Transforming Growth Factor-Beta Induces Senescence in Hepatocellular Carcinoma Cells and Inhibits Tumor Growth

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Senescence induction could be used as an effective treatment for hepatocellular carcinoma (HCC). However, major senescence inducers (p53 and $p16^{Ink4a}$) are frequently inactivated in these cancers. We tested whether transforming growth factor-β (TGF-β) could serve as a potential senescence inducer in HCC. First, we screened for HCC cell lines with intact TGF-β signaling that leads to small mothers against decapentaplegic (Smad)-targeted gene activation. Five cell lines met this condition, and all of them displayed a strong senescence response to TGF-β1 (1-5 ng/mL) treatment. Upon treatment, c-myc was down-regulated, $p21^{Cip1}$ and $p15^{Ink4b}$ were up-regulated, and cells were arrested at G_1. The expression of $p16^{Ink4a}$ was not induced, and the senescence response was independent of p53 status. A short exposure of less than 1 minute was sufficient for a robust senescence response. Forced expression of $p21^{Cip1}$ and $p15^{Ink4b}$ recapitulated TGF-β1 effects. Senescence response was associated with reduced nicotinamide adenine dinucleotide phosphate oxidase 4 (Nox4) induction and intracellular reactive oxygen species (ROS) accumulation. The treatment of cells with the ROS scavenger *N*-acetyl-L-cysteine, or silencing of the *NOX4* gene, rescued $p21^{Cip1}$ and $p15^{Ink4b}$ accumulation as well as the growth arrest in response to TGF-β. Human HCC tumors raised in immunodeficient mice also displayed TGF-β1–induced senescence. More importantly, peritumoral injection of TGF-β1 (2 ng) at 4-day intervals reduced tumor growth by more than 75%. In contrast, the deletion of TGF-β receptor 2 abolished *in vitro* senescence response and greatly accelerated *in vivo* tumor growth. *Conclusion:* TGF-β induces p53-independent and $p16^{Ink4a}$-independent, but Nox4-dependent, $p21^{Cip1}$-dependent, $p15^{Ink4b}$-dependent, and ROS-dependent senescence arrest in well-differentiated HCC cells. Moreover, TGF-β–induced senescence *in vivo* is associated with a strong antitumor response against HCC. (HEPATOLOGY 2010;52:966-974)

Cellular senescence is a permanent withdrawal from the cell cycle in response to diverse stress conditions such as dysfunctional telomeres, DNA damage, strong mitotic signals, and disrupted chromatin. Senescence is considered to be a major cause of aging, but also a strong anticancer mechanism.[1] The relevance of senescence in chronic liver diseases is poorly known, but it may play a central role. Hepatocyte telomeres undergo shortening during chronic liver disease progression,[2] and this is accompanied by a progressive decline of hepatocyte proliferation.[3] Senescence-associated β-galactosidase (SA-β-Gal)-positive cells have been detected in 3%-7% of normal liver, 50% of chronic hepatitis, 70%-100% of cirrhosis, and up to 60% of hepatocellular carcinoma (HCC) tissues.[2,4-7] Highly abundant senescence observed in cirrhosis has been confined to hepatocytes[2] and stellate cells.[8] Because telomere-deficient mice

Abbreviations: BrdU, bromodeoxyuridine; cDNA, complementary DNA; NAC, N-acetyl-L-cysteine; Nox4, NADPH oxidase-4; ROS, reactive oxygen species; SA-β-Gal, senescence-associated-β-galactosidase; siRNA, small interfering RNA; TERT, telomerase reverse transcriptase; TGF-β, transforming growth factor-β; TGF-βR1,TGF-β receptor 1.

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develop cirrhosis,[9] and cirrhotic hepatocytes display shortened telomeres, telomere dysfunction was proposed to cause senescence in cirrhosis.[2] It is assumed that HCC tumor cells bypass hepatocellular senescence to become immortalized. Frequent inactivation of *TP53* (encoding the tumor protein p53) and *CDKN2A* (cyclin-dependent kinase inhibitor 2A, encoding $p16^{Ink4a}$ protein) genes in these tumors supports this hypothesis.[10] Nevertheless, the detection of senescent cells in some HCC tumors suggests that transformed and presumably immortal hepatocytes have maintained the capacity to undergo senescence arrest under appropriate conditions.

With this regard, immortal HCC cell lines can spontaneously generate progeny that undergo replicative senescence[11]; murine HCC tumors generated by the expression of a mutant *Ras* gene in p53-deficient hepatoblasts can be cleared by a massive senescence response upon reactivation of p53 expression[12]; c-*myc* oncogene inactivation in murine HCCs results in senescence-mediated tumor regression.[13] One of our goals is to identify novel mechanisms of senescence induction in HCC cells. Here, we identify the transforming growth factor-beta (TGF-β) as a major cytokine that is able to trigger a massive senescence response in well-differentiated HCC cell lines. Reduced nicotinamide adenine dinucleotide phosphate oxidase-4 (Nox4) and reactive oxygen species (ROS) were key intermediates of TGF-β–induced growth arrest that was mediated by $p21^{Cip1}$ and $p15^{Ink4b}$.

