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Establishment of an Immortalized PARP-1$^{-/-}$ Murine Endothelial Cell Line: A New Tool to Study PARP-1 Mediated Endothelial Cell Dysfunction

Ana Carrillo,[1] Yolanda Monreal,[1] Pablo Ramirez,[1] Eduardo Suarez,[2] Pascual Parrilla,[1] Josiane Menissier-de Murcia,[3] Gilbert de Murcia,[3] Luis Alvarez-Vallina,[2] and José Yélamos[1,4]*

[1]Transplant Unit, University Hospital ''Virgen de la Arrixaca'', Murcia, Spain
[2]Department of Immunology, University Hospital Clinica Puerta de Hierro, Madrid, Spain
[3]Unité 9003 du CNRS, Ecole Supérieure de Biotechnologie de Strasbourg, Illkirch Cedex, Strasbourg, France
[4]Department of Biochemistry, Molecular Biology B and Immunology, University of Murcia, Murcia, Spain

Abstract Poly(ADP-ribose) polymerase-1 (PARP-1) plays a critical role in endothelial cell dysfunction associated with various pathophysiological conditions. To elucidate PARP-1 pathways involved in endothelial cell dysfunction, it is essential to establish ''in vitro'' experimental models using isolated endothelial cells. So far, two approaches have been used: primary endothelial cells from PARP-1$^{-/-}$ mice which have a limited life-span, being a major handicap if large quantities of cells are required; and pharmacological inhibition of PARP in PARP-1$^{+/+}$ endothelial cell lines, which is not specific for PARP-1 and would have biological effects different that genetic inhibition. To overcome these limitations, we have established an immortalized PARP-1$^{-/-}$ endothelial cell line (HYKO6) by transfection of primary cells with a plasmid containing the SV40 genome and selected on the basis of morphological and phenotypical features. The HYKO6 cell line exhibited endothelial characteristics, such as constitutive expression of CD105, CD31, ICAM-2, VCAM-1, and von Willebrand factor and formation of capillary-like structures (CLS) on Matrigel surface. However, expression of ICAM-1 antigen is lost in the HYKO6 cells. After TNF-α treatment, HYKO6 cells exhibited increased expression of E-selectin and VCAM-1. Likewise, NF-κB-dependent transcriptional activation was increased in the HYKO6 cell line in response to TNF-α at a level similar to that found for primary PARP-1$^{-/-}$ cells. This cell line should provide, for the first time, a valuable tool to study PARP-1 pathways in endothelial cell dysfunction. J. Cell. Biochem. 94: 1163–1174, 2005.

Key words: poly(ADP-ribose) polymerase-1; endothelial cell; cell line; SV40; TNF-α; NF-κB

Poly(ADP-ribose) polymerase 1 (PARP-1) is a highly conserved nuclear zinc-finger DNA-binding protein (113 kDa) that specifically detects DNA-strand breaks or nicks generated by different genotoxic agents [de Murcia and Menissier de Murcia, 1994]. PARP-1 belongs to a large family of enzymes [Smith, 2001] which, using NAD^+ as a substrate, synthesizes and transfers ADP-ribose onto aspartic and glutamic acid residues of acceptor proteins, including itself (automodification), histones, transcription factors, and DNA repair proteins [de Murcia and Shall, 2000]. Poly-ADP-ribosylation is terminated by the release of extensively poly-ADP-ribosylated (negatively charged) PARP molecules from DNA. ADP-ribose polymers are then subjected to degradation by poly-ADP-ribose-glycohydrolase [Lin et al., 1997]. Poly(ADP-ribosyl)ation is, therefore, an immediate covalent, but transient, post-translational modification of nuclear proteins, induced by DNA damage. In addition, it has been suggested that signals other than DNA lesions, including steroid hormones, stress and infection, may also activate PARP molecules at specific chromosome sites [Tulin and Spradling, 2003].

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*Correspondence to: José Yélamos, Department of Biochemistry, Molecular Biology B and Immunology, Facultad de Medicina, Campus de Espinardo, Apartado de Correos 4021, Universidad de Murcia, 30100-Murcia, Spain.
E-mail: jyelamos@um.es

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Recently, by using either genetically engineered mice lacking PARP-1 (PARP-$1^{-/-}$) or pharmacological inhibition of PARP, evidence has been provided that PARP-1 is involved in endothelial cell dysfunction associated with various pathophysiological conditions, such as reperfusion injury [Szabó et al., 2002], endotoxic shock [Szabó et al., 1997], diabetes [Garcia Soriano et al., 2001], and aging [Pacher et al., 2002]. The underlying mechanism of endothelial cell dysfunction mediated by PARP-1 could be attributed to PARP-1 overactivation in response to DNA damage induced by different genotoxic agents, such as peroxinitrite. NAD^+ consumption, due to PARP-1 overactivation, leads to cellular ATP depletion and functional alterations of the cell, with eventual necrotic-type cell death [Szabo and Dawson, 1998]. However, an alternative way in which PARP-1 may influence endothelial cell function could be through its transcription regulation function [Chiarugi, 2002; Kraus and Lis, 2003; Carrillo et al., 2004]. Thus, it has been demonstrated that PARP-1 is necessary for the induction of NF-κB-dependent gene expression after exposure to LPS, TNF-α, or hydrogen peroxide [Hassa and Hottiger, 1999; Oliver et al., 1999; Kameoka et al., 2000; Carrillo et al., 2004]. However, since translocation of NF-κB to the nucleus occurs in PARP-$1^{-/-}$ cells, as in wild-type cells [Oliver et al., 1999], a new level of control of NF-κB must occur after its translocation to the nucleus. It has been suggested that PARP-1 is an essential and novel transcription coactivator for κB-dependent gene expression [Hassa and Hottiger, 2002]. Recently, it has also been shown that PARP-1 is required for the activation of other inflammation-related transcription factors, such as AP-1, SP-1, Oct-1, YY-1, and STAT-1 [Ha et al., 2002; Andreone et al., 2003].

In order to clarify the PARP-1 pathway involved in endothelial cell dysfunction, it is of great importance to establish an "in vitro" experimental model using isolated endothelial cells. So far, two "in vitro" approaches have been used to study this pathway in endothelial cell injury. One approach has used primary endothelial cells derived from PARP-$1^{-/-}$ animals [Garcia Soriano et al., 2001; Jones et al., 2001]. However, this approach suffers from the drawback of using primary isolated cells. Primary cultures of endothelial cells can only be used for some passages in culture [Carrillo et al., 2002]. Therefore, such investigations require time-consuming repeated isolations of endothelial cells, which might render quantitative data subject to batch-to-batch variations [Saadi and Platt, 1995]. Moreover, the relatively short life span in culture of primary endothelial cells remains a major handicap if large quantities of cells are required for molecular and biochemical analyses. Furthermore, it is far more difficult to develop genetic engineering in primary cultures than in cell lines. The second approach has used pharmacological inhibition of poly-ADP-ribose synthesis in primary or immortalized PARP-$1^{+/+}$ endothelial cells from different species and sources [Garcia Soriano et al., 2001; Sharp et al., 2001; Du et al., 2003]. Immortalized endothelial cells have been maintained in culture for many passages without signs of senescence and with preserved characteristics of primary endothelial cells [Carrillo et al., 2002; Lidington et al., 2002; Mizuno et al., 2003]. However, this approach has several limitations. Pharmacological inhibition of PARP in endothelial cells is not specific for PARP-1, also affecting other members of the PARP family, such as PARP-2 [Smith, 2001]. Moreover, chemical inhibition of PARP-1 catalytic activity would have different biological effects than genetic inhibition. In fact, inhibition of NF-κB-dependent transcription activation has been demonstrated in PARP-$1^{-/-}$ endothelial cells, while pharmacological inhibition of PARP failed to suppress NF-κB activation in response to high glucose levels "in vitro" [Garcia Soriano et al., 2001]. Thus, it seems that the presence of PARP-1 (e.g., through physical association of PARP-1 with other nuclear proteins), rather than its catalytic activity, is the primary regulator of the activation of this transcription factor.

