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Intracellular Insulin-like Growth Factor-1 Induces Bcl-2 Expression in Airway Epithelial Cells

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Bcl-2, a prosurvival protein, regulates programmed cell death during development and repair processes, and it can be oncogenic when cell proliferation is deregulated. The present study investigated what factors modulate Bcl-2 expression in airway epithelial cells and identified the pathways involved. Microarray analysis of mRNA from airway epithelial cells captured by laser microdissection showed that increased expression of IL-1β and insulin-like growth factor-1 (IGF-1) coincided with induced Bcl-2 expression compared with controls. Treatment of cultured airway epithelial cells with IL-1β and IGF-1 induced Bcl-2 expression by increasing Bcl-2 mRNA stability with no discernible changes in promoter activity. Silencing the IGF-1 expression using short hairpin RNA showed that intracellular IGF-1 (IC-IGF-1) was increasing Bcl-2 expression. Blocking epidermal growth factor receptor or IGF-1-R activation also suppressed IC-IGF-1 and abolished the Bcl-2 induction. Induced expression and colocalization of IC-IGF-1 and Bcl-2 were observed in airway epithelial cells of mice exposed to LPS or cigarette smoke and of patients with cystic fibrosis and chronic bronchitis but not in the respective controls. These studies demonstrate that IC-IGF-1 induces Bcl-2 expression in epithelial cells via IGF-1-R and epidermal growth factor receptor pathways, and targeting IC-IGF-1 could be beneficial to treat chronic airway diseases. The Journal of Immunology, 2012, 188: 4581–4589.

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Abbreviations used in this article: AG1478, anilinoquinazoline; CF, cystic fibrosis; CS, cigarette smoke; EGFR, epidermal growth factor receptor; FA, filtered air; IGF-1, insulin-like growth factor-1; IL-1β, interleukin-1β; PPP, picropodophyllin; shRNA, short hairpin RNA.

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Human subjects and lung tissue analysis

Lung tissue specimens from subjects with cystic fibrosis were obtained under protocols approved by the University of North Carolina at Chapel Hill.
Office of Human Research Ethics Institutional Review Board. The lung specimens with the CFTR mutation were rapidly procured from individuals with the CFTR mutation were rapidly procured from individuals with chronic bronchitis and noncystic diseases (Table II) were obtained from Lung Tissue Research Consortium that is sponsored by the National Heart Lung and Blood Institute.

**Laboratory animals**
Specific pathogen-free F344/NCR male rats 6–8 wk age were obtained from the National Cancer Institute (Frederick, MD) and were housed until 8–10 wk age. The rats were housed in pairs and were provided food and water ad libitum, a 12-h light/dark cycle at 22.2 °C, and 30–40% humidity. Rats were weighed and randomly assigned to each experimental group. Pathogen-free wild-type C57BL/6J mice were purchased from The Jackson Laboratory. Mice were housed in isolated cages under specific pathogen-free conditions. All experiments were approved by the Institutional Animal Care and Use Committee and were conducted at Lovelace Respiratory Research Institute, a facility approved by the Association for the Assessment and Accreditation for Laboratory Animal Care International.

**Rat intratracheal instillation**
For intratracheal instillations, rats were briefly anesthetized with 5% isoflurane in oxygen and instilled intratracheally with 1000 µL (Pseudomonas aeruginosa serotype 10, lot 31K4122, 3,000,000 LPS units [endotoxin units/ml; Sigma-Aldrich, St. Louis, MO]) in 0.5 ml 0.9% pyrogen-free saline. Control rats were instilled with 0.5 ml 0.9% pyrogen-free saline.

**Laser-capture microdissection and microarray analyses**
The right lungs of F344/NCR male rats at 0 and 2 d after LPS instillation were snap-frozen and stored at −80°C after inflating with diluted (1:4 in PBS) Tissue-Tek OCT (EMS Biosciences, Hatfeild, PA). Frozen tissue sections (8 µm thick) were fixed, dehydrated, and air-dried, and epithelial cells from five large airways of each rat were captured using the laser onto Arcturus Capture HS laser-capture microdissection caps (Applied Biosystems, Foster City, CA). Total RNA was extracted from the cellular lysate in the cap using the PicoPure RNA isolation kit (Applied Biosystems) and mRNA were amplified using the RiboAmp RNA amplification kit (Applied Biosystems) as recommended by the manufacturer’s instructions. Epithelia microdissected from eight tissue sections resulted in an average 1000 ng amplified RNA. A second round of amplification was carried out and the biotinylated cRNA probes were then prepared. Microarray analysis was performed using a rat microarray chip RG-U34A (Affymetrix, Sunnyvale, CA). All datasets were deposited at the National Center for Biotechnology Information for the Assessment and Accreditation for Laboratory Animal Care.

