Crucial Role of TSC-22 in Preventing the Proteasomal Degradation of p53 in Cervical Cancer

Cheol-Hee Yoon1,2,3, Seung Bae Rho1,3, Seong-Tae Kim1, Seongho Kho1, Junsoo Park4, Ik-Soon Jang5, Seonock Woo6, Sung Soon Kim2, Je-Ho Lee6,7, Seung-Hoon Lee1*

1 Department of Life Science, Yonin University, Cheoin-gu, Yonin-si, Gyunggi-do, Korea, 2 Division of AIDS, Center for Immunology and Pathology, National Institute of Health, Cheongwon-gun, Chungbuk, Korea, 3 Research Institute, National Cancer Center, Ilsandong-gu, Goyang-si, Gyeonggi-do, Korea, 4 Division of Biological Sciences and Technology, Yonsei University, Wonju, Korea, 5 Korea Basic Science Institute, Daejeon Center, Daejeon, Korea, 6 South Sea Environment Research Department, Korea Ocean Research and Development Institute, Geoje, Korea, 7 School of Medicine, Sungkyunkwan University, Samsung Medical Center, Seoul, Korea

Abstract

The p53 tumor suppressor function can be compromised in many tumors by the cellular antagonist HDM2 and human papillomavirus oncogene E6 that induce p53 degradation. Restoration of p53 activity has strong therapeutic potential. Here, we identified TSC-22 as a novel p53-interacting protein and show its novel function as a positive regulator of p53. We found that TSC-22 level was significantly down-regulated in cervical cancer tissues. Moreover, over-expression of TSC-22 was sufficient to inhibit cell proliferation, promote cellular apoptosis in cervical cancer cells and suppress growth of xenograft tumors in mice. Expression of also TSC-22 enhanced the protein level of p53 by protecting it from poly-ubiquitination. When bound to the motif between amino acids 100 and 200 of p53, TSC-22 inhibited the HDM2- and E6-mediated p53 poly-ubiquitination and degradation. Consequently, ectopic over-expression of TSC-22 activated the function of p53, followed by increased expression of p21Waf1/Cip1 and PUMA in human cervical cancer cell lines. Interestingly, TSC-22 did not affect the interaction between p53 and HDM2. Knock-down of TSC-22 by small interfering RNA clearly enhanced the poly-ubiquitination of p53, leading to the degradation of p53. These results suggest that TSC-22 acts as a tumor suppressor by safeguarding p53 from poly-ubiquitination mediated-degradation.

Introduction

TGF-β stimulated clone 22 (TSC-22) was first identified as a TGF-β-inducible gene in mouse osteoblastic cells. TSC-22 expression is induced in a variety of cell lines by TGF-β, phorbol ester, serum, and progesterin and positively regulates the TGF-β signaling [1,2]. TSC-22 contains a leucine zipper-like motif, but it does not have a DNA-binding motif at the N-terminal region. TSC-22 can homodimerize and heterodimerize with TSC-22 homologous gene-1 (THG-1), and has transcriptional repressor activity [3].

Some researchers have identified the physiological roles of TSC-22 in the developmental process. TSC-22 is required for gastrulation during early embryogenesis in Xenopus laevis [4] and for oogenesis in Drosophila [5]. It has been also suggested that TSC-22 induces erythroid cell and Cardiac myofibril differentiation via activating the transcriptional activity of Smaβ3 and Smaβ4, and antagonizing the Smaβ7 in response to TGF-β-dependent signaling [6,7].

