TIS21 Negatively Regulates Hepatocarcinogenesis by Disruption of Cyclin B1–Forkhead Box M1 Regulation Loop

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A functional and biochemical interaction of TIS21/BTG2/PC3 with Forkhead box M1 (FoxM1), essential transcription factor for hepatocyte regeneration and a master regulator of mitotic gene expression, was explored. Growth of hepatocellular carcinoma (HCC), developed by a single injection of diethylnitrosamine (DEN), was the same in both the TIS21+/− and TIS21−/− mice until 6 months, whereas it was significantly higher in the TIS21−/− mice at 9 months. Expression of TIS21 was significantly lower in both human and murine HCCs than in the surrounding tissues. Forced expression of TIS21 impaired growth, proliferation, and tumorigenic potential of Huh7 cells. At the mechanistic level, TIS21 inhibited FoxM1 phosphorylation, a required modification for its activation, by reducing cyclin B1–cdk1 activity, examined by in vivo kinase assay and FoxM1 mutant analyses. These observations were further confirmed in vitro by the reciprocal control of TIS21 expression and FoxM1 phosphorylation in the diethylnitrosamine-induced HCCs and TIS21−/− mouse embryonic fibroblast (MEF), in addition to increased expression of cyclin B1 and cdk1 activity. Conclusion: TIS21 negatively regulated hepatocarcinogenesis in part by disruption of the FoxM1–cyclin B1 regulatory loop, thereby inhibiting proliferation of transformed cells developed in mouse and human livers. (HEPATOLOGY 2008;47:1533-1543.)
cell lines, and medulloblastoma. These findings support TIS21/BTG2/PC3 as a tumor suppressor.

Forkhead box M1 (FoxM1) is a transcription factor known to be essential for normal hepatocyte regeneration and a master regulator of mitotic gene expression, expressed in all replicating cells. Phosphorylation and recruitment of p300/CBP coactivators to FoxM1 is required for FoxM1 activation. FoxM1 phosphorylation is initiated by cyclin–cdk complexes in early G1 and continues to G2 and M phases of the cell cycle. Based on the putative consensus phosphorylation sites, cdk1, cdk2, and mitogen-activated protein kinase have been suggested to be involved in FoxM1 phosphorylation and increase transcriptional activity. Based on the report that cyclin B1 and cdc25B are target genes of p-FoxM1 and hepatocyte mitosis is associated with expression of cdc25B phosphatase and cyclin B1 accumulation, increased cyclin B1 may generate more cyclin B1–cdk1 complex and further activates FoxM1 in a positive-feedback loop, thus resulting in hepatocyte regeneration.

We already reported that TIS21 inhibited cdk1 activity and worked as a pan-cdk inhibitor in cancer cells. In the current study, therefore, we attempted to investigate a role of TIS21 in hepatocarcinogenesis, focusing on the regulation of FoxM1 activity. Proliferation of hepatocellular carcinoma (HCC) was increased in the TIS21–/– mice, whereas forced expression of TIS21 significantly reduced cell growth and in vitro tumorigenicity of Huh7 cells, as well as G2/M arrest of the cell cycle. Furthermore, expression of cyclin B1 and FoxM1 phosphorylation were markedly increased in mouse HCCs and TIS21–/– mouse embryonic fibroblast (MEF). We propose here that TIS21 negatively regulates hepatocarcinogenesis via disruption of the cyclin B1–FoxM1 regulatory loop, resulting in inhibition of cyclin B1 transcription.

Materials and Methods

Induction of HCC and Preparation of MEF. Diethylnitrosamine (DEN) (Sigma Chemical Co.; 20 mg/kg body weight) was injected intraperitoneally once to 12-day-old wild-type and TIS21–/– mice. The mice were sacrificed at 3, 6, and 9 months after the injection. Saline was injected as the control treatment. TIS21+/+ MEF and TIS21–/– MEF were prepared from 13.5 day-old embryos of the wild-type and TIS21–/– mice and then cultured in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum in a 37°C incubator with 5% CO2 in air.

Tumor Samples From Patients. During the period of 2003 to 2005, HCCs and surrounding control tissues were obtained from 15 patients at Ajou University Hospital after surgical resection with informed consent. The hard and firm tumor tissues were trimmed free of normal tissue and snap frozen in liquid nitrogen immediately after resection according to the specimen regulation of Ajou University Hospital. No patient in the current study received chemotherapy or radiation therapy before the surgery.

