Proc. Natl. Acad. Sci. USA
Vol. 81, pp. 2102–2106, April 1984
Cell Biology

Identification of glial filament protein and vimentin in the same intermediate filament system in human glioma cells

(immunoelectron microscopy/colloidal gold/protein A/cytoskeleton)

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Communicated by Igor Tamm, December 23, 1983

ABSTRACT **We have used a human glioma cell line (U-251MG) to study the expression and cytoplasmic organization of vimentin (decamin) and the glial filament protein (GFP). Four clones of the parental U-251 cultures were isolated and found to express GFP from 1–2% to 99% of the cells in the population. Double immunofluorescence microscopy with antibodies to vimentin and GFP has shown that, in all four clonal cell lines, vimentin-containing filaments are expressed in most cells as an organized network and, in GFP-positive cells, GFP and vimentin are associated with the same filament network. Immunoelectron microscopy with specific antibodies labeled with colloidal gold particles of various sizes shows that GFP and vimentin are localized in the same filaments. These findings confirm *in vitro* studies of the copolymerization of subunits of different biochemical nature into the same intermediate filament and suggest the *in vivo* probability of the coassembly of GFP and vimentin from a possible soluble pool of monomers.**

Intermediate filaments (IF), structurally identified as 10 nm in diameter, are an essential component of the cytoskeletal architecture of most eukaryotic cells. Although these filaments appear morphologically identical in different cell types, biochemical and immunological characterization has established five classes of IF, which include tonofilaments (keratin), neurofilaments, glial filaments, smooth muscle filaments (desmin), and fibroblastic filaments (vimentin) (1). The expression of the different filaments has been found to be highly tissue specific (2–9).

Such tissue specificity notwithstanding, biochemically distinct filaments have been observed in the same cell type. For example, in epithelial cells, vimentin-containing filaments form a cytoskeletal network independent of keratin filaments (10). In all reported cases, such dual presence is found with vimentin filaments as one of the constituents. However, in smooth muscle cells, immunofluorescence studies have shown that antibodies to vimentin and desmin show an identical distribution (6–8, 11–12). In some astrocytes, vimentin and glial filament protein (GFP) have been localized in the same cells (13). In biochemical studies, Steinert *et al.* (14) have demonstrated the *in vitro* copolymerization of vimentin and various keratin subunits into filaments 10 nm wide. Furthermore, tonofilaments are reported to be formed into filamentous polymers by two different types of keratin proteins (15, 16). Similar biochemical analysis shows that vimentin and desmin as well as vimentin and GFP can be cross-linked chemically to form heteropolymeric filaments (17, 18). These results indicate a high probability that vimentin can be expressed *in vivo* as a constituent coexisting with other members of the IF family of proteins in the same filaments.

The present paper reports that, in glial astrocytoma cells, GFPs can be physically distributed in the same filament system with vimentin. This finding suggests that the differentiation-associated glial filament proteins and vimentin can be assembled post-translationally into the insoluble IF scaffold. Similar results have been reported by Sharp *et al.* (19).§

MATERIALS AND METHODS

Cells. The human glial astrocytoma cell line (U-251) was originally from the biopsy specimen of a glioma (20). Four separate clones U-251-1, -4, -5, and -6 were obtained by several successive clonings. Clones 1 and 5 contain cells that show expression of GFP, while cells in clones 4 and 6 do not express GFP (20, 21). The cultures were grown in reinforced Eagle's medium/10% fetal calf serum in a humid atmosphere of 95% air/5% CO_2 at 37°C.

Fluorescence Microscopy. Cells grown on no. 1 coverslips (Corning) were processed for staining as described previously (22). Visualization of the filament distribution was done with a Zeiss photomicroscope III equipped with epifluorescence illumination and a 63× objective. In examining samples that had been processed for both fluorescein and rhodamine markers, a special filter of 546 nm was used to ensure the separation of the two light spectra.

Antibodies. Identification of vimentin-containing IF was done by incubating the cell specimens with a rabbit polyclonal antibody that recognizes vimentin (23). The GFP-containing filaments were identified by labeling with rabbit or guinea pig polyclonal antibodies to the GFP (24). Cell specimens were further incubated with fluorescein-conjugated goat anti-rabbit or goat anti-guinea pig immunoglobulin (IgG) or with rhodamine-labeled sheep anti-rabbit IgG.

