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CELL BIOLOGY – IMMUNOLOGY – PATHOLOGY

Isolation and characterization of conditionally immortalized mouse glomerular endothelial cell lines

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Isolation and characterization of conditionally immortalized mouse glomerular endothelial cell lines.

Background. The culture and establishment of glomerular cell lines has proven to be an important tool for the understanding of glomerular cell functions in glomerular physiology and pathology. Especially, the recent establishment of a conditionally immortalized visceral epithelial cell line has greatly boosted the research on podocyte biology.

Methods. Glomeruli were isolated from H-2K^b-tsA58 transgenic mice that contain a gene encoding a temperature-sensitive variant of the SV40 large tumor antigen, facilitating proliferative growth at 33°C and differentiation at 37°C. Glomerular endothelial cells were isolated from glomerular outgrowth by magnetic beads loaded with CD31, CD105, GSL I-B4, and ULEX. Clonal cell lines were characterized by immunofluorescence staining with antibodies/lectins specific for markers of endothelial cells, podocytes, and mesangial cells. Putative glomerular endothelial cell lines were analyzed for (*1*) cytokine-induced expression of adhesion molecules; (*2*) tube formation on Matrigel coating; and (*3*) the presence of fenestrae.

Results. As judged by immunostaining for Wilms tumor-1, smooth muscle actin (SMA), podocalyxin, and von Willebrand factor (vWF), we obtained putative endothelial, podocyte and mesangial cell lines. The mouse glomerular endothelial cell clone #1 (mGEnC-1) was positive for vWF, podocalyxin, CD31, CD105, VE-cadherin, GSL I-B4, and ULEX, internalized acetylated-low-density lipoprotein (LDL), and showed increased expression of adhesion molecules after activation with proinflammatory cytokines. Furthermore, mGEnC-1 formed tubes and contained nondiaphragmed fenestrae.

Conclusion. The mGEnC-1 represents a conditionally immortalized cell line with various characteristics of differentiated glomerular endothelial cells when cultured at 37°C. Most important, mGEnC-1 contains nondiaphragmed fenestrae, which is a unique feature of glomerular endothelial cells.

Key words: glomerular, endothelial, cell lines, conditionally immortalized.

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Endothelial cells play a role in many biologic and pathologic processes like coagulation, angiogenesis, and inflammatory responses. The morphology and biologic responses of endothelial cells vary depending on the vascular system they originate from, either within one organism or between different species. Endothelial cells play a key role during inflammation and their activation leads to the recruitment of leukocytes to the local area of inflammation, which involves various factors like chemokines, selectins, integrins, and heparan sulfates [1–4]. The glomerular endothelial cell layer is attached to the glomerular basement membrane (GBM), which is covered at the urinary side by visceral epithelial cells, also called podocytes. This triple sandwich of fenestrated endothelial cells, GBM, and podocytes constitutes the glomerular capillary filter, which is responsible for the charge and size-dependent filtration of plasma molecules [2, 5–7]. A characteristic feature of glomerular endothelial cells is the presence of numerous nondiaphragmed fenestrae [8], which in humans are about 100 nm in diameter [9].

The in vitro culture of the different types of glomerular cells (i.e., fenestrated endothelial cells, mesangial cells, podocytes and parietal epithelial cells) is an important tool to obtain a better understanding of their respective roles in renal physiology and pathology (including proteinuria, inflammation, sclerosis/fibrosis, and hyperglycemia). Techniques for the isolation and culture of glomerular cells, including endothelial cells, have been developed and successfully applied. A major draw back of the in vitro culture of isolated primary glomerular endothelial cells is their limited life span and the loss of characteristic features and/or differentiation markers after propagation [5, 10–12].

The development of genetically modified mice has allowed for the establishment of glomerular (endothelial) cell lines with a more constant phenotype and a longer life span. Initially, glomerular cell lines transgenic for the simian virus 40 (SV40) large T antigen have been established, which usually resulted in a cellular transformation

of cells to a more proliferative and less differentiated phenotype ("immortalized") compared to normal primary cells. Later on, the H-2K^b-tsA58 transgenic mouse ("immortomouse") was established [13], which expresses, depending on the temperature, SV40 large T antigen protein encoded by the temperature-sensitive (ts) A58 allele controlled by the interferon-γ (INF-γ)-inducible H-2K^b promoter. In cells derived from this mouse, the SV40 large T antigen protein is functionally expressed at the permissive temperature of 33°C, which results in cell proliferation. Exposure of the cells to the nonpermissive temperature of 37°C, leads to the degradation of the SV40 large T protein, which then results in a less proliferative and more differentiated phenotype that is comparable to that of primary cells. Therefore, these cell lines are designated as conditionally immortalized cells [13].

The H-2K^b-tsA58 transgenic mouse has been used to establish various cell lines, including mesangial cells [14], but also podocytes that are normally difficult to isolate and culture without loss of their differentiated phenotype. These conditionally immortalized podocyte cell lines have greatly boosted the research on podocyte biology and pathology [15, 16]. In addition, endothelial cells lines have been derived from the brain and the heart of these mice [17, 18]. However, thus far no glomerular endothelial cell lines have been described.

In this paper, we describe the isolation and characterization of the first conditionally immortalized glomerular endothelial cell line. We show that this cell line has all characteristics of a functional glomerular endothelial cell, including the presence of nondiaphragmed fenestrae. This cell line enables future studies on the role of glomerular endothelial cells in glomerular physiology and pathology.