The aim of the present study was to establish and characterize a PARP-1-deficient endothelial cell line. This cell line has been compared to primary PARP-$1^{-/-}$ endothelial cells for expression of surface antigens, for its ability to undergo morphologic differentiation into capillary-like structures (CLS) "in vitro" in short-term Matrigel assays, and its ability to respond to TNF-α stimulation. Furthermore, we have also generated stable PARP-1-expressing transfectants from our cell line that are able to recover the TNF-α induced NF-κB-dependent transcriptional activation of the cell line to similar levels of those found in PARP-$1^{+/+}$ primary endothelial cells. This cell line could be a very important tool to study the intrinsic

mechanism of PARP-1 mediated endothelial cell dysfunction, including the role of PARP-1 in molecular mechanisms controlling endothelial cell gene expression in response to inflammatory stimuli.

MATERIALS AND METHODS

Antibodies

Rat anti-mouse monoclonal antibodies (mAbs) recognizing CD102 (ICAM-2; clone 3C4), CD31 (PECAM-1; clone MEC 13.3), CD105 (Endoglin; clone MJ7/18), CD106 (VCAM-1; clone MVCAM.A), CD62E (E-selectin; clone 10E9.6), and MHC Class I (H-2D; clone 8F12) were from BD Pharmingen (San Diego, CA), and CD54 (ICAM-1; HB-233) was from the American Type Culture Collection (Rockville, MD). Rabbit polyclonal anti-von Willebrand factor (vWF) antibody and Texas-Red-conjugated goat anti-rabbit IgG were from Sigma (St. Louis, MO). Fluorescein isothiocyanate-conjugated goat anti-rat IgG (H + L) antibody was from Caltag (Burlingame, CA). Mouse anti-SV40 (sc-148) was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Animals

PARP-1 knockout (PARP-1$^{-/-}$) and their wild-type (PARP-1$^{+/+}$) littermate female mice, 9–10 weeks old, (strain 129/Sv × C57BL/6) [Menissier de Murcia et al., 1997] were used as a source of primary murine heart endothelial cells (MHEC).

Cell Isolation and Culture

MHEC were isolated by collagenase treatment and cell sorting of ICAM2-positive cells. Briefly, the hearts were washed extensively with cold phosphate-buffered saline (PBS) (BioWhittaker, Verviers, Belgium). Diced tissue was incubated in PBS supplemented with 0.5 mg/ml of collagenase (Roche, Foster City, CA) for 1 h at 37°C. After washing twice in PBS, cells were incubated for a further 10 min in 0.25% trypsin/0.04% EDTA solution (Life Technologies, Inc., Grand Island, NY), washed twice in PBS supplemented with 5% heat-inactivated fetal calf serum (FCS) (BioWhittaker), and incubated for 30 min at 4°C with rat anti-mouse CD31 and rat anti-mouse CD102 monoclonal antibodies. After washing twice with cold PBS, cells were incubated with sheep anti-rat Ig-conjugated microbeads (10^7 beads/ml) (Dynal, Oslo, Norway) for 15 min at 4°C. The magnetically labeled cells were collected with a Dynal magnetic particle concentrator. Positively selected cells attached to the Dynabeads were washed five times in PBS. After detachment, cells were resuspended in RPMI 1640 medium (BioWhittaker) supplemented with 20% FCS, 1 mM L-glutamine (Sigma), 1% penicillin/streptomycin (Sigma), 1 mM sodium pyruvate (Sigma), 20 mM HEPES (Sigma), 1% non-essential aminoacids (Sigma), 50 mM 2-mercaptoethanol (Sigma), 100 μg/ml endothelial cell growth supplement (Beckton Dickinson, Mountain View, CA), and 12 U/ml heparin (Rovi Laboratories, Madrid, Spain) and plated onto flasks coated with 1% gelatin (Sigma). Cells were split upon confluence by trypsinization (0.25% tripsin/0.04% EDTA), washed and incubated with rat anti-mouse CD102 Ab for 30 min at 4°C. After washing twice with cold PBS, cells were incubated in the same buffer containing fluorescein-conjugated goat anti-rat IgG (H + L) antibody. Following a 30 min incubation at 4°C in the dark, the samples were again washed twice with cold PBS, and resuspended in 500 μl of RPMI 1640 medium. Positive cells were collected by fluorescence-activated cell sorting (FACS) using a MoFlo® cell sorter (Cytomation, Inc., Fort Collins, CO) equipped with an Argon-ion blue laser (excitation 488 nm) and a Red Diode Laser (excitation 635 nm). Forward and side light scatter and specific fluorescence were used to establish sort regions by using Summit™ software (Cytomation) in a 1–2 drop single cell mode. Cells were plated and subcultured as described above. In all cases, the purified cells stained positively for cell surface CD102, displayed typical cobblestone morphology and were positive for other typical endothelial cell markers, such as CD105, CD31, CD54, and CD106, by flow cytometry analysis in a FACS cytofluorimeter (Becton Dickinson Labware, Bedford, MA) using Cell Quest software (Becton Dickinson).

Gene Constructs

The pRNS-1Hy construct is a modified version of the pRNS-1 plasmid described previously [Litzkas et al., 1984], in which the Neo-resistance gene was replaced by a hygromycin-resistance gene by cloning the *Dra*I fragment containing the hygromycin-resistance gene, from the pCI-hy-FasL plasmid [Rodríguez-Gago et al., 2001] into the pRNS-1 construct previously digested with *Sma*I and

*Bgl*II. To generate the pcDNAC-PARP-1-Zeo construct, the *Xho*I fragment from the pECV-PARP plasmid [Oliver et al., 1998] containing the complete human PARP-1 cDNA was cloned into pcDNA-4/HisMax C vector (Invitrogen, Carlsbad, CA) previously digested with *Xho*I.

Cell Transfection

Endothelial cells were plated onto 24-well plates (2×10^4 cells/well) the day before transfection. Cells were transfected by the liposome-mediated gene transfer method as previously described [Carrillo et al., 2002]. For stable transfection, selection was carried out 48 h later with MHEC growth medium supplemented with 125 μg/ml of hygromycin (selection for pRNS-1Hy) or 50 μg/ml zeocin (selection for pcDNAC-PARP-1-Zeo). Isolation of individual clones was carried out by limiting dilution and culture in MHEC growth medium.

