Endothelial cell DNA transfer and expression using Petri dish electroporation and the nonreplicating vaccinia virus/T7 RNA polymerase hybrid system

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The nonreplicating vaccinia virus MVA/T7 RNA polymerase hybrid system was tested with Petri dish electroporation for ectopic gene expression in human umbilical vein endothelial cells (HUVECs). A range of voltages (150–450 V), pulse times (10–40 ms), DNA concentrations (0–20 µg/ml) and infection levels (0–15 multiplicities of infection) were tested for effects on T7 promoter-directed chloramphenicol acetyltransferase (CAT) activity after MVA/T7RP infection. MVA/T7RP-directed expression was transient and at least 10 000-fold in excess of nonviral, cytomegalovirus enhancer-directed expression. Use of a Petri dish electrode with the MVA/T7RP system showed increased viability compared with a cuvette electrode. Overexpression of interleukin-2 alpha subunit (IL2R α) pro-

tein followed by anti-IL2R α -directed magnetic immunoaffinity cell sorting allowed isolation of the transfected population. The high fidelity of cellular sorting was shown by segregation of CAT activity in the IL2R α -sorted population after transfection of T7 promoter-directed bicistronic IL2R α /CAT DNA. Expression of a panel of proteins including the fluorophore green fluorescent protein as detected by fluorescence microscopy and p21^{cip1}, p27^{kip1}, pp60^{c-src}, FGF-1, pRb, p107 and pRb2/p130 proteins was also achieved. Thus, use of the nonreplicating vaccinia virus/T7 RNA polymerase expression system with Petri dish electroporation is feasible for certain applications for the manipulation of HUVECs by gene transfer.

Keywords: endothelial cell; vaccinia virus; electroporation; transfection; expression

Introduction

Cultured primary human umbilical vein endothelial cells (HUVECs) are attractive for the study of endothelial cell function *in vitro* because they: (1) are readily available and well characterized relative to endothelial cells of other human tissue origin; and (2) display growth factor and extracellular matrix-mediated phenotypes reflective of normal human endothelial cell growth and morphogenic pathways *in vivo*. However, HUVECs are resistant to conventional gene transfer techniques and selection methods relative to immortalized cell lines and non-human endothelial cells.^{1–5} Cultured HUVECs exhibit decreased transfection efficiencies, an intolerance to transfection and selection conditions and low expression levels after genes are delivered. The inefficiency of HUVEC transgene expression using nonviral, DNA transfection is illustrated by expression levels 1% relative to those in a commonly used human immortalized cell line and maximized transfection efficiencies reported as low as 0.68–2%.^{3,6} These barriers have limited the manipulation of HUVECs by the overexpression of both wild-type and mutant proteins or the application of 'knock-out/down' strategies using gene transfer. This

has hampered the study of endothelial cell function *in vitro* and the development of gene therapeutic methods *in vivo* where the endothelium is an attractive target.⁷ Improved approaches of gene delivery and expression are required to allow experimental and therapeutic manipulation of HUVECs and other normal human endothelial cells by ectopic gene overexpression. The use of viral methods as in adenoviral or retrovirus-mediated gene transfer offers attractive alternatives.^{8–10}

Another viral method for gene expression in mammalian cells utilizes vaccinia virus (VV). The nonreplicating vaccinia virus/T7 RNA polymerase hybrid expression system results in high expression levels in eukaryotic cells hundreds-of-fold in excess of conventional eukaryotic expression systems.^{11–14} This system utilizes infection with the cytoplasmic, nonreplicating VV-modified vaccinia Ankara engineered to express T7 RNA polymerase (MVA/T7RP). The T7RP expressed from a viral promoter drives transcription of transfected DNA vectors containing T7 promoter (PT7)-directed sequences.¹⁵ Thus, MVA/T7RP expression is less dependent on eukaryotic components than conventional expression systems and is potentially cytoplasmic in nature, bypassing nuclear barriers to transfection.

As compared with precipitation-based transfection methods, including liposome-mediated DNA transfection, advantages of electroporation include rapidity of use, avoidance of chemical toxicity and applicability *in vivo*.¹⁶ However, electroporation methods have yielded

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low transfection efficiencies and limited expression levels in HUVECs.^{1,3,17} Furthermore, cuvette electroporation requires manipulation of cells into a cuvette. This may introduce artifactual effects in addition to the receiving of an electric shock that may inhibit or delay subsequent gene expression and perturb cellular function. In contrast, a Petri dish electrode bypasses the necessary cellular manipulation for cuvette electroporation.