METHODS

Mice and genotyping

The H-2K^b-tsA58 transgenic mice [13] were obtained from Charles River Laboratories (CRL, Wilmington, DE, USA), and housed and handled according to the guidelines of the University Medical Center Nijmegen, The Netherlands.

Toe biopsies were lysed in lysis buffer [100 mmol/L Tris, pH 7.5, 5 mmol/L ethylenediaminetetraacetic acid (EDTA), 200 mmol/L NaCl, 0.2% sodium dodecyl sulphate (SDS), 5 μg protein K (Invitrogen Life Technologies, Breda, The Netherlands)] for 16 hours at 60°C. Subsequently, DNA was precipitated by isopropanol and centrifugation and finally dissolved in 100 μL TE buffer (10 mmol/L Tris, pH 8.0, and 1 mmol/L EDTA).

Polymerase chain reaction (PCR) was performed with the use of Platinum PCR SuperMix (Invitrogen Life Technologies) and a specific primer pair; forward primer (nucleotide position 1159–1179) 5′-GAGCGAATTCT GGATGGCTGGAGTTGCTTG-3′, reverse primer (nucleotide position 1441–1460) 5′-GATCGGATCCGAA GGCAAATCTCTGGACTC-3′ (Biolegio BV, Malden, The Netherlands). PCR cycling parameters were (*1*) denaturation of 94°C/5 minutes; (*2*) 35 cycles with 94°C/1 minute (denaturation), 55°C/1 minute (annealing), and 72°C/1 minute (elongation); and (*3*) elongation of 72°C/5 minutes using the Mastercycler 5330 (Eppendorf, Hamburg, Germany). PCR products were analyzed by agarose gel electrophoresis.

Isolating and cloning of mouse glomerular cells

Glomeruli were isolated from kidneys of 14 mice aged between 6 to 8 weeks under sterile conditions by gradually sieving with stainless steel sieves (150, 90, 75, 53, and 32 μm) (Endecotts, London, UK), which resulted in 95% pure glomeruli as judged by light microscopy inspection. These glomeruli were washed with 50 mL Dulbecco's modified Eagle's medium (DMEM) (Invitrogen Life Technologies) and subsequently treated with 2.5 mL 0.1% collagenase type II (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands) in DMEM at 37°C for $1^1/_2$ hours. The cell suspension was washed in 5 mL Hanks' balanced salt solution (HBSS) containing 10% fetal calf serum (FCS) (Invitrogen Life Technologies), and subsequently resuspended in 25 mL cloning medium, consisting of microvascular endothelial cell growth medium (EGM)-2MV (Cambrex Bioscience, Verviers, Belgium), supplemented with 100 μg/mL endothelial cell growth supplement (ECGS) (PeproTech, London, UK), 5 U/mL heparin (Sigma-Aldrich), 5% FCS, 10% newborn calf serum (NCS), 1% penicillin/streptomycin (Invitrogen Life Technologies), and 20 U/mL recombinant mouse IFN-γ (PeproTech). The cells were cultured in tissue culture flasks (Corning Life Sciences B.V., Schiphol-Rijk, The Netherlands) precoated with a mixture of 1% gelatin (Sigma-Aldrich) and human fibronectin (1 μg/cm^2) (Sigma-Aldrich) at the permissive temperature of 33°C in a humidified 5% CO_2 atmosphere. On days 7 to 14 after initial seeding, outgrowths of individual glomeruli were detached by trypsin-EDTA (Invitrogen Life Technologies) and glomerular endothelial cells were isolated using magnetic beads loaded with antibodies directed against endothelial markers and lectins using a MPC-50 magnet (Dynal, Hamburg, Germany). Details on these magnetic beads loaded with antibodies/lectins are given in Table 1. After culture for 1 week at 33°C, this procedure was repeated once. The retrieved cells were then subcloned by limiting dilution and by electronic sorting (Epics Elite Flowcytometer) (Beckman Coulter, Miami, FL, USA) and cultured in normal growth medium, consisting of DMEM:HAM-F12 medium (Invitrogen Life Technologies) supplemented with 10% FCS, 1%

Table 1. Magnetic beads loaded with anti-endothelial antibodies/lectins used to isolate glomerular endothelial cells

Magnetic beads	Catching antibodies/ lectins	Ratio beads: cells
Dynabeads with sheep antirat IgG,[a] 25 μL (1×10^7 beads/mL)	Rat antimouse PECAM-1 (CD31),[b] 1.5 μg/2.5×10^5 beads	3:1
	Rat antimouse endoglin (CD105),[b] 1.5 μg/2.5×10^5 beads	3:1
Biomag streptavidin ultraload particles,[c] 25 μL (12.5×10^7 beads/mL)	Biotinylated Griffonia simplicifolia isolectin B4 (GSL I-B4),[d] 7.5 μg/3×10^6 beads	50:1
	Biotinylated ULEX europaeus agglutinin I (ULEX),[d] 7.5 μg/3×10^6 beads	50:1

[a]Dynal, Hamburg, Germany.
[b]BD Biosciences, Alphen aan den Rijn, The Netherlands.
[c]Westburg, Leusden, The Netherlands.
[d]Vector Laboratories Inc., Burlingame, CA, USA.

penicillin/streptomycin, and 20 U/mL recombinant mouse IFN-γ at 33°C. Unless stated otherwise, this normal growth medium was used for all subsequent experiments.

