Gal-positive cells. Interestingly, a considerable fraction of ~20% positive cells was observed also in early-passage cells.

To confirm that hTERT transfection indeed resulted in TA we used RT-TRAP assay to measure the relative TA as normalized to HEK293 cells. While RPTECs and empty vector-transfected RPTECs displayed TA below the detection limit, RPTEC/TERT1 cells showed significant levels of activity that increased to 34% (SD ± 3%) of HEK293 (Fig. 1D). Accordingly, RPTEC/TERT1 cells were able to stabilize their telomere lengths, whereas in normal RPTECs telomere shortening measured by nuclear flow FISH from 21.5 kMESF (molecules of equivalent soluble fluorochromes) (SD ± 0.6) to 11.5 kMESF (SD ± 0.8) at PDL 22 became apparent (Fig. 1E).

Increased Formation of γ-H2AX in Senescent RPTECs Is Partially Suppressed by hTERT

To test whether the significant reduction (P < 0.01) in telomere length was associated with signs of DNA damage, we further investigated the presence of γ-H2AX foci (11, 52). Interestingly, γ-H2AX foci were already present in numerous early-passage cells (>85%), with an average of 4.3 foci per cell (Fig. 2A). However, in senescent cells the average cells the number (13.2) as well as the occurrence of large foci (Fig. 2B, arrowhead), possibly representing persistent, unrepairable lesions, increased (Fig. 2B). Interestingly, γ-H2AX focus formation in
RPTEC/TERT1 cells was not reduced to early-passage cell levels, with a number of 7.1 foci per cell (Fig. 2C).

**RPTEC/TERT1 Cells Retain Morphological and Biochemical Markers**

Immortalized RPTEC/TERT1 cells maintained the typical cobblestone appearance reminiscent of early-passage parental cells (Fig. 1C). At high cell densities both parental and TERT-immortalized cells formed characteristic domes (Fig. 3, A and B), and electron microscopy of a perpendicular section through a monolayer of RPTEC/TERT1 cells showed maintained ultrastructural organization with tight junctions and numerous microvilli (Fig. 3B). This indicates maintenance of functional cell polarization and an intact barrier, both prerequisites for vectorial transport of water and solutes as a key function of RPTECs (26). Furthermore, GGT activity was detected in immortalized cells (Fig. 3C), and GGT activity was increased in RPTEC/TERT1 cells (107.9 ± 0.3) compared with early-passage (20.0 ± 0.1) as well as late-passage (72.9 ± 2.0) RPTECs, indicating a cultivation time-dependent phenomenon. Cells also homogenously expressed APN, as observed for early-passage and late-passage parental RPTECs with immunofluorescent staining and flow cytometry (Fig. 3D). Both enzymes (APN and GGT) are located in the brush border of the tubule and are regarded as characteristic markers of RPTECs (43, 53).

Neither RPTECs nor RPTEC/TERT1 cells were capable of anchorage-independent growth, while HeLa cells as positive controls did form colonies as tested by soft agar assays (data not shown). Analysis of the karyotypes by Giemsa banding of mitotic spreads showed a normal diploid karyotype (46, XY) and no gross chromosomal aberration in RPTEC/TERT1 cells at PDL 39 and 85 after transfection, demonstrating genomic stability and the maintenance of a highly differentiated phenotype (data not shown).

**RPTEC/TERT1 Cells Form Densely Packed Microvilli and Solitary Cilia**

To visualize the basic characteristics of the RPTEC/TERT1 cell surface we analyzed RPTEC/TERT1 cells by scanning electron microscopy (SEM) compared with HK-2 cells, an established human proximal tubule cell line. RPTEC/TERT1 cells at lower magnification show a smooth, homogeneous surface (Fig. 4A), while HK cells are more irregularly shaped (Fig. 4B). At higher magnification, densely packed microvilli as well as solitary cilia are observed as described previously (9), while HK-2 cells show less dense but longer microvilli (Fig. 4, C and D).

**RPTEC/TERT1 Cells Form Tight Junctions and Respond to Parathyroid Hormone and Decreased pH**

Furthermore, we tested several primary RPTEC characteristic functions compared with RPTEC/TERT1 cells and the HK-2 cell line.