Proliferation Assay

Cells were seeded in 96-well plates (5×10^3 cells/well) and, after 72 h, proliferation was determined by using CellTiter 96 Aqueous One Solution Cell Proliferation Assay according to the manufacturer's protocol (Promega, Madison, WI). Experiments were performed in triplicate.

Western Blots

PBS-washed cells were lysed with lysis solution (1% SDS, 10 mM Tris-HCl, pH 7.4) preheated to 95°C and boiled for an additional 5 min. Viscosity was reduced by passing the sample several times through a 23 gauge needle and then centrifuged for 5 min. Protein concentrations in lysates were measured by the Bio-Rad protein assay (Bio-Rad Laboratories, München, Germany) and an equal amount (20 μg) was loaded with Laemmli sample buffer (Sigma) into each lane before separation by SDS–12% PAGE and, then transferred to a nitrocellulose membrane. Membranes, blocked in 5% nonfat dry milk in Tris-buffered saline/0.05% Tween-20 (Sigma) overnight at 4°C, were rinsed and incubated with an anti-SV40 mAb. Binding of primary antibody was revealed using horseradish peroxidase-coupled goat anti-mouse secondary antibody (Promega). Signals were developed by an enhanced chemoluminescence kit (Amersham Pharmacia Biotech, Buckinghamshire, UK), according to the manufacturer's instruction.

Matrigel Tube Formation Assay

Approximately 10^4 endothelial cells were plated on polymerized Matrigel (Becton Dickinson). Plates were incubated for 12–16 h at 37°C in a 5% CO_2 humidified atmosphere, and the formation of CLS was assessed under the microscope. Experiments were performed in triplicate.

Luciferase Activity

The transcriptional status of NF-κB was examined in transiently transfected primary and immortalized heart endothelial cells stimulated with mouse recombinant TNF-α (Sigma) for 6 h using a firefly-luciferase reporter plasmid under the control of a $3 \times$ κB consensus site from the HIV enhancer [Bachelerie et al., 1991]. As an internal control to normalize the values obtained with the firefly luciferase construct, cells were co-transfected with the Renilla luciferase expression vector pRL-CMV (Promega) at a DNA ratio of 20:1 (firefly:Renilla). After transfection, cells were cultured for 72 h, then stimulated or not with mrTNF-α for 6 h, and lysed with passive lysis buffer (Promega). Firefly and Renilla luciferase activities were measured and discriminated by using the Dual luciferase assay kit (Promega), as specified by the manufacturer in a Optocomp I luminometer (MGM Instruments, Inc., Hamden, CT). The ratio of firefly to Renilla activities was calculated to normalized the values and the ratio obtained for untreated cells was arbitrarily set at 1.

RT-PCR

Total RNA was prepared from endothelial cells using Rneasy Total RNA Isolation kit (Qiagen, Valencia, CA). cDNA was synthesized using oligo-$(dT)_{16}$ primer and the GeneAmp RNA-PCR kit (Roche). PCR was performed in a Mastercycler (Eppendorf, Hamburg, Germany) using Taq DNA polymerase (Roche). Primers used for PCR amplification were: hDBDfor (5′-TCTTCGGATAAGCTCTATCG-3′) and JYseback (5′-GGACTTGGCATACTCTGCTG-3′) for human PARP-1; and actin for (5′-TGGAATCCTGTGGCATCCATG-3′) and actin back (5′-TAAAACGCAGCTCAGTAACAG-3′) for mouse β-actin. The amplification conditions were 30 cycles of 1 min at 94°C for denaturation, 1 min at 55°C for annealing, and 1 min at 72°C for extension.

Analysis of vWF Expression

Cells (5×10^4), grown on glass coverslips, were washed with ice cold PBS, fixed for 10 min with

ice cold methanol:acetone (1:1) followed by washing three times with ice cold PBS/0.2% Triton/0.1%BSA. Cells were then incubated overnight at 4°C with rabbit polyclonal anti-vWF antibody (1:200). After three washes with ice cold PBS/0.1% Triton/0.1% BSA, cells were incubated for 2 h at 4°C with Texas-Red-conjugated goat anti-rabbit IgG (1:400). DNA was counterstained with DAPI. Immunofluorescence microscopy was performed using a Leica microscopy and the capture software Qfish (Leica).

Statistical Analysis

Data are expressed as mean ± SD. Statistical differences in the results for the different parameters between groups were evaluated by the two-tailed Student's *t*-test. A probability (P) value of <0.05 was considered significant.

RESULTS

Establishment of a PARP-1$^{-/-}$ Murine Heart Endothelial Cell Line

Primary PARP-1$^{-/-}$ murine heart endothelial cells (passage 3), isolated as indicated above, were transfected with a plasmid encoding for the complete genome of SV40 and, after selection in the presence of hygromycin, were cloned by limiting dilution. One clone (HYKO6) was selected on the basis of its staining for endothelial cell-associated antigens by flow cytometry (Fig. 1) and its cobblestone morphology (Fig. 2A). The HYKO6 cell line was compared to PARP-1$^{+/+}$ and PARP-1$^{-/-}$ primary MHEC for the expression of surface antigens by flow cytometry analysis. Constitutive expression of CD105, CD31, ICAM-2, VCAM-1, and MHC class I antigens, similar in both PARP-1$^{+/+}$ and PARP-1$^{-/-}$ primary MHEC, were conserved in the HYKO6 cell line, showing little differences from primary cells. Thus, the expression level of CD105 (a homodimeric endothelial cell membrane glycoprotein that plays a major role in vascular development and vascular tone) and ICAM-2 was higher in PARP-1$^{-/-}$ primary cells than in the immortalized cell line. However, expression of the ICAM-1 antigen was lost in our immortalized cell line (Fig. 1). Immunofluorescent staining for the presence of vWF show expression of this endothelial cell marker in the cytosol of the HYKO6 cell line (Fig. 2B).

The HYKO6 cell line was expanded up to 30 passages without signs of senescence, and preserved the characteristics of primary endothelial cells, as indicated above (Fig. 1). SV40 T-antigen was detected in protein extracts from HYKO6 cells, but not from primary cells by Western blot (Fig. 3). On the other hand, the proliferation rate of this cell line was three times higher than that of PARP-1$^{-/-}$ primary cells (Fig. 4). Likewise, we also found that the HYKO6 cell line is very efficiently transfected by the liposome-mediated gene transfer method.

"In Vitro" Angiogenesis

To determine whether HYKO6 cells were capable of morphologic differentiation into CLS, they were cultured on Matrigel. HYKO6 cells attached rapidly to Matrigel substrates, and after 12 h they showed abundant networks of branching and anastomosing cords of cells. HYKO6 tube formation paralleled that of primary PARP-1$^{-/-}$ endothelial cells (Fig. 5).