Characterization of glomerular cells by immunofluorescence

The cells were grown in slide flasks (Nunc, Roskilde, Denmark), precoated with 1% gelatin, for 1 week at 33°C (permissive temperature) or for 1 week at 37°C (nonpermissive temperature) in normal growth medium with or without IFN-γ, respectively, until they were 80% to 100% confluent. Cells were fixed for 10 minutes with 2% paraformaldehyde-phosphate-buffered saline (PBS) with 0.3% Triton X-100 (Sigma-Aldrich) for the evaluation of the SV40 expression or with 90% acetone for all other markers, and then blocked with 2% bovine serum albumin (BSA) (Sigma-Aldrich) in PBS for 30 minutes. Cells were incubated with antibodies (2 to 4 μg/mL) specific for SV40 large T antigen (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), platelet endothelial cell adhesion molecule (PECAM)-1 (CD31), endoglin (CD105) (BD Biosciences, Alphen aan den Rijn, The Netherlands), vascular-endothelium (VE)-cadherin (Santa Cruz Biotechnology), the biotinylated lectins *Griffonia simplicifolia* isolectin B4 (GSL I-B4) and *Ulex europaeus agglutinin I* (ULEX) (Vector Laboratories Inc., Burlingame, CA, USA), von Willebrand factor (vWF) (Dako, Glostrup, Denmark), and podocalyxin (0601) in 2% BSA-PBS for 45 minutes, rinsed with PBS and incubated with the appropriate Alexa 488–conjugated secondary antibody (Molecular Probes, Leiden, The Netherlands) in 2% BSA-PBS for 45 minutes at room temperature. Finally, the cells were rinsed in PBS, postfixed with 1% paraformaldehyde-PBS, rinsed in PBS, and embedded in VectaShield mounting medium H-1000 (Vector Laboratories). The slides were investigated by fluorescence microscopy (Zeiss Axioskop microscope equipped with an epi-illuminator) and photographed with a digital camera (Nikon Coolpix DXM 1200) (Bunnik, The Netherlands). To distinguish glomerular endothelial cells from other glomerular cells (mesangial cells and podocytes), we also stained for smooth muscle actin (SMA) (Sigma-Aldrich)(A-2547) and Wilms tumor-1 (Santa Cruz Biotechnology), respectively. As a positive control for mesangial cells and podocytes we used mouse mesangial cells (SV40 MES 13) (CRL-1927) (ATCC, Manassas, VA, USA) and the conditionally immortalized mouse podocyte cell line (MPC-5), which was cultured as described previously [15].

Uptake of acetylated low-density lipoprotein (LDL) by glomerular endothelial cells

For evaluation of uptake of acetylated LDL by the glomerular endothelial cells, the cells were grown in slide flasks, precoated with 1% gelatin, for 1 week at 37°C in normal growth medium without IFN-γ, until they were 80% to 100% confluent. The cells were incubated in normal growth medium without IFN-γ supplemented with 10 μg/mL Dil-labeled acetylated-LDL (Biomedical Technologies Inc., Stoughton, MA, USA) at 37°C. After 4 hours the cells were rinsed with PBS, fixed with 3% formaldehyde-PBS, rinsed with PBS, mounted in VectaShield, and evaluated by fluorescence microscopy. As controls we used mouse mesangial cells (SV40 MES 13) (ATCC, CRL-1927) and the conditionally immortalized mouse podocyte cell line (MPC-5) cultured for 1 to 2 weeks at 37°C.

Activation of glomerular endothelial cells by tumor necrosis factor (TNF)-α and interleukin (IL)-1β as determined in enzyme-linked immunosorbent assay (ELISA)

The glomerular endothelial cells were grown in 96-well plates (Corning Life Sciences) precoated with 1% gelatin for 1 week in normal growth medium without IFN-γ at 37°C until they were 100% confluent. The cells were then incubated with normal growth medium supplemented with murine TNF-α (10 ng/mL) and/or IL-1β (10 ng/mL) (PeproTech) for 18 hours at 37°C. After washing with PBS, the cells were fixed with 2% formaldehyde-PBS, washed with PBS, incubated with 2% BSA-PBS for 2 hours at room temperature, and washed with PBS containing 0.05% Tween 20 (PBS/Tween). Cells were incubated with rat antibodies against mouse intercellular adhesion molecule (ICAM)-1 or vascular adhesion molecule (VCAM)-1 in 2% BSA-PBS for 1 hour at room temperature, washed with PBS/Tween and incubated with mouse antirat IgG (H + L) conjugated with

horseradish peroxidase (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) in 2% BSA-PBS for 1 hour at room temperature. Finally, the cells were washed with PBS/Tween and incubated with tetramethylbenzidine (TMB) substrate solution (SFRI Laboratories, Berganton, France). After exactly 15 minutes, the reaction was stopped with 2 mol/L H_2SO_4 and absorption was measured at 450 nm using a Bio-Rad Multiplate Reader (Bio-Rad Laboratories Inc., Hercules, CA, USA).

Demonstration of fenestrae by scanning and transmission electron microscopy (TEM)

The glomerular endothelial cells were grown in normal growth medium without IFN-γ on glass coverslips precoated with 1% gelatin, for 1 week at 37°C, until they were 100% confluent. Cells were rinsed with basic DMEM medium and fixed in 2.5% glutaraldehyde dissolved in 0.1 mol/L sodium cacodylate buffer, pH 7.4, for 3 hours at 4°C. Subsequently, cells were washed in the same buffer, rinsed in palade buffer, pH 7.3, and postfixed in palade buffered 2% osmium (OsO_4) for 2 hours. Cells were dehydrated by subsequent incubations with 70%-90%-100% alcohol and acetone, critical point dried using a Polaron EMS 850 critical point drier (Balzers, Eindhoven, The Netherlands) and coated with gold by the use of an Edwards EMS 550 sputter coater (Aurion, Wageningen, The Netherlands). Cells were visualized with a Jeol 6310 scanning electron microscope (Jeol, Tokyo, Japan). As a control we used a microvascular endothelial cell line (EOMA) (ATCC, CRL-2586).

