Growth arrest and DNA damage 45G down-regulation contributes to Janus kinase/signal transducer and activator of transcription 3 activation and cellular senescence evasion in hepatocellular carcinoma

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Growth arrest and DNA damage 45G (GADD45G), a stress sensor with multiple implications in various biological processes, is down-regulated in a broad spectrum of cancers. However, little is known about the biological effects of GADD45G on hepatocellular carcinoma (HCC) cells and the related mechanisms. In the present study, we found that GADD45G was commonly down-regulated in oncogene-transformed mouse liver cells and in human and mouse HCC. Ectopic expression of GADD45G robustly elicited senescence in HCC cells and suppressed tumor growth in vivo. Furthermore, GADD45G-induced senescence occurred in HCC cells independently of p53, p16INK4a (p16), and retinoblastoma (Rb). Instead, the prompt inhibition of Janus kinase 2 (Jak2), tyrosine kinase 2 (Tyk2), and signal transducer and activator of transcription 3 (Stat3) activation was observed in cells undergoing senescence. Impairment of Jak-Stat3 activation caused by GADD45G expression was associated with activation of SH2 domain-containing protein tyrosine phosphatase-2 (Shp2). Expression of constitutively activated Stat3 or human telomerase reverse transcriptase (hTERT), as well as knockdown of Shp2, efficiently counteracted GADD45G-induced senescence. More important, in clinical HCC specimens, we found that GADD45G expression was inversely correlated with phosphorylated Stat3 expression in tumor cells and disease progression. Conclusion: GADD45G functions as a negative regulator of the Jak-Stat3 pathway and inhibits HCC by inducing cellular senescence. The decrease or absence of GADD45G expression may be a key event for tumor cells or premalignant liver cells to bypass cellular senescence. (HEPATOLOGY 2014;59:178-189)

Hepatocellular carcinoma (HCC) is one of the most malignant cancers and is listed as the second-most frequent cause of cancer deaths in men and sixth in women worldwide.1 HCC generally develops in patients with liver cirrhosis and chronic infection with hepatitis B virus (HBV) and hepatitis C virus (HCV). Emerging evidence has shown that cellular senescence provides a tumor-suppressive mechanism for preventing liver tumorigenesis through the cell-autonomous regulation of proliferation or by triggering immune surveillance.2-5 Consistently, genetic or functional inactivation of senescence-related proteins, such as p53 and p16/retinoblastoma (Rb), has been observed in human HCC specimens.6,7 Because initiation of the senescence program

Abbreviations: Akt, protein kinase; BrdU, bromodeoxyuridine; DEN, diethylnitrosamine; DOX, doxycycline; GADD45G, growth arrest and DNA damage 45G; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; hTERT, human telomerase reverse transcriptase; IHC, immunohistochemical; IL, interleukin; Jak, Janus kinase; LPS, liver progenitor cells; mRNA, messenger RNA; qRT-PCR, quantitative real-time polymerase chain reaction; Rb, retinoblastoma; SA-β-gal, senescence-associated β-galactosidase; SC, subcutaneous(ly); Shp2, SH2 domain-containing protein tyrosine phosphatase 2; siRNA, small interfering RNA; Stat3, signal transducer and activator of transcription 3; SV40 LT, SV40 large T antigen; Tet, tetracycline; TMA, tissue microarray; Tyk2, tyrosine kinase 2.

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in cells may require a reorganization of multiple events by adjusting their strength or functional presence, it is important to obtain more information regarding the mechanisms by which liver cells are transformed as well as how liver tumor cells bypass senescence.

