Association of hepatic lipase with proteoglycans stimulates the production of proteoglycans in vivo and in vitro

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Abstract **HL is synthesized in hepatocytes and functions while bound to heparan sulfate proteoglycans (HSPGs) in sinusoidal endothelial cells. The HL-mediated uptake of lipoprotein requires cell-surface HSPG. The present study tested whether HL plays a role in the production of HSPG. The production of HSPG in Chinese hamster ovary (CHO) cells was determined by measuring the incorporation of $^{35}SO_4$ into PGs. HL-producing HL-CHO cells showed approximately 30% more cellular PG than did wild-type (WT) cells. In contrast, PG production in cells producing a membrane-anchored HL-glycophosphatidylinositol (GPI) that was not bound to HSPG was virtually identical to that in WT cells. When purified HL was added to the WT- or HL-GPI cells, PG production increased significantly to a level similar to that of the HL-secreting cells, suggesting that the binding of HL to HSPG triggered the increased HSPG production. Heparin reduced PG production in HL-producing cells, confirming that PG production is stimulated only when HL is present as a ligand for HSPG. Real-time PCR and Northern blots demonstrated that PG production was significantly reduced in animals lacking HL. Together, these data suggest that the binding of HL to PG on the cell surface exerts a positive feedback on cellular PG production.**—Lee, S-J., S. Kadambi, C. David, A. D. Cooper, and S. Y. Choi. **Association of hepatic lipase with proteoglycans stimulates the production of proteoglycans in vivo and in vitro.** ***J. Lipid Res.*** **2004.** 45: **1266–1271.**

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Heparan sulfate proteoglycans (HSPGs) are metabolically active and abundant cell surface molecules that play roles in cell-cell adhesion (1, 2), cell-extracellular matrix adhesion (3), and activation of signaling receptors by growth factors such as basic fibroblast growth factor (FGF) (4, 5) and hepatocyte growth factor (6), as well as internalization of extracellular ligands. PGs also play a number of roles in lipoprotein metabolism. An established and very important HSPG function is anchoring the members of the lipase gene family, including lipoprotein lipase, HL, and endothelial lipase, to the surfaces of cells, where the enzymes hydrolyze the lipoproteins and anchor there to carry out receptor-mediated uptake. In addition to aiding cell surface receptors such as LDL receptors and LDL receptor-related protein (LRP), PGs may mediate the internalization of lipoproteins directly.

HL binds only to PGs of the liver, adrenal gland, and ovary. The LPL-binding sequence on PGs is distinct from the FGF-binding sequence (7, 8) or the antithrombin-binding sequence of PGs (9). However, the way in which the specificity is determined has not yet been explored in depth nor is the regulation of the quantity of HSPGs in particular organs well understood.

The present study was designed to test the hypothesis that lipase expression could stimulate the production of HSPGs. The effect of HL synthesis and cellular binding on the production of HSPGs was studied both in cell culture and in vivo. To do so, we prepared stably transfected Chinese hamster ovary (CHO) cells that produced HL in a native form or in a membrane-bound form via a glycophosphatidylinositol (GPI)-anchor (10). We also generated two lines of transgenic mice that expressed either a native HL or a membrane-bound form.

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Abbreviations: apo, apolipoprotein; CHO, Chinese hamster ovary; FGF, fibroblast growth factor; GAG, glycosaminoglycan; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GPI, glycophosphatidylinositol; HSPG, heparan sulfate proteoglycan; KO, knock-out; LRP, LDL receptor-related protein; McA, McArdle RH-7777; PG, proteoglycan; syn-1, syndecan-1; WT, wild-type.

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MATERIALS AND METHODS

Chemicals

Aqueous [^{35}S]sulfate solution was purchased from Amersham Pharmacia Biotech (Piscataway, NJ). All other chemicals were purchased from Sigma Co. (St. Louis, MO). Mouse syndecan-1 (syn-1) cDNA was kindly provided by the late Dr. M. Bernfield at the Children's Hospital, Harvard University, Boston, MA.