Indirect Immunoelectron Microscopy. Ultrastructural localization of the position of vimentin and GFP on the individual filaments were done by indirect labeling with protein A conjugated with colloidal gold particles of various sizes. Cells were initially grown on Mylar plastic coverslips. After fixation and extraction as described in the procedure for fluorescence microscopy, the cell specimens were incubated with guinea pig anti-GFP or rabbit antibody to vimentin at 37°C for 2 hr with constant shaking. These samples were then rinsed with three changes of phosphate-buffered saline containing bovine serum albumin at 5 mg/ml for 2 hr and incubated with goat anti-guinea pig IgG conjugated with 5-nm gold particles to localize the sites of reaction for guinea pig IgG in the specimens. For labeling the sites of reaction of rabbit antivimentin IgG, protein A conjugated with 20-nm gold particles was used as the secondary antiserum. Incubation of the colloidal gold-conjugated antiserum was also car-

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Abbreviations: IF, intermediate filament(s); GFP, glial filament protein.

§This work has been presented in abstract form. 1982 meeting for the American Society of Cell Biology, Baltimore, Maryland, November 1982.

ried out at 37°C for 2 hr. Afterward, the samples were rinsed with albumin-containing phosphate-buffered saline fixed with 1% glutaraldehyde in phosphate-buffered saline, treated with osmium tetroxide, dehydrated, and embedded as described (22). To ensure clear visualization of individual filaments, overnight *en bloc* uranyl acetate staining was done before dehydration.

For double immunoelectron microscopy, the samples were first incubated with guinea pig antibody to GFP and then labeled with goat anti-guinea pig IgG conjugated with 5-nm gold particles. The specimens were then processed for further labeling with rabbit antibody to vimentin and protein A conjugated with 20-nm gold particles. All antibody incubations were done at 37°C for 2 hr and the specimens were rinsed with albumin-containing phosphate-buffered saline before each incubation.

Colloidal gold particles were prepared according to the procedure of Faulk and Taylor (25). Particles 5 nm in diameter were conjugated with protein A (Sigma) following the procedure for gold–protein conjugation of Horrisberger *et al.* (26).

Immunoadsorption. The antivimentin or anti-GFP staining on the filaments was verified by adsorption of the antibodies with their respective antigens as described (24, 27). These adsorbed sera were then used as reagents for antibody labeling. In some instances, immunocompetition experiments were done to test the specificity of binding between antigens and antibodies. For example, rabbit antivimentin IgG was incubated with the Triton-insoluble cytoskeleton specimens in the presence of excess purified vimentin (2 mg/ml) isolated from cultured BHK-21 cells.

Biochemical Analysis. A crude cytoskeletal preparation, composed mainly of IF, was prepared as described by Burr *et al.* (28), except that a higher concentration (5%) of Nonidet P-40 was used to obtain better extraction of elements other than GFP and vimentin from the cell. For one-dimensional gel electrophoresis, 7.5% polyacrylamide gels were prepared by the method of Laemmli (29). Two-dimensional isoelectrofocusing $NaDodSO_4$/PAGE was carried out according to the procedure of O'Farrell (30). Immunoblotting was carried out by a modification of the method of Towbin *et al.* (31) as described elsewhere (23).

RESULTS

Biochemical Analysis. The two-dimensional isoelectrofocusing gel pattern of the cytoskeletal components of the cells from clone 5 is shown in Fig. 1. The major proteins of the cytoskeleton migrate with approximate M_r values of 50,000 and 58,000 (arrows). These two major proteins of the cytoskeleton of clone 5 focused to the position of vimentin and GFP as described (32).

Immunoblotting of the cell extract of clone 5 with antibodies to GFP and vimentin showed the specificity of the antibodies used in this study (Fig. 2). Monospecific antiserum to GFP reacted with the M_r 50,000 protein and several degraded fragments but not with vimentin (arrow, lane 1). Similarly, antiserum to vimentin reacted with the M_r 58,000 protein band and an apparent degradation product but not with GFP (arrow, lane 3).

