Proc. Natl. Acad. Sci. USA
Vol. 89, pp. 1224–1228, February 1992
Cell Biology

Immortalization of primary human smooth muscle cells

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Contributed by Earl P. Benditt, October 30, 1991

ABSTRACT **Primary human aortic and myometrial smooth muscle cells (SMCs) were immortalized using an amphotropic recombinant retroviral construct containing the E6 and E7 open reading frames (ORFs) of human papillomavirus type 16. The SMCs expressing the E6/E7 ORFs have considerably elevated growth rates when compared with nonimmortalized control cells and show no signs of senescence with long-term passage. The first SMC line derived in this study has been maintained in continuous tissue culture for >1 year (>180 population doublings). The immortalized SMCs have decreased cell size and decreased content of muscle-specific α-actin filaments as determined by indirect immunofluorescence. Southern blot analysis has demonstrated the stable integration of the E6/E7 ORFs in the retrovirally infected cells, and radioimmunoprecipitation has confirmed the continued expression of the E6 and E7 genes. Cytogenetic studies of the SMC lines have revealed essentially diploid populations except for the myometrial clonal line, which became aneuploid at late passage (>125 doublings). These cell lines were not tumorigenic in nude mice.**

Smooth muscle cell (SMC) proliferation in the intima of the aorta and medium-sized arteries is a major factor in the development of human atherosclerosis (1). These SMCs originate from the media and migrate into the intima, where their proliferation results in plaque progression (2, 3). What triggers severe atherosclerosis in individuals who lack known risk factors is not understood and several alternative hypotheses have been proposed (4). Glucose-6-phosphate dehydrogenase isoenzyme typing (5–7) and cytogenetic analysis (8) revealed that many atherosclerotic plaques were composed of a monoclonal population of SMCs. This phenomena was felt to be analogous to the monoclonal proliferation of benign neoplastic SMCs in uterine leiomyomas (9–11). Therefore, it has been proposed that a chemical mutagen (12) or a virus (13) could initiate some atherosclerotic lesions by altering the SMC genome in such a way as to provide the cell with a selective proliferative advantage (14).

A limited number of studies have suggested a role for the herpesvirus in the etiology of atherosclerotic plaque development. Pathogen-free chickens, when infected with a low-virulence strain of Marek disease virus (MDV), an avian herpesvirus, developed arterial lesions histologically similar to human atherosclerotic plaques, and MDV antigens were identified in the medial SMCs (15–17). As has been previously noted in human atherosclerotic plaque SMCs, these MDV-infected SMCs also accumulate excessive free and esterified cholesterol (18–20). Further experiments showed that this process in chickens was secondary to the inactivation of cytoplasmic cholesteryl esterase by the herpesvirus infection (21). In addition, when human and bovine arterial SMCs were infected with herpes simplex virus (HSV) *in vitro*, these cells also accumulated excessive cholesteryl esters (22–24). These experiments indicate that herpesviruses may induce atherogenesis by altering lipid metabolism.

In humans, HSV and cytomegalovirus nucleic acid sequences and antigens have been detected in atherosclerotic plaque and nondiseased medial SMCs by polymerase chain reaction, dot blot and *in situ* DNA hybridization, immunofluorescence, and immunocytochemical analyses (25–31). Seroepidemiologic studies have documented increased levels of cytomegalovirus antibody in patients with severe atherosclerosis (32, 33) and in those patients who developed accelerated coronary artery atherosclerosis following heart transplantation (34, 35). Despite the documented presence of human herpesviruses in lesional and nonlesional medial SMCs, it is not known what relationship the virus has with the host SMC. Human DNA viruses, particularly the herpesviruses, can produce either lytic infections with virus release, latent infections with reactivation, or host cell proliferation (36).

To demonstrate that viral genes alone may induce long-term SMC proliferation, we chose to create immortalized human SMC lines *in vitro* by infecting fetal aortic, adult aortic, and adult myometrial SMCs with a retroviral vector containing the E6/E7 open reading frames (ORFs) of human papillomavirus type 16 (HPV16). Although not expected to have a role in atherogenesis or leiomyoma formation, these DNA viral genes have been well characterized and have been shown to immortalize human keratinocytes and fibroblasts (37–40) by the inactivation of host proteins involved in cell cycle control. The E6 protein binds and promotes the degradation of the wild-type p53 protein (41, 42), and the E7 protein forms an inactivating complex with the product of the retinoblastoma tumor-suppressor gene (43, 44). These immortalized SMCs provide material to permit comparative studies of the genotypic and phenotypic properties of normal vessel wall versus atherosclerotic plaque SMCs, and myometrial versus leiomyoma SMCs.

