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J Clin Invest. 1995;95(2):464-468. https://doi.org/10.1172/JCI117686.

Research Article

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Extinction of Autonomous Growth Potential in Embryonic:Adult Vascular Smooth Muscle Cell Heterokaryons

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Abstract

Vascular smooth muscle cells (SMC) isolated from embryonic and early fetal (e13-e18) rat aortas exhibit an "embryonic growth phenotype" in culture (Cook, C. L., M. C. M. Weiser, P. E. Schwartz, C. L. Jones, and R. A. Majack. 1994. *Circ. Res.* 74:189–196). Cells in this growth phenotype exhibit autonomous, serum-independent replication, in contrast to SMC in the "adult" growth phenotype, whose proliferation in culture is dependent on exogenous mitogens. To determine which of these two phenotypes is genetically dominant, heterokaryons were constructed between adult and embryonic (day e17) rat aortic SMC. The fused cells were maintained in serum-free medium for 3 d, then were labeled with bromodeoxyuridine (BrdU) for an additional 24 h. Under these conditions, parental e17 SMC exhibited a high rate of self-driven DNA synthesis (73–85% BrdU-positive cells), while parental adult SMC showed minimal replication (13-21% BrdU-positive cells). Homokaryons of parental cells exhibited parental growth phenotypes and showed the expected mitogenic response when stimulated with serum. Heterokaryons between e17 and adult SMC exhibited a nonautonomous growth phenotype; the "adult" growth phenotype was calculated to be dominant in >89% of all true heterokaryons. The data suggest that adult SMC express molecules capable of genetically extinguishing or otherwise inhibiting the autonomous replication of embryonic SMC. (*J. Clin. Invest.* 1995. 95:464–468.) Key words: vascular smooth muscle • cell division • heterokaryons • cell fusion

Introduction

Vascular smooth muscle cells (SMC)[1] occupy a pivotal position in the pathogenesis of a number of vascular disease states including arteriosclerosis, systemic and pulmonary hypertension, and restenosis after angioplasties or vascular reconstructions. Each of these conditions may be characterized, to varying degrees, by the abnormal replication of vascular SMC. Typically, the proliferation of adult SMC during vascular disease is associated with the reiteration of immature patterns of gene expression (1–6, and Belknap, J. K., N. A. Grieshaber, P. E. Schwartz, M. A. Reidy, and R. A. Majack, manuscript submitted for publication). Despite extensive in vivo and in vitro research, little is actually known about the specific mechanisms which control either the replication or differentiated state of these cells under normal, developmental, or pathological conditions.

In most tissues, cellular differentiation or maturation is associated with dramatic changes in gene expression and replication rates and/or potential. During rat aortic development in vivo, SMC replication has been shown to decrease from an embryonic rate of ~ 80% per day (until day e18) (8) to an adult rate of < 0.06% per day (9, 10). A major change in replication potential also occurs: early in development (through day e18), aortic SMC exhibit autonomous (serum-independent) replication in culture; this autonomous growth potential appears to be completely lost by day e20 (8). The factors which drive replication of SMC in the embryonic growth phenotype, and the mechanisms underlying the developmentally timed loss of this phenotype, are unknown.

In this report we describe the growth phenotype of heterokaryons formed between autonomously replicating embryonic SMC and their adult counterparts. Analysis of such heterokaryons, in which nuclear integrity is maintained while cytoplasmic and cell-surface molecules are mixed, allows the identification of *trans*-acting and diffusible factors responsible for extinguishing or inducing specific traits in the nonexpressing parental cell. Specifically, in our studies, we sought to determine if adult SMC contain molecules which actively suppress autonomous replication.

Methods

Smooth muscle cells. Embryonic (day e17) aortic SMC were isolated, cultured, and characterized as described by Cook et al. (8). The isolates of e17 SMC used for these experiments were selected for their high capacity for autonomous replication (the cultures exhibit a 24-h bromodeoxyuridine [BrdU] labeling index, in serum-free medium, of 73–85%) and were used in the 10th through 17th passage. SMC cultured from adult rat aortas were used at similar passages. All cells were cultured in DME supplemented with 10% calf serum (CS). All tissue culture supplies were purchased from GIBCO BRL (Gaithersburg, MD).