Preparation of Cell Lines. Huh7 cells were transduced with TIS21 in pcDNA3-HA using Lipofectamine (Invitrogen, San Diego, CA), and selected with 975 µg/mL Geneticin 418 (Gibco BRL, Bethesda, MD) for 3 weeks. Adenoviruses of TIS21 (Ad-TIS21) and FoxM1B (Ad-FoxM1) were prepared by transfection of complementary DNAs (cDNAs) of TIS21 and FoxM1B inserted into replication-defective E1-adenoviral vectors and E3 adenoviral vectors, and then amplified in 293 cells. Adenovirus of bacterial β-galactosidase (Ad-LacZ) was prepared for the control experiment.

Clonogenic and Soft Agar Colony-Forming Assays. Huh7-TIS21 and Huh7-V cells were prepared by transfection of Huh7 cells with TIS21 and pcDNA3-HA, respectively. Cells (4 × 103) were seeded in a 35-mm plate and maintained for 9 days. Clones containing more than 50 cells were counted as positive after staining with crystal violet. For soft agar colony-forming assay, the cells (5 × 103) were plated in 0.6% agarose solution and then layered on 1.0% agarose bed. Colonies larger than 125 µm were counted as positive in 2 weeks.

Reverse Transcription Polymerase Chain Reaction and Real Time Polymerase Chain Reaction. Primer pairs used for reverse transcription polymerase chain reaction (RT-PCR) were sense 5′-gcggagacggcctgacgccagc-3′ and antisense 5′-ctggctgatccgcttgctgg-3′ for human cyclin B1, and sense 5′-gcggagacggcctgacgccagc-3′ and antisense 5′-ggctggagctgagcctggca-3′ for mouse cyclin B1. Real time PCR were sense 5′-gcatggctggctggccgaag-3′ and antisense 5′-ggctggagctgagcctggca-3′ for human cyclin B1, and sense 5′-ggctggagctgagcctggca-3′ for mouse cyclin B1, respectively. Human and mouse glyceraldehyde-3-phosphate dehydrogenase used for control expression were sense 5′-gcatggctggctggccgaag-3′ and antisense 5′-ggctggagctgagcctggca-3′, sense 5′-gcatggctggctggccgaag-3′ and antisense 5′-ggctggagctgagcctggca-3′, respectively. Primer pairs for real time PCR were sense 5′-gcatggctggctggccgaag-3′ and antisense 5′-ggctggagctgagcctggca-3′ for TIS21 expression, and sense 5′-gcatggctggctggccgaag-3′ and antisense 5′-tgccagagctgagcctggca-3′ for actin control.

Immunoprecipitation. Cell lysates (1.0 mg) were precleared with protein A–agarose beads (Invitrogen) for
1 hour at 4°C. Cyclin B1–associated and cyclin A–associated kinase assays, cyclin B1, FoxM1, and TIS21 were immunoprecipitated by incubating the cell lysates overnight at 4°C with antibodies against cyclin B1, cyclin A, FoxM1, and hemagglutinin (HA) (SantaCruz Biotechnology Inc., Santa Cruz, CA), respectively. Phosphoserine and phosphothreonine residues were detected by anti-phosphoserine (Zymed, San Francisco, CA) and anti-phosphothreonine (Zymed) antibodies. The immunoprecipitates were thoroughly washed twice with cell lysis buffer.

**Cyclin-Associated Kinase Assay.** Employing anti-cyclin B1 and anti-cyclin A antibodies, cdk1, and cdk2 immunocomplexes were prepared from Huh7 cells infected with either Ad-TIS21 or Ad-LacZ viruses. The cyclin-associated kinase activity was measured by incubating with histone H1 (5.0 μg) as a substrate and [γ-32P]adenosine triphosphate (ATP) (2.5 μCi/reaction) for 30 minutes at 30°C, and then terminated by adding 6 × sodium dodecyl sulfate sample buffer. Phosphorylation of the substrate was examined by autoradiography after sodium dodecyl sulfate gel electrophoresis. To further confirm inhibition of cdk1 activity by TIS21 in vitro, rTIS21 protein (5.0-10.0 μg) was added to the reaction mixture, and the kinase activity was assayed.