Materials and Methods

Detailed materials and methods are described in the Supporting Information Materials and Methods. Cell lines were tested under standard culture conditions in the presence of 10% fetal bovine serum. Total RNA was isolated using a NucleoSpin RNA II Kit (Macherey-Nagel, Duren, Germany), and first-strand complementary DNA (cDNA) was synthesized using RevertAid First Strand cDNA synthesis kit (MBI Fermentas, Leon-Rot, Germany). Genomic DNA was extracted as described,[11] and polymerase chain reaction (PCR) assays were done using appropriate primers. Quantitative PCR was performed using SYBR Green I (Invitrogen, Carlsbad, CA). Glyceraldehyde 3-phosphate dehydrogenase and β-actin were used as internal controls. The SA-β-Gal assay was performed as described.[11] Commercial and homemade antibodies were used. Western blot assays were performed as described,[11] using α-tubulin or calnexin as internal controls. For immunoperoxidase and immunofluorescence assays, cells were fixed with 4% formaldehyde, permeabilized with phosphate-buffered saline supplemented with 0.5% saponin and 0.3% TritonX-100 (Sigma, St. Louis, MO), and subjected to indirect immunofluorescence and immunoperoxidase assays. To test permanent cell cycle arrest, cells were labeled with bromodeoxyuridine (BrdU) for 24 hours in freshly added culture media, and the anti-BrdU immunofluorescence assay was performed as described.[11] Human $p15^{Ink4b}$ and $p21^{Cip1}$ were cloned into pcDNA3.1C/Neo and pcDNA3.1(+)/hygromycin (Invitrogen), respectively. Cells were transfected with Lipofectamine 2000 (Invitrogen) and selected with either Geneticin G418 (Gibco) or hygromycin-B (Roche, Indianapolis, IN) for 8 days. The *NOX4* gene was silenced using previously described Nox4-specific small interfering RNAs (siRNAs).[14] A negative control siRNA was used in parallel experiments. The siRNAs were transfected with Lipofectamine RNAiMAX (Invitrogen). The pSBE4-luc reporter was cotransfected with pRL-TK (plasmid Renilla luciferase, with thymidine kinase promoter; Promega, Madison, WI), using Lipofectamine 2000. The luciferase assay was performed using a Dual-Glo luciferase kit (Promega). For cell cycle studies, fixed cells were labeled with propidium iodide and analyzed using FACSCalibur Flow Cytometer (BD Biosciences, San Jose, CA). Intracellular ROS were detected with 2′,7′-dichlorofluorescein diacetate (DCFH-DA; Sigma), using MitoTracker Red (Invitrogen) as a counterstain. Apoptosis was tested by Negative in Apoptosis (NAPO)[15] and active caspase-3 antibody (Asp-175; Cell Signaling Technology, Danvers, MA) immunoassays. Subcutaneous human HCC tumors were obtained in CD1 *nude* mice using 5×10^6 live cells per injection. All animals received care according to the Guide for the Care and Use of Laboratory Animals. Results were expressed as mean $\pm$ standard deviation from at least three independent experiments. Data between groups were analyzed by one-tailed t test. A P value <0.05 was considered statistically significant. TGF-β1 expression in liver disease was analyzed using a publicly available global gene expression data,[16] which were normalized using JustRMA tool from the Bioconductor group.[17] A two-sample t test with random variance model was used with a 0.05 nominal significance level of each univariate test.

Results

Differential Expression of TGF-β1 in Normal Liver, Cirrhosis, and HCC. We first analyzed TGF-β1 expression in normal liver, cirrhosis, and HCC, using the publicly available clinical data sets.[16] TGF-β1 expression displayed a bell-shaped distribution with