For TEM, cells were cultured to confluency in normal growth medium on a 30 mm, 2 μ thick, foil bottom Willcodish (WillCo Wells BV, Amsterdam, The Netherlands) precoated with 1% gelatin, for 1 week at 37°C. After the filter was rinsed with culture medium, the cells were fixed with 2.5% glutaraldehyde in 0.1 mol/L sodium cacodylate buffer, pH 7.2, for 2 hours at 4°C. Subsequently, cells were rinsed several times in PBS, postfixed in cacodylate-buffered 1% OsO_4 for 30 minutes, rinsed in 50% alcohol, and incubated with 50% alcohol and 2% uranyl acetate for 20 minutes at 4°C. After the cells were rinsed in 50% alcohol, the cells were dehydrated via subsequent alcohol steps to alcohol 100%, alcohol 100%/epon 1:1 overnight, and finally embedded in epon 812. Ultrathin sections were cut on an ultratome, Reichert Ultracut S (Leica Microsystems, Heidelberg, Germany). The specimens were examined by electron microscopy (Jeol 1200 EX2) (Jeol).

Determination of the proliferation rate

Glomerular endothelial cells from one confluent 75 cm^2 flask, precoated with 1% gelatin, cultured at 33°C, were split into four and cultured in normal growth medium with or without IFN-γ at 33°C or 37°C, respectively, for 48 hours. Subsequently, cells were passaged into 96-well plates, precoated with 1% gelatin and/or fibronectin (1 μg/cm^2), at 10,000 cells/well. Cells were cultured for up to 96 hours before cell proliferation reagent WST-1, 10 μL/well, (Roche Diagnostics GmbH, Germany) was added. For the background control the same volume per well of growth medium and WST-1 was used. After 2 hours the absorbance was determined at 450 nm with a reference of 605 nm for the cleavage of the tetrazolium salt WST-1 to formazan.

Microtube formation assay

Glomerular endothelial cells (3×10^5) in 1 mL of normal growth medium with or without IFN-γ were plated on polymerized Matrigel (BD Biosciences), 600 μL/well, in 6 wells and cultured at 33°C or 37°C, respectively. After 20 hours tube formation was analyzed by phase-contrast microscopy and photographed (Nikon Coolpix 990).

RESULTS

Isolation and cloning of mouse glomerular endothelial cells (mGEnC)

Following the isolation with magnetic beads and subcloning, we finally obtained numerous clonal cell lines. The cell lines had different morphology and growth characteristics, especially when cultured at 37°C. Immunofluorescence staining with Wilms tumor-1 (podocyte marker), SMA (mesangial cell marker), vWF (endothelial cell marker), and podocalyxin (podocyte and endothelial cell marker) revealed potential podocyte, mesangial, and glomerular endothelial cell lines (not shown). We focused further on the characterization of seven potential glomerular endothelial cell lines (mGEnC-1 to -7), which positively stained for vWF and podocalyxin and negatively stained for SMA and Wilms tumor-1.

Morphology of mGEnC

The mGEnC had typical cobblestone morphology when grown on gelatin-coated tissue culture flasks at 33°C (Fig. 1A). The morphology of most mGEnC clones was different at permissive (33°C) or nonpermissive (37°C) culture conditions. The mGEnC cultured at permissive conditions had more active nuclei with an increased number of mitotic figures, were smaller in size, and grew faster than cells cultured at nonpermissive conditions. Similar to other mGEnC clones, clone mGEnC-1 cultured at 37°C appeared to be more flattened with a transparent appearance (Fig. 1B). Furthermore, mGEnC clones cultured on Matrigel rapidly formed the characteristic microtubes, especially apparent at 37°C (Fig. 2).

Since glomerular endothelial cells are one of the few types of endothelial cells that contain fenestrae, scanning