The high expression levels achieved by the MVA/T7RP hybrid system coupled with the benefits of Petri dish electroporation might overcome barriers to ectopic gene expression displayed by HUVECs. The following experiments evaluate the efficacy of such an approach. The result is a gene expression method that retains advantages of electroporation and allows manipulation of HUVECs by gene transfer.

Results

Optimization of electroporation conditions

The effects of voltage and pulse times were tested to establish optimized electroporation conditions for HUVECs using a Petri dish electrode. HUVECs were transiently transfected with a DNA plasmid containing the chloramphenicol acetyltransferase (CAT) gene under control of the T7 promoter (PT7/CAT) and infected with recombinant MVA modified to express T7RP (MVA/T7RP).¹⁴ The electroporation conditions tested were a single square-wave pulse at 150, 350 or 450 V each at 10, 20 or 40 ms (Figure 1). Readily detectable CAT activity was observed at 350 V and highest expression was observed at 450 V. Twenty milliseconds was utilized as a pulse length in all subsequent experiments.

Potential toxic effects of the transfection and infection conditions were assessed by quantification of viable HUVECs as measured by cell cytometry and trypan blue exclusion. For Petri dish electroporation HUVECs either remained untreated or were MVA/T7RP infected alone, electroporated alone at 350 or 450 V, or infected and elec-

troporated at 350 or 450 V (Figure 2). Results were compared with samples transfected by cuvette electroporation using optimized conditions. The cuvette electroporation conditions included removal of the cells from cultureware and placement into a 4-mm gap cuvette without or with a subsequent 180 V square-wave pulse for 20 ms using 5 μ g of PT7/CAT DNA in 800 μ l electroporation buffer. These conditions are similar to those previously published as optimal for HUVECs using cuvette electroporation.¹⁷ As typified by results shown in Figure 2a, both Petri dish and cuvette electroporation methods resulted in less viable cells compared with an untreated control. However, the Petri dish electrode showed 23% more viable cells relative to the cuvette electrode using the applied transfection and infection conditions (Figure 2a). For both electrodes, viable cell number was less after concomitant transfection and infection compared with electroporation or infection alone.

The results were compared with those achieved in samples using nonviral expression of CAT DNA under control of the powerful cytomegalovirus (CMV) enhancer after Petri dish or cuvette electroporation. Nonviral CMV-directed expression also displayed a decrease in viable cells relative to untreated control that, unlike the MVA/T7RP system, was similar regardless of the electrode type (Figure 2b).

The samples that produced the viability data displayed in Figure 2 were further tested for CAT expression. Similar amounts of MVA/T7RP-mediated expression were observed for Petri dish and cuvette electroporation using normalized amounts of protein (Figure 3). However, total protein recovery for the cuvette-electroporated sample was approximately one-half that of the Petri dish electrode (data not shown) consistent with the decreased number of viable cells displayed by the cuvette-electroporated sample (Figure 2). This is indicative of a decreased amount of total transgene expression after cuvette electroporation. Accordingly, quantification of CAT activity by imaging analysis showed a 48% increase in total CAT activity with the use of the Petri dish electrode relative to the cuvette electrode (data not shown).

The MVA/T7RP-mediated expression levels were also compared with nonviral expression after transfection of CMV/CAT DNA. Nonviral expression was undetectable under similar assay conditions (5 ng lysate for 30 min) regardless of the electrode used (Figure 3). However, the CMV/CAT DNA was fully functional as activity was detected using 10 000-fold more protein (50 μ g) and a six-fold longer assay time (3 h). Thus, activity of the nonviral CMV-mediated system was less than 0.0001% that achieved by the MVA/T7RP system under these conditions.

Effect of MVA/T7RP infection levels

The expression of transgenes in the nonreplicating MVA/T7RP system is dependent on viral infection.^{11,14} Not only is the virus involved in the delivery and expression of T7RP but other viral factors are required to enhance T7RP expression.^{11,18} After Petri dish electroporation and infection with a range from 1 to 15 multiplicities of infection (MOI) of MVA/T7RP, a linear dose-dependent increase in CAT activity was observed in HUVECs (Figure 4a). Standard conditions for future experiments were chosen at 10 MOI. This balanced the statistical probability of infecting all cells against unwanted cytopathic effects caused by over-infection.