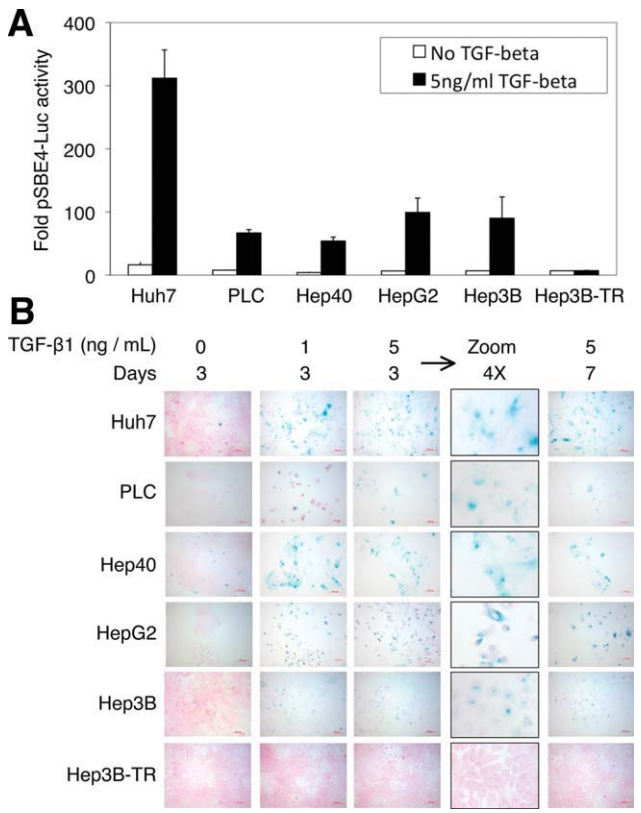

Fig. 1. Well-differentiated HCC cell lines are competent for TGF-β signaling activity and they respond to TGF-β by potent senescence-like growth arrest. (A) Cells were cotransfected with pSBE4-Luc and control pRL-TK plasmids, and treated with or without TGF-β1 (5 ng/mL) for 24 hours. The luciferase activity was measured and expressed as fold-activity of pSBE4-Luc/pRL-TK (mean $\pm$ standard deviation; n = 3). (B) Cells were plated at low density and treated with 1 or 5 ng/mL TGF-β1, and tested for SA-β-Gal activity (blue) at days 3 and 7. Counterstain: Fast Red. TGFBR2-deleted Hep3B-TR cells were used as negative controls in (A) and (B).

a sharp increase in cirrhosis (cirrhosis versus normal liver, $P < 0.001$), followed by a progressive decrease in early HCC (early HCC versus cirrhosis, $P < 0.02$) and advanced HCCs (Supporting Information Fig. 1). This expression pattern closely correlated with reported frequencies of SA-β-Gal activities in normal liver, cirrhosis, and HCC.[2,4-7]

TGF-β Is an Autocrine Cytokine Inducing a Senescence-Like Response in Well-Differentiated HCC Cell Lines. We hypothesized that TGF-β signaling can induce hepatocellular senescence response, because it is a potent inducer of G_1 arrest.[18] To test this hypothesis, we first formed a panel of "well-differentiated" HCC cell lines that display E-cadherin expression, epithelial-like morphology, and hepatocyte-like gene expression.[19] Well-differentiated cell lines also share the same TGF-β early response gene expression patterns with normal hepatocytes.[20] All selected cell lines expressed all critical components of TGF-β signaling including TGF-β1, TGF-β receptor 1 (TGFBR1), TGFBR2, small mothers against decapentaplegic homolog 2 (SMAD2), SMAD3, and SMAD4 (Supporting Information Fig. 2A). Hep3B-TR clone displaying homozygous deletion of *TGFBR2*[21] was used as a negative control (Supporting Information Fig. 2). All cell lines, except Hep3B-TR displayed intact TGF-β signaling activity (Fig. 1A), as tested by pSBE4-Luc reporter activity.[22] Treatment of cells with TGF-β1 (5 ng/mL) yielded 9-fold to 19-fold induction of pSBE4-Luc reporter activity in responsive cell lines. The expression of endogenous plasminogen activator inhibitor-1 (*PAI-1*), a well-known TGF-β target gene,[23] was also induced (Supporting Information Fig. 3). TGF-β1–treated cell lines were kept in culture with medium changes (without TGF-β1) every 3 days, examined morphologically, and subjected to SA-β-Gal assay. All cell lines tested, except Hep3B-TR, displayed growth inhibition associated with flattened cell morphology and >50% positive SA-β-Gal activity, as early as 3 days after TGF-β1 treatment (Fig. 1B).

Expression of TGF-β1 in all tested cell lines suggested that it could act as an autocrine cytokine. Therefore, we exposed Huh7 cells to either anti-TGF-β1 antibody (5 μg/mL) or TGF-β1 (5 ng/mL) and tested for total and SA-β-Gal–positive cells in isolated colonies 10 days later. Cells treated with anti-TGF-β1 antibody displayed two-fold increased colony size ($P < 0.04$) and 50% decreased SA-β-Gal activity ($P < 0.02$; Supporting Information Fig. 4). In contrast, ectopic TGF-β1 treatment caused a seven-fold decrease in colony size ($P < 0.005$) and five-fold increase in SA-β-Gal activity ($P < 0.0001$). Thus, Huh7 cells produced TGF-β1 acting as a weak autocrine senescence-inducing signal that was inhibited by anti-TGF-β1 antibody, and amplified by ectopic TGF-β1.

A Brief Exposure to TGF-β for a Robust Senescence Response. To test the shortest time of exposure to TGF-β1 for a full senescence response, three cell lines were treated with TGF-β1 for durations between <1 minute (~20 seconds) and 72 hours, and subjected to SA-β-Gal staining. To our surprise, <1 minute exposure was sufficient for a robust senescence response (Fig. 2). Thus, the senescence-initiating effect of TGF-β1 was immediate, even though the senescence phenotype (>50% SA-β-Gal–positive and flattened cells) was manifested 3 days later.

Lack of Evidence for TGF-β–Induced Apoptosis. Earlier studies indicated that TGF-β induces apoptosis in hepatocytes and some HCC cell lines under serum-free conditions.[24-27] Under our experimental conditions using 10% fetal bovine serum, all five cell lines tested failed to enter apoptosis following TGF-β