MATERIALS AND METHODS

Primary Cell Culture. The human aortic SMCs were a gift from C. M. Giachelli and S. M. Schwartz (University of Washington, Seattle) and originated from the media of two distinct nondiseased thoracic aortas, one from a 2½-month-old fetus (therapeutic abortion) and the second from a 51-year-old male heart transplant recipient. Under sterile conditions, the aortic media was dissected from the intima and adventitia. The SMCs were isolated by enzymatic digestion (fetal SMCs, 2–4 hr; adult SMCs, 16 hr) with collagenase type I (165 units/ml), elastase type III (15 units/ml), and soybean trypsin inhibitor (0.375 mg/ml) and were plated initially in Waymouth's me-

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Abbreviations: HPV16, human papillomavirus type 16; HSV, herpes simplex virus; LTR, long terminal repeat; Neo^r, neomycin resistance; ORF, open reading frame; P*n*, passage level *n*; SMC, smooth muscle cell; SV40, simian virus 40.

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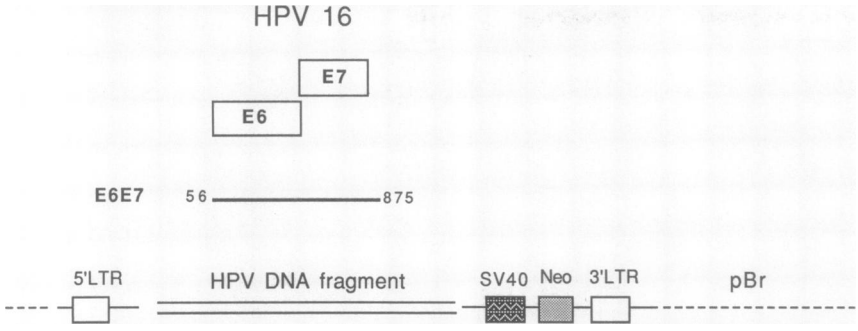

FIG. 1. Recombinant retroviral plasmid containing the HPV16 E6/E7 ORFs. (*Upper*) The HPV16 E6/E7 DNA fragment, nucleotides 56–875. (*Lower*) Retroviral vector pLXSN, with the HPV16 E6/E7 sequences, the Moloney murine leukemia virus promoter–enhancer sequences (LTR), the SV40 promoter, and the Neo^r gene. pBr, pBr322 plasmid sequence that contains the origin of replication and the gene conferring ampicillin resistance. Diagram was modified from Halbert *et al.* (47).

dium containing 20% fetal bovine serum and 2% endothelial cell growth supplement (Sigma). The myometrial SMCs were harvested from the uterus of a 49-year-old woman who underwent a hysterectomy for multiple leiomyomas. The myometrial tissue was selected from the middle third of the uterine wall, and leiomyoma cells were harvested from the center of medium-sized nodules. After fine mincing of the uterine tissue with scissors, the SMCs were dissociated by overnight digestion with type I (200 units/ml) collagenase (45). All the SMC lines and controls have been maintained in Dulbecco's modified Eagle's medium with high glucose (GIBCO) supplemented with 10% fetal bovine serum (HyClone), and antibiotics (penicillin, 100 units/ml; and streptomycin sulfate, 100 μg/ml; GIBCO). The SMCs are grown on 100-mm dishes, fed every 3 days, and when subconfluent, are divided into ratios of 1:3 (1.5 population doublings).