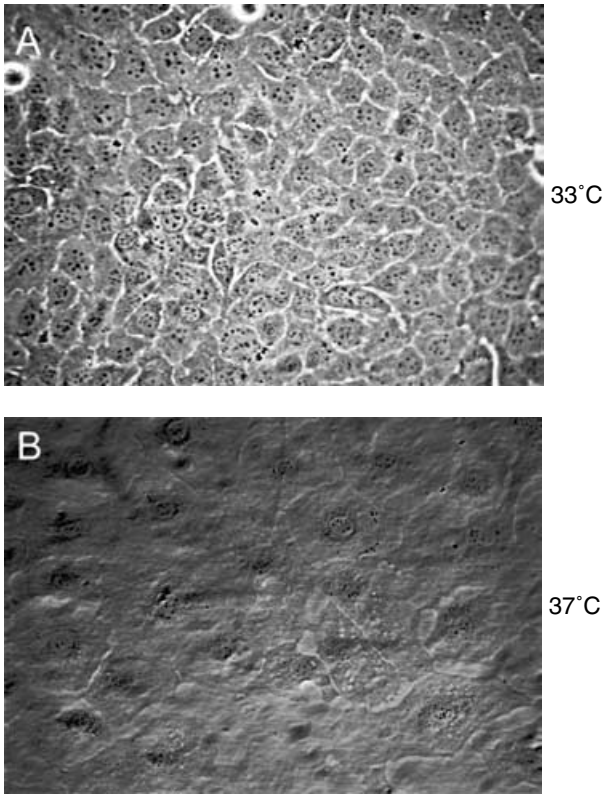

Fig. 1. Morphology of mouse glomerular endothelial cells (mGEnC-1). (*A*) mGEnC-1 [passage (P) 12] imaged under phase-contrast microscopy and cultured at 33°C in normal growth medium supplemented with 20 U/mL interferon-γ (IFN-γ), formed cobblestone monolayers on gelatin-coated plastic. (*B*) mGEnC-1 (P12) cultured at 37°C on gelatin in medium without IFN-γ demonstrated flattened cobblestone morphology with a transparent appearance (phase contrast ×200).

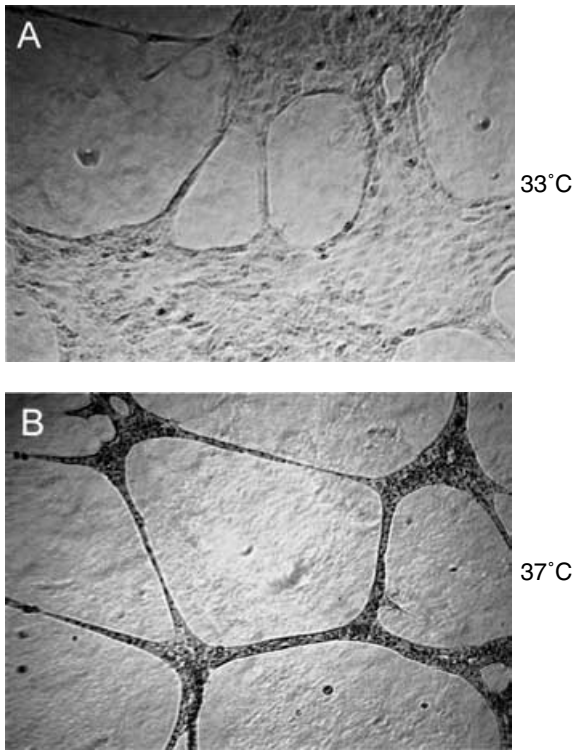

Fig. 2. Tube formation of mouse glomerular endothelial cells (mGEnC-1). mGEnC-1 (3×10^5) [passage (P) 14] cultured for 20 hours on Matrigel in a 6-well plates in normal growth medium under permissive conditions [with interferon-γ (IFN-γ) at 33°C] (*A*) formed less developed microtubes than under nonpermissive conditions (without IFN-γ at 37°C) (*B*) (phase contrast ×100).

electron microscopy was used to visualize these fenestrae in the mGEnC. As seen in Figure 3A and B, the mGEnC-1 cell line contains fenestrae, some of which are indicated by arrows, with a diameter between 50 and 100 nm. In contrast, the microvascular cell line EOMA did not contain these fenestrae (Fig. 3C). In addition, TEM was performed to show that the fenestrae in the mGEnC lacked a diaphragm as for example observed in peritubular capillary endothelial cells. We checked hundreds of cells by TEM for the presence of diaphragmed fenestrae. However, we never observed any diaphragm (Fig. 3D, E, and F).

Identification and characterization of mGEnC-1

As seen in Figure 4A, mGEnC-1 cultured at 33°C with IFN-γ demonstrated abundant nuclear staining for the mouse anti-SV40 large T antigen. However, after 1 week at 37°C in the absence of IFN-γ, the expression of the SV40 large T antigen was hardly detectable (Fig. 4B).

Indirect immunofluorescence microscopy was used to demonstrate the expression of endothelial markers by mGEnC-1. The vWF staining of the mGEnC-1 cell line cultured at 33°C (Fig. 4C) or at 37°C (Fig. 4D) nicely revealed the Weibel-Pallade bodies that are characteristic for endothelial cells. mGEnC-1 cultured at 33°C and 37°C stained also positive for podocalyxin (Fig. 4E and F) and for the markers used for the initial selection [i.e. PECAM-1 (CD31), endoglin (CD105), and the lectins GSL I-B4 and ULEX (not shown)]. The endothelial specific antigen VE-cadherin was present at the regions of intercellular contacts. Importantly, the VE-cadherin expression was not observed in mGEnC-1 grown at 33°C (Fig. 4G), but exclusively in mGEnC-1 grown at 37°C (Fig. 4H). In addition, the mGEnC-1 cell line was able to phagocytose acetylated-LDL (Fig. 5A), a function mediated by specific receptors typically found on endothelial cells and macrophages [19]. In contrast, mouse mesangial cells and the podocyte cell line MPC-5 were not able to internalize acetylated-LDL (Fig. 5B and 5C). When the mGEnC-1 cell line was subcultured for more then 30 passages, the cells remained positive for all these markers. The results shown for mGEnC-1 are representative for several other mGEnC cell clones that we have isolated.