Growth arrest and DNA damage 45G (GADD45G) is a member of the GADD45 family, which has been implicated in various biological processes, including integration of the cellular response to different stresses, regulation of development, cell differentiation, and survival. GADD45G expression is broadly down-regulated in a number of cancers, such as lymphoma, nasopharyngeal carcinoma, cervical carcinoma, esophageal carcinoma, lung carcinoma, and HCC. Mechanistically, decrease of GADD45G expression in these tumors is primarily attributed to hypermethylation of CpG islands. Although a ubiquitous down-regulation of GADD45G expression has been observed in various types of cancer, the molecular mechanisms by which GADD45G functions as a tumor suppressor are still unclear. In the present study, we show that GADD45G robustly elicits cellular senescence in HCC cells and remarkably suppresses tumor growth in vivo. We demonstrate, for the first time, that GADD45G induces HCC cell senescence independently of the functional presence of p16, p53, and Rb, and that down-regulation of Janus kinase (Jak)/signal transducer and activator of transcription 3 (Stat3) is the key event for GADD45G-induced cell senescence and tumor suppression. These findings provide new evidence for the involvement of the stress sensor, GADD45G, in the biological connection between cellular senescence evasion and hepatotumorigenesis.

**Materials and Methods**

**Human Tissue Specimens and Cell Lines.** A commercially available tissue microarray (TMA) containing 75 pairs of HCC was used, and the patient information provided by the manufacturer is listed in the Supporting Data. The TMA sections were used for immunochemistry staining. Another set of 45 paired HCC samples from the Qidong Liver Cancer Institute (Qidong, China) were processed for RNA and protein analysis. All human materials were approved by the institutional ethical review committee. The human HCC cell lines SK-Hep1 and Hep3B were purchased from the American Type Culture Collection (Manassas VA). The human HCC cell line SMMC-7721, embryonic kidney 293 cells were from the Chinese Academy of Sciences (Shanghai, China). Mouse fetal liver progenitor cells (LPCs) were isolated from p53-/- mice by the methods described previously and cultured for the retroviral delivery of H-Ras V12, myristoylated protein kinase B (Akt), and c-Myc.

**Animal Model.** Male BALB/c nude mice (6-8 weeks of age) received single subcutaneous (SC) flank injection of 5 or 6 × 10^6 Sk-Hep1 cells diluted in 200 µL of saline. Drinking water was supplemented with doxycycline (2 mg/mL) to induce GADD45G expression. Tumor growth was monitored by bidimensional measurements using a caliper. Tumor-bearing mice were sacrificed 50 days after inoculation, and then the tumors were removed for further study. All mice studies were conducted in accord with protocols approved by the Shanghai Medical Experimental Animal Care Commission.

**Statistical Analysis.** Statistical analyses were performed using SPSS 16.0 statistical software (SPSS, Inc., Chicago, IL). For in vivo studies, the percentage of tumor-free mice in each group was analyzed using Kaplan-Meier’s survival analysis and Tarone-Ware’s statistic was used for comparison of curves between groups. For in vitro studies, a two-tailed t test was used to determine significance. The statistical correlation between the clinical parameters of HCC and GADD45G staining levels in tissue sections was analyzed by the chi-square test, nonparametric test, and one-way analysis of variance. A P value less than 0.05 was considered statistically significant.

**Laboratory Methods.** See the Supporting Materials and Methods section for detailed experimental procedures.
Results

Identification of GADD45G as an Inducer of Cellular Senescence in HCC Cells. To explore the molecular basis of liver tumor initiation, we used a genome-wide transcriptional profile analysis to compare the transcriptional profile of nontransformed p53-deficient fetal LPCs (p53−/− LPCs) with those of the three types of oncogene-transformed p53−/− LPCs, namely, H-RasV12-LPCs, myristoylated-Akt1-LPCs, and c-Myc-LPCs. We focused on those differentially expressed genes potentially involved in cell-cycle regulation and found that the stress sensor, GADD45G, a member of the GADD45 protein family, was consistently down-regulated by these oncogenes (Supporting Fig. 1). Down-regulation of GADD45G in these oncogene-transduced cells was further validated by quantitative real-time polymerase chain reaction (qRT-PCR) analysis (Fig. 1A). Consistently, we also found that GADD45G messenger RNA (mRNA) expression was drastically decreased in human liver cancer cell lines, as compared with the immortalized liver cell line, Thle2 (Fig. 1B). Furthermore, an aberrant reduction in GADD45G expression was observed in primary mouse HCCs induced by diethylnitrosamine (DEN), in comparison with noncancerous mouse liver tissues (Fig. 1C). More important, a majority of clinical human HCC samples exhibited losses or substantial decreases in GADD45G expression, as demonstrated by an examination of transcripts from 45 paired clinical samples (Fig. 1D) and protein analysis of 12 paired HCC samples (Fig. 1E).