Figure 1 CAT expression as a function of voltage and pulse length. HUVECs were electroporated with a single square-wave pulse with 50 μ g of PT7/CAT DNA in 10 ml using a Petri dish electrode and the conditions shown. The cells were then infected with 10 MOI of MVA/T7RP and harvested 24 h after transfection. Gene expression was measured in HUVEC extracts by radioactive CAT assay. The unmodified [¹⁴C] chloramphenicol (CM) was separated from [¹⁴C] chloramphenicol 1-monoacetate (CM-1Ac) and [¹⁴C] chloramphenicol 3-monoacetate (CM-3Ac) by thin-layer chromatography and the resultant autoradiograph is shown.

Figure 2 Effect of infection and transfection conditions on cell number. (a) HUVECs were either untreated or MVA-T7RP infected alone or together with DNA transfection using Petri dish or cuvette electroporation as shown. Attached cells were harvested 16 h after transfection, stained with trypan blue and viable cells counted in triplicate by cell cytometry. The number of viable cells was compared with that obtained just before infection/transfection and expressed as a percentage as shown. The experiment was repeated three times with two different HUVEC isolates with similar results. (b) Using samples done in parallel with those in (a), HUVECs remained uninfected but were transfected with CMV/CAT DNA using either a Petri dish (50 µg DNA in 10 ml) or cuvette (5 µg DNA in 0.8 ml) electrode. Cells were harvested and the number of viable cells quantified as in (a). P, Petri dish electrode; C, cuvette electrode.

Figure 3 Effect of infection and transfection conditions on CAT expression. Cells used to produce the results depicted in Figure 2 were tested for gene expression using the CAT assay conditions shown. P, Petri dish electrode; C, cuvette electrode; CM, [¹⁴C] chloramphenicol; CM-1Ac, [¹⁴C] chloramphenicol 1-monoacetate; (CM-3Ac), [¹⁴C] chloramphenicol 3-monoacetate.

Effect of DNA concentration

DNA concentrations from 25 to 200 µg per 100-mm dish were tested for Petri dish electroporation (Figure 4b). Since a minimum buffer volume of 10 ml was required to immerse the Petri dish electrode completely, this represented 2.5–20 µg/ml of DNA. This is comparable with the range of DNA used in cuvette electroporation in our laboratory. Increasing the amount of DNA electroporated resulted in corresponding increases in CAT expression (Figure 4b).

Kinetics of expression

The replication-deficient MVA/T7RP system displays transient expression for 4–5 days.¹² In HUVECs MVA/T7RP-mediated expression was also detected for up to 4 days with peak expression occurring at 1–2 days

(Figure 4c). To determine whether such transient kinetics were a property of the MVA/T7RP system in general or of HUVECs in particular, the electroporation and infection conditions were applied to primary rabbit microvascular endothelial cells. These cells showed CAT expression that remained high for up to 2 weeks (data not shown).

Magnetic immunoaffinity cell sorting

The developed electroporation and infection techniques were coupled with a magnetic immunoaffinity cell sorting procedure to isolate the transfected HUVEC population.^{19,20} The alpha subunit of the interleukin-2 cell surface receptor (IL2Rα) was used as a sorting target. After square-wave Petri dish electroporation of PT7/IL2Rα DNA and infection with MVA/T7RP, distinctive anti-IL2Rα-directed magnetic bead binding was observed in IL2Rα- (Figure 5) but not mock-transfected (data not shown) cells as detected by light microscopy. The percent recovery of radiolabeled IL2Rα-transfected HUVECs isolated by anti-IL2Rα magnetic immunoaffinity cell sorting was 0.9 ± 0.3% and 15.9 ± 2.0% (n = 3) following 350 and 450 V pulses, respectively, for 20 ms. This agreed with microscopic observations.

The PT7/IL2Rα gene was also used to prepare a bicistronic IL2Rα/CAT DNA expression unit under PT7 control, a representation of which is shown in Figure 6a. Use of this DNA should result in expression of both the sorting target IL2Rα and the reporter gene CAT from the same vector. The bicistronic design was possible because of the internal ribosome entry site (IRES) preceding each gene in the IL2Rα/CAT expression unit. After square-wave, Petri dish electroporation of PT7/IL2Rα/CAT DNA and MVA/T7RP infection, HUVECs underwent anti-IL2Rα-targeted magnetic immunoaffinity cell sorting to separate the transfected from the nontransfected cell