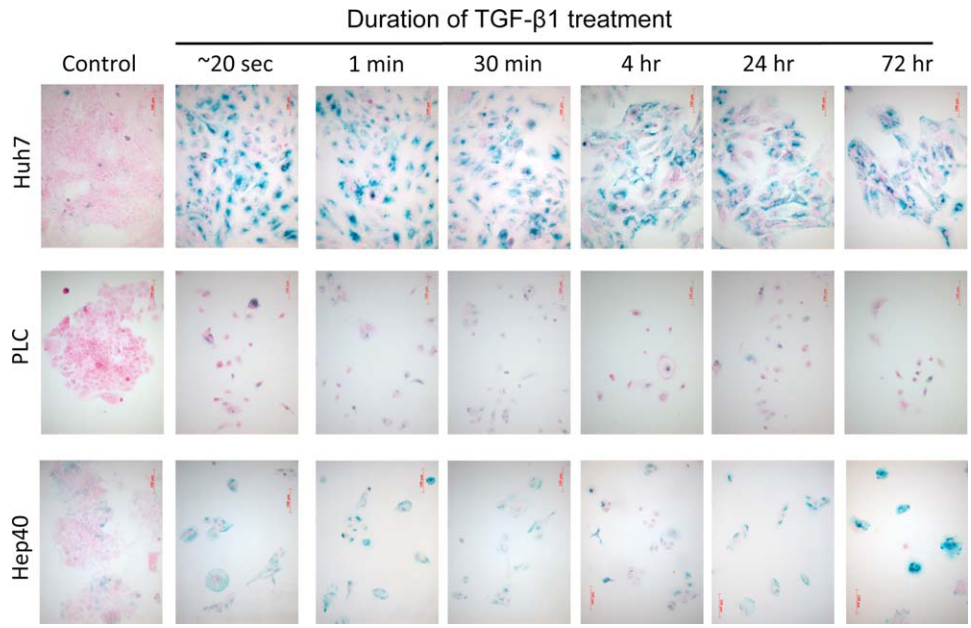

Fig. 2. Induction of a strong senescence-like response by TGF-β after a very short exposure. Cells were treated with TGF-β1 (5 ng/mL) for the indicated times, and SA-β-Gal activity (blue) was tested at 72 hours. Control: no TGF-β1 treatment. Counterstain: Fast Red.

treatment, as examined by NAPO antibody[15] and activated caspase-3–specific antibody tests (Supporting Information Figs. 5 and 6).

TGF-β–Induced Senescence Is Associated with Sustained Induction of $p21^{Cip1}$ and $p15^{Ink4b}$. Cellular senescence is usually associated with cell cycle arrest induced by p53, $p21^{Cip1}$, $p16^{Ink4a}$, and/or $p15^{Ink4b}$.[1,28] TGF-β1 caused c-myc repression and $p15^{Ink4b}$ and $p21^{Cip1}$ induction (Fig. 3; Supporting Information Fig. 7A). Decreased pRb phosphorylation, together with decreased p107 and increased p130 protein levels, was also observed. These changes in retinoblastoma family proteins correlate with exit from the cell cycle.[29] The TGF-β response was independent of p53. All HCC cell lines tested here, except HepG2, display p53 mutations.[30] The levels of total p53 did not change following TGF-β exposure, despite $p21^{Cip1}$ accumulation (Fig. 3; Supporting Information Fig. 7B). Moreover, we observed no phosphorylation of wild-type p53 in HepG2 cells, following TGF-β exposure (Supporting Information Fig. 7B). TGF-β also did not affect $p16^{Ink4a}$ levels (Fig. 3). Indeed, the *CDKN2A* gene is frequently silenced in HCC.[31] Accordingly, $p16^{Ink4a}$ protein levels were extremely low in all tested cell lines, except in pRb-deficient Hep3B and Hep3B-TR cells (Supporting Information Fig. 7C). On the other hand, our

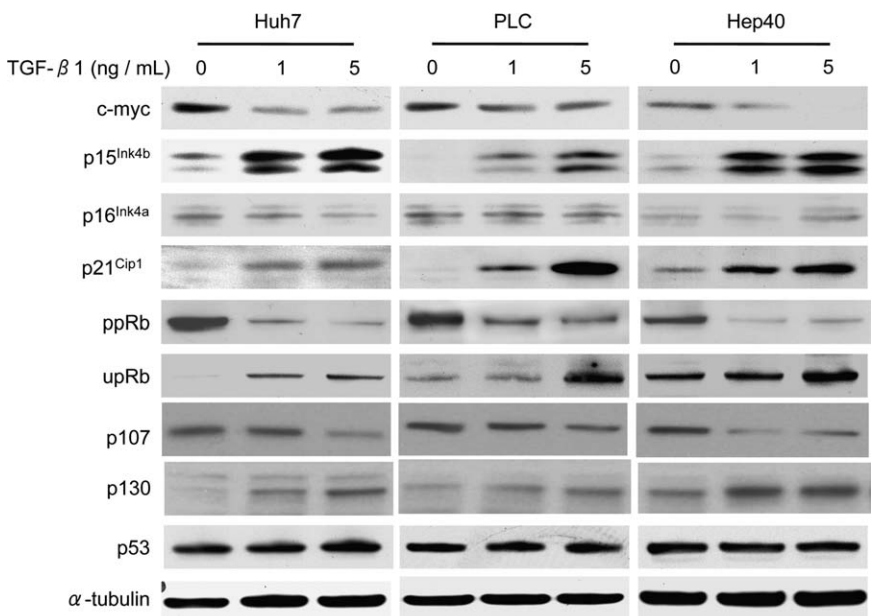

Fig. 3. TGF-β treatment of HCC cell lines causes the induction of $p15^{Ink4b}$ and $p21^{Cip1}$ that is associated with c-myc down-regulation, pRb underphosphorylation, p107 decrease and p130 increase. The levels of p53 and $p16^{Ink4a}$ did not change. Untreated and TGF-β1–treated cells were tested for indicated proteins by western blotting on day 3. ppRb: phospho-Ser^{807}/Ser^{811}-pRb, upRb: underphosphorylated pRb. The α-tubulin served as an internal control. $p16^{Ink4a}$ blots were overexposed to visualize weak expression.