We reason that the great consistency in GADD45G down-regulation or loss in expression among liver cancer cell lines and primary HCC tissues from mice and humans may suggest a suppressive function of this gene in HCC development. Hence, we extended our study by observing the influence of ectopic GADD45G expression on three human HCC cell lines (Sk-Hep1, SMMC-7721, and Hep3B). After 4 days of culture of cells with GADD45G expression, the senescence-related morphological changes, including the more flattened, enlarged, and irregular cell shapes, were readily observed in these cells. Indeed, approximately 50%-70% of these cells ectopically expressing GADD45G were stained positively for senescence-associated marker β-galactosidase (SA-β-gal;
Furthermore, mRNA levels of the senescence-associated secretory cytokine, interleukin (IL)-8, were also significantly elevated (Supporting Fig. 2). These cells were then subjected to both apoptosis and bromodeoxyuridine (BrdU) incorporation assays. We found that GADD45G expression did not induce cell apoptosis, but rather resulted in the substantial inhibition of cell proliferation in these cells (Fig. 2B). Next, we examined whether induction of endogenous GADD45G expression is contributable to drug-induced cellular senescence. We found that transient MG-132 treatment induced GADD45G expression in Sk-Hep1 cells (Supporting Fig. 3). Down-regulation of GADD45G by small interfering RNA (siRNA) remarkably alleviated MG132-induced senescence (Supporting Fig. 3), suggesting a significant role of GADD45G in certain drugs- or stresses-triggered cellular senescence. In addition, expression patterns of two other members of the GADD45 family, GADD45A and B, were also examined in transformed cells, HCC cells, and tumor tissues. Notably, expression of GADD45B was significantly down-regulated in human HCC specimens (Supporting Fig. 4). However, by ectopic expression of the GADD45 proteins in three different HCC cell lines, we found that only GADD45G could efficiently decrease cell proliferation and induce cellular senescence (Supporting Fig. 5).

Next, we further examined the consequence of GADD45G-induced liver cancer cell senescence in vivo. For tumor formation experiments, Sk-Hep1 cells were infected with lentiviruses carrying a tetracycline (Tet)-inducible GADD45G expression cassette (Tet-GADD45G) and the control (Tet-empty), respectively. We verified that doxycycline (DOX)-induced GADD45G expression efficiently triggered cell senescence in cultured cells, as indicated by SA-β-gal staining (Fig. 2C) and expression of senescence-related secretory proteins IL-6 and IL-8 (Supporting Fig. 6A,B). Consistently, levels of γ-H2AX were remarkably increased (Supporting Fig. 6C). These cells were SC inoculated into athymic nude mice. An inducible expression of GADD45G was achieved by feeding mice with DOX-containing water, and tumor growth was monitored for 50 days postinjection. GADD45G expression significantly increased the average latency of tumor appearance in vivo. Notably, by day 20 after injection, all mice infected with cells without ectopic GADD45G expression developed visible tumors, whereas only 2 of 5 mice injected with cells ectopically expressing GADD45G exhibited a palpable tumor mass (Fig. 2D). Moreover, the size of the generated tumors with ectopic GADD45G expression was remarkably smaller than tumors from control groups (Fig. 2E). Immunostaining results showed that induction of GADD45G in vivo resulted in a substantial reduction of Ki-67 expression in tumor cells (Fig. 2F). These data clearly demonstrate that GADD45G efficiently induces HCC cell senescence and suppresses tumor growth in vivo.