**Western blot analysis**
Protein was extracted from the right lungs of LPS instilled rats by homogenization in RIPA buffer (10 mM Tris [pH 7.4], 150 mM NaCl, 1% Triton X-100, 1% deoxycholate, 0.1% SDS, and 5 mM EDTA) supplemented with a protease inhibitor mixture (Sigma-Aldrich) at 1:100 final concentration. Protein concentration was determined using the BCA kit (Pierce/Thermo Fisher Scientific, Rockford, IL) and 100–150 µg protein lysate was analyzed by Western blotting. The Bcl-2, IGF-1, EGFR, and β-actin were detected using appropriate peroxidase-conjugated secondary Abs and visualized by chemiluminescence (PerkinElmer, Waltham, MA) using the Fujifilm image reader LAS-4000 (Fujifilm, Valhalla, NY).

**Lung tissue specimens**
Lung tissue specimens from subjects diagnosed with chronic lung disease and specimens from non-cystic fibrosis (CF) controls were then prepared. Microarray analysis was performed using quantitative real-time PCR using RT-PCR Master Mix (Applied Biosystems) in the ABI Prism 7900HT real-time PCR system. Relative quantities were calculated by normalizing averaged C(T) values to CDK1 or 18S to obtain ΔC(T), and the relative standard curve method was used for determining the fold change as described previously (23).

**mRNA half-life analysis**
Cells were treated with the RNA polymerase inhibitor 5,6-dichloro-1-β-D-ribofuranosilbenzimidazole (Sigma-Aldrich) at a final concentration of 100 ng/ml to block RNA polymerase activity and harvested at 0, 0.5, 1, 2, 4, 6, 8, 12, and 24 h posttreatment. The relative mRNA abundance was calculated using the ΔΔC(T) method, and mRNA half-life was calculated using the Greenberg Equation (24).

**Bcl-2 promoter constructs and transfection**
Generation of the P1–P2, P1, and P2 luciferase constructs has been previously described (25). The P1 promoter region is the main driving force for transcribing the Bcl-2 gene; and P1 activity is suppressed by the P2 region (25). The P2 region (681 bp) was cloned in pCR2.1 vector to obtain P2–TA construct and various deletion mutants were generated by using standard techniques. The full-length mutant P2 construct was generated by purine–pyrimidine substitutions (Mutagenex) to fully destroy the consen-
sus Brm3a-interacting region at residues 93–99. All transient transfections were carried out in 24-well-plates (USA Scientific, Orlando, FL) using Fugene 6.0 reagent (Roche Applied Biosystems, Indianapolis, IN) as described previously (25).

**LPS instillation**

Male C57BL/6 mice at 8–10 wk age were briefly anesthetized with 5% isoflurane in oxygen and instilled intranasally with 60 μg LPS (Pseudomonas aeruginosa serotype 10, lot 31K4122, 3,000,000 LPS units [endotoxin units/mg; Sigma-Aldrich] in 0.05 ml 0.9% pyrogen-free saline. Control mice were instilled with 0.05 ml 0.9% pyrogen-free saline. Mice were sacrificed 3 d after instillation and lung tissues were processed and analyzed.

**Exposure to cigarette smoke**

Male C57BL/6 mice at 8–10 wk age were exposed whole body to mainstream cigarette smoke (CS) or filtered air (FA) as described previously (26). Mice were acclimated to the CS exposure at 100 mg/m³ total particulate material for the first week and then exposed to CS concentration at 250 mg/m³ total particulate material for the following 22 wk. CS was generated from 2R4F filtered research cigarettes (University of Kentucky Tobacco Research and Development Center, Lexington, KY) using the AMESA type 1300 smoking machine. Male C57BL/6 mice at 8–10 wk age were briefly anesthetized with 5% isoflurane in oxygen and instilled intranasally with 60 μg LPS (Pseudomonas aeruginosa serotype 10, lot 31K4122, 3,000,000 LPS units [endotoxin units/mg; Sigma-Aldrich] in 0.05 ml 0.9% pyrogen-free saline. Control mice were instilled with 0.05 ml 0.9% pyrogen-free saline. Mice were sacrificed 3 d after instillation and lung tissues were processed and analyzed.