Figure 4 Characterization of infection and transfection conditions. (a) HUVECs were square-wave electroporated with 50 µg of PT7/CAT DNA using a Petri dish electrode at 350 V for 20 ms. The cells were then infected with the indicated levels of MVA/T7RP. Twenty-four hours after transfection, HUVECs were lysed and analyzed for CAT activity. The resultant autoradiogram of separated chloramphenicol products is shown. (b) HUVECs were transfected with the indicated amounts of PT7/hCAT DNA using a Petri dish electrode at 350 V for 20 ms and then infected with 10 MOI of MVA/T7RP. Cell extracts were analyzed for CAT activity as described. (c) HUVECs were transfected with 50 µg of PT7/CAT DNA using a Petri dish electrode at 350 V for 20 ms and then infected with 10 MOI of MVA/T7RP. At the indicated times, cells were harvested and extracts were analyzed for CAT activity. The resultant autoradiograph of TLC-separated reaction products is shown. [¹⁴C] chloramphenicol (CM); [¹⁴C] chloramphenicol 1-monoacetate (CM-1Ac); [¹⁴C] chloramphenicol 3-monoacetate (CM-3Ac).

populations. Expression from the bicistronic IL2Rα/CAT DNA sequence and fidelity of the sorting technique was verified by over 100-fold enrichment of CAT activity in the positively sorted cell population (Figure 6b).

To determine whether such bicistronic expression was applicable to more physiological genes besides the common reporter CAT, a similar strategy was used to achieve expression of bicistronic T7 promoter-directed IL2Rα/FLAG-tagged retinoblastoma protein family member sequences. Expression of each of FLAG-tagged pRb, p107 and pRb2/p130 proteins could be detected by anti-FLAG immunoblotting in the anti-IL2Rα-targeted, magnetic immunoaffinity cell sorted HUVEC population (Figure 7).

Expression of green fluorescent protein

To examine whether the established electroporation and infection conditions were sufficient for expression of

Figure 5 Anti-IL2Rα-directed magnetic immunoaffinity cell sorting. HUVECs were electroporated with PT7/IL2Rα DNA and infected with MVA/T7RP. IL2Rα expression was detected 16 h later by incubating the cells with mouse anti-IL2Rα antibody and goat anti-mouse antibody-conjugated magnetic beads. The cells were imaged in normal phase. The arrows indicate HUVECs that bound beads. The inset shows two IL2Rα-expressing HUVECs imaged in bright field to enhance the appearance of the bound magnetic beads.

Figure 6 Fidelity of magnetic immunoaffinity cell sorting. (a) A T7 promoter (PT7)-directed expression unit was prepared containing the IL2Rα and CAT genes each preceded by an internal ribosome entry site (IRES) from the encephalomyocarditis virus. A 3' untranslated region derived from the *Xenopus laevis* β globin gene followed by a 30 residue adenine tract completes the expression unit as shown. (b) HUVECs were electroporated with a DNA vector containing the T7 expression unit shown in panel a. Transfected and infected HUVECs that bound beads (positive sort) were separated from those that did not (negative sort) using anti-IL2Rα-targeted magnetic affinity cell sorting. Normalized protein from each population was analyzed for CAT activity. Unmodified [¹⁴C] chloramphenicol (CM) was separated from [¹⁴C] chloramphenicol 1-monoacetate (CM-1Ac) and [¹⁴C] chloramphenicol 3-monoacetate (CM-3Ac) by thin-layer chromatography and the resultant autoradiograph is shown.

genes other than the reporter gene CAT and the sorting marker IL2Rα, a T7 expression unit was prepared for overexpression of green fluorescent protein (GFP).²¹ This PT7/GFP DNA vector was transfected into HUVECs using the established electroporation and MVA/T7RP infection conditions. Transfected, viable HUVECs were readily evident as detected by fluorescence microscopy (Figure 8). Interestingly, HUVEC GFP expression appeared somewhat nucleophilic.

Figure 7 Expression of bicistronic IL2R/FLAG-tagged retinoblastoma protein family members. T7 promoter (PT7)-directed expression units were prepared similar to that shown in Figure 5a with either FLAG-tagged pRb, p107 or pRb2/p130 genes in place of the CAT gene. MVA/T7RP-infected HUVECs were electroporated with 50 μ g of PT7/IL2R α DNA vector (-) or PT7/IL2R α /FLAG-pRb, PT7/IL2R α /FLAG-p107, PT7/IL2R α /FLAG-pRb2/p130. Twenty-hour hours after transfection, the cells were harvested and equivalent amounts of extract were analyzed by SDS-PAGE and immunoblotting with anti-FLAG antibodies. Immunoreactive proteins were detected by chemiluminescence. Calculated molecular weights for FLAG-pRb, FLAG-p107 and FLAG-pRb2/p130 are 107, 122 and 130 kDa, respectively.