Immunofluorescence Microscopy. The presence of vimentin in all four clonal cultures (1, 4, 5, and 6) and the absence of GFP in clones 4 and 6 was also shown by immunofluorescence staining of formaldehyde-fixed acetone-extracted cell specimens with antibodies against the two IF proteins. An elaborate network of filaments was observed when the samples of clone 5 cells were stained with guinea pig anti-GFP IgG followed by fluorescein-conjugated goat anti-guinea pig IgG (Fig. 3*a*). An identical filamentous network was observed when the same specimens were incubated with rabbit

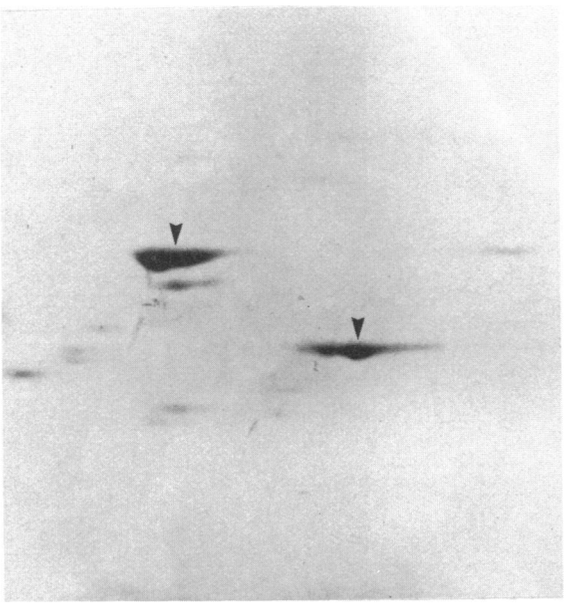

FIG. 1. Two-dimensional isoelectric focusing $NaDodSO_4$/polyacrylamide gel profile of the cytoskeletal proteins of U-251-5 glioma cells. Isoelectric focusing was done in the first dimension with a pH gradient of pH 5 to pH 7 (left to right). The second dimension was a 7.5% polyacrylamide/$NaDodSO_4$ gel.

antivimentin IgG in combination with rhodamine-conjugated sheep anti-rabbit IgG (Fig. 3*b*). These results show that vimentin and GFP coexist in the cytoplasm of clone 5 in one and the same filamentous network. Similar observations were made with clone 1 cells. In contrast, staining of clone 4 cell specimens with guinea pig anti-GFP IgG failed to reveal such a network of filaments (Fig. 3*c*), although subsequent staining with rabbit antivimentin IgG showed a distinct reticular distribution of filaments in the cytoplasm (Fig. 3*d*). Seemingly, clone 4 and 6 cells resemble fibroblasts of mesenchymal origin as they express only vimentin-containing filaments; however, unlike the fibroblasts, these clones express a glial astrocyte-specific surface antigen. Previous studies of the four clonal lines (1, 4, 5, and 6) of U-251 have

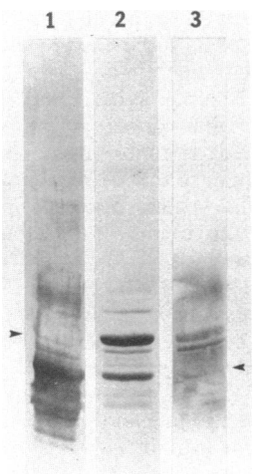

FIG. 2. Immunoblotting experiment. Antibodies against GFP (lane 1) and vimentin (lane 3) were allowed to react with the cytoskeletal extract from U-251-5 glioma cells. The SDS/polyacrylamide gel electrophoresis pattern of the cytoskeletal extract is shown in lane 2. Rabbit anti-GFP IgG reacts strongly with the M_r 50,000 protein but not with vimentin (lane 1, arrow). Rabbit antivimentin reacts with the M_r 58,000 protein but not with GFP (lane 3, arrow).