Cells

CHO cells were transfected with rat HL cDNA as previously described (11). CHO-K1 [wild-type (WT)] cells were purchased from the American Type Culture Collection (Manassas, VA). PG-deficient CHO cells (745) were kindly provided by Dr. J.D. Esko, of the University of Alabama at Birmingham (12). In some experiments, CHO cells expressing rat HL in a GPI-anchored form were used (10). McArdle RH-7777 (McA) cells transfected with human HL were kindly provided by Dr. R. Mahley of the Gladstone Institute of Cardiovascular Disease, San Francisco, CA.

Animals

WT C57/BL mice and mice deficient in HL were purchased from Jackson Laboratories (Bar Harbor, ME). The HL-deficient mouse was bred with C57/BL mice. All protocols were approved by the Institutional Animal Care and Use Committee (IACUC), Palo Alto Medical Foundation-Research Institute (PAMF-RI), Palo Alto, CA. The animal facility at PAMF-RI is AALAC accredited.

Preparation of rat HL

CHO cells transfected with rat HL cDNA were grown in flasks in Dulbecco's modified Eagle's medium-Coon's F-12 (1:1) plus 10% fetal calf serum at 37°C in an atmosphere of 5% CO_2 until just subconfluent, and then the medium was replaced with an induction medium containing 30 μM $ZnSO_4$ (11). The enzyme was purified as previously described (11). Briefly, a medium from rat HL-producing CHO cells was collected, and HL was purified on octyl-sepharose (Amersham Pharmacia Biotech) followed by heparin-sepharose columns. Fractions containing lipase activity were concentrated using Centricon-30 filters and were stored in liquid nitrogen. Triglyceride lipase activity was measured by the method of Nilsson-Ehle and Schotz (13) using [^{3}H]triolein. Its molecular mass was approximately 57 kDa as previously reported (11).

Labeling of cells

Cells were incubated with 50 μCi/ml [^{35}S]sulfate at 37°C for 8 h. The medium was removed, and the cells were washed 3× with PBS. The cells were dissolved in 0.25 ml NaOH (0.1 N), and an aliquot was removed for protein determinations by the method of Lowry, Rosbrough, and Randall (14). The amount of radiolabeled PG was determined by counting the radioactivity in the cell lysate. In some experiments, intracellular PG levels were determined by the method previously described by Edwards and Wagner (15).

Northern blot analysis

Total liver RNA from WT and HL knock-out (KO) C57/BL/6 mice was prepared using a kit from Qiagen, Inc. (Valencia, CA). Twenty micrograms of total RNA was electrophoresed on a 1% formaldehyde-agarose gel and transferred to a nylon membrane. The filter was baked, prehybridized, and then hybridized with a [^{32}P]dCTP-labeled mouse syn-1 probe. The filter was washed, air dried, and autoradiographed overnight. The film was scanned using a phosphoimager (Molecular Dynamics, Sunnyvale, CA). Protein sequences of syn-1 between mouse (16), human (17), hamster (18), and rat (19, 20) are virtually identical.

Real-time PCR

Total RNA was prepared from livers obtained from WT and KO mouse lines using a kit from Qiagen, Inc., and a reverse transcriptase reaction was performed on 1 μg of RNA using random hexamer primers and reverse transcriptase (Gibco BRL, Life Technologies, Vienna, Austria). The primers and probe for mouse syn-1 were designed using Primer Express 1.5 (Applied Biosystems, Foster City, CA). The probe for syn-1 was labeled with a reporter dye (FAM) and quencher dye (TAMRA). The primers and TaqMan™ probe for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were purchased from ABI. The probe for GAPDH was labeled with a reporter dye (VIC) and TAMRA. Real-time PCR (RT-PCR) was performed using 40 amplification cycles (95°C, 15 s; 55°C, 1 min; 72°C, 30 s). The level of GAPDH RNA was quantified and used to normalize the concentration of syn-1 RNA in each sample.

Statistics

Statistical analysis was done by nonpaired Student's *t*-test.