Figure 8 Expression of green fluorescent protein in HUVECs. Cells were transfected with 50 μ g of PT7/GFP DNA using a Petri dish electrode and infected with 10 MOI of MVA/T7RP. Twenty four hours later cells were washed and visualized by epifluorescence microscopy.

Overexpression of a panel of proteins

To examine further use of the expression system with a variety of genes, T7 expression vectors were prepared containing either the cyclin-dependent kinase inhibitors p21^{cip1} or p27^{kip1}, the protein tyrosine kinase pp60^{c-src}, members of the retinoblastoma family pRb, p107 and

pRb2/p130, and fibroblast growth factor (FGF)-1.²²⁻²⁸ Each of these gene products was readily detected by immunoblotting after using the established electroporation and infection conditions (Figure 9). Each product migrated at the expected apparent molecular weight and was specifically observed in the sample-transfected HUVECs (Figure 9).

Discussion

The MVA/T7RP expression system and a cellular sorting method were coupled with square-wave electroporation using a Petri dish electrode for gene expression in primary cultures of HUVECs. Results showed: (1) high, but transient, transgene expression after transfection of T7 promoter-directed target genes into MVA/T7RP-infected cells; (2) selective isolation of the transfected cell population by magnetic immunoaffinity cellular sorting against a co-expressed cell surface protein; and (3) successful application of a Petri dish electrode with resultant increased HUVEC viability and overall expression. The success of the system was manifested by the overexpression of a variety of genes, including those for non-reporter proteins, in HUVECs.

The nonreplicating MVA/T7RP recombinant vaccinia virus used in this study provides high T7RP-directed expression with diminished cytopathic effects.¹²⁻¹⁴ The MVA strain has been used successfully as an expression vehicle for the immunotherapeutic treatment of infectious disease and cancer in animal models and was successfully safety tested in humans.²⁹⁻³⁴ In the approach described here, a T7 promoter-directed transgene is introduced by transfection and the T7 RNA polymerase is expressed by the virus. This obviates the need to prepare recombinant viruses for each desired transgene.

Using this approach, gene expression was examined

Figure 9 Immunoblots of proteins expressed in HUVECs using the electroporation and infection conditions. Cells were transfected with 50 μ g of an empty DNA vector (-) or the same vector with the indicated genes under control of a T7 promoter (+). The transfected HUVECs were infected with 10 MOI of MVA/T7RP. Twenty-four hours after transfection, the cells were harvested and equivalent amounts of extract were analyzed by SDS-PAGE and immunoblotting. Immunoreactive proteins were detected by chemiluminescence and the resultant autoradiographs are shown.

after varying the voltage applied during electroporation, the MVA/T7RP infection level and PT7/target gene DNA concentration. A single 350 or 450 V pulse for 20 ms with 50 μ g of DNA in 10 ml electroporation buffer and viral adsorption with 10 MOI was sufficient for readily detectable expression of CAT activity using the Petri dish electrode. Higher CAT expression was achieved by increasing the voltage, the amount of transfected PT7-containing DNA or the MVA/T7RP infection level within tested limits.

Use of the MVA/T7RP system with the Petri dish electrode resulted in higher overall CAT expression and increased numbers of viable cells relative to a cuvette electrode using respective optimized electroporation conditions. Expression achieved was at least 10 000-fold higher than that with CMV/CAT expression based on the dilution of lysates required for CAT assay.

Optimizing any expression method involves maximizing expression balanced against resultant cytotoxic effects. Petri dish electroporation resulted in a higher number of viable cells compared with cuvette electroporation after application of the MVA/T7RP system. The combination of infection and electroporation appeared somewhat synergistic in decreasing the number of viable cells that was less severe for the Petri dish electrode. Decreases in the number of viable cells may be due to cell death or, since untreated cells exhibited proliferation, inhibition of cell growth. The level of cell viability achieved here is similar or improved over those reported for HUVECs and other cell types using comparable methods.^{1,35}

The additional cellular manipulation required of the cuvette electrode in addition to viral infection may be detrimental. Consistent with this, similar viability was observed regardless of electrode type during nonviral CMV/CAT expression.