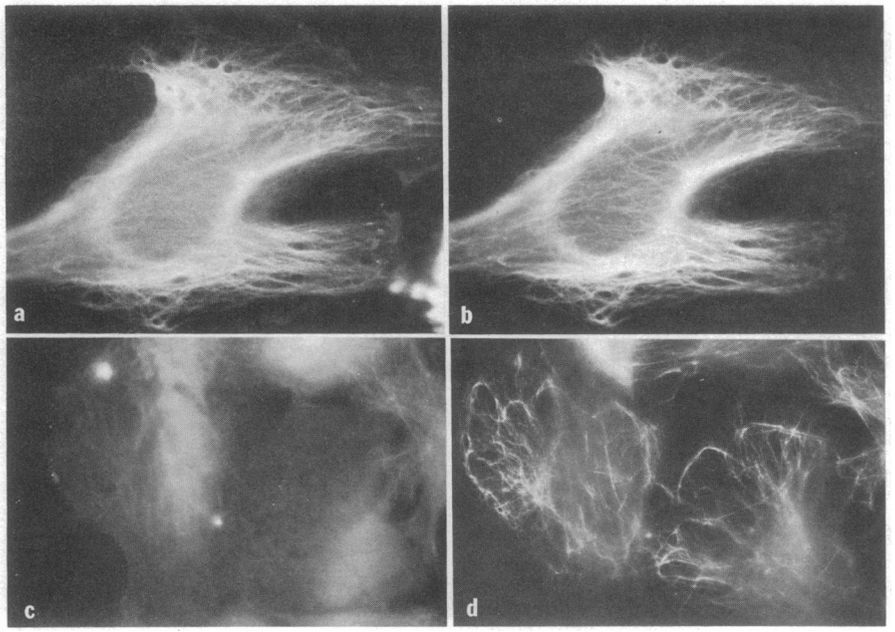

FIG. 3. Simultaneous localization of vimentin and GFP in clonal lines of U-251 astrocytes. Formaldehyde-fixed acetone-extracted cell specimens were first incubated with guinea pig anti-GFP IgG and then labeled with fluorescein-conjugated goat anti-guinea pig IgG; subsequently, they were incubated with rabbit antivimentin IgG and then with rhodamine-conjugated sheep anti-rabbit IgG. Specimens were examined under epi-illumination with filters for fluorescence (485 nm) and rhodamine molecules (546 nm). An added filter (520- to 540-nm) was used to ensure complete separation of the two different-wavelength spectra. (*a*) GFP-containing filament network in clone 5 cells (485-nm filter); (*b*) the same cell contains vimentin-containing filaments in an identical network (546-nm filter); (*c*) absence of GFP-containing filaments in clone 4 cells (485-nm filter); (*d*) in contrast to *c*, there is an elaborate network of vimentin-containing filaments in the cytoplasm of clone 4 cells (546-nm filter). (*a* and *b*; ×630; *c* and *d*, ×495.)

shown that all four express a surface antigen detected by monoclonal antibody (AO10) (20).

Immunoelectron Microscopy. We have investigated further the localization of the two proteins, GFP and vimentin, in clone 5 and 1 cultures by indirect immunoelectron microscopy with protein A conjugated with colloidal gold particles of various sizes. The formaldehyde-fixed acetone-extracted cell specimens of these clonal lines were incubated with guinea pig anti-GFP IgG and then stained with goat anti-guinea pig IgG conjugated with 5-nm gold particles. The linear arrangement of gold particles superimposed on the intermediate filaments, which reveals the positions of the GFP antigen, is shown in Fig. 4*a*. Similar patterns of alignment of gold particles were observed when the specimens were incubated with rabbit antivimentin and then labeled with protein A conjugated with 20-nm gold particles (Fig. 4*b*). No decoration was observed when guinea pig anti-GFP IgG was applied to the extracted specimens of clone 4 and 6 cells (Fig. 4*c*). Decoration similar to that in Fig. 4*b* was observed when the specimens of clone 4 and 6 cells were processed for decoration with rabbit antivimentin in combination with 20-nm gold-conjugated protein A.

Gold particles of two different sizes (5 and 20 nm) were observed along the same filaments when the cytoskeletal samples of clone 5 cells were incubated with guinea pig anti-GFP IgG and 5-nm gold particles conjugated to goat anti-guinea pig IgG. Afterward, these specimens were incubated with rabbit antivimentin IgG and then with 20-nm gold particles conjugated with protein A. As shown in Fig. 4*d*, the specific filament positioned in the center of the micrograph was decorated by both types of gold particles. There are a few gold particles of both sizes scattered in the cytoplasm, associated with either organelles or other filaments. This may result from structural interaction of intermediate filament with cell organelles (22) or filaments that are physically present in the adjacent sections. Another example of particles of both sizes positioned in linear orientation, representing decoration on the same filaments, is shown in Fig. 4*e*. At higher magnification, gold particles of two different sizes were observed in consecutive order superimposed on the filament in the background (Fig. 4*f*). The cluster formation of the 5-nm gold particles results from the less-than-optimal reaction during the initial conjugation of the colloidal gold particles and the goat anti-guinea pig IgG molecules.