Cytoplasmic and nuclear labeling. Cultures in T-75 flasks were cytoplasmically labeled with either FITC-labeled or nonfluorescent latex microspheres (0.22 μm; Polysciences, Inc., Warrington, PA) for 24 h before cell fusions. Microspheres were sterilized in 70% EtOH, resuspended in two volumes of PBS, and added to cultures at a 1:30 dilution. Cultures were washed three times with serum-free medium (SFM) before fusions. The number of beads per parental cell ranged from 10 to > 100. Cells containing FITC-conjugated microspheres were easily detected under UV fluorescence using a fluorescein filter set; cells containing non-fluorescent microspheres were detected under dark-field illumination, under which the microspheres appeared either white or pink

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Received for publication 11 August 1994 and in revised form 10 October 1994.

1. *Abbreviations used in this paper:* BrdU, bromodeoxyuridine; CS, calf serum; SFM, serum-free DME; SMC, smooth muscle cells.

J. Clin. Invest.

0021-9738/95/02/0464/05 $2.00
Volume 95, February 1995, 464–468

(see below). BrdU immunocytochemistry was used as a marker for DNA synthesis. BrdU (10 mM/ml) was added to cultures for 24 h before fixation in ice-cold methanol containing 0.3% H_2O_2, and immunocytochemistry was performed as previously described. As shown below, this protocol allowed the simultaneous detection, under concurrent fluorescence-dark field illumination, of FITC-labeled and unlabeled microspheres and BrdU-positive and BrdU-negative cells.

Construction of heterokaryons and experimental design. After cytoplasmic labeling of adult and e17 SMC, T-75 cultures were extensively washed to remove any extracellular microspheres. Typically, cells from both parental populations were trypsinized, pelleted, and combined in a small volume (125 μl). Over a 1-min period, 62 μl 42% polyethylene glycol (PEG) (MW 8,000) (Sigma Chemical Co., St. Louis, MO) was added followed by 62 ml 25% PEG, added over a 2-min period. 500 μl of DME was then added and the cells were pelleted, resuspended in DME containing 10% CS, and plated on Lab-Tek one-well chamber slides (Nunc, Inc., Naperville, IL). Cells were allowed to attach and spread for 24 h. The cultures were then maintained for 72 h in serum-free DME (SFM), then were labeled with BrdU as described above for an additional 24 h in the absence or presence of 10% CS. For each experiment, parallel control cultures of parental cells were labeled with microspheres, plated onto chamber slides, maintained in SFM for 72 h, then labeled with BrdU for 24 h to determine basal replication rates.

Data analysis. A total of five e17:adult fusion experiments were performed, with qualitatively identical results. A minimum of 120 fusion cells were identified and counted for each individual test condition per experiment. Cells were classified as heterokaryons if they contained two nuclei and if they contained a significant number of both nonfluorescent microspheres (appearing pink under UV fluorescence-dark field illumination) and FITC-labeled microspheres (appearing green under UV fluorescence illumination). Identification of any particular cell as a heterokaryon therefore depended on the simultaneous occurrence of two unambiguously scored independent markers. Heterokaryons were considered to be positively labeled with BrdU if either nucleus of the dikaryon contained peroxidase reaction product. In practice, > 80% of the heterokaryons labeled as "positive" contained two BrdU-labeled nuclei. Rare heterokaryons containing more than two nuclei were not included for analysis. As controls, homokaryons of e17 parents and of adult parents were constructed and analyzed in an identical fashion.

To determine the per cent dominance of the adult (nonautonomous) phenotype, it was necessary to adjust the data for contributions due to heterogeneity within the parental cells (see reference 11 for a more complete discussion). Assuming random fusion of the parental cells, the data were adjusted as follows (11). The frequencies of all parental phenotypes (BrdU positive or negative after 72 h in SFM) were determined. No differences were observed in labeling indices of parental cell populations cultured individually, after treatment with PEG, or in coculture with the other parental population. Adult SMC replicated at a low but significant level when maintained under serum-free conditions (for the cells used in these experiments, this value was 0.13–0.21); these replicating cells therefore represent a potentially autonomous subpopulation of adult SMC. Similarly, ~ 0.15–0.27 of the e17 parental cells failed to replicate DNA during the 24-h labeling period; they therefore appear as nonautonomous cells and potentially exhibit the adult phenotype. The predicted frequency for each false heterokaryon class possibly contributing to any given phenotype was then determined, and this value was subtracted from the observed fraction of heterokaryons exhibiting the expected behavior for that phenotype. This corrected dominance frequency was then divided by the predicted frequency of heterokaryons derived from parental cells exhibiting the "true" BrdU phenotype (BrdU-negative for adult SMC, BrdU-positive for e17 SMC). For example, for the data presented in Fig. 3 (*Experiment 1* in Table I), the calculation of per cent dominance of the adult phenotype in e17:adult heterokaryons was as follows. The observed frequency of heterokaryons with no labeled nuclei was 0.82. Since 0.15 of e17 cells behaved as adult cells, exhibiting nonautonomous replication, the corrected frequency of dominance of the adult (nonautonomous) phenotype becomes 0.67. The percent dominance of the adult cell phenotype was then calculated by dividing 0.67 by 0.71 (the predicted frequency of "true" heterokaryons (the frequency of "true" adult cells [0.83] × the frequency of "true" autonomous cells [0.85]). Thus the nonautonomous state was calculated, for this experiment, to be the dominant phenotype in 94.4% of true e17:adult SMC heterokaryons.