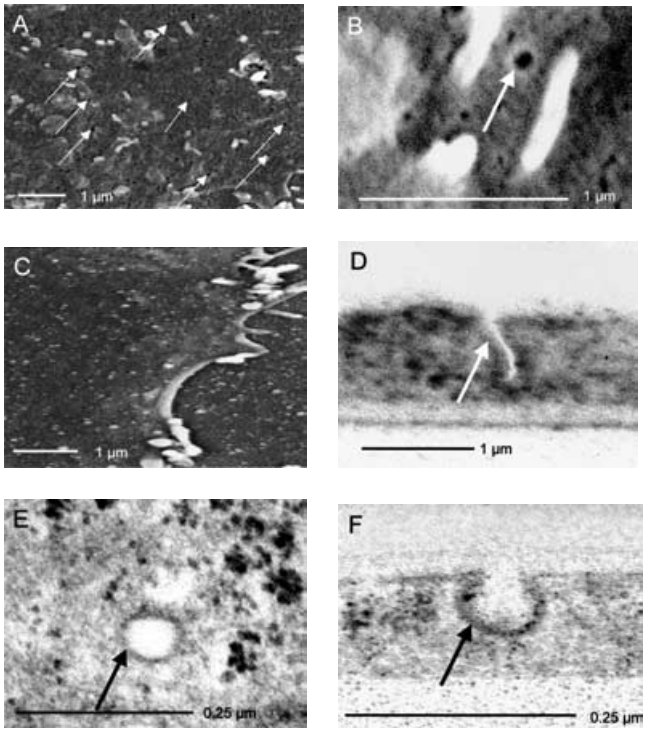

Fig. 3. Presence of fenestrae in mouse glomerular endothelial cells (mGEnC-1). mGEnC-1 [passage (P) 15] cultured at 37°C on glass coverslips and foil bottom Willco-dishes were analyzed by scanning (*A*, *B*, and *C*) and transmission electron microscopy (*D*, *E*, and *F*), respectively. Some fenestrae (A) and one fenestra (B) are indicated by arrows. The microvascular endothelial cell line EOMA was used as negative control (C). Nondiaphragmed fenestrae are indicated by arrows (D, E, and F). The bars represent 1 µm (A, B, C, and D) and 0.25 µm (E and F).

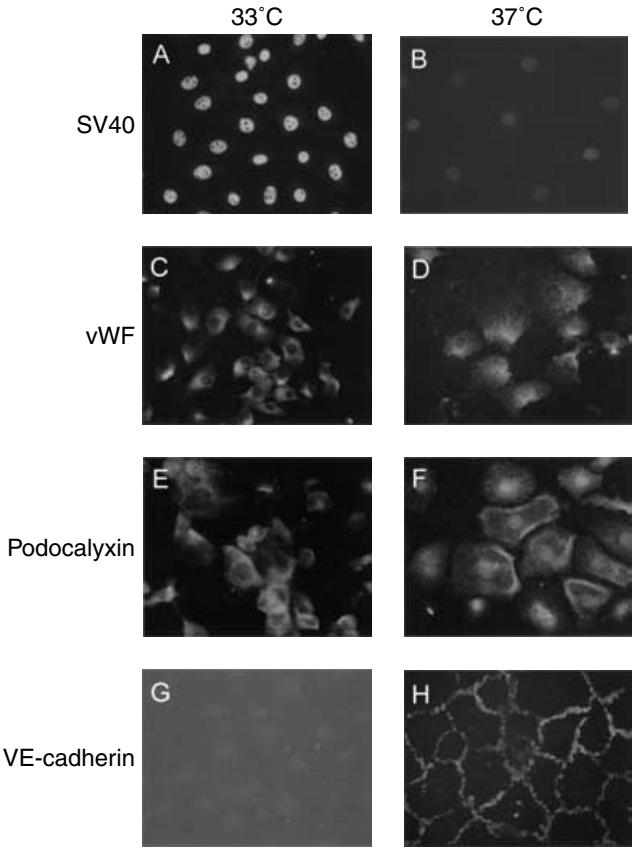

Fig. 4. Expression of the SV40 large T antigen and endothelial markers by mouse glomerular endothelial cells (mGEnC-1). mGEnC-1 [passage (P) 14] cultured in slide flasks at 33°C in medium supplemented with 20 U/mL interferon-γ (IFN-γ) (*A*) or at 37°C in normal growth medium (*B*) for 1 week were fixed, stained with the antibody against mouse SV40 large T antigen followed by an ALEXA 488–conjugated secondary antibody, and visualized by fluorescence microscopy. mGEnC-1 (P14) cultured in slide flasks for 1 week in normal growth medium with or without IFN-γ under permissive or non-permissive conditions, respectively, were fixed and stained with the antibodies against von Willebrand factor (vWF) (*C* and *D*), podocalyxin (*E* and *F*), and VE-cadherin (*G* and *H*) followed by an ALEXA 488–conjugated secondary antibody, and visualized by fluorescence microscopy (×250).

Proliferation of mGEnC-1

Comparison of endothelial cell proliferation using the cell proliferation reagent WST-1 demonstrated a clear difference between several cell lines cultured at 33°C with IFN-γ and those cultured at 37°C without IFN-γ. A minimum of 24 hours was required for the mGEnC clones to adapt to the change in temperature, and therefore the mGEnC clones derived from a single flask grown at 33°C were cultured for 48 hours in tissue culture flasks that were precoated with 1% gelatin and/or fibronectin in normal growth medium with or without IFN-γ at 33°C and 37°C, respectively, before the proliferation experiments started. As shown in Figure 6, mGEnC-1 maintained under permissive conditions, entered the log phase of growth and proliferated rapidly. In contrast, the same cells cultured at 37°C in the absence of IFN-γ showed a markedly reduced proliferation rate. mGEnC-1 grown in wells precoated with both 1% gelatin and fibronectin grew faster than mGEnC-1 grown in wells precoated with gelatin alone (Fig. 6). Fibronectin also resulted in a distinct change in cellular morphology, inducing cellular extensions when subconfluent.