Response of the HYKO6 Cell Line to TNF-α Activation

TNF-α is an important pro-inflammatory mediator released following pro-inflammatory stimulus such as LPS or bacterial infection, which initiates signalling pathways to the nucleus in endothelial cells, mainly mediated by the transcription factor NF-κB, which reprograms gene expression [Kalogeris et al., 1999]. In order to know whether our cell line was able to respond to cytokine stimulation, we examined the surface expression of VCAM-1 and E-selectin, two NF-κB-dependent proteins [Soares et al., 1998] which are up-regulated in endothelial cells after TNF-α stimulation [Bevilacqua et al., 1987; Osborn et al., 1989]. As shown in Figure 6, the HYKO6 cell line was able to up-regulated the expression of E-selectin and VCAM-1 in response to 4 and 16 h of TNF-α stimulation, respectively.

We have also analysed the transcriptional status of NF-κB in response to 6 h of TNF-α treatment in HYKO6 cells (passage 6) and compared to that of PARP-1$^{-/-}$ primary cells (passage 4) by transient transfection with a luciferase reporter plasmid under the control of a 3 × κB consensus site from the HIV enhancer [Bachelerie et al., 1991]. Our results show that NF-κB-dependent transcriptional activation was increased in the HYKO6 cell line in response to 6 h of treatment with TNF-α, at a level similar to that found for primary PARP-1$^{-/-}$

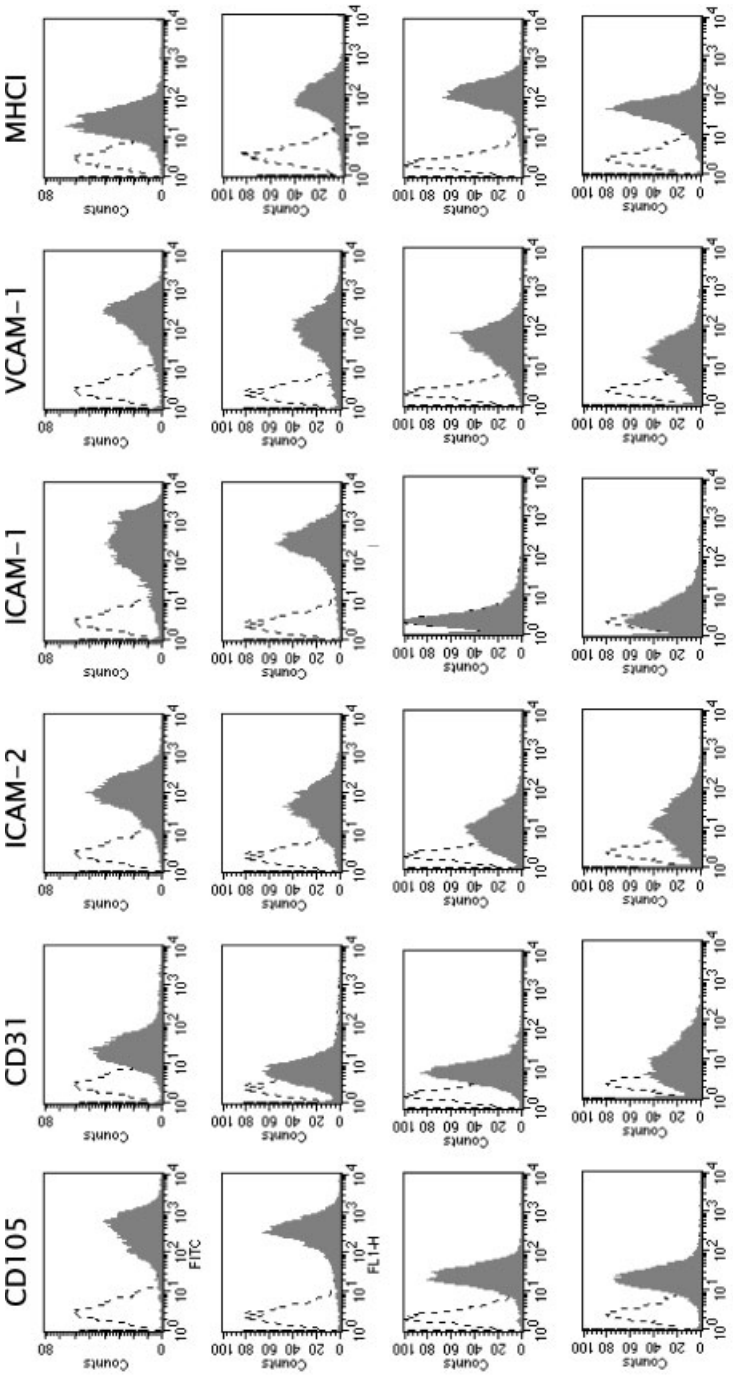

Fig. 1. Flow cytometry analysis of surface antigens in poly(ADP-ribose) polymerase-1 (PARP-1)$^{+/+}$ and PARP-1$^{-/-}$ primary endothelial cells (passage 6) and the immortalized PARP-1$^{-/-}$ HYKO6 cell line (passages 6 and 30). Primary and immortalized cells were stained with the antibodies directed to the molecules indicated within each graph (gray-filled histograms), as described in the ''Materials and Methods.'' Isotype-matched irrelevant antibodies were used as a control (dotted line). A representative of three experiments is shown.