Retroviral Infection. The amphotropic helper-free retrovirus vector pLXSN was provided by A. D. Miller (Fred Hutchinson Cancer Research Center, Seattle). This replication-defective retrovirus construct contains the gene conferring resistance to neomycin (Neo^r) under the control of the simian virus 40 (SV40) promoter (46). As previously described, the HPV16 E6/E7 ORFs were inserted downstream of the 5′ long terminal repeat (LTR) promoter–enhancer sequences of the Moloney murine leukemia virus (47). A diagram of this construct is shown in Fig. 1. The human fetal and adult aortic SMCs were infected at passage level 5 (P5) and the human adult myometrial SMCs at P3. Control cells for each line included the noninfected parent SMCs with and without G418 treatment and the parent SMCs infected with the retroviral vector containing the LTR–Neo^r gene alone. The SMCs were infected with high titers of retrovirus, and the infection medium contained Polybrene at 4 μg/ml (46). The cells expressing the vector sequences were selected with medium containing the neomycin analogue G418 (GIBCO) at 0.75 mg/ml for 10 days. The pH of the medium was adjusted with 7.5% $NaHCO_3$ (5 μl/ml). After G418 selection, up to 300 colonies appeared on each retrovirally infected plate, and 18 clones were picked and frozen from each line. The noninfected parent SMCs treated with G418 did not survive at this drug concentration. For each retroviral SMC line, one clone and the remaining pooled population were then maintained continuously in tissue culture along with the untreated, noninfected parent SMCs.

Southern and Radioimmunoprecipitation Analyses. High molecular weight cellular DNA was isolated by standard procedures (48). DNA (10 μg) was digested with restriction enzymes *Eco*RI and *Bam*HI and electrophoresed in a 1.2% agarose gel. The DNA was transferred onto a Hybond-N nylon membrane (Amersham) and probed with a ^{32}P-labeled fragment of HPV16 E6/E7 released from a pUC18 plasmid. Radioimmunoprecipitation was performed as described (49). Cellular proteins were labeled with cysteine-free, methionine-free medium containing 200 μCi of [^{35}S]cysteine (Amersham) and 100 μCi of [^{35}S]methionine (Express-Mix, New England Nuclear). The cell lysates were incubated with rabbit polyclonal antisera generated against bacterial fusion proteins containing the HPV16 E6 and E7 polypeptides (50). Immunoprecipitated proteins were separated by SDS/17.5% polyacrylamide gel electrophoresis and detected by autoradiography.

Immunofluorescence. The SMCs were grown on glass chamber slides, washed in phosphate-buffered saline with 0.5% Tween 20 (Sigma), fixed in cold absolute methanol for 5 min at 4°C, and rinsed in cold acetone. The presence of muscle-specific α-actin was analyzed by indirect immunofluorescence with the monoclonal antibody HHF35 (Enzo Di-

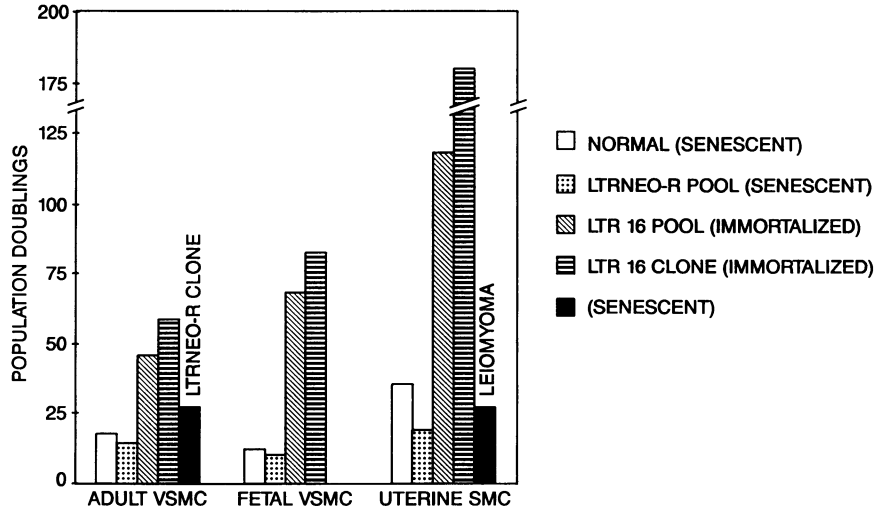

FIG. 2. Population doublings of immortalized HPV16 E6/E7 and control SMCs. Data were calculated from the time of retroviral infection (P0) up to present. Differences among the SMC types reflect when they were initially infected. The adult myometrial (uterine) HPV16 E6/E7 (LTR 16) clonal line has been in continuous culture for >1 year. VSMC, vascular (aortic medial) SMC.