GADD45G Induces Liver Cancer Cell Senescence Independently of p53 and p16/Rb Pathways. Mounting evidence demonstrates that the major cell-cycle regulators, p53, p16, and Rb, are critically implicated in the induction of cell senescence.15-17 In the present study, we found that GADD45G overexpression in p16-deficient, but p53-competent, Sk-Hep1 cells did not significantly affect the protein levels of p53, p21, p27, or Rb throughout the induction of cell senescence (Fig. 3A). To explore the possibility that GADD45G-induced senescence may not necessarily require the functional presence of p53/p21 and p16/Rb, different strategies were employed in the experiments. We first transfected human Sk-Hep1 cells with lentiviral vector harboring SV40 large T antigen (SV40 LT), which can inactivate p53 function by direct binding.18,19 The efficiency of SV40 LT expression entirely blocked up-regulation of p53 and downstream p21 expression in Sk-Hep1 cells treated with the chemotherapy agent, doxorubicin (Fig. 3B). We found clear evidence that SV40 LT expression did not rescue GADD45G-induced cell senescence in p16-deficient Sk-Hep1 cells (Fig. 3C and Supporting Fig. 7A), indicating that GADD45G-induced senescence efficiently occurs in cells deficient in both p16 and p53 function. We further verified roles of p53/p21 and p16/Rb in GADD45G-induced senescence by knocking down these proteins. SK-Hep1 cells were separately transfected with small interfering RNAs (siRNAs) against p21, p27, p53, and Rb and then cultured for 3 days in the presence or absence of GADD45G induction. Our results showed that knockdown of these proteins did not affect induction of cell senescence and cell arrest by GADD45G (Fig. 3D and Supporting Fig. 7B). The efficiency of the siRNAs for the down-regulation of the target proteins was confirmed by western blotting (Supporting Fig. 7C). Moreover, in p53−/− mouse LPC-H-RasV12 cells, GADD45G also efficiently induced cellular senescence (Supporting Fig. 7D,E). Finally, we tested whether the combinatory deficiency of p53, Rb, and p16 could modulate the process of senescence initiated by GADD45G. We showed that human hepatoma Hep3B cells deficient in both p53 and Rb had a similar vulnerability to GADD45G-induced senescence when treated with siRNA specific
Fig. 2. GADD45G induces cellular senescence and inhibits tumor growth of HCC cells. (A and B) Sk-Hep1, SMMC-7721, and Hep3B cells lentivirally transduced with GADD45G (pSin-GADD45G) and their control (pSin-Vector) were cultured for 4 days. (A) Representative light microscopy showing SA-β-gal staining (left panel). Percentage of positive cells is depicted (right panel). (B) Cell apoptosis (upper panel) and proliferation (lower panel) were examined by Annexin V/7-aminoactinomycin D and BrdU fluorescence-activated cell sorting staining, respectively. Values represent the mean ± SD of three independent experiments (*P < 0.05; **P < 0.01). (C) Tet-GADD45G- and Tet-empty-Sk-Hep1 cells were cultured for 4 days in the presence (Tet⁺) or absence (Tet⁻) of 0.5 μg/mL of DOX for GADD45G induction. Representative images showing SA-β-gal staining (left panel) and the calculation of the percentage of positive cells (right panel) are shown. (D-F) Groups of 5 nude mice were SC injected with 5 x 10⁶ Sk-Hep1 cells as indicated. (D) Kaplan-Meier's analysis of tumor onset. The group of GADD45G; Tet⁺ mice versus the control groups of mice (*P < 0.05). (E) Volumes of tumor were measured at the indicated time intervals. Statistical data are shown as mean ± SD of tumor volumes by day 50 postinjection (**P < 0.01). (F) Tumor sections were subjected to H&E staining and IHC for Ki-67. Original magnification, ×400. H&E, hematoxylin and eosin.
for p16, as compared with cells treated with control siRNA (Fig. 3E and Supporting Fig. 7F). The efficiency of siRNA in p16 knockdown was confirmed by western blotting (Fig. 3F). Collectively, these results indicate that GADD45G functions as an inducer of senescence in liver cancer cells through p53- and p16/Rb-independent mechanisms.