The mGEnC-1 cell line shows increased adhesion molecule expression in response to TNF-α and/or IL-1β

In mGEnC-1 the expression of ICAM-1 and VCAM-1 was detectable on resting cells and was up-regulated at least threefold after activation by TNF-α and/or IL-1β (Fig. 7). Further analysis of mGEnC-1 at later passages (up to 30 tested) showed that the cells retain their sensitivity for cytokine-induced up-regulation of adhesion molecules. The results shown for mGEnC-1 are representative for other mGEnC clones.

DISCUSSION

In this study, we describe the isolation and characterization of conditionally immortalized mouse glomerular endothelial cell lines. The establishment of glomerular

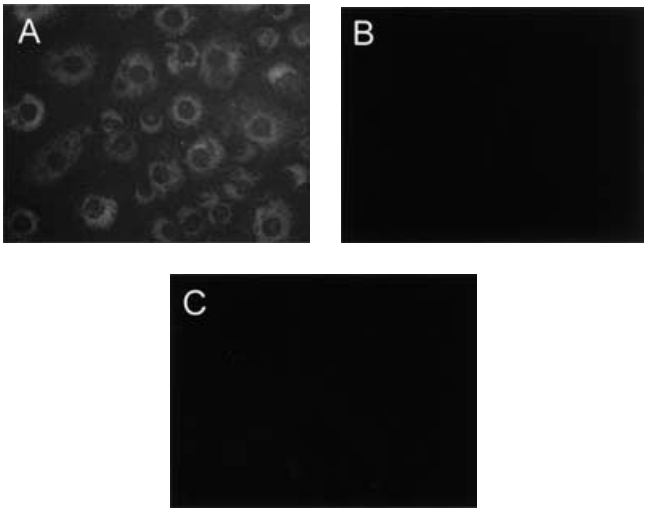

Fig. 5. Uptake of acetylated-low-density lipoprotein (LDL) by mouse glomerular endothelial cells (mGEnC-1), mesangial cells and podocytes. (*A*) mGEnC-1 [passage (P) 14] cultured in slide flasks for 1 week at 37°C in normal medium were incubated with Dil-labeled acetylated-LDL for 4 hours, fixed and visualized by fluorescence microscopy at 570 nm. As negative controls we used mouse mesangial cells (*B*) and the mouse podocyte cell line MPC-5 cultured for 2 weeks at 37°C (*C*) (×250).

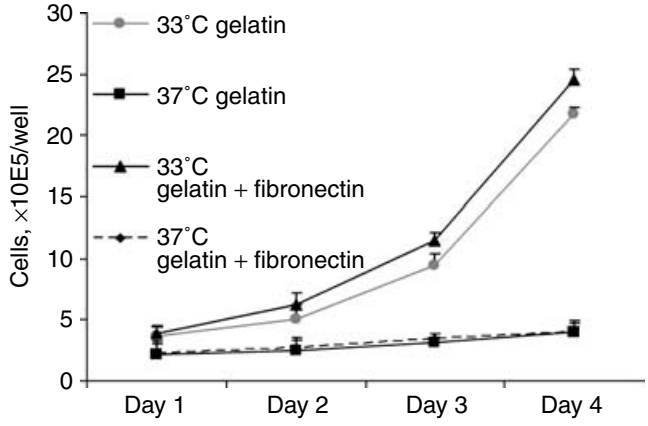

Fig. 6. Proliferation rates of mouse glomerular endothelial cells (mGEnC-1) cultured on different coatings and at different temperatures. The cleavage of tetrazolium salts WST-1 to formazan in mGEnC-1 [passage (P) 15] cultured at 33°C in growth medium supplemented with 20 U/mL interferon-γ (IFN-γ) or at 37°C in growth medium alone on 1% gelatin or 1% gelatin and 1 μg/mL fibronectin for 1 to 4 days. Data are expressed as the optical density (OD) in nm (means ± SD) ($N = 3$) and are representative of three experiments.

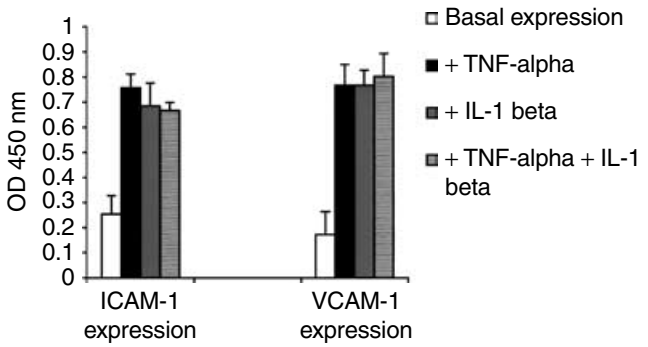

Fig. 7. Surface antigen expression on resting and cytokine-stimulated mouse glomerular endothelial cells (mGEnC-1). Monolayers of mGEnC clones [passage (P) 14 to 16], cultured for 1 week at 37°C in 96-well plates, were stimulated for 18 hours with normal growth medium alone or supplemented with 10 ng/mL tumor necrosis factor-α (TNF-α) and/or interleukin (IL)-1β and analyzed by enzyme-linked immunosorbent assay (ELISA) for the expression of intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1). The results are expressed as mean ± SD optical density (OD) for triplicate wells. The figure is representative of four experiments.