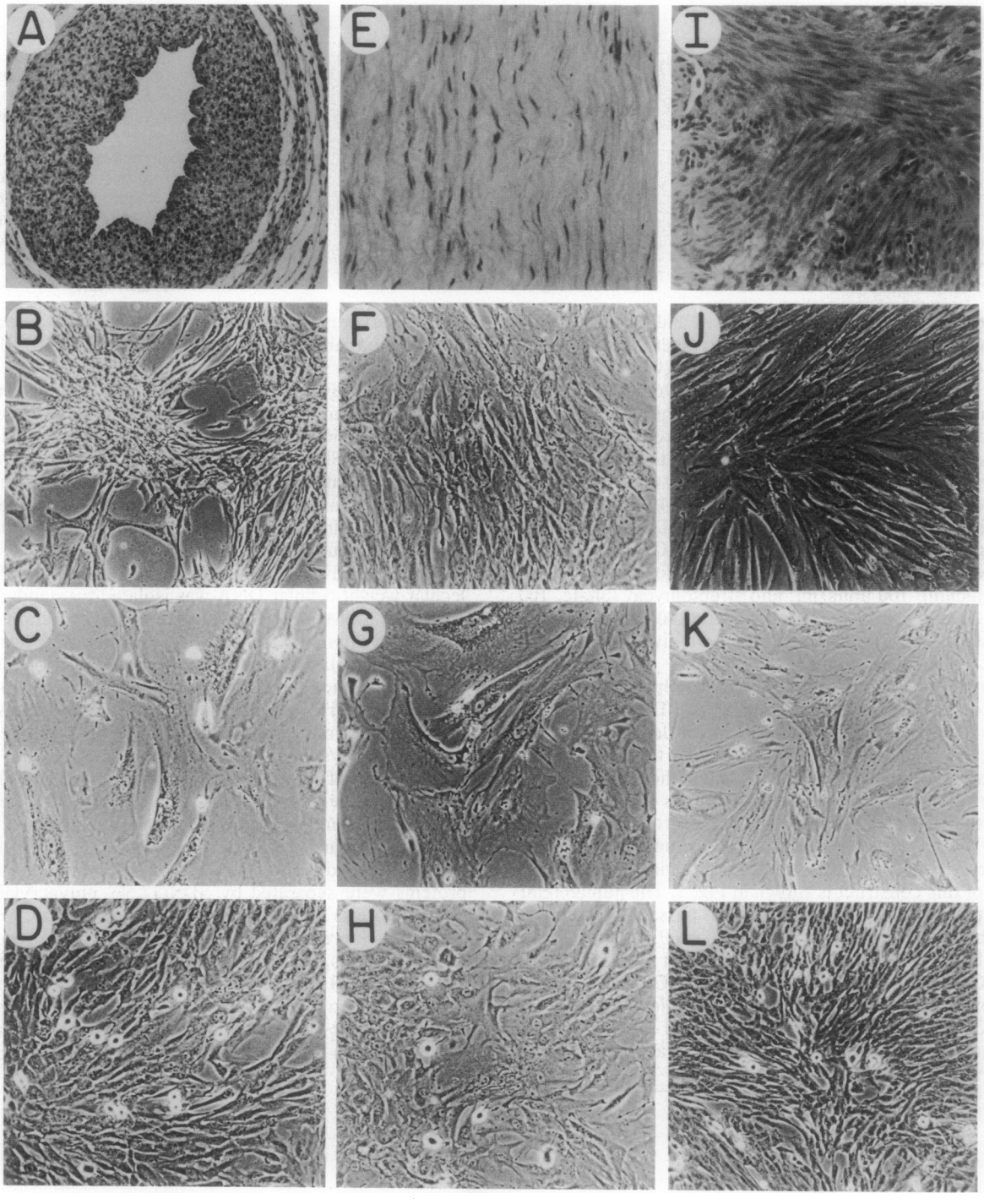

FIG. 3. Comparison of phase-contrast morphology of immortalized HPV16 E6/E7 SMCs versus parent SMCs. (*Left*) Human fetal thoracic aorta: tissue photomicrograph (*A*); noninfected early-passage fetal SMCs, P3 (*B*); noninfected senescing fetal SMCs, P8 (*C*); and HPV16 E6/E7 fetal SMCs, P23 (*D*). (*Middle*) Human adult thoracic aorta: tissue photomicrograph (*E*); noninfected early-passage adult SMCs, P3 (*F*); noninfected senescing adult SMCs, P11 (*G*); and HPV16 E6/E7 adult SMCs, P15 (*H*). (*Right*) Human adult myometrium: tissue photomicrograph (*I*); noninfected early-passage adult myometrial SMCs, P6 (*J*); noninfected senescing adult myometrial SMCs, P13 (*K*); and HPV16 E6/E7 adult myometrial SMCs, P35 (*L*). [Tissues (*A*, *E*, and *I*), ×200; cultured SMCs, ×100.]

agnostics, New York) at a dilution of 1:100. The secondary antibody, fluorescein isothiocyanate-conjugated goat anti-mouse IgG (Organon Teknika, West Chester, PA), was added at a 1:25 dilution.

Cytogenetic Studies. All SMC lines and controls were analyzed *in situ*. Chromosome modal number and aberrations were determined on 16 or more G-banded metaphases.

Tumorigenicity Assay. Five-week-old male BALB/c *nu/nu* athymic mice were irradiated with a ^{60}Co source [300 rads (3 Gy)] to reduce the possibility of rejection from host natural killer cells. Twenty-four hours after irradiation, the mice were injected subcutaneously in the scapular region with 3–9 × 10^6 middle-passage HPV16 E6/E7 and control SMCs. The mice were examined weekly for 6 months for the presence of tumors and then autopsied for further examination.

RESULTS

Growth Properties. A number of phenotypic differences between SMCs containing HPV16 E6/E7 and control cells

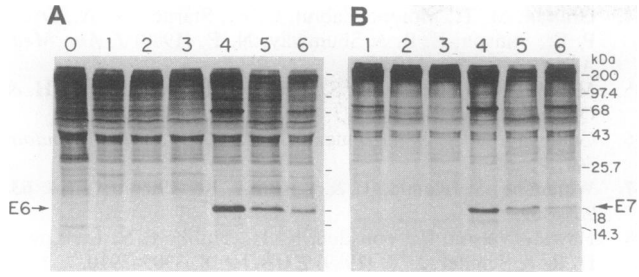