**Inhibition of the JAK/Stat3 Pathway in GADD45G-Induced Senescence.** The strength or functional presence of multiple signal pathways may be fundamentally reorganized in cells undergoing senescence. Therefore, we analyzed the alterations in several components of different signaling pathways in cells with or without GADD45G overexpression. Intriguingly, we found that the constitutive phosphorylation of Stat3 (Tyr^{705}) was substantially inhibited by GADD45G expression in the human liver cancer cell lines, Sk-Hep1, SMMC-7721, and Hep3B (Fig. 4A). More specifically, in Sk-Hep1 cells, we found that levels of Tyr^{705}-phosphorylated Stat3 rapidly decreased, starting within 2 hours after GADD45G induction; coincidently, levels of phosphorylated Jak2 and tyrosine kinase 2 (Tyk2), the upstream activators of Stat3, were also severely down-regulated (Fig. 4B). We extended our observation to two subclones (numbers 1 and 2) of mouse LPC-H-RasV12 cells, wherein GADD45G could significantly induce cell senescence (Fig. 4C). Consistently, levels of phosphorylated Stat3, Jak2, and Tyk2 were also substantially decreased in these cells (Fig. 4D). Moreover, we observed a similar down-regulation in Stat3 activation in colon cancer cells in the presence of GADD45G (data not shown). These results suggest that JAK-Stat3 inhibition is an early event in GADD45G-mediated cell arrest and cell senescence.

Considering that Stat3, Jak2, and Tyk2 can be directly dephosphorylated by SH2 domain-containing protein tyrosine phosphatases (SHPs), we then tested whether inhibition of phosphatase activity is sufficient to block GADD45G-mediated senescence. The phosphatase inhibitor, sodium orthovanadate (Na_{3}VO_{4}), was added to culture 30 minutes before DOX induction of GADD45G expression. Strikingly, treatment with the phosphatase inhibitor resulted in a dose-dependent attenuation of GADD45G-mediated senescence in both human Sk-Hep1 cells and mouse LPC-H-RasV12 cells (Fig. 4E). Accordingly, the reduced expression in phosphorylated Stat3, Jak2, and Tyk2 was largely restored (Fig. 4F). We further examined the status of the phosphatases, Shp1 and 2, in Sk-Hep1 cells and LPC-H-RasV12 cells after GADD45G induction. Results showed that phosphorylated Shp2 at Tyr^{580} was promptly increased in cells upon GADD45G induction (Fig. 5A), whereas the basal level of Shp1 was undetectable in these cells (data not shown). Intriguingly, levels of p-Stat3 and p-Tyk2 and p-Jak2 were constantly down-regulated from days 1 to 4 in Sk-Hep1 cells with GADD45G expression, coincidently with the persistent up-regulation of p-Shp2 and p-Erk expression (Supporting Fig. 8). We further employed the SHP inhibitor, NSC 87877, and siRNA against Shp2 to confirm whether Shp2 activation is essential for GADD45G-mediated inhibition of Stat3. Indeed, treatment with NSC 87877 significantly increased the levels of phosphorylated Stat3, Jak2, and Tyk2 in both Sk-Hep1 and LPC-H-RasV12 cells (Fig. 5B). Furthermore, we found that knockdown of Shp2 by siRNA efficiently abrogated GADD45G-induced dephosphorylation of Stat3 (Fig. 5C) and profoundly inhibited senescence induction, as indicated by SA-β-gal and cell-cycle analysis (Fig. 5D,E). In accord with the observation, we also found that JAK/Stat3 inhibition, either by their inhibitors (LLL12 and WP1066) or the siRNAs targeting Stat3, Jak2, and Tyk2, effectively restored GADD45G-induced senescence in cells pretreated with sodium orthovanadate (Supporting Figs. 9 and 10). Collectively, we demonstrate that the stress sensor, GADD45G, negatively regulates JAK-Stat3 phosphorylation in the early stage of senescence induction, whereas inhibition of phosphatase Shp2 entirely rescues GADD45G-induced cell senescence.