RESULTS

Production of PGs in CHO cells secreting HL

Given the importance of the interaction between HSPG and HL, the effect of HL binding on the production of HSPG was assayed using several CHO cell lines. PG synthesis was determined by measuring the incorporation of SO_4 into cellular PG. WT (K1) cells, CHO cells producing HL in a secreted form (D121), PG-deficient (745) cells, and CHO cells expressing a membrane-bound form of HL (244.6) were incubated with [^{35}S]sulfate for 8 h. Radiolabeled PGs in the medium, intracellular, and pericellular pools were measured. As shown in **Fig. 1**, HL-secreting CHO cells

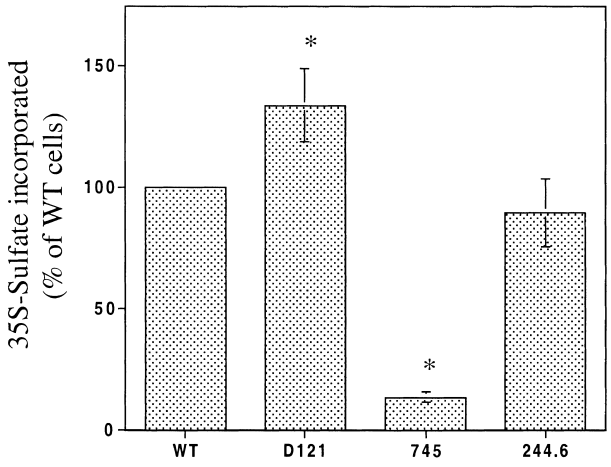

Fig. 1. Intracellular proteoglycan (PG) levels. Chinese hamster ovary (CHO) cells were incubated with 50 uCi/ml [^{35}S]sulfate for 8 h, and cellular PG levels were determined by the method previously described by Wagner et al. (15). 244.6, CHO cells producing HL as a membrane bound form; 745, CHO cells lacking in PGs; D121, CHO cells secreting rat HL; WT, wild-type CHO cells. Data are expressed as mean ± SD (n = 4). * $P < 0.01$.

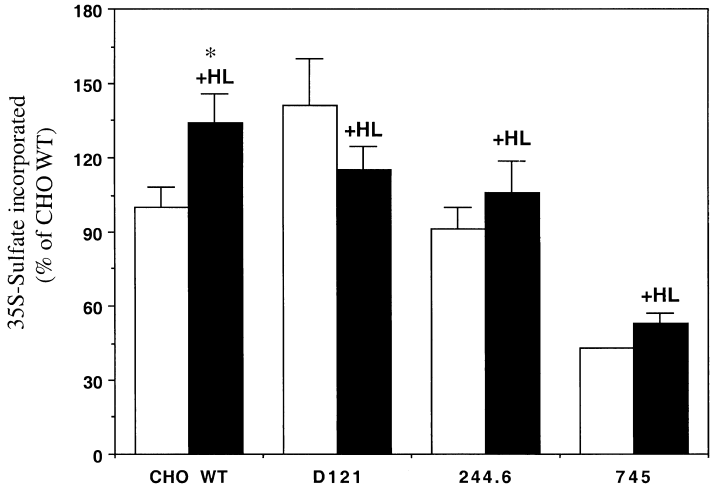

Fig. 2. Effect of purified HL on PG production. Rat HL was purified, 1.0 μg/ml of lipase was added to CHO cell lines, and production of PGs was determined as described under Materials and Methods. Data are expressed as percent of WT (mean ± SD, n = 4). * $P < 0.01$.

(D121) had about 30% more newly synthesized PG in the intracellular pool. In contrast, the expression of a membrane-anchored HL (244.6) did not affect PG synthesis. As expected, PG-deficient cells (745) had a dramatically lower rate of PG synthesis than did WT (K1) cells. Measurement of radiolabeled PGs in the medium and pericellular pools also showed increased incorporation of radiolabeled sulfate in D121 cells as compared with that in WT or 244.6 cells (data not shown). These data suggest that cells expressing HL in a secreted form that is able to bind to HSPG have higher levels of HSPG synthesis. Thus, the association of HL with cell surfaces, and not the synthesis of HL, is responsible for the increase in PG production.

Effects of purified HL on the production of PG in CHO cells producing membrane-anchored HL

To further test our hypothesis, we added purified rat HL to cells and determined PG production. When purified HL was added to WT CHO cells, SO_4 incorporation was significantly increased (**Fig. 2**). An identical result was observed when HL was added to 244.6 cells that express HL in a GPI-anchored form, but this did not reach statistical significance. In contrast, PG production was not altered in HSPG-deficient 745 cells. These studies provide further evidence that HL binding to HSPG, and not its synthesis, is responsible for the increase in PG synthesis.