To investigate whether TIS21 can inhibit FoxM1 phosphorylation in vitro, immunoprecipitation (IP)-kinase assays were performed: Cell lysates of Huh7, infected with Ad-FoxM1B virus (100 moi) for 2 days, were immunoprecipitated overnight with anti-FoxM1 antibody, and the precipitate was used as a substrate, while using cyclin B1 immunocomplexes as a kinase source. Kinase assay was carried out in the presence of glutathione S-transferase TIS21 fusion protein (rTIS21) or glutathione S-transferase (GST) (0-10 μg) for 30 minutes at 30°C.

**FoxM1 Mutants by Site-Directed Mutagenesis.** Wild-type FoxM1 (wtFoxM1) cDNA was isolated from Huh7 cells, cloned into pCMV-Flag vector (Sigma), and used for FoxM1 mutants construction (Supplementary Table 1). FoxM1ΔC, C-terminal deletion mutant, was prepared by PCR with ΔC-sense and ΔC-antisense primers with wtFoxM1 7cDNA as template. To prepare N-terminal deletion mutant of FoxM1 (FoxM1ΔN cDNA as template. To prepare N-terminal deletion mutant of FoxM1 (FoxM1ΔN cDNA as template. To prepare N-terminal deletion mutant of FoxM1 (FoxM1ΔN, NΔ-sense and NΔ-antisense primers were used for construction of FoxM1 mutants in order to confirm the phosphorylation sites in transactivation domain of FoxM1. The FoxM1ΔN-S657A template, and NΔ-sense and megaprimer 3 primers with the FoxM1ΔN-T585A template, respectively. FoxM1 triple mutants (FoxM1ΔN-T585A,S657A; FoxM1ΔN-S507A,T585A; FoxM1ΔN-S507A,T585A and FoxM1ΔN-S507A,T585A,S657A) were amplified with NΔ-sense and megaprimer 1 primers the with FoxM1ΔN-T585A,S657A template, AN-sense S507A and megaprimer 1 primers with the FoxM1ΔN-T585A,S657A template, AN-sense S507A and AN-antisense primers with the FoxM1ΔN-T585, S507, S657A template, respectively.

**Cyclin B1 Promoter Analysis.** Reporter construct containing cyclin B1 promoter (−950 base pairs) was generated in basic pGL3 vector (Promega, Madison, WI), and transfected to Huh7 cells using lipofectamine. Thymidine kinase promoter-driven Renilla luciferase plasmid (Promega) was employed as control DNA. Transfection activity was measured by TD 20/20 luminometer (Turner BioSystems, Sunnyvale, CA) according to the instructions for the Dual-Luciferase Reporter Assay System (Promega). All transfection experiments and luciferase assays were carried out in triplicate and repeated more than twice.

**Results**

**Rapid Growth of HCC in Livers of TIS21−/− Mice.** No spontaneous tumor developed in either TIS21+/+ or TIS21−/− mice until 9 months by saline treatment; however, single injection of DEN developed HCCs in both wild-type and null mice (Fig. 1A). Severity of the HCCs in TIS21+/+ mice was less than that of the TIS21−/−

| Table 1. Inhibition of Tumor Growth Developed by a Single Injection of DEN to TIS21+/+ and TIS21−/− Mice |
|---------------------------------------------------------------|
| **DEN-Treated Number** | **Tumor Size** |
| No. of Mice | <0.2 cm² | 0.2-1.0 cm² | >1.0 cm² |
| 3M | TIS21+/+ | 8 | 0 | 0 | 0 |
| | TIS21−/− | 8 | 0 | 0 | 0 |
| 6M | TIS21+/+ | 8 | 2.3 ± 1.8 | 0.1 ± 0.3 | 0.1 ± 0.3 |
| | TIS21−/− | 8 | 2.4 ± 2.1 | 0.1 ± 0.3 | 0.4 ± 1.0 |
| 9M | TIS21+/+ | 8 | 13.1 ± 11.6 | 0.7 ± 0.8 | 0.2 ± 0.4 |
| | TIS21−/− | 12 | 26.5 ± 8.9* | 1.5 ± 0.8* | 0.9 ± 0.8* |

Number and size of HCC were significantly increased in the TIS21−/− mice at 9 months after DEN injection. *P < 0.05 versus TIS21+/+.
mice. The number and size of tumors increased markedly in the TIS21\(^{+/+}\) mice compared with the TIS21\(^{-/-}\) (Table 1).