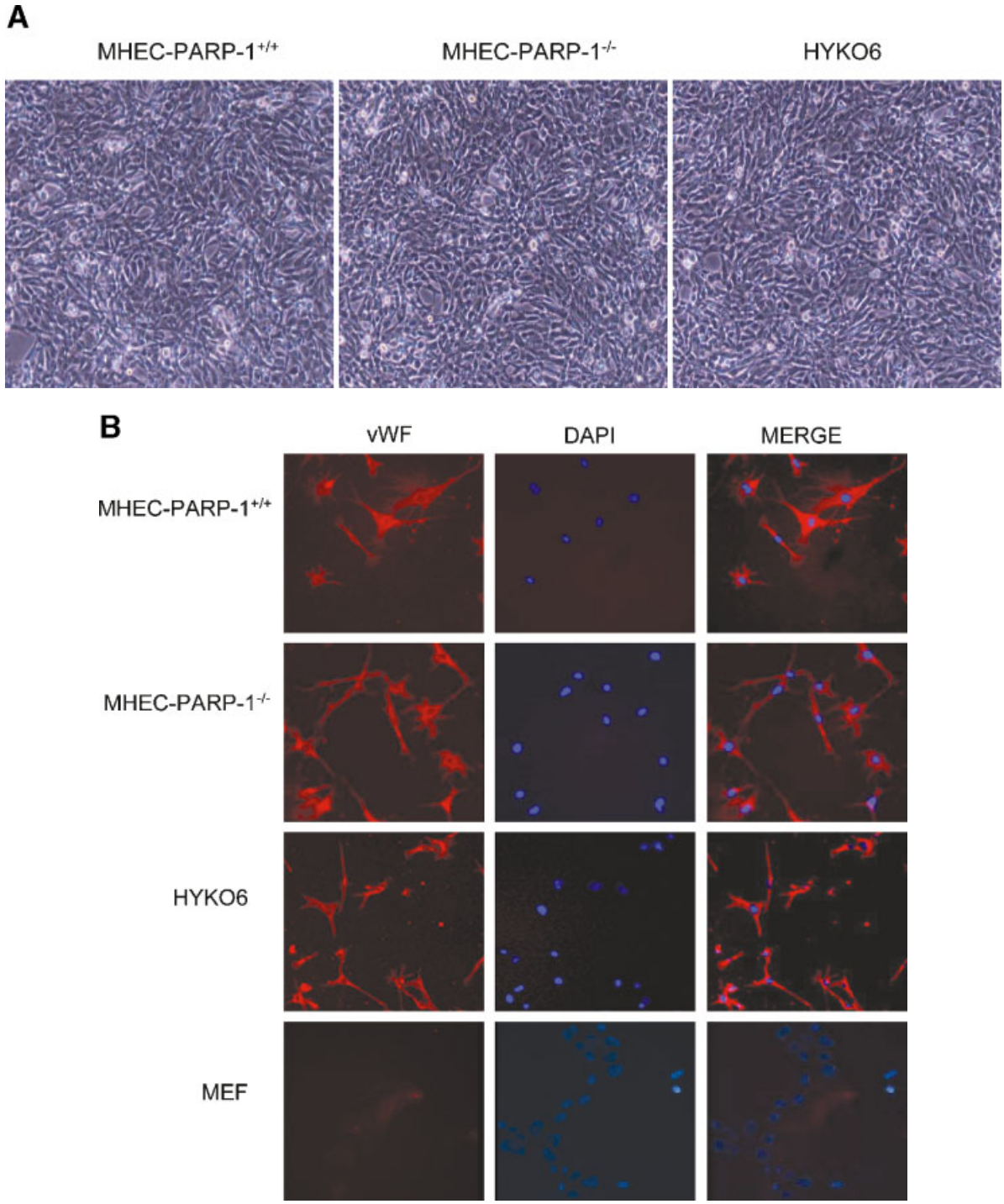

Fig. 2. Morphological and phenotypical characteristics of the HYKO6 cell line by phase contrast microscopy. **A**: Morphological appearance of PARP-1$^{+/+}$ and PARP-1$^{-/-}$ primary endothelial cells and the immortalized PARP-1$^{-/-}$ HYKO6 cell line. **B**: vWF expression in PARP-1$^{+/+}$ and PARP-1$^{-/-}$ primary endothelial cells and the HYKO6 cell line. Cytosolic expression of vWF was identified with a polyclonal rabbit anti-vWF and Texas-Red-conjugated goat anti-rabbit IgG. DAPI staining (blue) indicated nuclei of the cells. Murine embryonic fibroblasts (MEF) were used as negative control. Magnifications ×40. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

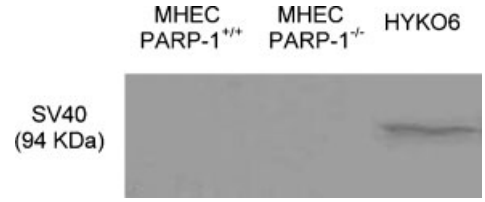

Fig. 3. SV40-T antigen expression in immortalized PARP-$1^{-/-}$ cells by Western blot. Cell lysates were fractionated by SDS–PAGE, and transferred to a nitrocellulose membrane. The membrane was incubated with anti-SV40 antibody and stained with horseradish peroxidase-coupled goat anti-mouse second antibody.

endothelial cells (Fig. 7). We have previously demonstrated that NF-κB-dependent transcriptional activation was partially inhibited in PARP-1-deficient primary endothelial cells in response to TNF-α, compared to that in PARP-$1^{+/+}$ endothelial cells [Carrillo et al., 2004]. To know whether PARP-1 expression could restore NF-κB-dependent transcriptional activation in our cell line to the level found in PARP-$1^{+/+}$ cells, we generated stable transfectants of HYKO6 expressing full length human PARP-1 cDNA (HYKO6-pcDNA/PARP-1) (Fig. 8). As shown in Figure 7, NF-κB-dependent transcriptional activation was increased significantly in

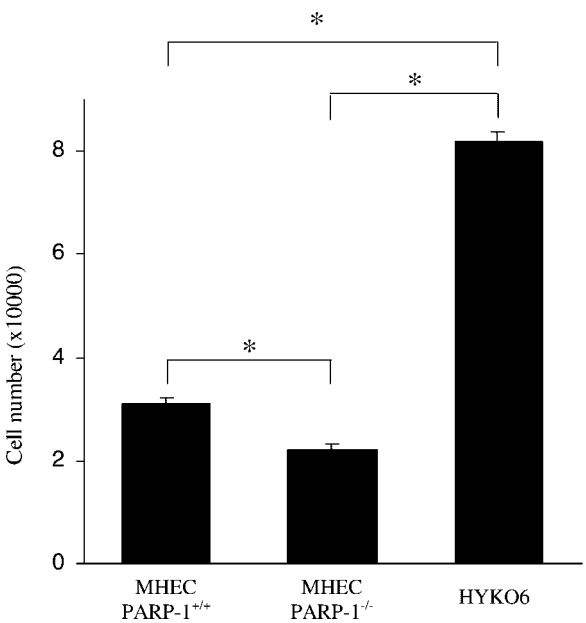

Fig. 4. Proliferation of PARP-$1^{+/+}$ and PARP-$1^{-/-}$ primary endothelial cells and the immortalized PARP-$1^{-/-}$ HYKO6 cell line. Cells were seeded in 96-well plates (5×10^3 cells/well) and after 72 h, proliferation was determined by using CellTiter 96 Aqueous One Solution Cell Proliferation Assay. Experiments were performed in triplicate. The results are expressed as mean ±SD. *Statistically significant difference ($P < 0.05$).

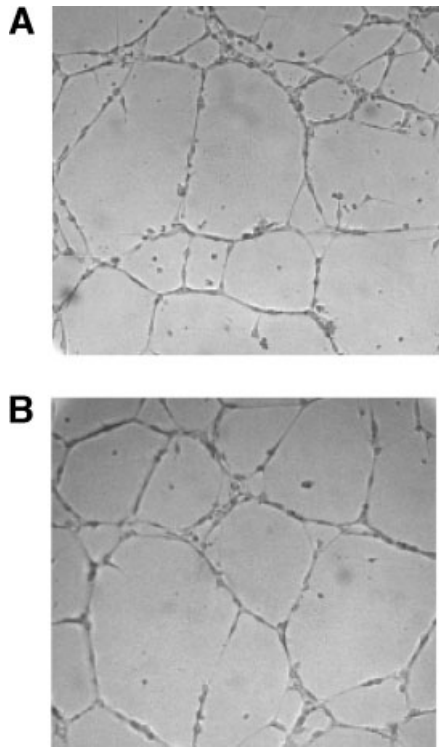

Fig. 5. Phase-contrast photomicrograph of HYKO6 (**A**) and primary PARP-$1^{-/-}$ endothelial cells (**B**) cultured on Matrigel for 16 h show tube formation by both cell types.