**Stat3 Reactivation Counteracts GADD45G-Induced Cellular Senescence.** Next, we tested whether Stat3 reactivation can counterbalance GADD45G-induced senescence in liver tumor cells. A constitutively active Stat3 mutant that substitutes cysteine residues for A661 and N663 (Ca-Stat3) was lentivirally transduced into Sk-Hep1 cells (Supporting Fig. 11A). Prominently, Ca-Stat3 almost entirely diminished the cellular senescence and cell-cycle arrest induced by GADD45G (Fig. 6A,B and Supporting Fig. S11B). Meanwhile, we found that GADD45G expression significantly impaired human telomerase reverse transcriptase (hTERT) expression, and that Ca-Stat3 expression partially rescued the inhibitory effects (Fig. 6C). Furthermore, overexpression of hTERT remarkably attenuated GADD45G-induced cellular senescence (Fig. 6D and Supporting Fig. 11C).

We further substantiated the aforementioned observation by *in vivo* tumor growth experiments. We found that fewer than 30% (2 of 8) of mice developed tumors at day 50 after SC inoculation of Sk-Hep1 cells with the inducible expression of GADD45G (Tet^{+} GADD45G/Vector group). However, in the
presence of Ca-Stat3 expression, 90% (7 of 8) of mice (Tet+ GADD45G/Ca-Stat3 group) developed tumors (Fig. 6D). GADD45-induced delay of tumor onset was significantly rescued by Ca-Stat3 expression. In addition, the average volume of tumors from the group (Tet+ GADD45G /Ca-Stat3) was comparable to that of the control (Tet−) groups, and tumors were much larger than in the Tet+ GADD45G/Vector group (Fig. 6E). Histological analyses showed that the tumor cells expressing both GADD45G and Ca-Stat3 displayed a robust augmentation in Ki-67 expression, when compared with tumors that expressed GADD45G alone (Fig. 6F). These results clearly demonstrate that Stat3 activation counteracts the GADD45G-mediated inhibition of tumor growth in vivo.

**GADD45G Expression Is Inversely Correlated With Stat3 Phosphorylation in Primary Human HCCs.** To further investigate whether dys-regulated GADD45G-mediated regulation of Stat3 is relevant to human HCC development, we tested the association between GADD45G expression and Stat3 activation in human HCC samples. We did immunoblotting assay using another set of human HCC samples, and found that most of the human liver tumors with decreased or absent GADD45G expression displayed activated Stat3 (Fig. 7A). Immunohistochemical (IHC) staining of GADD45G and p-Stat3 in an independent cohort of
75 pairs of HCC specimens demonstrated that a decreased expression of GADD45G protein accompanied a higher p-Stat3 staining in continuous tissue sections from the same patient in HCC sections (Fig. 7B; Supporting Table 2). Positive staining of GADD45G (defined as a score greater than 4) was detected in approximately 89.3% (67 of 75) of adjacent nontumor regions, whereas only 25.3% (19 of 75) of HCC samples were positive for GADD45G staining ($P < 0.001$). In contrast, p-Stat3 staining was positive (score, 4-12) in approximately 4% (3 of 75) of nontumor sections and in 49.3% (37 of 75) of tumor areas (Fig. 7C). Statistical analysis showed a significant difference in p-Stat3 expression in HCC regions between GADD45G-negative regions, whereas only 25.3% (19 of 75) of HCC samples were positive for GADD45G staining ($P < 0.001$). In contrast, p-Stat3 staining was positive (score, 4-12) in approximately 4% (3 of 75) of nontumor sections and in 49.3% (37 of 75) of tumor areas (Fig. 7C). Statistical analysis showed a significant difference in p-Stat3 expression in HCC regions between GADD45G-negative
and -positive groups (Fig. 7D, left). When categorized with p-Stat3 expression in HCC sections, the positive group had significantly lower GADD45G expression than the negative group (Fig. 7D, right). Moreover, GADD45G expression was significantly lower in poorly or moderately differentiated tumor tissues than in well-differentiated tissues (Fig. 7E). Overall, the IHC scores of GADD45G and p-Stat3 in HCC cells were inversely correlated in the cohort. Altogether, these results indicate that GADD45G expression is inversely correlated with Stat3 phosphorylation and tumor progression in human HCC.