**Results**

**Microarray analysis of laser-capture microdissected airway epithelia**

Previously, we observed increased number of Bcl-2–positive epithelial cells in rats exposed to LPS, specifically on day 2 after LPS challenge (7). Thus, to identify the mediators involved in upregulation of Bcl-2 expression, we microdissected airway epithelial cells by laser-capture microscopy (Fig. 1A) from lungs of noninstilled rats or 2 d after LPS exposure. Microarray profiling of the mRNA isolated from the dissected epithelial cells on rat RGU34A chips revealed 800 differentially expressed genes (p < 0.05), and the heat map of the differentially expressed genes displayed consistent gene expression patterns among individual rats in the same treatment group (Fig. 1B). Among the 800 genes, there were 137 growth factors, 51 cytokines, and 44 apoptosis-related genes (Fig. 1C). The most significantly induced genes (2- to 6-fold) were IGF-1, IL-1α, IL-1β, eotaxin, MCP-1, MIP-2, and MIP-1α; however, IGFBP3 levels were downregulated by 3-fold in LPS-treated rats. Highly induced expression of IGF-1 and IL-1β along with that of elevated MUC5AC levels was validated by quantitative RT-PCR (Fig. 1D). Consistent with previous observations, Bcl-2 mRNA was also found to be 1.9-fold higher in LPS-exposed rats compared with noninstilled controls (Fig. 1D).

**IL-1β and IGF-1 induce Bcl-2 expression in airway epithelial cells**

We first tested the effect of the candidate inflammatory factors, IL-1β and IGF-1, on Bcl-2 expression by treating AALEB cells, a human airway epithelial cell line, with recombinant human IL-1β and IGF-1. A significant induction of Bcl-2 mRNA levels was consistently observed following IGF-1 (Fig. 2A) and IL-1β (Fig. 2B) treatments. Similar results were observed in SPC-1 cells, a rat airway epithelial cell line, following treatment with recombinant rat IL-1β and IGF-1 (Supplemental Fig. 1). Accordingly, 100 ng/ml IGF-1 and 5 ng/ml IL-1β were chosen for further analysis and resulted in increased Bcl-2 protein levels as analyzed by Western blot (Fig. 2C) and immunofluorescent staining (Fig. 2D) analyses.

**Intracellular IGF-1 mediates Bcl-2 upregulation via EGFR and IGF-1R pathways**

The EGFR pathway has been shown to be essential in cell survival and proliferation pathways, and therefore we tested whether IL-1β– and IGF-1–mediated induction of Bcl-2 requires the EGFR pathway. For this purpose, AALEB cells were treated with IGF-1 or IL-1β in the presence and absence of a specific EGFR kinase inhibitor, AG1478. The pretreatment of cells with AG1478 abolished the IGF-1– and IL-1β–mediated increase in Bcl-2 mRNA levels (Fig. 3A). Pretreatment with other EGFR kinase inhibitors, PD153035 and EKB-569, a potent irreversible inhibitor, also showed identical results (Supplemental Fig. 2). Because IL-1β was previously shown to induce IGF-1 (27), we hypothesized that IL-1β mediates IGF-1 and Bcl-2 expression. To test this hypothesis, AALEB cells were pretreated with specific IGF-1R tyrosine kinase inhibitor, PPP, followed by IGF-1 or IL-1β treatment. Both IGF-1– and IL-1β–mediated increases in Bcl-2 mRNA levels were abolished with PPP pretreatment (Fig. 3A).

Furthermore, both IGF-1 and IL-1β treatment increased the immunopositivity for intracellular IGF-1 (IC-IGF-1) and Bcl-2 by 4–5-fold, and IC-IGF-1 and Bcl-2 were colocalized in the same cells (Fig. 3B). Bcl-2 was absent in cells lacking IC-IGF-1 expression, and pretreatment with either AG1478 or PPP suppressed this effect (Fig. 3B). Similar results were obtained when IGF-1R and EGFR were inhibited in SPC-1 cells treated with rat IGF-1 and rat IL-1β (Supplemental Fig. 3).