Results

The parental cells used in these studies were: (*a*) highly replicative cultures of embryonic (day e17) rat aortic SMC, exhibiting a 24-h BrdU labeling index of 73–85% after 72 h in SFM (Fig. 1, *a* and *b*) matched cultures of adult (3 mo) rat aortic SMC, exhibiting a 24 h BrdU labeling index of 13–21% after 72 h of serum deprivation (Fig. 1 *b*). The purpose of these experiments was to construct heterokaryons of autonomously replicating e17 SMC and nonautonomous adult cells, then to assess their ability to replicate in SFM (by definition, an assay for autonomous, self-driven replication) to determine which phenotype (autonomous or adult) was genetically dominant. Parental cells were fused as described, plated on glass slides, growth-arrested for 72 h in SFM, then assayed for DNA synthesis in individual heterokaryon nuclei by BrdU immunocytochemistry. Homo/heterokaryons were defined as such based on two criteria: (*a*) the presence of two nuclei, and (*b*) the presence of significant quantities of both cytoplasmic labels (Fig. 2). Cells were considered BrdU-positive if either or both nuclei was labeled. Over 80% of all fused cells scored as BrdU-positive contained two labeled nuclei.

We first constructed homokaryons of parental SMC, as controls to assess the effects of PEG treatment on the ability of fused SMC to replicate. As shown in the experiment presented as Fig. 3, adult SMC maintained in SFM for 96 h (labeled with BrdU for the final 24 h) exhibited a labeling index of 17%. Stimulation of these cells with 10% CS for the final 24 h increased the labeling index to 68%. Adult:adult homokaryons exhibited a labeling index of 15%. Importantly, these cells were fully responsive to the mitogenic effects of 10% CS, which increased the replication rate to 72%. Embryonic SMC (e17) in this experiment exhibited a basal replication rate of 85%; this level of DNA synthesis was maintained in e17:e17 homokaryons. The data therefore demonstrate that PEG-treated, fused SMC are viable, do not lose the ability to replicate DNA in response to normal growth stimuli, and exhibit the expected parental growth phenotypes.

In serum-deprived e17:adult heterokaryons, the labeling index (for the experiment shown) (Fig. 3) was found to be 18%, roughly identical to that of adult parental cells and adult:adult heterokaryons. After stimulation with 10% CS, the replication index increased to > 80%, demonstrating the viability of these heterokaryons. The data demonstrate that adult SMC contain or produce molecules capable of repressing the autonomous replication of the e17 nuclei.

The percent dominance of the adult (BrdU-negative after 72 h in SFM) phenotype in homo/heterokaryons was calculated as described in Methods. In adult:adult homokaryons, the adult phenotype was dominant in > 90% of the fused cells. In e17:e17 homokaryons, the per cent dominance of the adult phenotype was 0%. In five separate e17:adult fusion experiments, the adult phenotype was dominant in 94.4, 96.7, 86.7, 80.3, and 88.2% of heterokaryons (see Table I). The autonomous DNA synthesis observed in e17-derived nuclei therefore was repressed in the context of e17:adult SMC heterokaryons. We conclude that