HYKO6-pcDNA/PARP-1 cells (passage 6), compared with that of the control HYKO6 cells transfected with the pcDNA-4/HisMax C vector (passage 6), recovering NF-kappaB dependent

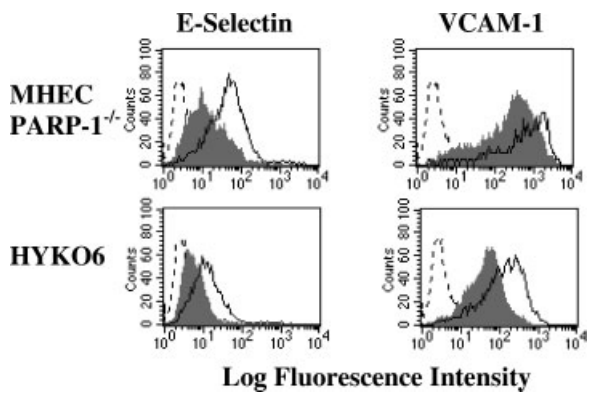

Fig. 6. Increased expression of E-selectin and VCAM-1 in TNF-α-stimulated PARP-$1^{-/-}$ primary endothelial cells and the HYKO6 PARP-$1^{-/-}$ cell line. Surface expression of E-selectin and VCAM-1 was determined by flow cytometry in untreated (gray-filled histograms) or after 4 h (E-selectin) or 16 h (VCAM-1) TNF-α treatment (unfilled histograms). Isotype-matched irrelevant antibodies were used as a control (dotted line). A representative of three experiments is shown.

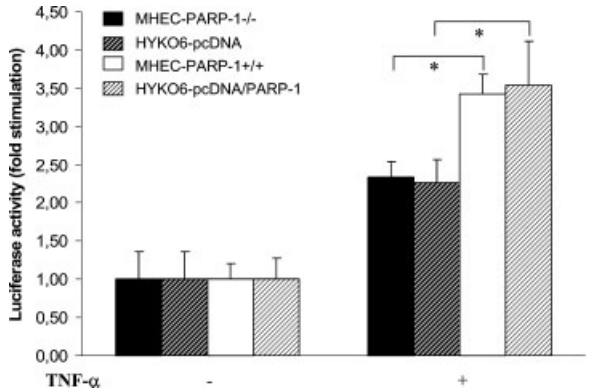

Fig. 7. NF-κB-dependent transcriptional activation in TNF-α-stimulated PARP-1$^{+/+}$ and PARP-1$^{-/-}$ primary endothelial cells and the HYKO6 PARP-1$^{-/-}$ cell line. Cells were transient co-transfected with a luciferase reporter plasmid under the control of a 3 × κB promoter element and the Renilla luciferase expression vector pRL-CMV. After 72 h, untreated or treated (for 6 h with TNF-α) cells were lysated and firefly and Renilla luciferase activities were measured. Results were normalized as indicated in ''Material and Methods.'' The ratio obtained for untreated cells was arbitrarily set at 1. A representative of three experiments, performed in quatriplicate is shown. The results are expressed as mean ± SD. *Statistically significant difference ($P < 0.05$).

transcriptional activation in the HYKO6 cell line expressing PARP-1 cDNA to that level found in primary PARP-1$^{+/+}$ cells (passage 4). Similar results were found by using HYKO6-pcDNA/PARP-1 and HYKO6-pcDNA transfected cells at passage 30 (data not shown).

DISCUSSION

Endothelial cell dysfunction plays a critical role in uncontrolled inflammatory conditions, such as ischemia/reperfusion injury, sepsis, and multiorgan dysfunction syndrome [Aird, 2003]. In all of these processes, PARP-1 seems to play a critical role, although the underlying mechanisms of endothelial cell injury mediated by

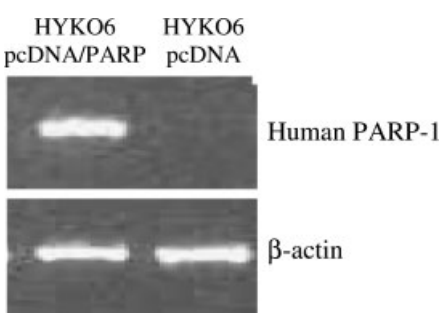

Fig. 8. RT-PCR analysis of PARP-1 expression in the HYKO6 cell line transfected with pcDNA/PARP-1 or the control pcDNA plasmids.

PARP-1 are unknown. An important tool for the elucidation of the PARP-1 signaling pathways involved in endothelial cell dysfunction could be the development of PARP-1$^{-/-}$ endothelial cell lines. Here, we have described for the first time a PARP-1$^{-/-}$ endothelial cell line that it has been established by SV40 transfection of primary PARP-1$^{-/-}$ murine heart endothelial cells.

Transformation with SV40 might lead to dedifferentiation and, therefore, to variations in phenotype and functional assays [Choi et al., 1988]. Thus, it is essential to carry out phenotypical and functional studies before utilizing such cell line as substitutes for primary MHEC in vitro. Specific heart endothelial cell surface antigens, such as CD105, CD31, ICAM-2, and VCAM-1, were detected in our HYKO6 cell line, as they were in primary PARP-1$^{-/-}$ endothelial cells. However, surface expression of the ICAM-1 molecule was lost in our cell line. Transformation of cells by SV40 is induced by two oncoproteins encoded in the early region of the viral genome, the large tumor antigen (Tag) [Simmons, 2000] and the small tumor antigen (tag) [Rundell and Parakati, 2001]. The main activity of Tag for cell transformation is to target key cellular proteins, such as the tumor suppresor p53, inactivating their functions [Pipas and Levine, 2001]. Recently, it has been demonstrated that expression of ICAM-1 is abolished in p53-deficient cell lines [Gorgoulis et al., 2003]. Indeed, we found that expression of ICAM-1 was also abrogated in a PARP-1$^{+/+}$ endothelial cell line generated by SV40 transformation (data not shown). Endothelial cells seeded on Matrigel underwent morphological differentiation and organized rapidly into networks of CLS. This model reflects the latter aspects of the angiogenic response, i.e., endothelial cell alignment, multicellular organization, and differentiation as evidenced by lumen formation [Kubota et al., 1988]. The results of our "in vitro" angiogenesis assays show that both the primary and our immortalized PARP-1$^{-/-}$ cell line are able to form capillary-like networks "in vitro." This finding indicates that the HYKO6 cell line maintains a functional endothelial cell phenotype.

