Proc. Nat. Acad. Sci. USA
Vol. 10, No. 12, Part I, pp. 3669-3673, December 1973

Manipulation of Fatty Acid Composition in Animal Cells Grown in Culture*

(LM cells/desthiobiotin/serum-free medium/membranes/detergents)

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Communicated by P. D. Boyer, July 3, 1973

ABSTRACT The fatty acid composition of animal cells cultured in serum-free medium can be manipulated when the synthesis of endogenous fatty acids is inhibited by a biotin analog and fatty acids are supplied in tbe medium as detergent esters of Tween. When mouse LM cells were grown in medium supplemented with Tween-19:0 (an ester of Tween and nonadecanoic acid), odd chain fatty acid content of cellular phospholipids and neutral lipids increased from 1% to 75%. Concurrently, the saturated fatty acid content increased from 27% to 85%. Similar alterations in fatty acid content have been observed when BHK_{21} cells are subjected to the same enrichment regime. The ability to control the fatty acid composition of cultured animal cells is a prerequisite to investigations into the role of the membrane lipid physical state in processes unique to these cells.

Microbial fatty acid auxotrophs have been used to modify the fatty acid composition of biomembrane lipids in order to study the effect of lipid composition on membrane structure, function, and assembly. Research with fatty acid-requiring mutants of *Escherichia coli* has yielded significant insight into the nature of membrane assembly (1, 2). These mutants have also been used to demonstrate a dependence of transport rate on the physical state of membrane lipids (3-8).

Animal cells have many functions with no counterpart in microorganisms. They undergo oncogenic transformation and cell-cell fusion. Cell-cell fusion has been implicated in the release of dormant tumor viruses (9). Membrane-enveloped viruses such as herpes, parainfluenza, influenza, and RNA tumor viruses infect by virtue of their ability to undergo membrane fusion with their host cells, or as a result of pinocytosis (10). Some type of membrane alteration is implicated in all these processes.

In order to manipulate the physical properties of animal cell membranes, as has already been done in bacterial and fungal systems (11-13), we have systematically searched for procedures to force cultured animal cells to incorporate exogenously-supplied fatty acids into membrane lipids. By manipulating the fatty acid composition of these cells, we could analyze the effects of altered lipid fluidity on numerous membrane functions, e.g., cell-cell fusion, virus-cell fusion, antigenic mixing, and phagocytosis.

To date, no one has succeeded in grossly enriching the phospholipids of cultured animal cells with a defined fatty acid supplied exogenously. The problems are as follows: (*1*) Growth media typically contain serum or other non-defined materials; many defined media contain Tween 80 (fatty acid composition similar to olive oil) and cholesterol. (*2*) Unesterified (free) fatty acids supplied in ethanol or on an albumin carrier lead to lipid accumulation (steatosis) and irreversible cell degeneration (14, 15). (*3*) Endogenous fatty acid synthesis must be arrested, or alternatively, lipid-requiring mutants must be isolated. (*4*) Endogenous fatty acid desaturation must be controlled. By eliminating or minimizing these factors, we were able to enrich cultured animal cells with fatty acids supplied exogenously, and to alter the degree of saturation in membrane lipid fatty acids.

MATERIALS AND METHODS

Organism. Mouse LM cells derived from NCTC clone 929 (L cells) and adapted to growth on medium 199 plus 0.5% peptone were obtained from the American Type Culture Collection.

Media. Maintenance medium (MEM+P) was Eagle's minimal essential medium with Earle's salts (medium MEM, GIBCO) plus 0.5% bacto-peptone (Difco). Chemically-defined medium (MEM+GV) consisted of MEM modified to contain twice the normal concentrations of glutamine and minimal essential vitamins. Medium MEM+GV (biotin-free) was supplemented with *d*-biotin (20 μg/liter) to prepare MEM+GVB, with avidin (2 mg/liter, 13 units/mg) to prepare MEM+GVA, and with *dl*-desthiobiotin (2000 μg/liter) to prepare MEM+GVdB. All media contained 2.2 g/liter of sodium bicarbonate, and were adjusted to pH 7.3 with HCl before filtration.