**Loss of TIS21 Expression in Liver Tumors of Mouse and Human.** When TIS21 expression in liver was measured by RT-PCR, the level was variable in the saline-treated mice, whereas it was much less in every case of HCC obtained at 9 months after a single injection of DEN (Fig. 1B). It was approximately 30% of the control level, examined by real time PCR (Fig. 1C). Expression of BTG2\(^{+/+}\) was found in 1 of 15 human HCC cases, as opposed to 7 of 15 surrounding tissues (Fig. 1D). These data indicate that TIS21/BTG2 expression is significantly reduced in tumor compared with its surrounding tissue and suggest possible involvement of TIS21 in hepatocarcinogenesis.

**Inhibition of Tumorigenesis of Huh7 Cells by TIS21.** When the role of TIS21 in HCC cells was investigated, TIS21 messenger RNA (mRNA) and protein expressions were found in Huh7-TIS21 cells, but not in Huh7-V, by RT-PCR and immunoblot analyses (Fig. 2A), and growth of Huh7-TIS21 was significantly lower than that of Huh7-V (Fig. 2B). In contrast, however, growth of TIS21\(^{-/-}\) MEF was much higher than that of TIS21\(^{+/+}\) MEF, suggesting regulation of tumor cell growth by the TIS21 gene. To evaluate the effect of TIS21 on tumorigenicity, clonogenic and soft agar colony-forming abilities were examined; Huh7-TIS21 revealed one third the clonogenicity (Fig. 2C) and soft agar colony-forming abilities (Fig. 2D) of the Huh7-V. These data strongly suggest that TIS21 might negatively work in hepatocarcinogenesis.

**Regulation of Cyclin B1 Expression by TIS21.** Based on the reduced cell growth and in vitro tumorigenicity of Huh7-TIS21 cells, changes of cyclin proteins were evaluated in the TIS21 expressers by immunoblot analyses; mRNA and protein expression of cyclin B1, but not cyclin A and cyclin D1, were markedly reduced in Huh7-TIS21 cells, whereas pRB and FoxM1 expressions were slightly decreased (Fig. 3A). In contrast, expressions of cyclin A and cyclin B1 were higher in TIS21\(^{-/-}\) MEF than in TIS21\(^{+/+}\) (Fig. 3B), further confirming a role of
TIS21 in regulation of cyclin B1 expression. To test a possible effect of stable transfection of TIS21 on the reduced cyclin B1 expression, we performed Ad-TIS21 virus infection and found reduced protein and mRNA levels of cyclin B1 and G2/M arrest of the cells (Fig. 3C,D). These data support our hypothesis that expression of TIS21 inhibits cell growth and tumorigenicity of HCC cells by inhibiting cyclin B1 expression.