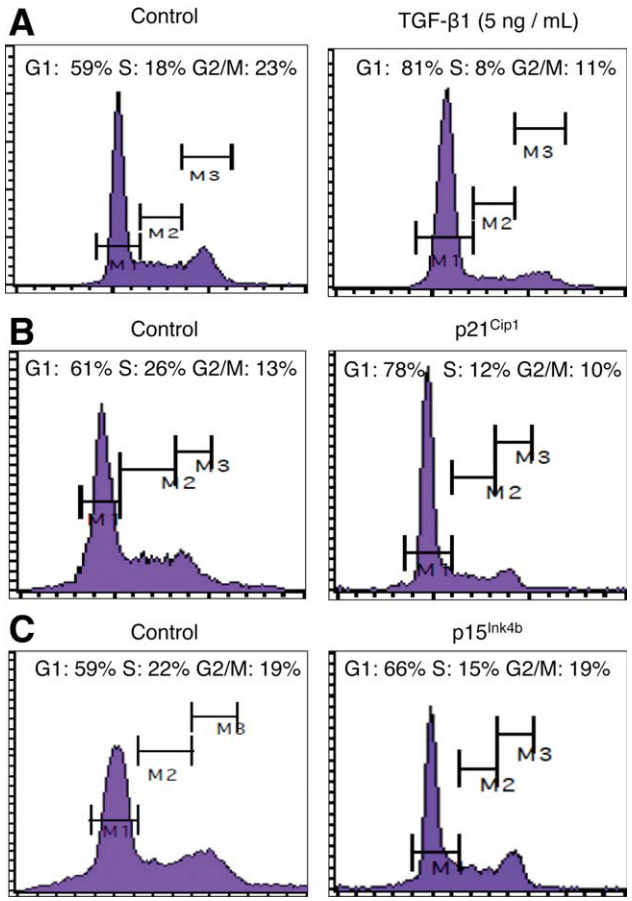

Fig. 4. G_1 arrest induced by TGF-β treatment can be recapitulated by ectopic expression of $p21^{Cip1}$ and $p15^{Ink4b}$. (A) Control and TGF-β1–treated Huh7 cells were subjected to cell cycle analysis after 3 days of culture. (B, C) Huh7 cells were transiently transfected with (B) $p21^{Cip1}$ and (C) $p15^{Ink4b}$ expression vectors, and subjected to cell cycle analysis after 8 days of culture. Control: cells transfected with empty plasmid vectors (B,C). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

observation of senescence arrest in Hep3B cells suggests that pRb expression is also dispensable for TGF-β–induced senescence in HCC cells. Taken together, these findings suggested that TGF-β was able to induce senescence in HCC cells independent of p53, $p16^{Ink4a}$, or pRb status.

TGF-β Induces a Permanent G_1 Arrest that Can Be Reproduced Either by $p21^{Cip1}$ or $p15^{Ink4b}$. Cellular senescence is defined as an irreversible arrest of mitotic cells at the G_1 phase, but some cancer cells enter senescence at the G_2 or S phases.[1] Initially, we used Huh7 cells for cell cycle studies. These cells accumulated at G_1 phase (from 59% to 81%) with a concomitant depletion of S phase cells (from 18% to 8%), after TGF-β1 exposure (Fig. 4A). Similar results were obtained with PLC/PRF/5 cells (Fig. 5) and other cell lines (data not shown). These observations suggested that $p21^{Cip1}$ and/or $p15^{Ink4b}$ are involved in TGF-β–mediated G_1 arrest and senescence response. Therefore, we tested respective contributions of $p21^{Cip1}$ and $p15^{Ink4b}$ in these responses, by transient transfection assays using Huh7 cells. The $p21^{Cip1}$-transfected and $p15^{Ink4b}$-transfected cells demonstrated highly increased $p21^{Cip1}$ protein (Supporting Information Fig. 8A) and moderately increased $p15^{Ink4b}$ expression (Supporting Information Fig. 8B), respectively. The $p21^{Cip1}$-overexpressing cells accumulated at G_1 (from 61% to 78%), together with a depletion of S phase cells (from 26% to 13%; Fig. 4B). In association with these changes, SA-β-Gal activity was increased (Supporting Information Fig. 9A) and BrdU