Culture Procedures. Monolayer cultures were established in 250 ml Falcon flasks containing 15 ml of medium. Incubation was at 37° with humidified air and 6% CO_2. At confluency (approximately 10^8 cells per flask), cells were removed by rinsing the monolayer with 3 ml of 0.005% trypsin (Sigma: type 2, pancreatic; 1500 BAEE units/mg) diluted with Tris-saline (Tris, 3 g/liter; NaCl, 8 g/liter; Na_2HPO_4, 0.1 g/liter; and 12 N HCl, 1.75 ml) at pH 7.4. Trypsin was removed immediately and the flask incubated for 5 min. Then, 3 ml of MEM was added and the monolayer dispersed by agitation. Aliquots of this cell suspension (0.1-0.25 ml) were used for inoculating the flasks. Medium was changed twice weekly. When some cultures were first shifted to Tween-supplemented medium, they yielded viable, floating cells. These cells subsequently grew in monolayers when the medium containing them was added to 15 ml of MEM

Abbreviations: Saturated, aliphatic, straight chain fatty acids are designated 16:0 for hexadecanoic acid, 17:0 for heptadecanoic acid, etc. Similarly, a fatty acid designated 16:1 is a 16 carbon straight chain fatty acid containing one *cis* double bond.

* A preliminary report of this work has been published [Wisnieski, B. & Fox, C. F. (1973) *Fed. Proc.* 32, 556Abstr].

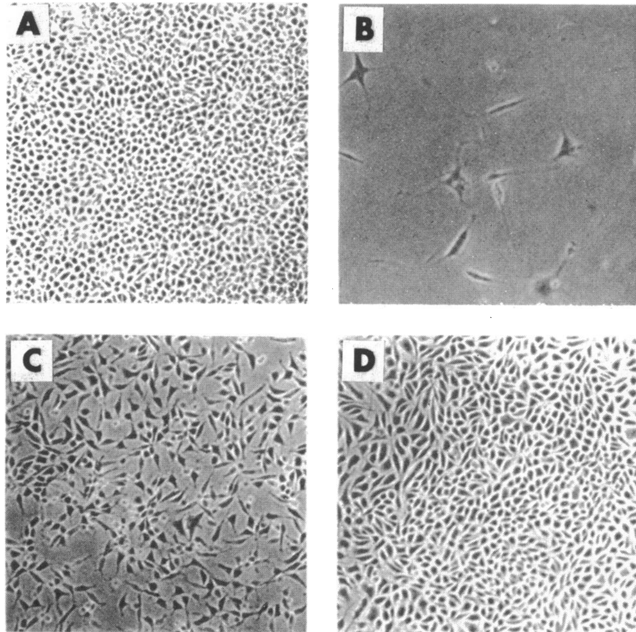

FIG. 1. Photomicrographs (×100) of LM cells starved for biotin by growth in MEM+GVdB for 4 days and then shifted to (*A*) MEM+P, (*B*) MEM+GVdB, (*C*) MEM+GVdB + Tween-16:0 (50 μg/ml; approximately 10% 16:0 by weight), and (*D*) MEM+GVdB + Tween-19:0 (100 μg/ml; approximately 5% 19:0 by weight). Cultures were incubated in the specified media for 7 days (*Methods*) prior to visual assessment of growth.

in a new flask. After cell attachment the MEM was replaced with a Tween-supplemented medium.

Fatty Acid Supplements. Tween-acetate was synthesized by reacting 5 g of Tween 20 with 20 ml of glacial acetic acid in 100 ml of *t*-butyl alcohol plus 10 ml of 12 N HCl. The reaction mixture was refluxed under N_2 for 4 hr. Solvents were removed by vacuum distillation at 50°.

Tween-fatty acid esters were synthesized by transesterification of commercially available Tweens with excess fatty acid (approximately 1:40 molar ratio) and also by transesterification of Tween-acetate (see above) with a moderate excess of fatty acid (1:2–1:5 molar ratio Tween-acetate to fatty acid). A specific example of each method follows. *Method 1:* Tween-palmitate (16:0) was prepared by reacting 4 g of 16:0 and 1 g of Tween 40 in 25 ml of *t*-butyl alcohol plus 2.5 ml of 12 N HCl. The reaction mixture was refluxed under N_2 for 4 hr and solvents removed by vacuum evaporation. *Method 2:* Tween-nonadecanoic acid (19:0) was prepared by refluxing 0.5 g of 19:0 and 1.0 g of Tween-acetate in 10 ml of *t*-butyl alcohol plus 1 ml 12 N HCl. All fatty acids were purchased from the Hormel Institute (Austin, Minn.) and were more than 99% pure. Commercial grade Tweens are heterogeneous with respect to the composition of the fatty acid moiety.