FIG. 4. Expression of the HPV16 E6 (*A*) and E7 (*B*) proteins as detected by radioimmunoprecipitation. Arrows indicate the location of the E6 and E7 proteins, and the molecular size markers are presented to the right. Lane 0, preimmune serum of E6. Lanes 1–3, noninfected parent: fetal aortic SMCs, adult aortic SMCs, and adult myometrial SMCs. Lanes 4–6, HPV16 E6/E7 immortalized lines at middle passage: fetal aortic SMCs, adult aortic SMCs, and adult myometrial SMCs.

have been observed. These include immortalization of all three HPV16 E6/E7 SMC lines with no signs of senescence. Fig. 2 illustrates the population doublings of cell lines and controls since the time of retroviral infection (P0). The noninfected SMCs and the LTR–Neo^r pools and clones senesced between P7 and P14 (41–107 days) from the day of infection. In contrast, the HPV16 E6/E7 SMC lines have remained in continuous culture. The myometrial SMCs were the first cells to be retrovirally infected and have been in continuous tissue culture for >1 year (>180 population doublings), whereas the fetal and adult aortic SMCs were infected more recently.

From the time of infection, the HPV16 E6/E7 SMCs immediately manifested a much increased growth rate, with passage once every 3 days versus once a week for the control cells. Cell size is markedly decreased and growth occurs in a sheetlike, random pattern unlike the control cells, which form the characteristic "hills and valleys" pattern of SMCs in tissue culture (Fig. 3). No further growth factor supplementation is required and they reach higher cell densities than controls and continue to divide without growth inhibition at confluency. In addition, they survive in serum-depleted medium (0.5% fetal bovine serum). At approximately middle passage (P30), the HPV16 E6/E7 SMCs decreased in growth rate, with passage once every 7–10 days, but within 2 months had resumed their initial rapid doubling rate.