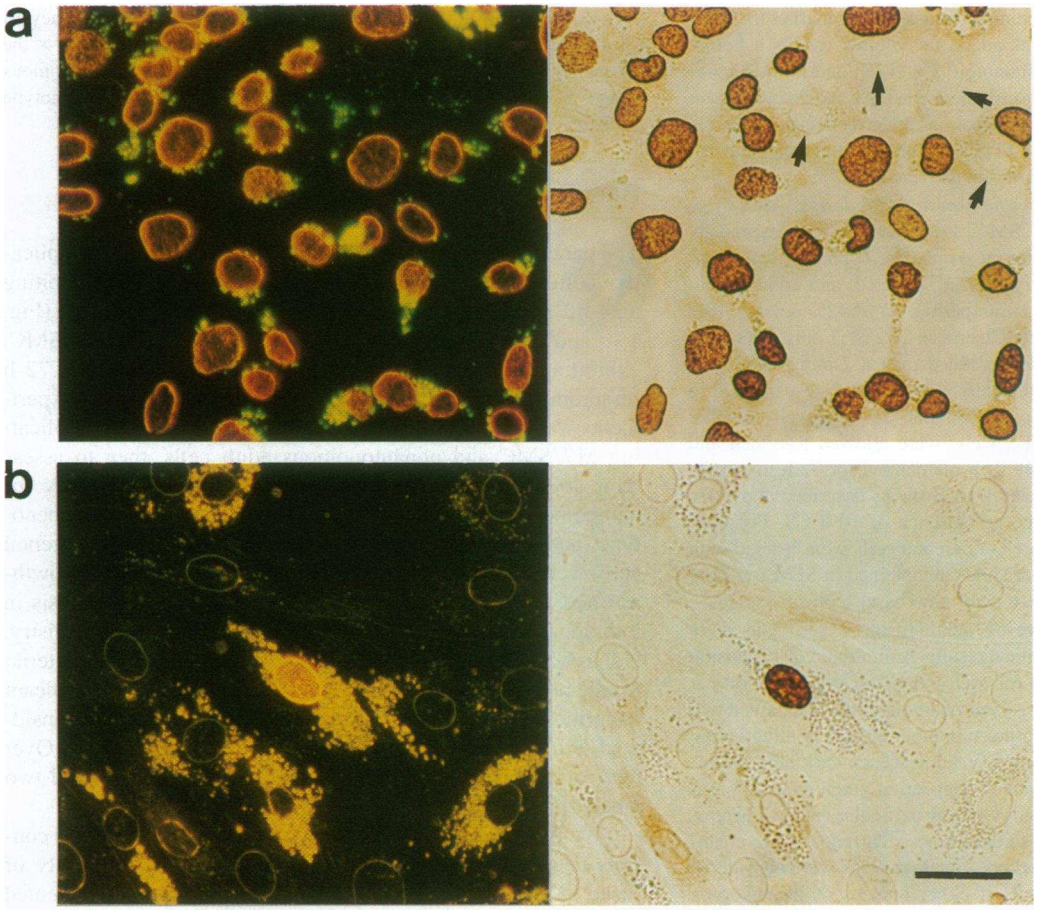

Figure 1. Characteristics of parental SMC heterokaryons. (*a*) Cultures of e17 vascular SMC, labeled cytoplasmically with fluorescein-labeled microspheres, maintained in SFM for 72 h, labeled with BrdU for an additional 24 h, then immunocytochemically stained with an antibody against BrdU to detect replicating cells. BrdU-positive nuclei appear orange under dark field–UV fluorescent illumination (*left*) and brown under phase–contrast or bright field illumination (*right*). Arrows in *a* indicate BrdU-negative nuclei. (*b*) Cultures of adult SMC, treated as described above after cytoplasmic labeling with nonfluorescent microspheres. Bar, 50 μm.

adult SMC contain a nuclear or cytoplasmic factor which is capable of suppressing or blocking the autonomous replication of embryonic SMC.

Discussion

Progressive and sequential changes in gene expression, cellular phenotype, and replication potential drive and regulate most developmental and morphogenetic processes. Appropriate controls over morphogenesis during the development and repair of the vascular system appear to be particularly crucial given the pathological consequences of abnormal blood vessel structure. Examples of vascular pathologies exacerbated or caused by abnormal SMC replication include hypertension (characterized by an increased thickness of the tunica media), vascular malformations (characterized by an underdeveloped tunica media), and arteriosclerosis and restenosis (characterized by the development of a neointima). Little is known concerning the factors which positively or negatively regulate SMC replication during blood vessel development and morphogenesis. The goal of our research is to elucidate the morphogenic mechanisms which determine why SMC stop replicating once blood vessel structure is established; it is our hope that a knowledge of the endogenous growth-suppressive mechanisms operative in the normal blood vessel wall will lead to the eventual development of therapeutic agents effective against vascular disease states involving unregulated SMC proliferation.