Effect of heparin on PG production

To further confirm that the association of HL with cell-surface HSPG is necessary for the HL-mediated production of HSPG, heparin was used to inhibit the association of HL with PGs. When heparin was added to HL-secreting CHO cells (HL+Hep), the increase in HSPG production was reduced to the levels observed in WT cells (**Fig. 3A**). Because liver cells are more physiologically relevant for HL studies, HSPG production in the liver cell line McA, a rat hepatoma cell line, was tested. Consistent with the results in CHO cells, HL-secreting hepatic cells (HHL McA) had greater PG synthesis than did control cells, and heparin reduced PG synthesis to levels equivalent to those in WT McA cells (Fig. 3B). Together, these data strongly support the concept that the binding of HL to HSPG induces HSPG synthesis.

Northern blot analysis and RT-PCR of syn-1 mRNA

To determine whether HL affects PG synthesis at the transcriptional level, the relative quantities of mouse syn-1

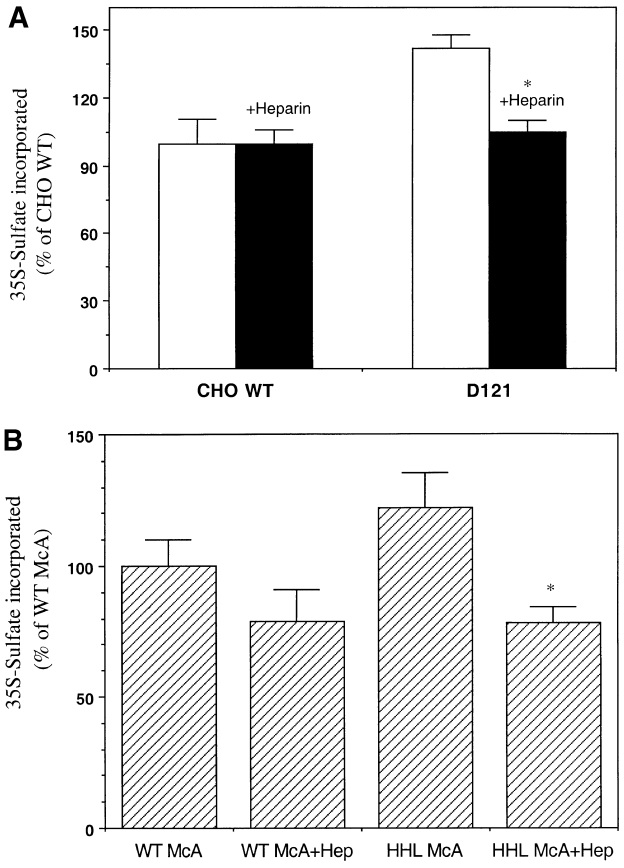

Fig. 3. Effect of heparin in PG production. CHO cells (A) or rat hepatoma, McArdle RH-7777 cells (B) were incubated with media containing 50 μCi/ml [^{35}S]sulfate and 3 units/ml heparin for 6 h, and cellular production of [^{35}S]sulfate-labeled PGs was measured as described above. Values are mean ± SD (n = 4). Hep, heparin treatment. * $P < 0.01$.

mRNA in the cell lines were measured. Furthermore, to study whether production of PG can be stimulated by HL, in vivo livers from several animal models were also studied.

Northern blot analysis was used to compare the expression of PGs (syn-1) using RNA prepared from CHO cells and mouse liver. Syn-1 mRNA levels were increased by about 30% in the CHO cell lines that secreted HL (D121) as compared with levels in WT CHO cells (**Fig. 4A**). Furthermore, syn-1 mRNA levels were about 43% less in HL KO mice than in WT mice (Fig. 4B).

Similarly, expression of syn-1 mRNA as determined by RT-PCR was significantly higher in the livers of animals producing rat HL (HL-NL) than in WT animals (WT) (**Fig. 5**). The production of syndecan in mice expressing a GPI-anchored rat HL was moderately lower than that in HL-NL mice. Strikingly, animals deficient in HL (HL KO) expressed significantly lower levels of syndecan than did the WT. The amount of HL mRNA in animals producing rat HL was similar to that in animals producing rat GPI-anchored HL (data not shown). Thus, the data obtained from RT-PCR and Northern blot analysis suggest that the occupancy of PGs by HL on the cell surface causes a positive feedback in PG production, and that HL regulates PG production by increasing mRNA levels.