In order to further characterize the HYKO6 cell line, we analyzed NF-κB-dependent transcriptional activation upon TNF-α stimulation. Many TNF-α-induced genes, mediators of the inflammatory response, are NF-κB target genes

[Pahl, 1999]. Our results show that our HYKO6 cell line is able to respond to TNF-α stimulation, performing a similar level of NF-κB-dependent transcriptional activation as that of the primary PARP-$1^{-/-}$ endothelial cells. We previously observed that NF-κB-dependent transcriptional activation is hampered in primary PARP-$1^{-/-}$ endothelial cells, compared to primary PARP-$1^{+/+}$ endothelial cells, in response to TNF-α [Carrillo et al., 2004]. Similar results have been previously reported by Garcia Soriano et al. [2001] after high-glucose stimulation of PARP-$1^{+/+}$ and PARP-$1^{-/-}$ endothelial cells and by Oliver et al. [1999] and Hassa and Hottiger [1999] in TNF-α-activated fibroblasts from both genotypes. Here, we have also established a stable transfectant of HYKO6 expressing a full PARP-1 cDNA. The PARP-1 complemented HYKO6 cell line is able to recover endothelial cell wild-type NF-κB-dependent transcriptional activity. These results suggest that the genomic integration of SV40 in our cell line has not significantly affected NF-κB-dependent gene transcription. Therefore, our cell line in combination with the PARP-1 stable transfectant thereof, seems to be a good system for studying the role played by PARP-1 in endothelial cell transcriptional regulation. PARP-1 might modulate gene expression through different mechanisms: (i) physical interactions with other proteins, especially transcription factors; (ii) direct binding to the gene-regulating sequences; and (iii) transient post-translational modifications of nuclear proteins by poly (ADP-ribosyl)ation [Yélamos and Oliver, 2002]. Recently, Soldatenkov et al. [2002] have demonstrated transcriptional repression of PARP gene expression by binding of PARP to its own promoter sequences. Likewise, Nirodi et al. [2001] have suggested that PARP may be a coactivator of CXCL1 transcription. It has also been shown that PARP-1 binds the IL-6/glucocorticoid-responsive element of *Reg* gene, forming the active transcriptional DNA/protein complex for Reg gene expression [Akiyama et al., 2001]. Thus, PARP-1 appears to have dual functions in the regulation of transcription, working as a silencing or enhancing transcription factor. On the other hand, many of the pathways in response to TNF-α have been proved to be cell type-specific, requiring that observations made in other cell types should be confirmed or ruled out in endothelial cells [Madge and Pober, 2001; Carrillo et al., 2004].

Important tools for investigating cell physiology pathways have involved over expression or invalidation of selected genes "in vitro." The HYKO6 cell line described here is efficiently transfected using liposomes, and might be genetically engineering in a stable manner, allowing the constitutive or conditional expression of activating or blocking genes. Thus, our cell line could be useful to identify new proteins related to the PARP-1 pathway in endothelial cell dysfunction, which might be potential targets for new therapeutic drugs.

In conclusion, the HYKO6 cell line is the first established PARP-$1^{-/-}$ endothelial cell line phenotypically and functionally similar to primary PARP-$1^{-/-}$ endothelial cells. This cell line should provide a valuable tool to study the PARP-1 endothelial cell injury pathway by using both genetic and pharmacological approaches.

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REFERENCES

Aird WC. 2003. The role of the endothelium in severe sepsis and the multiple organ dysfunction syndrome. Blood 101:3765–3777.

Akiyama T, Takasawa S, Nata K, Kobayashi S, Abe M, Shervani NJ, Ikeda T, Nakagawa K, Unno M, Matsuno S, Okamoto H. 2001. Activation of *Reg* gene, a gene for insulin-producing β-cell regeneration: Poly(ADP-ribose) polymerase binds Reg promoter and regulates the transcription by autopoly(ADP-ribosyl)ation. Proc Natl Acad Sci USA 98:48–53.

Andreone TL, O'Connor M, Denenberg A, Hake PW, Zingarelli B. 2003. Poly(ADP-ribose) polymerase-1 regulates activation of activator protein-1 in murine fibroblasts. J Immunol 170:2113–2120.

Bachelerie F, Alcami J, Arenzana SF, Virelizier JL. 1991. HIV enhancer activity perpetuated by NF-κB induction on infection of monocytes. Nature 350:709–712.

Bevilacqua MP, Pober JS, Mendrick DL, Cotran RS, Gimbrone MA. 1987. Identification of an inducible endothelial–leukocyte adhesion molecule. Proc Natl Acad Sci USA 84:9238–9242.

Carrillo A, Chamorro S, Rodríguez-Gago RA, Alvarez B, Molina MJ, Rodríguez-Barbosa JI, Sánchez A, Ramírez P, Muñoz A, Domínguez J, Parrilla P, Yélamos J. 2002. Isolation and characterization of immortalized porcine

aortic endothelial cell lines. Vet Immunol Immunopathol 89:91–98.

Carrillo A, Monreal Y, Ramírez P, Marin L, Parrilla P, Oliver FJ, Yélamos J. 2004. Transcription regulation of TNF-α-early response genes by poly(ADP-ribose) polymerase-1 in murine heart endothelial cells. Nucleic Acids Res 32:757–766.

Chiarugi A. 2002. Poly(ADP-ribose) polymerase: Killer or conspirator? The "suicide hypothesis" revisited. Trends Pharmacol Sci 23:122–129.

Choi YW, Lee JC, Ross SR. 1988. Requirement for the simian virus 40 small tumor antigen in tumorigenesis in transgenic mice. Mol Cell Biol 8:3382–3390.

de Murcia G, Shall S. 2000. From DNA damage and stress signaling to cell death: Poly (ADP-ribosylation) reactions. Oxford: Oxford University Press.

de Murcia G, Menissier de Murcia J. 1994. Poly(ADP-ribose) polymerase: A molecular nick-sensor. Trends Biochem Sci 19:172–176.

Du X, Matsumura T, Edelstein D, Rossetti L, Zsengellér Z, Szabó C, Brownlee M. 2003. Inhibition of GAPDH activity by poly(ADP-ribose)polymerase activates three major pathways of hyperglycemic damage in endothelial cells. J Clin Invest 112:1049–1057.

Garcia Soriano F, Virág L, Jagtap P, Szabó E, Mabley JG, Liaudet L, Marton A, Hoyt DG, Murthy KGK, Salzman AL, Southan GJ, Szabó C. 2001. Diabetic endothelial dysfunction: The role of poly(ADP-ribose) polymerase activation. Nat Med 7:108–113.

Gorgoulis VG, Zacharatos P, Kotsinas A, Kletsas D, Mariatos G, Zoumpourlis V, Ryan KM, Kittas C, Papavassiliou AG. 2003. p53 activates ICAM-1 (CD54) expression in an NF-κB-independent manner. EMBO J 22:1567–1578.

Ha HC, Hester LD, Snyder SH. 2002. Poly(ADP-ribose) polymerase-1 dependence of stress-induced transcription factors and associated gene expression in glia. Proc Natl Acad Sci USA 99:3270–3275.

Hassa PO, Hottiger MO. 1999. A role of poly(ADP-ribose) polymerase in NF-κB transcriptional activation. Biol Chem 380:953–959.

Hassa PO, Hottiger MO. 2002. The functional role of poly(ADP-ribose)polymerase 1 as novel coactivator of NF-kappaB in inflammatory disorders. Cell Mol Life Sci 59:1534–1553.