To understand the kinetics of Bcl-2 induction, we determined the expression of Bcl-2 in primary human airway epithelial cells by immunofluorescence staining of cells treated with IGF-1 or IL-1β for 1, 2, 3, 4, 8, and 12 h. Compared to untreated cells, both IGF-1- and IL-1β–treated cells showed increase in Bcl-2–positive cells already at 1 h posttreatment with further increases in Bcl-2–positive cells at later time points (Fig. 3C). However, there were no significant differences between the two treatments. The role of IC-IGF-1 in inducing Bcl-2 expression was investigated by blocking its expression using shRNA specific for IGF-1. The IL-1β–mediated induction of both IC-IGF-1 and Bcl-2 were completely suppressed compared with control shRNA transfected cells (Fig. 3D).

Because IC-IGF-1 appeared to be central in increasing Bcl-2 expression, we examined whether IGF-1 induces Bcl-2 expression in airway epithelial cells in vivo by instilling rats with IGF-1 intratracheally and harvesting lungs at 24 h for immunostaining. Bcl-2 positivity of airway epithelial cells was increased by 2-fold compared with the vehicle-instilled group (Fig. 3E). Thus, IGF-1 induced Bcl-2 expression in airway epithelial cells both in vitro and in vivo.

Collectively, these data suggest that Bcl-2 expression is upregulated by IC-IGF-1 via convergence of EGFR and IGF-1R pathways.

**IGF-1 increases Bcl-2 mRNA stability**

We tested whether IGF-1 induces Bcl-2 promoter activity by transflecting AALEB cells with various Bcl-2 promoter luciferase reporter constructs described earlier (25). However, no changes...
in luciferase activity were observed following IGF-1 treatment (Supplemental Fig. 4A), suggesting that transcriptional activity was not affected. However, IGF-1 increased Bcl-2 mRNA half-life by ∼4.6-fold in AALEB cells (Fig. 4A). Because the Bcl-2 mRNA contains the P2 region upstream of the transcriptional start site (25), we investigated whether merely transfection with plasmids containing this upstream region may compete for mRNA degrading factors and thereby affect Bcl-2 mRNA levels in IGF-1–treated cells. Interestingly, cells transfected with the constructs containing only the P2 promoter region consistently showed enhanced increase in Bcl-2 mRNA levels compared with those transfected with empty vector when treated with IGF-1 (Fig. 4B). Similar results were obtained when cells transfected with P2 promoter constructs were treated with IL-1β (Supplemental Fig. 4B). These highly reproducible findings suggested that P2 region may interact with mRNA destabilizing factors that facilitate the IGF-1–mediated stability of Bcl-2 mRNA.

To identify the minimum possible subregion within P2 that was responsible for facilitating IGF-1–induced Bcl-2 mRNA stability, we generated various P2 deletion constructs and a mutant P2 construct with substitutions at the 93–99 region that would destroy the Brn3a-interacting site (28) (Fig. 4C). Following IGF-1 treatment, the cells transfected with D1 (1–356) and D2 (1–126) deletion constructs showed an IGF-1–induced increase in Bcl-2 mRNA levels compared with those transfected with empty vector when treated with IGF-1 (Fig. 4B).
mRNA whereas cells transfected with D3 deletion construct (1–106) and the mutant P2 construct failed to show any change (Fig. 4C). These findings suggest that the region 91–108 previously shown to interact with Brn3a (29) is involved with IGF-1–induced stabilization of Bcl-2 mRNA.

**IC-IGF-1 and Bcl-2 are coexpressed in epithelial cells of chronic inflammatory airways**

We next tested whether IC-IGF-1 and Bcl-2 are expressed in animal models and, more importantly, in subjects with chronic airway diseases. As reported earlier (7–9), Bcl-2 mRNA levels were increased significantly in LPS- compared with saline-instilled rats (Fig. 5A). The airway epithelial cells when analyzed for IC-IGF-1 and Bcl-2 immunopositivity showed a 9- and 11-fold increase, respectively, in LPS-instilled mice compared with saline-instilled controls (Fig. 5B). Additionally, in CF the percentage of Bcl-2–positive epithelial cells is significantly increased compared with nondiseased controls (7). Because the present study shows that IC-IGF-1 is crucial for inducing Bcl-2 expression, we immunostained airway tissues from CF and controls with Abs to IC-IGF-1 and Bcl-2.