Inhibition of cdk1 Activity In Vivo and In Vitro by TIS21. Based on our earlier report that TIS21 binds to cdk1,11 interaction of TIS21(HA) with cyclin B1 was examined. Thus, Huh7 cell lysates infected with either Ad-LacZ or Ad-TIS21 viruses for 2 days were subjected to IP and immunoblot analyses using anti-cyclin B1 and anti-HA antibodies. As expected, cyclin B1 was bound to TIS21(HA), and vice versa (Fig. 4A). To evaluate regulation of cyclin-associated kinase activity by TIS21, a kinase assay was performed with [γ-32P]ATP and histone H1 after IP with either anti-cyclin B1 or anti-cyclin A antibodies. Phosphorylation of histone H1 by cdk1 in the Ad-TIS21–infected cells was found to be inhibited by 70% (Fig. 4B), whereas cyclin A–associated activity was inhibited by only 10%. To prove whether TIS21 directly inhibited the activity, in vitro kinase assay and autoradiography were performed in the presence of rTIS21 protein (0-10 μg); approximately 40% of cdk1 activity was inhibited by adding rTIS21 protein, but not GST (Fig. 4C). The results indicate that TIS21 inhibits in vivo and in vitro cdk1 activity via direct interaction with cyclin B1-cdk1.
Inhibition of FoxM1 Phosphorylation and Its Transcriptional Activity by TIS21. Based on reports that cdk1 and cdk2 are involved in FoxM1 phosphorylation and increase its transactivation activity,22,23 the possibility of regulating FoxM1 phosphorylation by TIS21 was investigated. FoxM1 was isolated by IP of Huh7-TIS21 and Huh7-V cell lysates, and the degree of p-FoxM1 was measured by immunoblot analyses. FoxM1 phosphorylation was significantly reduced in Huh7-TIS21 compared with Huh7-V (Fig. 5A). To further investigate whether TIS21 regulates FoxM1 phosphorylation, in vitro kinase assay was performed with IP of cyclin B1–associated kinase and FoxM1. FoxM1 phosphorylation was concentration-dependently inhibited in Huh7-TIS21 compared with Huh7-V (Fig. 5A). To further investigate whether TIS21 regulates FoxM1 phosphorylation, in vitro kinase assay was performed with IP of cyclin B1–associated kinase and FoxM1. FoxM1 phosphorylation was concentration-dependently inhibited in the presence of rTIS21, indicating direct inhibition of the phosphorylation by TIS21 (Fig. 5B). To examine the phosphorylation residues in FoxM1, deletion mutant analyses were extensively performed using IP and immunoblot analyses with FoxM1, Flag, p-Ser, and p-Thr antibodies (Fig. 5C): FoxM1 phosphorylation on Ser and Thr residues was inhibited by Ad-TIS21 infection (the 1st row); however, it was lost in both the Ad-LacZ–infected and Ad-TIS21–infected cells after transfection of C-terminal deletion mutant of FoxM1 (FoxM1ΔC, the second row), suggesting that the phosphorylated residues are on the C-terminus. When N-terminal deletion mutant (FoxM1ΔN) was expressed, p-Ser and p-Thr were detected in the Ad-LacZ–infected but not in Ad-TIS21–infected cells (the third row), suggesting inhibition of phosphorylation by TIS21. When the Ser and Thr residues were singly mutated to Ala (S507A, S657A, T585A, and T596A), phosphorylation was same as that of FoxM1ΔN; however, double (S507,657A, T585,596A) and triple mutants (T585,596A, S507,657A, T585,596A; S507A,T585,657A, T585,596A) revealed loss of phosphorylation in both the Ad-LacZ and Ad-TIS21 cells, suggesting the phosphorylation on the two Ser and the two Thr residues (the fourth to thirteenth rows). Mutant analyses indicate that TIS21 inhibits phosphorylation of the two Ser and two Thr residues of FoxM1. When cDNA of FoxM1B was transfected to Huh7 cells, promoter activity of cyclin B1 increased 2.7 times from that of the vector-transfected cells. However, when TIS21 cDNA was cotransfected together with FoxM1B, the promoter activity was significantly reduced, as compared with the FoxM1B–alone–treated group (Fig. 5D), which suggests that TIS21 down-regulates the transcriptional activity of FoxM1B.

To investigate a possibility of direct interaction of TIS21 with FoxM1, IP with anti-FoxM1 and anti-HA antibodies, and immunoblot with anti-HA and anti-
FoxM1 antibodies, respectively, were performed. The results confirmed that TIS21 and FoxM1 do not directly interact with each other (Supplementary Fig. 1).

**In Vivo Phosphorylation of FoxM1 in DEN-Treated Mice and TIS21**

To examine whether expression of cyclin B1 and cdk1 activities were changed in TIS21-/- hepatocytes, immunoblot analyses were performed with liver homogenates of TSI21+/+ and TIS21-/- mice, and significant increases in cyclin B1 expression (Fig. 6A) and cdk1 activity (Fig. 6B) were found in the TIS21-/- mice compared with the wild-type.

To evaluate the activity of FoxM1 in normal liver and HCC, we measured p-FoxM1 in mouse liver treated with either DEN or saline. Expression of p-FoxM1 was low in TIS21+/+ mice, but increased twice after DEN treatment (Fig. 6C). The p-FoxM1 was already increased in TIS21-/- mice regardless of DEN injection. Not only in liver but also in MEF, expressions of cyclin B1, p-FoxM1, and cdk1 activity were significantly higher in TIS21-/- than TIS21+/+, evidenced by immunoblot, IP-immunoblot analyses, and autoradiography (Fig. 6D). Expression of FoxM1 was the same; however, p-Ser in FoxM1 was significantly higher in TIS21+/+ MEF than in wild-type. These data indicate negative regulation of FoxM1 phosphorylation in vivo by TIS21.

**Discussion**

HCC is the 3rd most common cancer in Korean men. However, liver transplantation remains the only viable treatment for HCC. Even though cancer is not a disease regulated by a single gene expression, malignancy of HCC is significantly reduced by regulating TIS21 expression; TIS21 inhibits cdk1 activity via interaction with the cyclin B1/cdk1 complex, which in turn inhibits FoxM1 phosphorylation and transcription of cyclin B1 (Fig. 7). TIS21 forms a negative regulation loop with FoxM1 and cyclin B1. Indeed, TIS21 expression was significantly reduced in
human and mouse HCC compared with the surrounding tissue. Thus, induction of TIS21 appears to be an attractive alternative to handle HCC by genetic manipulation.