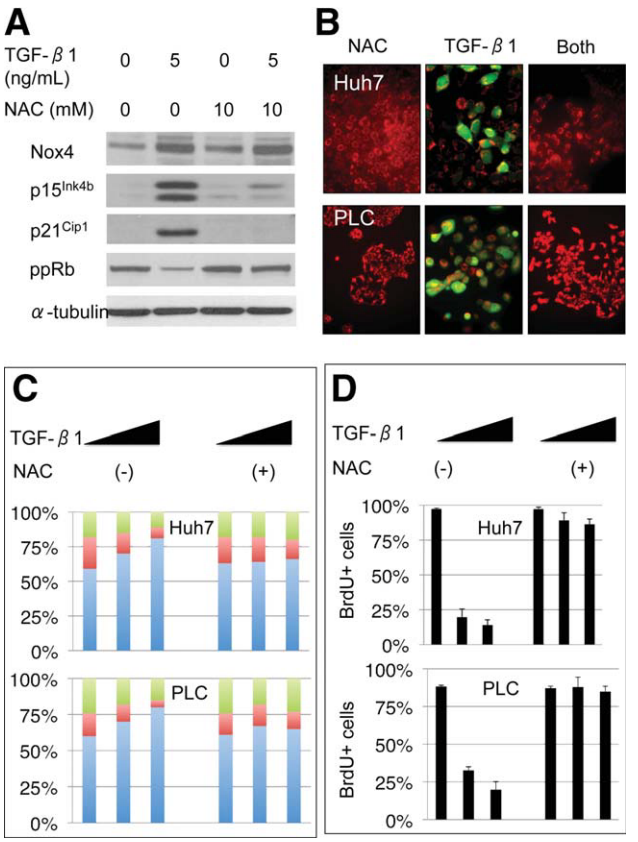

Fig. 5. Implication of Nox4 induction and ROS accumulation in TGF-β-induced growth arrest. (A) TGF-β1 treatement induces the expression of Nox4, $p15^{Ink4b}$, and $p21^{Cip1}$ together with ppRb down-regulation; ROS scavenger NAC inhibits $p15^{Ink4b}$ and $p21^{Cip1}$ induction, and ppRb down-regulation, but not Nox4 accumulation. Cell lysates were collected at day 3, following treatment with TGF-β1 and/or NAC, and tested by western blotting. (B-D) ROS accumulation observed in TGF-β1–treated cells is inhibited by NAC cotreatment (B), and this results in (C) inhibition of G_1 arrest, and (D) restoration of BrdU incorporation into cellular DNA. (A) PLC/PRF/5 cells were treated for 3 days with either 10 mM NAC or 5 ng/mL TGF-β1 alone, or in combination, and tested for Nox4, $p15^{Ink4b}$, $p21^{Cip1}$, and ppRb by western blotting. (B) Huh7 and PLC/PRF/5 (PLC) cells were treated with either 10 mM NAC or 5 ng/mL TGF-β1 alone, or in combination, and tested for ROS accumulation using a green fluorescent ROS indicator, and a red fluorescent mitochondrial marker as counterstain. The effects of 10 mM NAC cotreatment on growth inhibition by TGF-β1 (0, 1, or 5 ng/mL) were tested by (C) cell cycle analysis, and (D) BrdU incorporation assay. Blue, red, and green columns in (C) represent cells at G_1, S, and G_2/M, respectively.

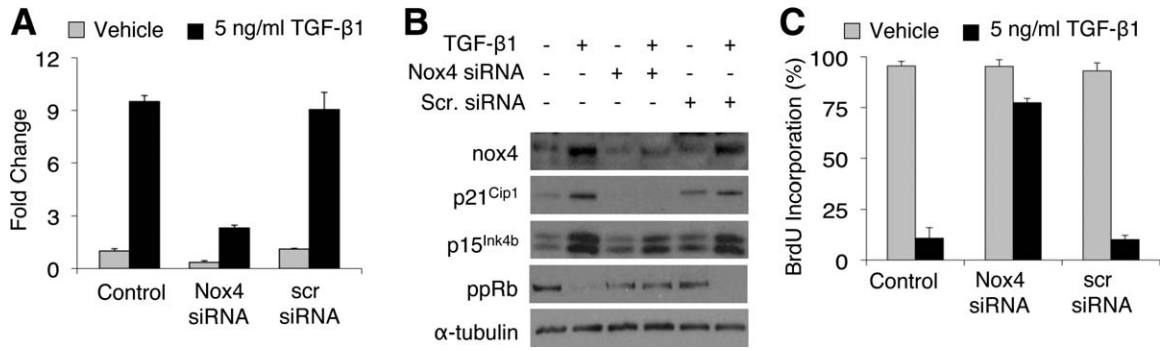

Fig. 6. Rescue of TGF-β-induced $p21^{Cip1}$ and $p15^{Ink4b}$ accumulation and growth arrest by *NOX4* gene silencing. (A) TGF-β-induced accumulation of Nox4 transcripts was strongly inhibited by Nox4 siRNA, but not by control siRNA. Transcript analysis was performed by quantitative reverse transcription PCR. (B) *NOX4* gene silencing rescued TGF-β-induced Nox4, $p21^{Cip1}$, and $p15^{Ink4b}$ protein accumulation, and the inhibition of pRb phosphorylation, as tested by western blotting. Compared to others, the inhibition of $p15^{Ink4b}$ accumulation was modest. (C) *NOX4* gene silencing also rescued TGF-β-induced inhibition of DNA synthesis, as tested by BrdU incorporation. Cells were labeled with BrdU for 24 hours prior to day 3, and percent BrdU-positive cells were counted manually.

incorporation into cellular DNA was inhibited ($P < 0.001$; Supporting Information Fig. 9B). The $p15^{Ink4b}$ overexpression caused a moderate response (G_1 cells rising to 66% from 59%; S phase cells decreasing from 22% from 15%; Fig. 4C). However, $p15^{Ink4b}$ overexpression was also associated with increased SA-β-Gal activity (Supporting Information Fig. 9C) and decreased BrdU incorporation ($P < 0.001$; Supporting Information Fig. 9D).