Tweens were separated from free fatty acids by column chromatography. MN-silica gel, 70-325 mesh ASTM (E. Merck Div.), was washed three times with glass distilled water, and fine particles which remained in suspension were removed after each wash. The gel was then filtered, washed with methanol, refiltered and activated overnight at 110°. Columns were packed with silica gel (10 g/g of sample) in diethyl ether and washed with three column volumes of ether. Samples were applied in ether containing sufficient methanol to solubilize the Tween portion of the sample. Fatty acids were eluted with four column volumes of ether and the Tween was eluted with four column volumes of methanol.

The purity of the Tween preparations was determined by thin-layer chromatography and gas-liquid chromatography; absence of free fatty acids was verified by thin-layer chromatography and purity of fatty acid composition by gas-liquid chromatography. None of the Tweens contained detectable free fatty acids. Tweens synthesized by *Method 1* were at least 90% pure in esterified fatty acid composition; Tweens made by *Method 2* were at least 98% pure. Methanol-free Tween preparations were solubilized in glass-distilled water (100 mg/ml) and autoclaved at 15 psi for 15 min.

Lipid Extraction. Trypsinized cell suspensions were diluted 1:10 with Tris-saline, pelleted by centrifugation (12,100 × *g* for 15 min), washed once with Tris-saline and again pelleted. Cells were extracted with chloroform:methanol (2:1 v/v). The extract was filtered to remove debris, dried under N_2, and suspended before application to predeveloped TLC plates (250 μm of silica gel G; Analtech). Chloroform:methanol:ammonium hydroxide (65:35:10 by volume) was used to resolve the phospholipids. Tweens migrated with the solvent front in this system.

Lipids were visualized by iodinating an exposed edge of the plate. Areas containing phosphatidylethanolamine and phosphatidylcholine were scraped and eluted with three volumes of methanol followed by one volume of chloroform: methanol (2:1). Lipids were transmethylated with BF_3 (14% in methanol).

Gas-Liquid Chromatography. Gas-liquid chromatography of methylated fatty acids was performed on a Perkin Elmer 990 gas chromatograph. The analytical columns were $^1/_8$ inch by 6 ft stainless steel packed with 100/120 mesh (DMSC) Chromosorb W coated with 10% EGSS-X. Runs were temperature programmed from 150° to 200° at 4°/min. Peak areas and retention times were calculated using an Infotronics digital integrator.

RESULTS

The protocol developed to enrich LM cells with exogenously-supplied fatty acids involved growing the cells in modified Eagle's minimal essential medium (see *Methods*) containing no serum, cholesterol, or extraneous lipids. Fatty acid-detergent esters (Tweens) which act as nontoxic carriers of fatty acid supplements were chemically synthesized to contain a defined fatty acid moiety. To inhibit endogenous fatty acid synthesis, cells were starved for biotin in medium MEM+GV ("biotin-free") containing avidin or desthiobiotin. Desthiobiotin treatment proved to be the more effective method. Since fatty acid desaturase activity is sensitive to fatty acid chain length, the ratio of saturated to unsaturated fatty acids in the cells was manipulated by using Tween-fatty acid esters of appropriate chain length.

Cultures maintained continuously in biotin-starvation medium die within 1–2 weeks. However, the effects of a 4–7 day starvation for biotin are reversible if cultures are shifted to growth in MEM + 0.5% peptone or to MEM+GVdB + Tween-fatty acid supplement (Fig. 1).

Cells shifted to Tween-19:0 medium were analyzed for enrichment of 19:0 in phosphatidylcholine and phosphatidyl-