A variety of previous studies have utilized heterokaryons to study mechanisms involved in tumor suppression (12, 13), cellular differentiation (14–19), apoptosis (20), and replication potential (11). Typically, when cultured cells expressing differentiated functions are fused, the differentiated functions of the parental cells are "extinguished" by factors from the other parental cell type (16, 17), leading to the concept that differentiated functions must be actively maintained (18, 19). When cells of the same developmental lineage are fused (e.g., when undifferentiated myoblasts are fused with differentiated muscle cells), the heterokaryons tend to express the more differentiated functions (11, 14). We have used this technology to study the relative dominance of specific growth phenotypes differentially expressed by embryonic and adult vascular SMC.

During rat aortic development in utero, SMC maturation is associated with the loss of capacity for autonomous replication (8). Aortic SMC isolated from embryos and fetuses aged e13-e18 exhibit a significant capacity for self-driven replication in culture, and replicate freely under serum-free conditions. The

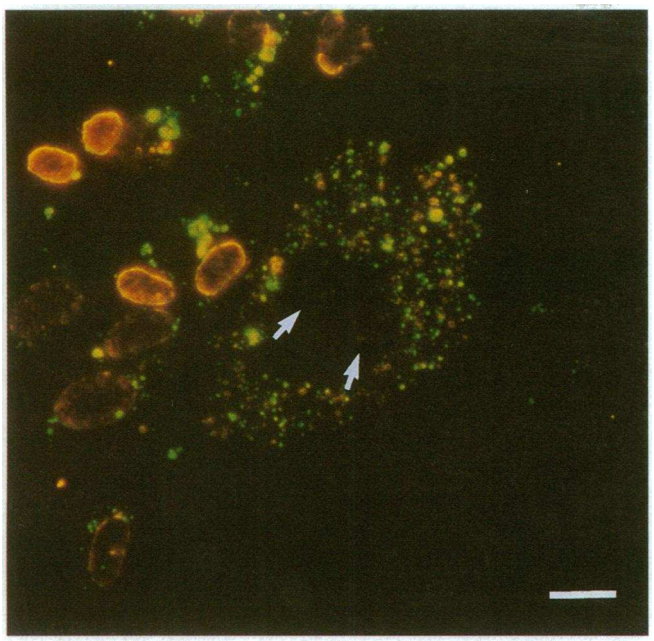

Figure 2. Identification of e17:adult heterokaryons. Heterokaryons were formed between autonomously replicating, fluorescein-labeled e17 SMC and their adult counterparts labeled with nonfluorescent microspheres. After fusion, cells were plated and allowed to attach and spread overnight in 10% CS, then were maintained in serum-free DME for a total of 96 h; BrdU was present in the cultures for the final 24 h. Cultures were immunocytochemically stained for BrdU; labeled nuclei appear orange under dark field illumination. Cells containing two nuclei and significant amounts of both cytoplasmic labels were classified as heterokaryons. Note that both nuclei (indicated by arrows) of the e17:adult SMC heterokaryon shown are negative for BrdU staining. Bar, 25 μm

molecular basis for this self-driven, embryonic growth phenotype remains unknown. By day 20 of fetal life, the capacity for autonomous replication is completely lost and SMC of older gestational ages exhibit an "adult," serum-dependent growth phenotype in culture (8). SMC replication during vascular morphogenesis therefore appears to be associated with the successive expression of at least two distinct growth phenotypes. The changes in gene expression which occur across this developmentally timed switch, and the mechanisms controlling these changes, remain under investigation.

The results of the experiments described herein demonstrate that, in embryonic:adult SMC heterokaryons, the adult or nonautonomous growth phenotype is dominant. The results are not due to non-specific growth-inhibitory effects of PEG treatment, since PEG-treated e17 SMC homokaryons maintain the ability to synthesize DNA autonomously in culture, and since adult:adult homokaryons and e17:adult heterokaryons replicate DNA normally in response to serum stimulation. The data therefore indicate that adult SMC acquire, during development, a factor which suppresses or inhibits the autonomous growth potential of embryonic SMC.