TGF-β–Induced Senescence Depends on Nox4 Induction and Intracellular Accumulation of ROS. TGF-β induces Nox4 expression and ROS accumulation in hepatocytes.[32-34] Because ROS have been implicated in Ras-induced senescence,[35] we tested whether TGF-β–induced senescence was associated with Nox4 induction and ROS accumulation. TGF-β1 induced Nox4 protein expression (Fig. 5A; Supporting Information Fig. 10A), as well as ROS accumulation (Fig. 5B). First, we used *N*-acetyl-L-cysteine (NAC) as a physiological ROS scavenger[36] to test the role of ROS in TGF-β–induced senescence. The cotreatment of 5 ng/mL TGF-β1–treated cells with 10 mM NAC completely suppressed the accumulation of ROS (Fig. 5B) and TGF-β1 effects on $p15^{Ink4b}$, $p21^{Cip1}$, and pRb, but not Nox4 expression (Fig. 5A). More importantly, NAC cotreatment rescued cells from TGF-β1–induced senescence response (Supporting Information Fig. 10B) and growth arrest (Fig. 5C,D; Supporting Information Fig. 11). Next, we silenced *NOX4* gene in Huh7 cells using a previously described NOX4-specific siRNA.[14] NOX4-specific siRNA inhibited the accumulation of Nox4 transcripts (~75%; Fig. 6A) and protein (Fig. 6B) under TGF-β1 treatment. This resulted in a strong inhibition of $p21^{Cip1}$ accumulation and a moderate inhibition of $p15^{Ink4b}$ accumulation in association with restoration of pRb phosphorylation (Fig. 6B). More importantly, Nox4 inhibition was sufficient to restore cell proliferation under TGF-β treatment (Fig. 6C).

TGF-β–Induced Senescence and Antitumor Activity In Vivo. We tested *in vivo* relevance of TGF-β–induced senescence in human HCC tumors raised in

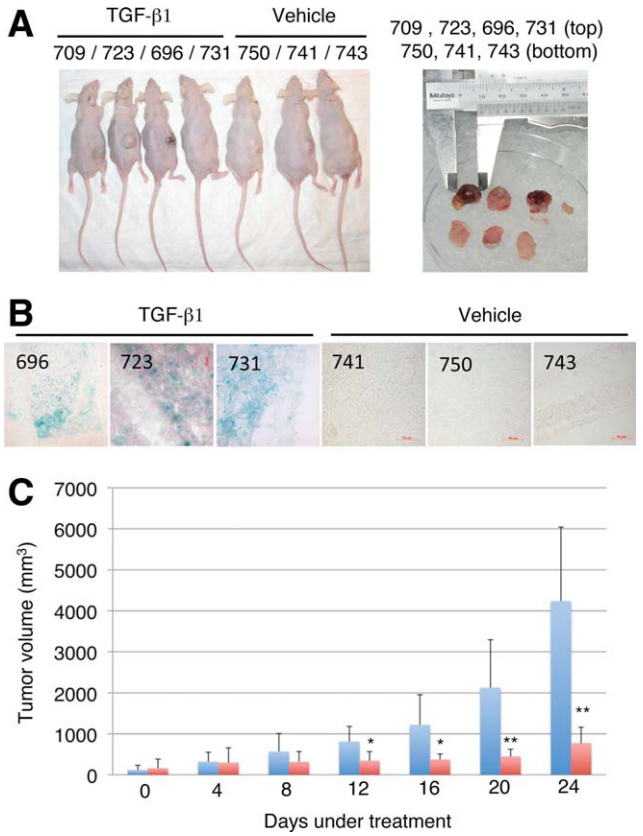

Fig. 7. TGF-β induces senescence and inhibits the growth of Huh7 tumors in *nude* mice. (A,B) TGF-β1–induced SA-β-Gal activity in Huh7 tumors. Huh7 tumors were obtained in *nude* mice and treated with TGF-β1 (~0.5 ng) or a vehicle only. (A) Animals were sacrificed 7 days later to collect tumor tissues. (B) Cryostat sections from freshly frozen tumors were subjected to SA-β-Gal staining (blue). Counterstain: Fast Red. (C) Huh7 tumors were treated with 2 ng TGF-β or vehicle only at 4-day intervals and tumor sizes were measured. TGF-β–treated tumors were growth arrested, resulting in >75% inhibition of tumor growth. *$P < 0.05$; **$P < 0.01$.

immunodeficient mice. TGF-β1 ($\sim$50 μL of a 10 ng/mL solution)-injected Huh7 tumors were removed 1 week later (Fig. 7A) and subjected to SA-β-Gal staining. TGF-β1 induced local but expanded SA-β-Gal activity in three of four tumors tested; three tumors treated with vehicle only were negative (Fig. 7B).