The improved viability and resultant increased overall expression in addition to the minimization of HUVEC perturbation justifies use of the Petri dish electrode with the MVA/T7RP system. The short transfection (ms) time and avoidance of chemicals typical of electroporation contrast with the extended transfection times of 6 h or more for maximal DNA uptake during lipofections³⁶ that are often accompanied by cytotoxic effects. Petri dish electroporation is further desirable as it also avoids disruption of the cell monolayer inherent to cuvette electroporation. This may maintain cellular stability that is more reflective of the state of endothelial cells *in vivo*.

Higher applied voltages appear necessary to achieve adequate expression for Petri dish *versus* cuvette electroporation. This may be required to overcome a larger voltage drop between the Petri dish electrode and the cells that is caused by a larger buffer volume and increased target area relative to a cuvette electrode. The lack of significant cytotoxic effects even though using relatively higher voltages with a Petri dish electrode may also be partly explained by a larger voltage drop.

The kinetics of expression were measured and CAT activity was detectable from 1 to 4 days. Highest expression was at 1–2 days. The subsequent decrease in expression may be due to transience or dilution of the expression components (PT7 DNA, T7RP enzyme), a loss of transfected cells, or loss of CAT protein over time. Importantly, extended CAT expression for up to 2 weeks was achieved in primary cultures of rabbit microvascular

endothelial cells. This suggests that the T7 expression kinetics are cell-type specific and highlights the magnitude of the differences in expression commonly observed between HUVECs and other types of cells.

Expression of the cell surface protein IL2R α was used as a molecular target to isolate the transfected population by magnetic immunoaffinity cell sorting. Transfection efficiency was quantified at 0.9% and 15.9% at 350 or 450 V, respectively, for 20 ms. The increase in transfection efficiency as a function of voltage potentially explains the overall increase in CAT expression caused by increasing the voltage applied.

As the number of transfected cells is in the minority, the ability to isolate the transfected cell population enables enrichment for cells harboring the transgene. Cellular sorting overcomes low transfection efficiencies and allows analysis in a background of diminished non-transfected cells and endogenous gene products. Here, isolation of the transfected cell population was achieved with high recovery and fidelity by anti-IL2R α -targeted magnetic immunoaffinity cell sorting. A bicistronic expression unit allowed production of both the sorting marker IL2R α and the reporter gene CAT from the same DNA vector. This obviated the necessary cellular colocalization of both genes if expressed from two different vectors and potentially resulted in improved sorting efficiency. Accordingly, CAT activity expressed from the bicistronic T7 promoter-directed IL2R α /CAT expression unit was specifically segregated into the positively sorted cell population.

A similar strategy was used to achieve expression of DNA sequences containing bicistronic T7 promoter-directed IL2R α /FLAG-tagged retinoblastoma protein family members. Expression of each of FLAG-tagged pRb, p107 and pRb2/p130 proteins could be detected by anti-FLAG immunoblotting in the anti-IL2R α -targeted, magnetic immunoaffinity cell sorted HUVEC population. Thus, bicistronic IL2R α /target gene expression coupled with the sorting method appears feasible for efficient and specific isolation of the expressing cell population for physiological genes in addition to the reporter gene CAT.

The electroporation and infection conditions chosen were sufficient for the overexpression of a variety of proteins. The fluorophore GFP was readily detected and appeared nucleophilic in HUVECs. In addition, a panel of ectopically expressed proteins was detected by immunoblotting and included the cyclin-dependent kinase inhibitors p21^{cip1} and p27^{kip1}, the protein tyrosine kinase pp60^{c-src}, members of the retinoblastoma family pRb, p107 and pRb2/p130, and the angiogenic polypeptide FGF-1. Interestingly, although the human proteins expressed from the ectopic genes were readily detected, their endogenous counterparts were not with the number of HUVECs analyzed and under the exposure conditions used. This appears indicative of the superphysiological expression levels achieved by the coupled viral T7 RNA polymerase system which can achieve levels as high as 10% of cellular protein.¹⁸

Use of the MVA/T7RP system in HUVECs appears to favor experimental and therapeutic applications requiring extremely high expression occurring during transient expression periods. Possible applications of this system include biochemical analysis of the ectopically expressed gene or affected molecules and short-term cell cycle analysis. The high expression afforded this system would

also appear optimal for high-output production of secreted ectopic proteins from endothelial cells or over-expression of proteins from endothelial cells for subsequent purification purposes. This system may be desirable for the application of knockout, dominant negative or antisense technology where ultra-high expression is required. In addition, the system may be useful in rapidly screening expression of different genes as the preparation of recombinant viruses for each desired transgene is not required of this approach. Use of the nonreplicating vaccinia virus/T7 RNA polymerase expression system with square wave, Petri dish electroporation appears feasible for certain applications for the study of endothelial cells by gene transfer.