The factor may act as a nuclear or *trans*-acting factor to suppress the transcription of specific genes involved in driving autonomous replication. The wild-type Wilms tumor gene product, for example, is known to suppress transcription of the A-type platelet-derived growth factor and insulin-like growth factor-1 genes (21, 22). While neither of these gene products are believed to be involved in driving the replication of SMC in the "embryonic growth phenotype" (reference 8 and unpublished data), a similar mechanism, involving the repression of embryo-specific gene expression by an adult-specific *trans*-acting factor, may be operative. Alternatively, the factor responsible for the extinction of the self-driven growth properties of embryonic SMC may act cytoplasmically or on the cell surface. For example, an adult-specific phosphatase, responsible for reversing phosphorylation events involved in driving cellular replication, could maintain normal growth control in adult SMC and consequently in e17:adult heterokaryons. A variety of other models can also be postulated and will be investigated in future studies.

The heterokaryon data also reveal information concerning the molecular mechanisms which drive embryonic SMC growth. These and previous co-culture studies (8) suggest that autonomously replicating e17 SMC do not secrete factors capable of mitogenically stimulating adult SMC. The results of the present study support this concept: if embryonic self-driven replication were due to the overexpression of a mitogen capable of stimulating adult SMC replication, the e17:adult heterokaryons would have been expected to exhibit e17-like growth characteristics.

Regardless of the mechanism of extinction, it is important to recognize that the mechanism responsible for the repression of autonomous replication potential during SMC maturation appears to be actively maintained in the adult. This is apparent given that the inhibitory factor is present in adult SMC (aged 3 mo), long after self-driven growth is initially suppressed during development. Like tumor suppressor genes, expression of this developmentally regulated extinguisher locus may therefore be continuously essential for normal SMC growth control. Loss of

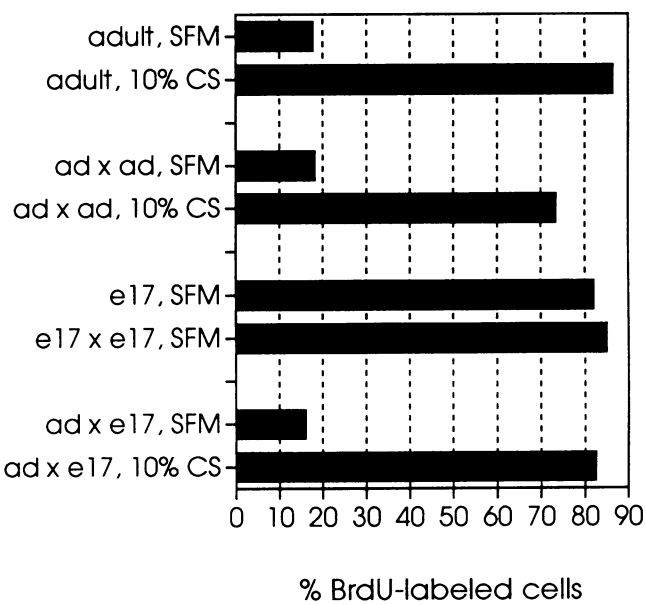

Figure 3. Replication potentials of parental SMC and of homo- and heterokaryons. Parental adult (*ad*) and embryonic (*e17*) SMC, e17 × e17 and ad × ad homokaryons, and e17 × ad heterokaryons were maintained in SFM for 72 h, labeled with BrdU for an additional 24 h, then were processed for BrdU immunocytochemistry to detect replicating nuclei. As indicated, 10% CS was added to specific cultures to determine the ability of the cells to respond to normal growth stimuli. See text for further details.

Table I. Dominance of the "Adult" (Nonautonomous) Growth Phenotype in Adult:Embryonic SMC Heterokaryons

	Percentage cells containing BrdU-labeled nuclei after 72 h in SEM			Percentage dominance of "adult" phenotype
	Adult	e17	e17:adult heterokaryons	
Experiment 1	0.17	0.85	0.18	94.4
Experiment 2	0.21	0.85	0.20	96.7
Experiment 3	0.17	0.75	0.21	86.7
Experiment 4	0.13	0.73	0.22	80.3
Experiment 5	0.15	0.80	0.18	88.2

this suppressor in adult vascular SMC, by mutation, altered regulation, or reversion of the cells to a less differentiated state, may lead to the reexpression of autonomous growth potential in pathological conditions such as hypertension, restenosis, and arteriosclerosis.

Acknowledgments

This work was supported by grants HL-46481 and HL-47685 from the National Institutes of Health.

R. A. Majack is a Genentech-American Heart Association Established Investigator.

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