In addition to the immunoblotting results, the specificity of the antibody reaction was verified in various control experiments including absorption with purified antigen prior to use, employment of preimmune serum in place of the monospecific antiserum, and an immunocompetition assay with excess purified antigen. In all cases, the specificity of the antibodies to GFP and vimentin was verified by the reduction in the abundance of gold particles in the cytoplasm as well as the lack of correlation between the positions of the particles and the filaments. The lack of particle decoration along the filaments when the samples were incubated with rabbit anti-vimentin in the presence of excess vimentin (2 mg/ml) purified from the BHK-21 cells is shown in Fig. 4*g*. A few 20-nm gold particles were observed randomly scattered in the cytoplasm.

DISCUSSION

The results in this paper show the existence of both vimentin and GFP in two clonal lines (1 and 5) of glioma cells (U-251). Their presence in the same individual filaments was verified by immunoelectron microscopy. At the light microscopic level, identical filamentous networks containing vimentin and GFP were observed.

Polymerization of different IF proteins into 10-nm filaments has been observed in mixtures for different monomeric subunits (e.g., vimentin and keratin *in vitro*) (33). It was not, however, shown whether the polymers that formed were hetero- or homopolymers. Our results show that vimentin and GFP can indeed be integrated in the same hetero-

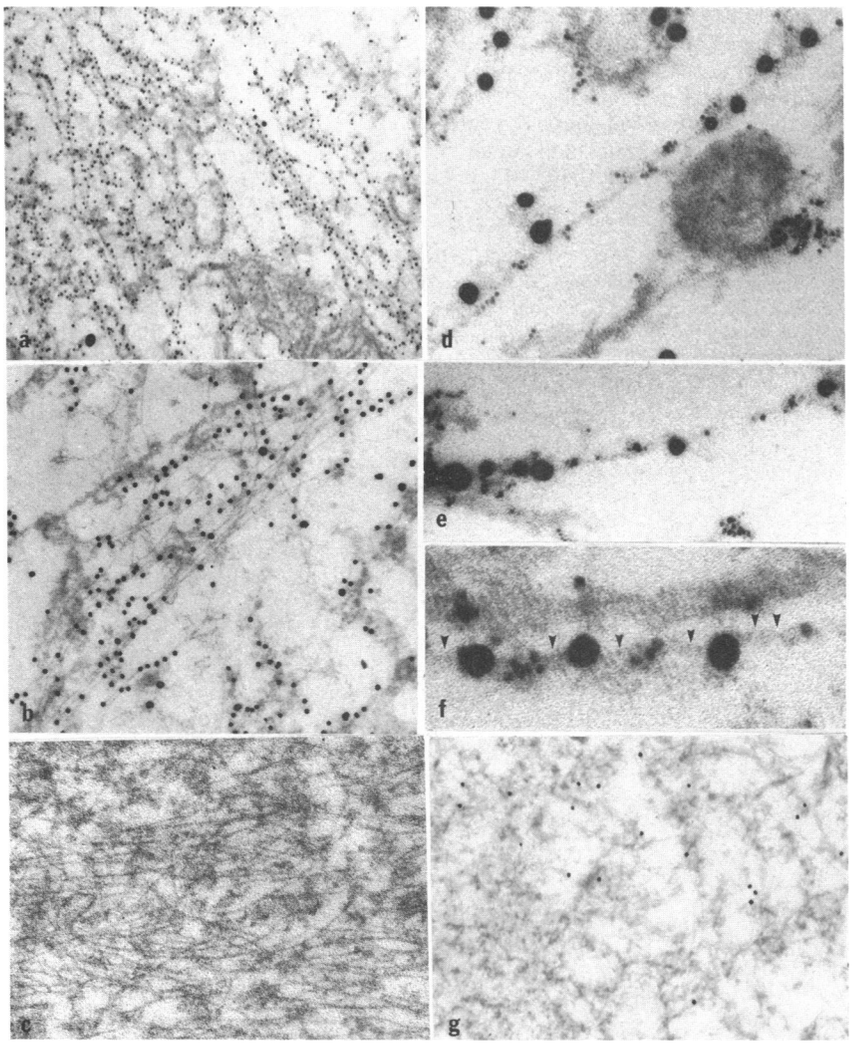

FIG. 4. Ultrastructural colocalization of vimentin and GFP on the same filament in U-251 cells. Formaldehyde-fixed acetone-extracted samples were stained with guinea pig antibody to GFP and then incubated with goat anti-guinea pig IgG conjugated with 5-nm colloidal gold particles. After extensive washing, the specimens were then further stained with rabbit antibody to vimentin and labeled with protein A conjugated with 20-nm colloidal gold particles. (*a*) Linear arrays of gold particles decorate most of the 10-nm filaments in a clone 5 cell that had been incubated with guinea pig anti-GFP and stained with colloidal gold (5-nm)-conjugated goat anti-guinea pig IgG. (*b*) Similar arrays of particle-decorated 10-nm filaments were found when the cell specimen was incubated with rabbit antivimentin in combination with colloidal gold (20-nm)-conjugated protein A. (*c*) When cells of clone 4 cultures were incubated with the combination of antibodies used in *a*, decoration by gold particles was absent. (*d*) Decoration of *A* particular filament by two sizes of gold particles. Several particles of both sizes are scattered in random fashion in the cytoplasm, possibly resulting from association between intermediate filaments and organelles or particles decorating filaments in adjacent sections. (*e*) Another example of a random sequence of particles of two sizes in linear orientation, reflecting the decorating pattern on the same filament. (*f*) Higher magnification shows that gold particles of two sizes indeed overlie a particular filament in the background (arrow). The large particles represent the locations of vimentin