Materials and methods

Human umbilical vein endothelial cells

Human umbilical cords were obtained through the Card-eza Foundation for Hematologic Research, Thomas Jefferson University. HUVECs were isolated as described³⁷ and grown on human fibronectin-coated plates (2 µg/cm²)^{38,39} in complete growth medium (M199 (Mediatech, Herndon, VA, USA) containing 10% (v/v) fetal bovine serum (Hyclone, Logan, UT, USA), 100 µg/ml endothelial cell growth supplement,⁴⁰ 10 U/ml heparin (Elkins-Sinn, Cherry Hill, NJ, USA) and antibiotic-antimycotic) in 95% humidified air, 5% CO₂ at 37°C. The endothelial identity of the cells was verified by positive immunostaining for the endothelial cell-specific marker PECAM-1. The number of viable HUVECs was measured after incubation in a 1:1 (v/v) mixture of resuspended cells and 0.4% trypan blue. Unstained HUVECs were counted using a hemacytometer.

Plasmid construction

DNA vector reagents containing T7 expression units were prepared using standard procedures with the pMINI plasmid as the vector backbone.¹¹ Each T7 expression unit contained the cDNA of interest with flanking 5' and 3' structures that enhance the expression of T7 transcripts in eukaryotic cells. The region flanking the 5' end of the gene consisted of a T7 promoter (PT7) for T7 RNA polymerase-mediated expression and an internal ribosome entry site (IRES) derived from the encephalomyocarditis virus for cap-independent translation.⁴¹ The region flanking the 3' end of the transgene contained the untranslated region from the *Xenopus laevis* β globin gene followed by 30 adenine residues (A₃₀).⁴² Genes chosen for expression were the chloramphenicol acetyl-transferase enzyme (CAT), green fluorescent protein (GFP, green lantern 'humanized' GFP, GIBCO/Life Technologies, Rockville, MD, USA), p21^{cip1}, p27^{kip1}, pp60^{c-src}, pRb, p107, pRb2/p130 and FGF-1.^{21–28,43}

A T7 expression unit was also prepared containing the alpha subunit of the interleukin-2 receptor (IL2Rα).⁴⁴ IL2Rα expression acts as a surface marker for anti-IL2Rα-directed magnetic immunoaffinity cellular sorting. The PT7/IL2Rα DNA was then used to prepare bicistronic PT7/IL2Rα/CAT (see Figure 6a) and PT7/IL2Rα/retinoblastoma family member expression units.

Transfection and infection

Transfections were conducted by square-wave electroporation using a T820 Electrosquare Porator

(BTX/Genetronics, San Diego, CA, USA). HUVECs were seeded at 400 000 cells per 100 mm dish in complete growth medium the day preceding the transfection and were at sub-confluence at the time of infection and transfection (see Figure 5 for the approximate degree of confluence). Cells were washed once with filter-sterilized electroporation buffer (20 mM Hepes, pH 7.05, 137 mM NaCl, 5 mM KCl, 0.7 mM Na₂HPO₄, 6 mM dextrose, 1 mg/ml bovine serum albumin⁴⁵) and incubated for 15 min at room temperature in 2 ml electroporation buffer with 50 µg DNA or other amount as stated. DNA was purified either by CsCl-double band ultracentrifugation or by anion exchange chromatography (MaxiPrep, 5 Prime → 3 Prime, Boulder, CO, USA). The electroporation volume was increased to 10 ml and placed in contact with a 100-mm sized, 2-mm gap Petri dish electrode with stainless steel plates (BTX/Genetronics). The electrode plates were cleaned by water-bath sonication and 70% ethanol rinse. The complete electrode was sterilized by exposure to ethylene oxide. After the indicated electroporation conditions were applied, the DNA solution was aspirated and the nonreplicating vaccinia virus strain modified vaccinia virus Ankara expressing T7RP (MVA/T7RP) (a gift of L Wyatt and B Moss, National Institutes of Health, Bethesda, MD, USA) was added at indicated infection levels in 2 ml reduced serum medium (OPTI-MEM I; Life Technologies) for 30 min at 37°C. After the adsorption period, the viral solution was removed and cells were placed in complete growth medium for 16–24 h unless indicated otherwise.