**FIGURE 4.** IGF-1 induces Bcl-2 expression by increasing Bcl-2 mRNA stability in a mechanism that involves the Bcl-2 P2 promoter region. (A) The calculated half-life of Bcl-2 mRNA in AALEB cells treated with nothing as control or with IGF-1 and transfected with nothing, empty vector, or a vector carrying the P2 construct (681 bp). Transfected and nontransfected (NT) cells were treated with IGF-1 for 4 h and Bcl-2 mRNA levels were analyzed by quantitative RT-PCR and compared with the media-treated NT cells (denoted by empty bar) (n = 4). (B) Bcl-2 mRNA levels following IGF-1 treatment of AALEB cells transfected with various deletion constructs of P2 promoter region and a mutant P2 construct (mutP2). Left panel illustrates the full-length P2 construct, various deletion constructs, and the mutP2 construct; the detailed description of constructs is in Materials and Methods. All cells were treated with recombinant human IGF-1 for 4 h and Bcl-2 mRNA was determined by quantitative RT-PCR normalized to empty TA-cloning vector-transfected cells (n = 4). Data are shown as means ± SEM. *p < 0.05, **p < 0.01.
Bcl-2. We found that in subjects with CF the number of IC-IGF-1– and Bcl-2–positive airway epithelial cells/mm BL was increased 3- and 7-fold, respectively, compared with normal subjects (Fig. 5C).

In a mouse model of chronic CS exposure, we observed a 4-fold increase in Bcl-2 mRNA levels in lung tissues of mice instilled with LPS or saline as quantified by quantitative RT-PCR. (A) Bcl-2 mRNA levels in lung tissues of mice instilled with LPS or saline as quantified by quantitative RT-PCR. (B) Analysis of IC-IGF-1– and Bcl-2–positive airway epithelial cells in lung tissues of mice instilled with LPS or saline. Mouse lung tissues were harvested 3 d after LPS or saline instillation. (C) IC-IGF-1– and Bcl-2–positive epithelial cells in airways of subjects with CF or no lung disease. (D) Bcl-2 mRNA levels in lung tissues of mice exposed to CS or FA controls for 22 wk. (E) IC-IGF-1– and Bcl-2–positive airway epithelial cells in lung tissues of mice exposed to CS or FA. (F) IGF-1– and Bcl-2–positive airway epithelial cells in lung tissue from former smokers with chronic bronchitis (CB) compared with former smokers with no CB. Lung tissue sections from former smokers with no CB (left panels) and with CB (right panels) were coimmunostained for IGF-1 (top panels) and Bcl-2 (middle panels). The bottom panels illustrate the merged image showing the coexpression of IGF-1 and Bcl-2 in airway epithelial cells of CB (n = 4 subjects/group). For (B), (C), (E), and (F), lung tissue sections were immunostained for IC-IGF-1 (top panels) and Bcl-2 (bottom panels) and the numbers of positive cells per millimeter of basal lamina were quantified (n = 4/group). Scale bars, 10 μm. Results are shown as means ± SEM. *p < 0.05, **p < 0.01, ***p < 0.0001.

Discussion

The present study identifies IL-1β and IGF-1 as the principal inflammatory mediators that induce Bcl-2 expression in airway epithelial cells. Induction in Bcl-2 expression involved both EGFR and IGF-1R pathways and required the expression of IC-IGF-1. Several observations suggest that not the “secreted IGF-1” but the IC-IGF-1 mediates induction in Bcl-2 expression. First, IGF-1 treatment of airway epithelial cells increased IC-IGF-1 and Bcl-2 levels both in vitro and by intratracheal instillation of rats in vivo. Second, immunofluorescent analysis of IL-1β–treated cells showed that IC-IGF-1 and Bcl-2 were colocalized in the same cells, whereas cells without IGF-1 positivity were Bcl-2–negative. Third, IL-1β treatment of airway epithelial cells resulted in robust IC-IGF-1 expression and blocking IC-IGF-1 expression using shRNA suppressed Bcl-2 expression. Collectively, these data show that IL-1β and IGF-1 lead to induced Bcl-2 expression via upregulating IC-IGF-1. The mechanism by which IL-1β induces IC-IGF-1 is not clear. However, similar results were observed in prostate epithelial cells and in cardiac cells, where IL-1β induces anti-apoptotic and proliferative phenotype by upregulating IGF-1 transcription using the JAK–STAT pathway (27, 30). The molecular mechanisms by which IL-1β induces IC-IGF-1 in airway epithelial cells are yet to be defined.