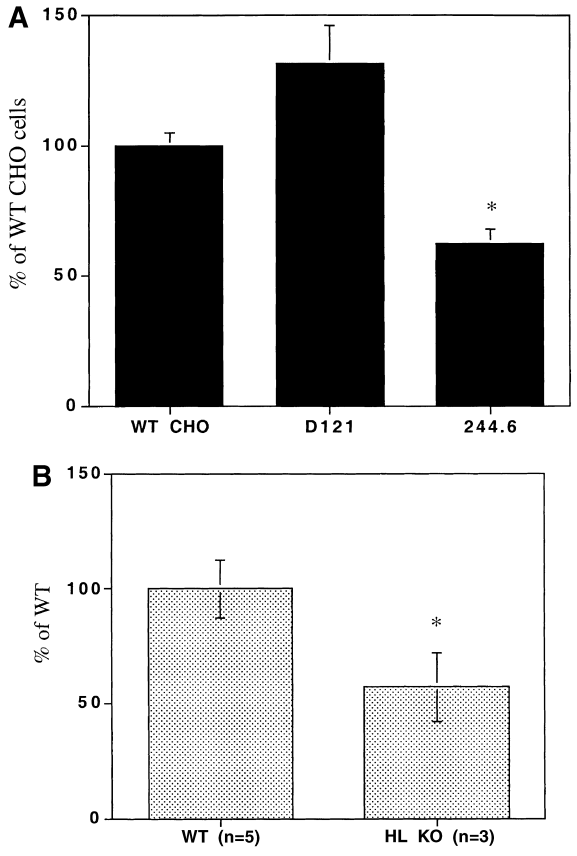

Fig. 4. Northern blot analysis of syndecan RNA in HL-producing CHO cells (A) and mice lacking HL (B). Twenty micrograms of total liver RNA was subject to a Northern blot analysis as described under Materials and Methods. Values are mean ± SD [n = 5 for WT and n = 3 for HL knock-out (KO) mice]. * $P < 0.01$.

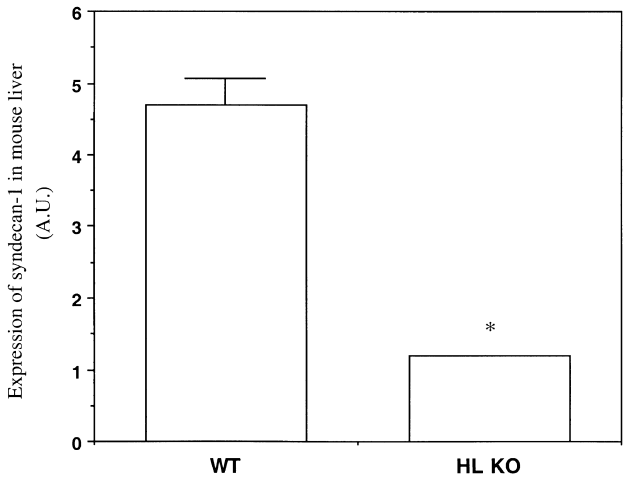

Fig. 5. Real-time PCR (RT-PCR) analysis of syndecan-1 in mouse liver. RT-PCR was performed using total liver RNA prepared from WT mice (WT, C57 BL) and HL KO. Values are mean ± SD (n = 5). * $P < 0.01$.

DISCUSSION

The present study establishes a relationship between HSPG synthesis and the binding of HL.

PGs are ubiquitous components of cell membranes. They consist of a core protein with one or more glycosaminoglycan (GAG) chains covalently attached (21). GAGs are linear, sulfate-substituted carbohydrates that interact with proteins with clusters of positively charged amino acids. This property allows PGs and GAGs to interact with a wide variety of proteins (21, 22, 23, 24), including growth factors, enzymes, cytokines, chemokines, lipoproteins, and viruses. Among the PGs, HSPGs have been implicated in playing a major role in lipoprotein metabolism, in part because of their role in anchoring lipases to the surface of specific cells (23, 24). By using quantitative RT-PCR and Northern blotting to measure mRNA levels both in vivo and in vitro, we demonstrated for the first time that HL stimulates the production of cell surface PGs.