RPTEC/TERT1 cells and primary RPTECs exhibited a continuous belt of occludin and E-cadherin around the individual

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**Fig. 5.** RPTEC/TERT1 cells display functional characteristics of RPTECs. *A:* immunofluorescent staining of E-cadherin and occludin in RPTECs, RPTEC/TERT1 cells, and HK-2 cells. Original magnification ×630. *B:* transepithelial electrical resistance (TEER) was measured on cells cultured on microporous filters. The time course of TEER stabilization is shown for RPTEC/TERT1 (top), and the stabilized values are shown for all 3 cells (bottom). *C:* cellular cAMP was measured after stimulation with arginine vasopressin (AVP) or parathyroid hormone (PTH). Dose response to AVP and PTH is shown for RPTEC/TERT1 cells (top), and response to 100 nM AVP and 10⁻⁷ M PTH is shown for all 3 cell types (bottom). *D:* the ability of all 3 cell types to respond to altered extracellular pH by enhancing ammonia production is shown. *P < 0.05, **P < 0.01, ***P < 0.001 by 1-way ANOVA with a Dunnett’s posttest.
cells of the monolayer (Fig. 5A). TEER of RPTEC/TERT1 cells grown on aluminum oxide filter inserts sharply increased at day 10 and subsequently reached a maximum at day 15 (Fig. 5B). This dynamic further confirms the ability to form an intact functional barrier. RPTEC/TERT1 cells compared well to primary RPTEC cells with regard to TEER, even though TEER in primary cells is slightly higher than in immortalized cells. HK-2 cells, however, exhibited much lower TEER and expressed much less E-cadherin than RPTECs and RPTEC/TERT1 (Fig. 5, A and B).

Proximal tubule cells in vivo respond to PTH by enhancing cAMP production, whereas arginine vasopressin (AVP) has this effect in distal cells (58). Treatment with PTH but not AVP induced cAMP production in a dose-dependent manner in RPTECs and RPTEC/TERT1 cells (Fig. 5C). In contrast, HK-2 cells increased cAMP production in response to AVP and not PTH.

In proximal tubular cells a decline in blood pH elicits a rapid production and excretion of ammonia. All three cell types responded to decreasing extracellular pH by enhanced ammonia genesis (Fig. 5D).

Transport Activity of RPTEC/TERT1 Cells is Retained

Since transport functions are a further characteristic of RPTECs, we tested for expression and functionality of RPTEC specific sodium-dependent phosphate transporters (16). Indeed, SLC34A3 mRNA, for example, was detected by RT-PCR in parental RPTECs as well as in RPTEC/TERT1 cells. As positive controls HK-2 cells as well as kidney tissue were used, while HeLa cells served as negative control (Fig. 6A). Accordingly, RPTECs, RPTEC/TERT1 cells, as well as HK-2 cells showed sodium-dependent phosphate uptake as described for primary cells of the proximal tubules. Substitution of sodium

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**Fig. 6.** RPTEC/TERT1 cells retain sodium-dependent phosphate uptake and protein endocytosis. **A:** transcripts of SLC34A3 are detected by RT-PCR. As positive control the housekeeping gene β-actin (ACTB) was used. NTC, no template control. **B:** highly significant reduction of phosphate uptake by substitution of sodium chloride with choline chloride demonstrates that the uptake is sodium dependent (*P < 0.05, **P < 0.01 by Student’s t-test of triplicate measurements). In addition, phosphate uptake is time dependent as indicated by the difference between 1-min uptake (NaCl short) vs. 10-min uptake (NaCl) in the presence of sodium. **C:** transcripts of cubilin are detected by RT-PCR. **D:** indirect immunofluorescence of megalin expression with rabbit anti-megalin antibody and a Leica confocal microscope. Left: RPTEC/TERT1 (top) is compared with L2 cell line (bottom). Right: corresponding phase-contrast pictures. Original magnification ×1,260. **E:** endocytosis of Alexa Fluor 633-labeled aprotinin into RPTEC/TERT1 is dose- and temperature dependent.
by choline significantly decreased phosphate uptake (Fig. 6B). The uptake is even enhanced in RPTEC/TERT1 cells compared with RPTECs or HK-2 cells, in line with the finding that telomerase overexpression sometimes enhances functionality (22). A second specialized transport system highly expressed in the renal proximal tubule is the megalin/cubilin multiligand receptor. A considerable number of ligands have been identified, establishing its essential role for endocytotic uptake of filtered proteins by the proximal tubule (7).