Expression of Viral DNA and Protein. Southern blot analysis detected the released 819-base-pair fragment of the HPV16 E6/E7 ORFs in the retrovirally infected SMCs at middle passage (data not shown). The control SMCs and leiomyoma cells did not contain HPV16 sequences. Radioimmunoprecipitation at middle passage confirmed the production of the E6 and E7 proteins in all the HPV16 E6/E7 SMCs lines (Fig. 4).

Immunofluorescence. Cells harvested from primary tissues were confirmed to be of SMC origin by the presence of muscle-specific α-actin filaments as shown by indirect immunofluorescence (51). The α-actin filaments in the HPV16 E6/E7 SMCs stained less intensely and were distributed in a more irregular pattern.

Chromosomal Analysis. Karyotyping of the control SMCs and leiomyoma cells revealed diploid cell populations. The HPV16 E6/E7 SMCs lines have remained essentially diploid, with only occasional, random chromosome loss. However, the myometrial HPV16 E6/E7 clonal line did become aneuploid with several marker chromosomes at late passage (>125 population doublings).

Tumorigenicity. None of the control SMCs nor HPV16 E6/E7 SMC lines formed tumors in irradiated nude mice after 6 months.

DISCUSSION

We have described the establishment of permanent human aortic and myometrial SMC lines. These SMCs were immortalized using the HPV16 E6/E7 ORFs in a defective retrovirus vector containing a selectable marker gene. The retroviral vector system was chosen to ensure high gene-transfer efficiency and SMC viability. Stable integration and expression of the HPV16 E6/E7 DNA sequences were observed. At this time, the immortalized cells have up to 7 times the population doublings and 4 times the life-span of the control cells and show no signs of senescence. The first retrovirally infected SMC line has been in continuous culture for >1 year. In addition, these cells have an increased growth rate, decreased cell size, and changes in SMC α-actin filament distribution and staining intensity. Chromosomal analysis of the HPV16 E6/E7 SMC lines has revealed near-diploid populations with some random chromosome loss, whereas the late-passage myometrial clonal line has become aneuploid with several marker chromosomes. Neither of the three SMC lines nor control SMCs are tumorigenic in irradiated nude mice. These features suggest that the immortalized human SMCs have retained expression of SMC α-actin and that they may have acquired characteristics comparable to those of *in vivo* benign proliferating SMCs.

Since the HPV16 E6/E7 ORFs are known to be sufficient to immortalize human keratinocytes and fibroblasts (37–40), we chose these DNA viral genes to attempt to immortalize human SMCs. Characterization of these viral gene products has revealed that they interact with host cell cycle regulatory proteins (41–44). Previous attempts to immortalize SMCs with viral DNA sequences have been achieved only in rat and rabbit aortic medial SMCs, by using SV40 and HSV-2 (52–54). When SV40 early-region genes were transfected into human umbilical artery SMCs, transformation occurred without immortalization (55). Human leiomyoma cells transformed with these SV40 genes were shown to retain their original chromosomal translocation throughout the pre-crisis period (56). The SMCs in many of the above experiments share several features in common with the immortalized human SMC lines described in this paper. These include an increased growth rate, altered morphology, and decreased SMC α-actin content.

Apart from immortalized animal SMC lines, the phenotype of benign proliferating human SMCs has been difficult to study in tissue culture because atherosclerotic plaque SMCs and leiomyoma SMCs have an *in vitro* life-span and doubling rate equal to or less than those of normal parent tissue (57). This decreased proliferative capacity in plaque SMCs is presumed to be secondary to a greater number of *in vivo* cell divisions (58). For these reasons, we chose to immortalize human SMCs from aortic media and myometrium in order to create an *in vitro* model of long-term proliferation that would mimic the atherosclerotic plaque and leiomyoma SMCs and allow further characterization of their altered phenotypic properties (59, 60). In addition, we have shown that DNA viral genes alone are sufficient to induce continual human SMC proliferation *in vitro*.

We thank Drs. Stephen C. Conroy and Lenora R. Garrett for scientific advice and critical reading of the manuscript. This research was supported by National Institutes of Health Grants HL03174, HL07312, and CA42792.

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