ethanolamine. These contain greater than 80% of the total fatty acids in LM cell phospholipids (16). Odd chain fatty acids provided a marker for gas-liquid chromatography since the cells synthesize primarily even chain fatty acids. The fatty acid compositions of phospholipids from LM cells maintained on MEM+P medium (0.3 μg of biotin per g of peptone) and from cells shifted to MEM+GVdB supplemented with Tween-19:0 are described in Fig. 2. Essentially no odd chain fatty acids were synthesized *de novo* in the presence of biotin (Fig. 2*a*). Cells in biotin-starvation medium containing Tween-19:0 incorporated 19:0 into phospholipids at the expense of even chain fatty acids (Fig. 2*b*). Biotin starvation is necessary to maximize the incorporation of exogenous fatty acid into phospholipid. In an experiment done in duplicate under conditions identical to those described for Fig. 2 (MEM+GVdB + 100 μg/ml of Tween-19:0), 33.4 and 35.9% of the fatty acids in phosphatidylcholine plus phosphatidylethanolamine were derived from Tween-19:0. Under conditions identical to those described for Fig. 2*a* (MEM+P), except for the addition of 100 μg/ml of Tween-19:0, only 9.7 and 11.0% of the fatty acids in phosphatidylcholine plus phosphatidylethanolamine were derived from Tween-19:0. The predominant unsaturated fatty acid in the phospholipids of control cultures is 18:1; the level of this fatty acid was reduced in cells enriched for odd chain fatty acids. Although there was always some desaturation of 19:0, the level of desaturation was greatly reduced compared to that of 18:0. Consequently, the saturated fatty acid content increased proportionately to 19:0 enrichment. However, metabolic products of 19:0 (15:0, 17:0, 17:1, and 19:1) increased when the period of growth with Tween-19:0 was lengthened (data not shown).

Tables 1, 2, and 3 describe the fatty acid compositions of phospholipids extracted from control LM cultures (Table 1), and from LM cultures grown with various concentrations of Tween-19:0 (Table 2) and Tween-16:0 (Table 3). Medium

TABLE 1. *Fatty acid composition of phospholipids* extracted from control cultures*

	Culture medium†			
Fatty acid	MEM+ P (%)	Mem+ GVdB (%)	MEM+ GVB (%)	MEM+ GVB +Tw-acetate‡ (%)
19:1	0	0	0	0
19:0	0	0	0	0
18:1	59.8	56.7	45.9	46.8
18:0	10.0	9.9	12.7	18.2
17:1	0.6	0.2	0.3	0
17:0	0.1	0.1	0.3	0.9
16:1	9.4	8.6	7.6	5.8
16:0	15.8	18.7	27.3	21.9
15:0	0.5	0.8	0.8	0
14:0	1.4	2.0	3.0	3.3
12:0	0.2	0.4	0.3	0.2
Other§	2.2	2.6	1.8	2.9
% odd/% even	1.2/98.8	1.1/98.9	1.4/98.6	0.9/99.1
% sat./% unsat.	28.0/72.0	31.9/68.1	44.4/56.4	44.5/56.5

* Phosphatidylcholine plus phosphatidylethanolamine.

† See *Methods* for details; incubation periods for biotin starvation and for growth were as indicated for Fig. 1.

‡ Tween-acetate concentration was 50 μg/ml.

§ Fatty acids included in Other were even chain and unsaturated. These consisted primarily of 20:1.

TABLE 2. *Fatty acid composition of phospholipids* extracted from Tween-19:0 cultures*

	Tween-19:0 concentration (μg/ml)†			
Fatty acid	100 μg (%)	250 μg (%)	375 μg (%)	500 μg (%)
19:1	2.0	7.1	5.8	0.5
19:0	13.6	19.9	33.4	72.9
18:1	36.3	35.9	28.6	11.9
18:0	8.7	4.1	5.8	2.6
17:1	1.4	3.0	3.2	0.4
17:0	1.0	0.5	1.3	1.0
16:1	10.2	11.5	6.5	2.7
16:0	17.4	12.2	11.3	5.3
15:0	1.4	1.2	0.9	0.4
14:0	5.1	3.5	2.7	1.5
12:0	1.6	0.1	0	0.8
Other‡	1.3	1.0	0.5	0
% odd/% even	19.4/80.6	31.7/68.3	44.6/55.4	75.2/24.8
% sat./% unsat.	48.8/51.2	41.5/58.5	55.4/44.6	84.5/15.5

* Phosphatidylcholine plus phosphatidylethanolamine.

† Tween-19:0 contained 5% 19:0 by weight. Procedures were as described in *Methods* and Fig. 1.

‡ Fatty acids included in Other were even chain and unsaturated. These consisted primarily of 20:1.