For cubilin, RT-PCR similar to that for SLC34A3 was performed, and again RPTECs, RPTEC/TERT1 cells, as well as kidney tissue showed cubilin transcription, while HeLa cells did not. HK-2 cells seem to transcribe less cubilin mRNA (Fig. 6C), although quantitative PCR would have to be performed for confirmation. Megalin was stained by immunofluorescence with anti-megalin antibody 459 directed against the cytoplasmatic tail of megalin (10). A characteristic staining in punctate structures at the membrane was observed (Fig. 6D). As control, L2 rat yolk sac carcinoma cells, which were previously shown to express high levels of megalin (34), showed similar staining, but also some intracellular staining.

Furthermore, we tested the functionality of megalin/cubilin transport. Indeed, fluorescently labeled aprotinin, which is one of the ligands for megalin (29), was shown to accumulate in RPTEC/TERT1 cells in a dose-dependent manner. As control, uptake was tested at 4°C, and only slight uptake was observed, indicating active endocytosis (Fig. 6E).

Finally, even after prolonged cultivation RPTEC/TERT1 cells did not express URO-5, a marker of the distal tubule (data not shown).

These data therefore suggest that RPTEC/TERT1 cells highly maintain a proximal tubular differentiated phenotype up to 90 PDs, which seems much more pronounced than that of the established HK-2 cell line and compares favorably to primary proximal tubular cells.

DISCUSSION

Human RPTECs represent a valuable in vitro model for toxicology, drug screening and development, as well as tissue engineering. Since all of these applications are hampered by the limited replicative potential of these cells in vitro, several approaches have been performed to establish continuously growing RPTEC cell lines with a high degree of differentiation. However, so far this has been achieved by introduction of viral oncogenes or combinations of SV40 and hTERT only (24, 35, 39, 44).

To circumvent introduction of viral oncogenes, we established simple and defined culture conditions for RPTECs that allow passaging of the cells to replicative senescence. The cultivation conditions reported here are based on observations with RPTECs of animal and human origin in a hormonally defined medium (38, 48, 54–56). Indeed, the terminal growth arrest under these conditions was telomere dependent and characterized by morphological changes, telomere shortening, >95% SA-β-Gal staining, increased γ-H2AX focus formation, G1 arrest, and very little residual DNA synthesis. In consequence, ectopic expression of hTERT was sufficient to immortalize the cells and to prevent all senescence-like characteristics analyzed, resulting in the continuously growing cell line RPTEC/TERT1.

RPTEC/TERT1 closely resembles primary counterparts in all functional assays performed so far, showing the intact vectorial transport, hormone responsiveness, and excretory capacity of RPTEC/TERT1 cells. They show typical morphology in SEM with densely packed microvilli and solitary cilia, form tight junctions and domes, are stimulated by PTH but not by AVP, and react with enhanced ammonia genesis on lowering of the environmental pH. Furthermore, RPTEC specific sodium-dependent phosphate uptake is intact (3, 9), as well as the megalin/cubilin transporter system, when aprotinin is used as model protein (29, 33).

In addition, by proving telomere-dependent senescence, RPTECs also provide an additional novel cell model for aging research. This is of special interest, since senescent kidney cells have been found to increase with age in vivo (6, 28) or with time after kidney transplantation, which inversely correlates with the functionality of the graft (5). The kidney as a whole undergoes dramatic structural changes (47) and shows increased sensitivity to physiological and xenobiotic stressors (15) and abnormalities in sodium and electrolyte homeostasis during aging, functions in which RPTECs are clearly involved.

In conclusion, we propose RPTEC/TERT1 as a general tool for detailed investigations in basic cell biology, but also for toxicology, drug screening, and aging and development, as well as a potential source for tissue engineering and cell-based therapy approaches.

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GRANTS

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REFERENCES


12. Finkel T,.SystemColors(10.220.33.1 on June 8, 2017 http://ajprenal.physiology.org/ Downloaded from)