cell lines (podocytes, parietal epithelial cells, mesangial cells, endothelial cells) will improve our understanding of their roles in glomerular function both in health and disease. Until now, several (conditionally) immortalized podocyte, parietal epithelial, mesangial, and endothelial cell lines derived from different species have been described (Table 2). Especially, the conditionally immortalized mouse podocyte cell line has greatly boosted research on podocyte physiology and pathology in recent years [15, 20–22]. As far as we know no conditionally immortalized glomerular parietal epithelial and endothelial cell lines have been described. For glomerular endothelial cells, several cell lines have been described containing the intact SV40 large T antigen [23–26] or another immortalizing gene [24]. Primary glomerular endothelial cells have also been isolated from cow and human [2, 6, 9, 10, 27–29]. However, the isolation of normal mouse glomerular endothelial cells from BALB/c mice is almost impossible, since the primary mouse glomerular endothelial cells hardly proliferate or differentiate and die after a few days (own experience). This problem can be overcome through the use of the immortomouse, which allows the generation of continuously proliferating cell lines capable of differentiation after degradation of the immortalizing protein. The H-2K^b-tsA58 transgenic mice have the oncogene in all cells at the same locus [30–33]. The presence of the inducible tsA58 SV40 large T antigen allows mGEnC-1 to expand rapidly under permissive conditions (33°C + IFN-γ), to obtain sufficient glomerular endothelial cells for analysis, before switching to the untransformed state for experimentation by culture at 37°C in the absence of IFN-γ. Thus, these cells proliferate much slower and differentiate when cultured at 37°C in the absence of IFN-γ. This was confirmed by the lack of detectable SV40 tsA58 large T antigen and by the reduction in proliferation of mGEnC-1 cultured under nonpermissive conditions compared to mGEnC-1 cultured under permissive conditions. Our mGEnC cells expressed SV40 large T antigen mRNA at 33°C and 37°C, but the SV40 large T antigen protein could be observed only at 33°C by Western blot analysis (data not shown). The mGEnC-1 cell line has been cultured under permissive

Table 2. Several podocyte, mesangial, endothelial, and parietal epithelial cell lines derived from different species

Glomerular cell line	Immortalizing gene	Species	Reference
Podocytes	Intact SV40 large T antigen	Rat	[40]
		Human	[41, 42]
		Mouse	[23]
	Temperature sensitive SV40 large T antigen	Mouse	[15]
		Human	[16]
Mesangial cells	Intact SV40 large T antigen	Human	[43, 44]
		Rat	[45, 46]
	Temperature sensitive variant SV40 large T antigen	Mouse	[14]
Endothelial cells	Intact SV40 large T antigen	Mouse	[23]
		Cow	[25, 26]
	Oncogenic type 31 adenovirus	Rat	[24, 25]
Parietal epithelial cells	Intact SV40 large T antigen	Rat	[45, 47, 48]

conditions for more than 30 passages with no signs of senescence and has retained the temperature and IFN-γ responsiveness for large T antigen expression. However, after 2 weeks of culture under nonpermissive conditions, they showed signs of senescence. This phenomenon has also been described in another study [17].

The morphologic appearance of the glomerular capillary endothelial cell clones we have isolated is similar to that described by others for capillary endothelial cells derived from a variety of sources [2, 6, 9, 10, 17, 27–29, 34–36]. The appearance of tubular capillary-like structures has also been observed [37], although this morphologic characteristic is not specific for capillary endothelial cells, since endothelial cells from human umbilical vein also form tube-like structures [38]. Most important, the only difference between glomerular (and hepatic) endothelial cells and endothelial cells from other sources is the presence of nondiaphragmed fenestrae [8, 9], which indeed are present in our mGEnC clones based on scanning electron microscopy and TEM (Fig. 3).

The endothelial nature of mGEnC-1 was further confirmed by the expression of PECAM-1 (CD31), endoglin (CD105), vWF in the Weibel-Pallade bodies, podocalyxin, binding of GSL I-B4 and ULEX, and uptake of acetylated-LDL [2, 18, 39]. VE-cadherin did stain the intercellular junctions of mGEnC-1 cultured at 37°C and seems to be a differentiation marker for our endothelial cells since mGEnC-1 cultured at 33°C with IFN-γ did not express VE-cadherin.

Fibronectin coating results in a significant higher proliferation rate of mGEnC-1 cultured at 33°C and induced cellular extensions when the cells were subconfluent. The difference in growth and morphology of glomerular endothelial cells cultured on different coatings has also been observed in other studies [6, 24].

We isolated mGEnC-1 for the study of interaction between leukocytes and glomerular endothelial cells. Therefore, the expression of cellular adhesion molecules on resting and cytokine-stimulated mGEnC-1 was studied. ELISA analysis of resting mGEnC-1 confirmed basal expression of ICAM-1 and VCAM-1 and this expression was at least threefold up-regulated by cytokines.

CONCLUSION

We have described the isolation of conditionally immortalized mouse glomerular endothelial cells that can be expanded rapidly under permissive conditions before switching to the differentiated state for experimentation. The mGEnC-1 cell line retains the phenotypic characteristics of glomerular endothelial cells and can be used in further adhesion studies to study the role of heparan sulfate proteoglycans in glomerular inflammation. In addition, co-culture experiments with podocytes can be done to study the functional roles of these cells in inflammation, proteinuria, and sclerosis/fibrosis.

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