TIS21 did not bind to FoxM1 (Supplementary Fig. 1), but rather indirectly regulated FoxM1 activity by inhibiting cyclin B1–cdk1 activity. Nevertheless, size and number of HCCs were not significantly different between
TIS21^{+/+} and TIS21^{-/-} mice until 6 months after a single injection of DEN. This is in accordance with an earlier report^{29} and can be explained by a previous report^{30}; kinetics between doses of DEN and HCC development show a linear response, indicating only 1 hit, whereas time–response requires 4 hits to develop HCC, regardless of the dose used. Therefore, it takes more than 6 months to develop HCC after a single injection of DEN in both
TIS21^{+/+} and TIS21^{-/-} mice. However, a lack of the TIS21 gene may provide a growth advantage to the transformed cells, thus forming more HCCs in the TIS21^{-/-} mice at 9 months. A possible mechanism of the reduced expression of TIS21 in HCC may be selection of the clone transformed by DEN injection. Moreover, transfection of TIS21 to Huh7 cells significantly reduced in vitro tumorigenicity and tumor cell growth. These findings strongly suggest in vivo and in vitro effects of TIS21 on the negative regulation of HCC. In addition to TIS21, lack of the antiproliferative gene Tob also induces spontaneous tumors in liver, lung, and lymph nodes,\(^31\) supporting a tumor suppressor role of TIS21.

We propose that TIS21 works as an important regulator of HCC growth in mouse and human by inhibiting FoxM1 phosphorylation by cyclin B1/cdk1. The hypothesis is well supported by other reports that FoxM1 is an essential transcription factor for liver regeneration\(^{20,32}\) and that cyclin-mediated/cdk-mediated phosphorylation of FoxM1 is required for transactivation potential of FoxM1\(^{22,24}\) and tight regulation of cyclin B1 expression by FoxM1.\(^{33}\) A close interaction between TIS21 binding to cyclin B1, FoxM1 phosphorylation, and transcription of cyclin B1 is suggested here based on the following evidence: When Fig. 3A is compared with Fig. 3C, cyclin B1 expression was significantly lower in the Huh7-TIS21 (TIS21-stable expresser) than in the Ad-TIS21 cells (TIS21-transient expresser). This might be attributable to a transient effect of adenovirus in Ad-TIS21 cells, as opposed to continuous selection of Huh7-TIS21 cells with Geneticin 418. Moreover, it was increased in the TIS21^{-/-} MEF compared with TIS21^{+/+} (Fig. 3B).

The observation that TIS21 partially inhibited FoxM1-induced transcriptional activity can be supported by an earlier study on the FoxM1 phosphorylation at Ser and Thr residues to maintain transcriptional activity of FoxM1.\(^{22-25}\) Because synthesis and degradation of cyclin B1 are essential for progression of mitosis,\(^{34,35}\) indirect regulation of FoxM1 activity by TIS21 appears to be a very effective and clever way to inhibit carcinogenesis.

Based on the reports that FoxM1 protein is highly expressed, especially in all replicating cells and cancer tissues,\(^{19,20,36}\) and that sustained expression of TIS21 induces G2/M arrest by its binding to cdk1,\(^{11}\) inhibition of FoxM1 phosphorylation by TIS21 could be another function of TIS21 as a cell cycle inhibitor. Epidermal growth factor–induced cell death starts with TIS21 phosphorylation and Pin-1 binding in U937 cells under enforced expression of TIS21, which results in mitochondrial cell death.\(^{12}\) Therefore, epidermal growth factor–induced growth inhibition of various cancer cells, such as A431, HN6\(^{37}\), breast cancer,\(^{38}\) and U937 cells,\(^{39}\) could be explained. Here, we propose that loss of TIS21 expression in mouse liver by a single injection of DEN may be 1 of the critical hits.\(^{29}\)

In conclusion, TIS21, a pan-cell cycle regulator, inhibits FoxM1 activation by binding to the cyclin B1–cdk1 complex, thereby reducing cell growth, proliferation, and clonogenicity of transformed cells containing one critical hit by DEN injection.

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