monomers and the clusters of small particles represent the positions of GFP monomers. (*g*) No correlation between gold particles and filaments was found when clone 5 cultures were incubated with rabbit antivimentin in the presence of excess purified vimentin isolated from cultured BHK-21 cells. A few gold particles of 20-nm in size, are found randomly scattered in the cytoplasm. (*a*, ×62,000; *b*, ×46,500; *c*, ×49,600; *d*, ×175,600; *e*, ×151,400; *f*, ×351,200; *g*, ×31,000.)

polymers in the cytoplasm. Most of the IF proteins, once assembled, are insoluble under physiological conditions. However, Blikstad and Lazarides (34) have recently reported that the newly synthesized vimentin subunits exist in the soluble pool and are rapidly incorporated into the insoluble cytoskeletal scaffold. Their results of pulse–chase experiments indicate that the half-life for vimentin in the soluble fraction is as short as 7 min. It therefore appears likely that soluble pools for both vimentin and GFP may be present in the GFP-positive cells used for the present study and coassembled *in situ* into the insoluble fraction of IFs shortly after it is synthesized.

Many reports have described the biochemical mechanism that IF subunits assemble into protofibrils by the α-helical polypeptide strands intertwined in a coiled-coil configuration that is then laterally as well as longitudinally connected to form the filaments (for review, see ref. 35). The α-helical domains of the different IF subunits contains the most highly conserved sequence of all IF molecules. Therefore, it is possible that, in the *in vivo* environment, the helical domains of vimentin and GFP can be assembled together to form the protofibril strands following the coiled-coil configuration and then forming the protofilaments and finally the filaments. Nevertheless, our results do not exclude the possibility that two different subunits are assembled individually into either vimentin- or GFP-containing protofibrils and then connected laterally and lengthwise into the individual filaments, as suggested for the end-to-end linkage during formation of the elongation for protofibrils and for side-to-side connection during the bundling of several fibrils into 8- to 10-nm filaments. If the *in vivo* coassembly of vimentin and GFP indeed follows the same mechanism as has been found in *in*

vitro studies, the protein sequences of both the helical and nonhelical regions determine the efficiencies of both the initial polymerization of fibrils and the subsequent spatial arrangement of them into filaments. This was recently found in an *in vivo* study in which the arginine residues in the rod domain were replaced by canavanine, resulting in the inhibition of vimentin assembly but not its synthesis (36).

We would like to express our deepest gratitude for the excellent technical assistance of Mses. Susan Nornes, Amy Berg, and Susan Hutchison and Messrs. Phil Huie and Steve Chin. We would also like to thank Dr. Igor Tamm for his reading and comments of this manuscript. This work was supported by National Institutes of Health Grants AG03020 to E.W. and EY03849 to R.K.H.L.

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