To test antitumor effects of TGF-β, early Huh7 tumors were treated with peritumoral injection of $\sim$2 ng TGF-β at 4 days of intervals. Vehicle-treated tumors displayed exponential growth to reach 4 cm^3 volume on average within 24 days. In contrast, TGF-β–treated tumors were growth arrested throughout the experiment and remained <1 cm^3 on average at the same time period. Tumor inhibition was significant for at least 24 days ($P < 0.01$ to $P < 0.05$). The TGF-β treatment was stopped at day 24 and animals were observed for an additional period of 4 weeks. All vehicle-treated and four TGF-β–treated animals died, whereas complete remission was observed in two TGF-β–treated animals (data not shown). We also compared *TGFBR2*-deleted Hep3B-TR cells[21] with parental Hep3B cells. Hep3B-TR cells formed palpable tumors 2 weeks after subcutaneous injection, and host animals died within 4-6 weeks. In contrast, Hep3B cells formed tumors with a latency of 6-7 weeks (Supporting Information Fig. 12).

Discussion

Our findings provide strong evidence for senescence as a major response of HCC cells to TGF-β. Senescence-associated changes included flattened morphology, $p21^{Cip1}$ and $p15^{Ink4b}$ accumulation, and positive SA-β-Gal activity. This response has not been noticed previously, probably because of its late occurrence, at least 3 days after TGF-β treatment. The primary findings of our mechanistic studies on TGF-β–induced senescence in HCC cells are outlined in Fig. 8. TGF-β-induced senescence response was associated with $p21^{Cip1}$-mediated and $p15^{Ink4b}$-mediated G_1 arrest, independent of p53 or $p16^{Ink4a}$. This correlates with the earlier observations showing that TGF-β uses $p21^{Cip1}$ and $p15^{Ink4b}$, but not $p16^{Ink4a}$ nor p53 to induce G_1 arrest in other cell types.[18] Although TGF-β–induced senescence had been described many years ago,[37] its mechanisms are poorly understood. Here, we show that the overexpression of $p21^{Cip1}$, and $p15^{Ink4b}$ to a lesser degree, recapitulates TGF-β–induced senescence response. Thus, $p21^{Cip1}$ and $p15^{Ink4b}$ are able to induce G_1 arrest and senescence response in HCC cells, as it occurs in other cell types.[10] Our most interesting finding was the implication of both Nox4 and ROS in the induction of $p21^{Cip1}$ and $p15^{Ink4b}$, and

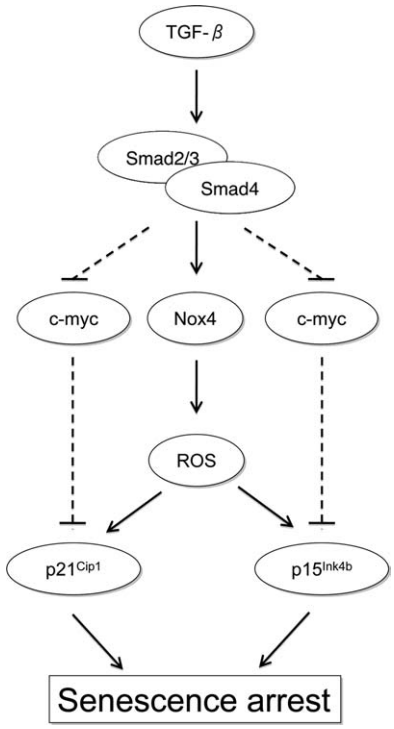

Fig. 8. A hypothetical model summarizing major components of TGF-β–induced senescence in HCC cells.

G_1 arrest by TGF-β. Either *NOX4* gene silencing or ROS scavenging was sufficient to interrupt the TGF-β signaling toward growth arrest mediated by $p21^{Cip1}$ and $p15^{Ink4b}$ induction. Thus, the accumulation of both Nox4 protein and ROS is a critical step for $p21^{Cip1}$ and $p15^{Ink4b}$ accumulation in TGF-β–exposed HCC cells (Fig. 8). Inhibition of $p21^{Cip1}$-mediated ROS accumulation has been previously shown to rescue senescence,[38] and a feedback between $p21^{Cip1}$ and ROS production was necessary for stable growth arrest.[39]

Our findings also provided preliminary evidence for antitumor activity of TGF-β against HCC. This effect was associated with *in vivo* induction of SA-β-Gal activity in tumor cells. Thus,TGF-β–induced senescence in human HCC cells, similar to p53-induced senescence in mouse HCC cells,[12] may be a potent tumor suppressor mechanism. The accelerated tumorigenesis of TGFBR2-deleted Hep3B-TR cells supports this hypothesis. Previous studies indicated that the disruption of TGF-β signaling in mice through dominant-negative Tgfr2 (transforming growth factor receptor 2) accelerates chemically induced hepatocarcinogenesis.[40] A similar disruption in β-spectrin embryonic liver fodrin knockout mice also leads to hepatocellular cancer.[41,42] However, our findings are limited to well-differentiated HCC cells that represent early forms of HCC.[43] Poorly differentiated HCC cell lines appear to

be resistant to TGF-β-induced senescence (S. Senturk and M. Ozturk, unpublished data). Nevertheless, TGF-β treatment might be an attractive therapeutic option for early HCCs.

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