The stimulation of PG production by HL was first assessed in a cell culture system by determining the rate of precursor incorporation into HSPG. CHO cells that secreted native HL showed enhanced production of PGs as determined by $^{35}SO_4$ incorporation. In contrast, $^{35}SO_4$ incorporation in CHO cells that expressed membrane-anchored HL was no different from that in WT CHO cells. The anchored enzyme was generated by linking GPI to the carboxyl-terminal moiety of HL (10). This technique requires the modification of the carboxyl-terminus of the protein, and the C terminus does not affect the catalytic activity of the enzyme (25). However, the putative heparin-binding domain is located in the C-terminal region. Thus, the interaction between the GPI-anchored HL and the cell surface HSPG would presumably be reduced or eliminated as compared with the interaction between native HL and HSPG. The inability of the anchored form to induce PG synthesis was not due to a defect in the syn-

thetic pathway, as the addition of purified HL to these cells stimulated PG production to the same extent as in control cells.

Together, these data suggest that the occupancy of PGs by HL on the cell surface has a positive feedback on cellular PG production. The level of mRNA of a major HSPG, syndecan, was higher in cells expressing HL than in control cells, suggesting that the increase in HSPG production occurs at a pretranslational level. This finding provided a means of determining whether the phenomenon observed in cultured cells was also true in vivo. Indeed, syndecan mRNA levels were substantially lower in mice that did not express HL than in those that did, even though in mice the major portion of HL circulates in the blood and does not interact with PGs due to the defects in heparin binding affinity (26). This suggests that HL binding and syndecan synthesis are not strictly coupled. As the amount of cell-surface-bound HSPG in cells overexpressing HL may not be rate limiting for the binding of HL, it is possible that increased PG synthesis is induced to serve a function other than HL binding.

It was previously proposed that an interaction between apolipoprotein E (apoE) and HSPG is required for the uptake of β-VLDL mediated by LRP (27), and that HSPG is involved in the LPL-mediated uptake of lipoproteins (28); Goldberg et al. (29) reported that the N-terminal region of apoB in lipoproteins is involved in the association of lipoprotein with GAGs. Furthermore, the association of both LDL and chylomicron remnants with cells was decreased in PG-deficient cells independent of lipases (data not shown). Based on these observations, it is reasonable to suggest that HSPG and lipases act together to maximally enhance receptor-mediated lipoprotein removal, although either one alone can do so.

The stimulation of HSPG synthesis in response to the binding of HL could be a way of providing more HL binding sites. Alternatively, the present data suggest that it could be a mechanism for sensing that a cell is to be active in lipoprotein and sterol removal, and, thus, additional HSPG may be needed for direct lipoprotein binding to further facilitate removal. Previously, Obunike et al. (30) reported that LPL and apoE increase PG production in cell culture as measured by [^{35}S]sulfate incorporation. Both apoE and LPL increased GAG synthesis and decreased its degradation by protecting PGs from heparanase digestion. Similarly, binding of PGs to FGFs has been suggested to induce conformational changes and/or stabilize the protein configuration. Thus, inhibition of HS binding to FGFs may modulate cellular responses to FGFs.

In the present study, by using RT-PCR and Northern blotting, we demonstrated that when HL is a ligand of HSPG, PG synthesis is induced at the mRNA level by a positive feedback mechanism. Furthermore, the relevance of the phenomenon to the intact organism was demonstrated by the fact that HSPG mRNA levels were reduced in HL KO mice. Thus, HL relies on HSPG for its physiological function and also regulates the level of HSPG expression.

The authors are indebted to Dr. Jeffrey Esko for providing us with proteoglycan-deficient Chinese hamster ovary cells (745), Dr. Robert Mahley for HL-expressing McArdle RH-7777 cells, and the late Dr. M. Bernfield for syndecan-1 cDNA. The authors thank Dr. Sivaram Pillarisetti and Dr. Loren Fong for scientific discussions. This research was supported by funds provided by Grants HL-58037 (S.Y.C.), DK-38318, and DK-56339 (A.D.C.) from the National Institutes of Health.

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