CAT assays

Gene expression was measured in the cell lysates by assaying for CAT activity using radiolabeled chloramphenicol as substrate. Analysis was by thin-layer chromatography (TLC).⁴⁶ Unless otherwise stated, extracts were prepared from transfected HUVECs 16–24 h after transfection. For quantification, if conversion of greater than 80% of chloramphenicol occurred, the assay was considered out of the linear range and lysates were diluted appropriately for analysis.

Magnetic immunoaffinity cell sorting

The cell surface expression of IL2Rα has been used as a target for subsequent isolation of transfected cells by magnetic immunoaffinity cell sorting.^{19,20} HUVECs were electroporated with either PT7/IL2Rα DNA or a bicistronic PT7/IL2Rα/CAT DNA and infected with MVA/T7RP. The following day the HUVECs were incubated in OPTI-MEM with magnetic beads (M-5, Dynal, Lake Success, NY, USA) coated with goat anti-mouse antibodies directed against mouse anti-IL2Rα antibodies (Upstate Biotechnology, Lake Placid, NY, USA). The cells were lifted from the plates and subjected to a magnetic force to separate the cells that bound beads from those that did not as described previously.^{19,20}

To determine transfection efficiency the anti-IL2Rα-directed magnetic immunoaffinity cell sorting procedure was applied to PT7/IL2Rα-transfected and infected HUVECs that were then radiolabeled with 0.1 mCi/ml ¹⁴C-thymidine (New England Nuclear, Boston, MA, USA). Quantification was by scintillation counting of the trichloroacetic acid-precipitable material from the sorted relative to the total population. Transfection efficiency was expressed as a percentage of the counts sorted rela-

tive to the total counts. HUVECs transfected with an empty pMINI vector served as a negative control.

Fluorescence microscopy

Green fluorescent protein (GFP) isolated from the bioluminescent jellyfish *Aequorea victoria* absorbs light and fluoresces without additional cofactors or substrates and is a useful reporter for gene expression in living cells.²¹ GFP expression was detected by placing the tissue culture plate with cells and medium under an Axiovert 100A inverted epifluorescence microscope (Carl Zeiss, Thornwood, NY, USA) with a HB100W mercury short arc lamp (Atto Instruments, Rockville, MD, USA). The filter set was a standard fluorescein isothiocyanate set (Chroma Technologies, Brattleboro, VT, USA) with excitation and emission centered at 480 nm (range 460–500 nm) and 535 nm (range 510–560 nm). Image acquisition was conducted with a CCD video camera (Grundig, Fürth, Germany), Scion Frame Grabber hardware (Applied Scientific Instrumentation, Eugene, OR, USA) and NIH Image software.

Immunoblotting

Transfected HUVECs were removed from tissue culture dishes, isolated by centrifugation and lysed in 66 mM Tris-HCl, pH 6.8 containing 2% (w/v) SDS and 10 mM EDTA for 10 min at 95°C. Equal amounts of lysate were resolved by sodium dodecyl sulfate/polyacrylamide gel electrophoresis under reducing conditions and transferred to nitrocellulose membranes (Amersham, Piscataway, NJ, USA). The membranes were incubated with TBS/Tween 20 (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% (v/v) Tween 20) containing 5% (w/v) nonfat dry milk to block nonspecific binding. The membranes were incubated with primary antibody in TBS/Tween 20 containing 1% (w/v) nonfat dry milk, washed with TBS/Tween 20, and then incubated with horseradish peroxidase-conjugated secondary antibody (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA; or Jackson Laboratories, Bar Harbor, ME, USA) in TBS/Tween 20 containing 1% (w/v) nonfat dry milk. After washing with TBS/Tween 20, immunoreactive proteins were visualized using enhanced chemiluminescence detection (Pierce, Rockford, IL, USA) with exposure on to autoradiographic film. Primary antibodies were anti-IL2R α and anti-aFGF (Upstate Biotechnology), anti-FLAG (Kodak, New Haven, CT, USA), anti-p27^{kip1} (Transduction Laboratories, Lexington, KY, USA), anti-pRb (Pharmingen, San Diego, CA, USA) and anti-p21^{cip1}, anti-p107, anti-pRb2/p130 and anti-pp60^{c-src} (Santa Cruz Biotechnology Santa Cruz, CA, USA).

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