Jones CB, McIntosh J, Huang H, Graytock A, Hoyt DG. 2001. Regulation of bleomycin-induced DNA breakage and chromatin structure in lung endothelial cells by integrins and poly(ADP-ribose) polymerase. Mol Pharmacol 59:69–75.

Kalogeris TJ, Laroux FS, Cockrell A, Ichikawa H, Okayama N, Phifer TJ, Alexander JS, Grisham MB. 1999. Effect of selective proteasome inhibitors on TNF-induced activation of primary and transformed endothelial cells. Am J Physiol 276:856–864.

Kameoka M, Ota K, Tetsuka T, Tanaka Y, Itaya A, Okamoto T, Yoshihara K. 2000. Evidence for regulation of NF-κB by poly(ADP-ribose) polymerase. Biochem J 346:641–649.

Kraus WL, Lis JT. 2003. PARP goes transcription. Cell 113: 677–683.

Kubota Y, Kleinman HK, Martin GR, Lawley TJ. 1988. Role of laminin and basement membrane in the morphological differentiation of human endothelial cells into capillary-like structures. J Cell Biol 107: 1589–1598.

Lidington EA, Rao RM, Marelli-Berg FM, Jat PS, Haskard DO, Mason JC. 2002. Conditional immortalization of growth factor-responsive cardiac endothelial cells from H-2K(b)-tsA58 mice. Am J Physiol Cell Physiol 282:C67–C74.

Lin W, Ame JC, Aboul-Ela N, Jacobson EL, Jacobson MK. 1997. Isolation and characterization of the cDNA encoding bovine poly (ADP-ribose)-glycohydrolase. J Biol Chem 272:11895–11901.

Litzkas P, Jha KK, Ozer HL. 1984. Efficient transfer of cloned DNA into human diploid cells: Protoplast fusion in suspension. Mol Cell Biol 4:2549–2552.

Madge LA, Pober JS. 2001. TNF signaling in vascular endothelial cells. Exp Mol Pathol 70:317–325.

Menissier de Murcia J, Niedergang C, Trucco C, Ricoul M, Dutrillaux B, Mark M, Oliver FJ, Masson M, Dierich A, LeMeur M, Walztinger C, Chambon P, de Murcia G. 1997. Requirement of poly(ADP-ribose) polymerase in recovery from DNA damage in mice and in cells. Proc Natl Acad Sci USA 94:7303–7307.

Mizuno R, Yokoyama Y, Ono N, Ikomi F, Ohhashi T. 2003. Establishment of rat lymphatic endothelial cell line. Microcirculation 10:127–131.

Nirodi C, NagDas S, Gygi SP, Olson G, Aebersold R, Richmond A. 2001. A role for poly(ADP-ribose) polymerase in the transcriptional regulation of the melanoma growth stimulatory activity (*CXCL1*) gene expression. J Biol Chem 276:9366–9374.

Oliver FJ, de la Rubia G, Rolli V, Ruiz-Ruiz MC, de Murcia G, Menissier de Murcia J. 1998. Importance of poly(ADP-ribose) polymerase and its cleavage in apoptosis. J Biol Chem 273:33533–33539.

Oliver FJ, Menissier de Murcia J, Nacci C, Decker P, Andriantsitohaina R, Muller S, De la Rubia G, Stoclet JC, de Murcia G. 1999. Resistance to endotoxic shock as a consequence of defective NF-κB activation in poly(ADP-ribose) polimerase-1 deficient mice. EMBO J 18:4446–4454.

Osborn L, Hession C, Tizard R, Vassallo C, Luhowskyj S, Chi-Rosso G, Lobb R. 1989. Direct expression cloning of vascular cell adhesion molecule 1, a cytokine-induced endothelial protein that binds to lymphocytes. Cell 59: 1203–1211.

Pacher P, Mabley JG, Soriano FG, Liaudet L, Komjati K, Szabo C. 2002. Endothelial dysfunction in aging animals: The role of poly(ADP-ribose) polymerase activation. Br J Pharmacol 135:1347–1350.

Pahl HL. 1999. Activator and target genes of Rel/NF-κB transcription factors. Oncogene 18:6853–6866.

Pipas JM, Levine AJ. 2001. Role of T antigen interactions with p53 in tumorigenesis. Semin Cancer Biol 11: 23–30.

Rodríguez-Gago MA, de Heredia A, Ramírez P, Parrilla P, Aparicio P, Yélamos J. 2001. Human anti-porcine γδ T-cell xenoreactivity is inhibited by human FasL expression on porcine endothelial cells. Transplantation 72: 503–509.

Rundell K, Parakati R. 2001. The role of the SV40 small t antigen in cell growth promotion and transformation. Semin Cancer Biol 11:5–13.

Saadi S, Platt JH. 1995. Transient perturbation of endothelial integrity induced by natural antibodies and complement. J Exp Med 181:21–31.

Sharp C, Warren A, Oshima T, Williams L, Li JH, Alexander JS. 2001. Poly ADP ribose-polymerase inhibitors prevent the upregulation of ICAM-1 and E-selectin in response to Th1 cytokine stimulation. Inflammation 25:157–163.

Simmons DT. 2000. SV40 large T antigen functios in DNA replication and transformation. Avd Virus Res 55:75–134.

Smith S. 2001. The world according to PARP. Trends Biochem Sci 26:174–179.

Soares MP, Muniappan A, Kaczmarek E, Koziak K, Wrighton CJ, Steinhauslin F, Ferran C, Winkler H, Bach FH, Anrather J. 1998. Adenovirus-mediated expression of a dominant negative mutant of p65/RelA inhibits proinflammatory gene expression in endothelial cells without sensitizing to apoptosis. J Immunol 161: 4572–4582.

Soldatenkov VA, Chasovskikh S, Potaman VN, Trofimova I, Smulson ME, Dritschilo A. 2002. Transcriptional repression by binding of poly(ADP-ribose) polymerase to promoter sequences. J Biol Chem 277:665–670.

Szabo C, Dawson V. 1998. Role of poly(ADP-ribose) synthetase in inflammation and ischaemia-reperfusion. Trends Pharmacol Sci 19:287–298.

Szabó C, Cuzzocrea S, Zingarelli B, O'Connor MO, Salzman AL. 1997. Endothelial dysfunction in a rat model of endotoxic shock. Importance of the activation of poly(ADP-ribose) synthetase by peroxynitrite. J Clin Invest 100: 723–735.

Szabó G, Bährle S, Stumpf N, Sonnenberg K, Szabó E, Pacher P, Csont T, Schulz R, Dengler TJ, Liaudet L, Jagtap PG, Southan GJ, Vahl CF, Hagl S, Szabó C. 2002. Poly(ADP-ribose) polymerase inhibition reduces reperfusion injury after heart transplantation. Cir Res 90:100–106.

Tulin A, Spradling A. 2003. Chromatin loosening by poly(ADP)-ribose polymerase (PARP) at *Drosophila* puff loci. Science 299:528–529.

Yélamos J, Oliver FJ. 2002. Role of poly(ADP-ribose) polymerase-1 (PARP-1) in the inflammatory response. Inmunologia 21:219–227.