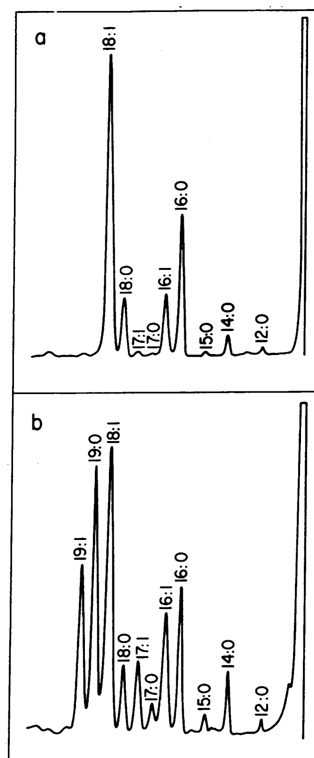

FIG. 2. Fatty acid composition of combined phosphatidylcholine and phosphatidylethanolamine fractions from cells grown in (a) MEM+P and (b) MEM+GVdB + Tween-19:0 (100 μg/ml; approximately 5% 19:0 by weight). The time periods for initial biotin starvation (4 days) and for growth in the specified media (7 days) were as described in Fig. 1.

TABLE 3. *Fatty acid composition of phospholipids* extracted from Tween-16:0 cultures*

	Tween-16:0 concentration (μg/ml)†			
Fatty acid	50 μg (%)	100 μg (%)	200 μg (%)	250 μg (%)
19:1	0	0	0	0
19:0	0	0	0	0
18:1	38.0	51.3	48.8	55.8
18:0	11.0	13.2	10.8	9.2
17:1	0.2	0.4	0.4	0.3
17:0	0.6	0.7	0.4	0
16:1	16.0	10.0	19.2	11.5
16:0	27.7	17.8	15.4	17.8
15:0	1.6	1.4	1.7	0.7
14:0	4.0	3.0	1.0	1.5
12:0	0	0	0.1	0
Other‡	0.9	2.2	2.2	3.2
% odd/% even	2.4/97.6	2.5/97.5	2.5/97.5	1.0/99.0
% sat./% unsat.	44.9/55.1	36.1/63.9	29.4/70.6	29.2/70.8

* Phosphatidylcholine plus phosphatidylethanolamine.

† Tween-16:0 contained 10% 16:0 by weight. Procedures were as described in *Methods* and Fig. 1.

‡ Fatty acids included in Other were even chain and unsaturated. These consisted primarily of 20:1.

MEM+P was used to maintain stock cultures; MEM+GVdB was used for biotin starvation and was subsequently supplemented with Tween-fatty acid esters to effect enrichment. Biotin supplementation of MEM+GV medium (i.e., MEM+GVB) did not suffice to maintain cell growth indefinitely, nor did it replace Tween-fatty acid esters in promoting the growth of biotin-starved cells. Our present information cannot account for this phenomenon.

We can manipulate the degree of enrichment for an exogenously supplied fatty acid by increasing the growth time in medium supplemented with a Tween-fatty acid ester supplied at constant molarity, or by increasing the concentration of the Tween-fatty acid ester supplement. The extent to which we can enrich cells for 19:0 is demonstrated in Table 2. An increase in the concentration of the fatty acid supplement effected increases in enrichment for both odd chain and saturated fatty acids. When a high concentration of Tween-19:0 was supplied (500 μg of Tween-19:0 per ml), 75% of the fatty acids in phospholipids were derived from 19:0. Enrichment in 19:0 alone was 73%. The saturated fatty acid content increased from 27% in cells grown in MEM+P to 85% in cells grown with 500 μg/ml of Tween-19:0. The neutral lipid fractions derived from these cells had a fatty acid content similar to that of the phospholipids (data not shown). Cell lysis was encountered in medium supplemented with higher concentrations of Tween-19:0 (approximately 750 μg/ml). Although we have not succeeded in totally blocking endogenous fatty acid synthesis, high concentrations of Tween-19:0 apparently inhibit a portion of this background synthesis.

The data in Table 3 indicate that cells apparently have an increased capacity for elongating 16:0 to C_{18} fatty acids when the concentration of 16:0 supplement is increased. Whatever the reason for this, cells grown in medium containing the highest concentration of Tween-16:0 compatible with cellular growth (approximately 250 μg of Tween-16:0 per ml) have a fatty acid profile similar to that of cells grown in control cultures (Table 1).