Discussion

The present study demonstrates that GADD45G functions as a senescence inducer and has a robust capability for inhibiting Jak-Stat3 activation in HCC cells. In line with our observation, compelling evidence demonstrates that Stat3 hyperactivation functions downstream of many pro-oncogenic signals, including oncogenes and growth factors, to promote tumor development. In human HCC, Stat3 is constitutively activated and correlated with tumor progression. Therefore, decreased GADD45G expression, concurrently with Stat3 activation in human HCC, strongly suggests the functional relevance of GADD45G-mediated senescence in attenuating HCC development.

Loss of function of the key effectors in senescence induction, such as p53, Rb, and INK4a-ARF, frequently occurs in primary HCC. Recently, cellular senescence has been programmed in the absence of activation of the key cycle checkpoints. For example, p16/RB- and p53-independent senescence is elicited by oncogenic
RAS signaling, wherein transforming growth factor beta signaling is required. Down-regulation of p300 histone acetyltransferase activity induces senescence independently of p53, p21, and p16 function. We found that GADD45G-induced senescence did not require the presence of p53, p16, and Rb in HCC cells. These findings highlight that, in addition to classical initiators of cell senescence, such as p53, Rb, and INK4a-ARF, other proteins may trigger cellular senescence to inhibit tumor development in an independent manner or as a complementary mechanism. Dys-regulation of these nonclassical senescence inducers may be essential for efficient tumorigenesis. In this regard, GADD45G down-regulation is considered to be one of the important events that lead to senescence evasion in premalignant liver or HCC cells. Notably, using a mouse model, Tront et al. demonstrated that GADD45A, a member of the GADD45 family, suppresses Ras-driven mammary tumors by triggering cell apoptosis and senescence, although we found that GADD45A and B did not induce senescence in various HCC cell lines. These findings suggest that identification of a new senescence inducer is significant for understanding the mechanism of tumor development.

Cells undergoing senescence require a highly coordinated regulation of multiple signaling pathways. We found that GADD45G expression promptly resulted in the dephosphorylation of Stat3 in different HCC cell lines as well as other types of tumor cell lines (data not shown). Phosphorylated Tyk2 and Jak2 were
down-regulated upon GADD45G induction. Coincidentally, GADD45G efficiently activated the phosphatase, Shp2, which dephosphorylates Stat3, Tyk2, and Jak2. Alterations in activities of Shp2 and Stat3 are functionally relevant in that either down-regulation of Shp2 or restoration of Stat3 activity can efficiently counteract GADD45G-induced senescence. Importantly, a recent study demonstrates that hepatic deficiency of Shp2 in mice dramatically promotes DEN-induced HCC development and that decreased Shp2 expression occurs in certain cases of human HCC specimens.\textsuperscript{30} Interestingly, a recent study also describes a role of Stat3 in driving cell senescence in nontransformed cells.\textsuperscript{31} Therefore, the cell context-dependent roles of Stat3 in cellular senescence remain a paradox in the field, most likely because of the diversities in Stat3 targets or in cross-talk with other signals in these cells. One interesting aspect of the study is that GADD45G suppresses hTERT expression. This observation is supported by previous studies that show that hTERT expression confers cellular senescence resistance.\textsuperscript{32,33} In fact, we found that restoration of hTERT was sufficient to block GADD45G-induced senescence. The clinical relevance of GADD45G down-regulation or absence in HCC was further addressed in this study. We found that GADD45G was down-regulated in approximately 75% of HCCs and was significantly correlated with tumor staging. More importantly, GADD45G levels in tumor tissues were inversely correlated with Stat3 activation in HCC. In summary, this study provides novel insight into a function of GADD45G in inhibiting the Jak-Stat3 pathway and attenuating growth of HCC cells through induction of tumor cell senescence. The present findings identify a
link between stress sensor-initiated senescence and HCC inhibition that may enhance our understanding of human hepatotumorigenesis and lead to the development of novel treatment strategies.

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