In airway epithelial cells, the EGFR pathway has been identified as a common denominator for proinflammatory responses that aid in epithelial cell hyperplasia and mucous cell metaplasia (19, 31–33). Tyner et al. (19) proposed that the EGFR pathway is associated with survival of airway epithelial cells in response to a
viral challenge. However, the mechanism of EGFR-induced survival airway epithelial cells was not identified. Our data show that EGFR activation by inflammatory mediators results in increased Bcl-2 expression and present a new paradigm that EGFR activation sustains airway epithelial cells by inducing the antiapoptotic protein Bcl-2. These findings are consistent with other studies that report that TGF-α–induced activation of EGFR mediates Bcl-2 induction in NCI-H292 cells (34) and in gastric mucosal cells (35). Moreover, the external stimuli such as exposure to ozone, LPS, CS, or allergen sensitization that upregulate Bcl-2 expression (36) are also known to activate EGFR in airway epithelial cells (37, 38). EGFR and IGF-1R pathways and their ligands have been shown to cross-talk, but mostly in transformed cells, with IGF-1 induction resulting in secretion of the EGFR ligands TGF-α and amphiregulin, and blocking of EGFR activation abolished the IGF-1R–stimulated intracellular pathways (39, 40). Additionally, continuous EGF stimulation required a functional IGF-1R pathway that resulted in ERK activation in mouse fibroblasts (41, 42). The observed increase in Bcl-2 immunopositivity as early as an hour after treatment indicates rapid convergence of these pathways leading to Bcl-2 induction.

Besides epithelial cell proliferation and survival signaling, EGFR mediates MUC5AC synthesis following exposure to LPS, CS, or infection with Pseudomonas aeruginosa (31, 37, 38). Interestingly, EGFR activation causes MUC5AC and Bcl-2 expression (33, 34), and Bcl-2 expression sustains epithelial cell hyperplasia (7). Future studies will investigate whether Bcl-2 expression directly mediates MUC5AC expression. Moreover, Bcl-2 binds and suppresses NALP1, an integral component of inflammasomes, to reduce caspase-1, originally identified as an interleukin–converting enzyme, and thus inhibits IL-1β production (43, 44). Taken together, the present data suggest that the inflammatory mediators such as IL-1β and IGF-1 stimulate EGFR and IGF-1R pathways that converge to induce IC-IGF-1 to help upregulate Bcl-2 expression in epithelial cells, and induced Bcl-2 expression could, in a negative feedback mechanism, suppress inflammation (Fig. 6).

Bcl-2 expression is regulated at the transcriptional and posttranslational levels. The P1 region harbors the positive regulatory elements for Bcl-2 transcription (25, 45), and the P2 region suppresses the P1 activity (25). Transcriptional factors such as the CREB and SP-1 (46, 47) interact with the P1 region to upregulate its transcriptional activity, whereas factors such as nuclear NF-CREB and SP-1 (46, 47) interact with the P1 region to upregulate its transcription (25, 45), and the P2 region suppresses the P1 activity (25). Transcriptional factors such as the CREB and SP-1 (46, 47) interact with the P1 region to upregulate its transcriptional activity, whereas factors such as nuclear NF-CREB and SP-1 (46, 47) interact with the P1 region to upregulate its transcription (25, 45), and the P2 region suppresses the P1 activity (25). Transcriptional factors such as the CREB and SP-1 (46, 47) interact with the P1 region to upregulate its transcriptional activity, whereas factors such as nuclear NF-CREB and SP-1 (46, 47) interact with the P1 region to upregulate its transcription (25, 45), and the P2 region suppresses the P1 activity (25).
Disclosures

The authors have no financial conflicts of interest.

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