BHK_{21} cells adapted to growth in serum-free medium (MEM+P) and starved for biotin in MEM+GVdB have also been enriched for 19:0 after growth in MEM+GVdB supplemented with Tween-19:0. The protocol we have established for enriching LM cells with specific fatty acids may be applicable to other cell lines as well.

DISCUSSION

We have established a protocol for enriching phospholipids of animal cells grown in culture with fatty acids supplied exogenously. Cells are first starved for biotin in a lipid-free minimal medium until growth is inhibited. The medium is subsequently supplemented with a fatty acid esterified to Tween and growth resumes.

Incorporation of exogenous fatty acids into cellular phospholipids was initially verified with a radioactive fatty acid esterified to Tween. To verify enrichment, Tween-19:0 (an ester of Tween and nonadecanoic acid) was employed as a medium supplement. This supplement was used since the cells can not synthesize a significant amount of odd chain fatty acids, and since odd and even chain fatty acids and their desaturation products are easily resolved by gas-liquid chromatography. Odd chain fatty acids were incorporated into both phospholipids and neutral lipids when cells were grown in medium containing a Tween-19:0 supplement. The fatty acid compositions of both these fractions contained as much as 73% 19:0. The results from published *in vitro* studies predicted that desaturation of 19:0 would be minimal (17, 18). As expected, the proportion of saturated fatty acids was greatly increased, from 27% in controls, to 85% in 19:0-enriched cells.

Experiments with fatty acid auxotrophs of *E. coli* have demonstrated that parallel changes occur in transport activity and membrane lipid fluidity at characteristic temperatures determined by the fatty acid composition of membrane phospholipids (3–8). The characteristic temperatures for transport correlate with characteristic temperatures defining the end points (upper and lower) of the course of lateral phase separations in membrane lipids as monitored by electron spin resonance spectroscopy (8, 19) and x-ray diffraction (Blasie, K., Linden, C. D. & Fox, C. F., to be published). In addition, the lateral mobility of membrane lipids is apparently arrested below the lower characteristic temperature (t_l) (ref. 2; Hubbell, W., unpublished observations), and at least some membrane assembly processes are abortive below t_l (1). Membranes with a high saturated fatty acid content have a high t_l and membranes with a low saturated fatty acid content, a low t_l. Since we can now control the ratio of saturated to unsaturated fatty acids in established lines of animal cells (Tables 1, 2, and 3), we hope to determine how alterations in t_l, and thus in membrane lipid fluidity, affect membrane functions unique to these cells.

Alterations in animal cell membranes are implicated in the etiology of many disease states and in aging. Membranes are targets for viruses and bacteria, and for certain hormones and drugs. Feedback between the cellular genome and membranes appears to regulate density-dependent cell growth (i.e., contact inhibition). Cell–cell and virus–cell fusion are certainly membrane phenomena. A wealth of in-

formation exists linking membrane alterations with these processes and others, such as oncogenic transformation.

The protocol we have developed enables us to take novel approaches to studying these problems. Our ability to manipulate the fatty acid composition of BHK_{21} cells may allow us to investigate possible interrelationships between membrane fluidity and the rate and/or degree of oncogenic transformation. The lipid compositions of cells and enveloped virus can be varied independently by harvesting virus from cells with a fatty acid composition different from that of the experimental host cells. It should thus be possible to assess independently the role of cellular and viral membrane fluidity in the infiltration of cells by enveloped viruses.

Since some membrane-associated enzymes are sensitive to membrane phase behavior (6, 20, 21), we should be able to monitor fatty acid enrichment in several different membrane fractions (e.g., mitochondrial, plasma, etc.) and proceed to determine how lipid fluidity affects enzyme activity and regulatory phenomena. The known lipid dependence of adenyl cyclase activity is of considerable interest here (22–25). Immunological response may also be affected by membrane lipid fluidity. The clustering of antigen receptors on lymphocytes is apparently a prerequisite to subsequent mitogenic response (26–28). This clustering undoubtedly requires membrane fluidity.

This work was supported by Research Grants GM-18233 and AI-17033 from the United States Public Health Service, Grant DRG-1153 from the Damon Runyon Fund, and Grant BC-79 from the American Cancer Society. C.F.F. is the recipient of United States Public Health Service Career Development Award GM-42359; B.J.W. is a Postdoctoral Fellow of the Damon Runyon Fund (DRF-749).

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