Additionally, several studies have focused on the tumor suppression functions of TSC22. TSC22 is thought to be a potent tumor suppressor in salivary cancer cells [8,9] human gastric carcinoma cells [10], hepatic carcinoma [11], human astrocytic tumors [12], and large granular lymphocyte leukemia [13]. The detailed mechanisms of the tumor suppressor function of TSC22 have been reported along with the hypothesis that TSC-22 represses the expression of the anti-apoptotic genes Gadd45b and Lzts2 [11], negatively regulates Ras/Raf signaling [14] and involved in the TGF-β-mediated gastric carcinoma cell death in a caspase3-dependent manner [10]. On the other hand, TSC22-mediated apoptotic activity is inhibited by the interaction between TSC22 and fortillin, followed by leading to TSC22 destabilization [15].

p53 is a well-known tumor suppressor gene that acts by activating the transcription of its targeted genes such as p21, PUMA, Pkris, PCD and BAX [16–18]. p53 functions are regulated by post-translational modifications such as phosphorylation, acetylation, and ubiquitination. It is well-understood that p53 regulation of p53 expression is correlated with tumorigenesis through infection by human papilloma virus expressing E6, which leads to ubiquitin-mediated p53 degradation [21,22]. Even though the regulation of p53 has been examined via multiple
Figure 1. Expression of TSC-22 was decreased in human cancer tissue. (A) Total RNA was prepared from patients’ cancer specimens and the TSC-22 mRNA level was then evaluated with real-time RT-PCR. Cx; serial number of patient tissue samples. (B) HeLa and Caski cells were plated on 6-well plates. After 24 h, the cells were infected with Ad-TSC-22 or Ad-LacZ. At the indicated time points, cell numbers were determined by MTT assay to analyze cell proliferation rates. (C) HeLa cells infected with Ad-TSC22 or Ad-LacZ were cultured for the indicated times, and the cells were then stained with propidium iodide (PI). The sub-G1 cell population (dead cell) and cell cycle profile of the PI-stained cells were analyzed by flow cytometry after PI-staining. (D) DNA fragmentation assay was performed by isolating chromosomal DNA from $1 \times 10^6$ number of HeLa and Caski cells infected with Ad-TSC22 or Ad-lacZ for 72 hrs (left). After 3 days infection of Ad-TSC-22, p53, Puma, p21, E6 and TSC22 expression were analyzed by Western hybridization (right).

doi:10.1371/journal.pone.0042006.g001

routes related to tumorigenesis, many questions remain about the tumor inhibition mechanism of the p53 pathway.

Since the mechanism underlying the network of tumor suppressor genes has not yet been explicitly elucidated, we conducted a cDNA microarray analysis of the gene expression profile in cervical cancer tissue to find novel tumor-related genes. We found that TSC-22 expression was significantly decreased in cervical cancer tissues compared to normal tissues. Subsequently, we explored the novel function of TSC-22 during tumorigenesis.

We therefore performed a yeast two-hybrid assay to screen for novel TSC-22-binding proteins. From this, p53 was identified as a TSC-22-binding protein. We also found that TSC-22 could enhance the activities of p53 through the inhibition of HDM2 and E6-mediated ubiquitination by directly binding to p53. On the other hand, TSC-22 over-expression stabilized p53 protein level, leading to increased cell death and inhibition of cell proliferation. Finally, tumor growth rate was strongly reduced by the expression of TSC-22 in a xenograft tumor model. Taken together, these results indicate that TSC-22 plays a crucial role in the inhibition of tumor growth through the regulation of p53 ubiquitination.

Results

TSC22 Inhibits Tumor Growth

In order to analyze the specific gene expression profile of cervical cancer, we conducted cDNA microarray analysis with cDNA prepared from patients’ cervical cancer tissues. Interestingly, our microarray data showed that TSC-22 gene expression was noticeably decreased in all cancer samples (data not shown). We then conducted real-time PCR (RT-PCR) analysis to confirm the microarray results. As shown in Figure 1A, TSC-22 mRNA expression levels in the patients’ cancer tissues were significantly reduced compared to those in normal tissue.

These results led to further questions. The first question was whether TSC-22 could suppress tumor cell proliferation or not. Therefore, we infected HeLa (HPV-18) and Caski (HPV-16) cells with adenovirus expressing TSC-22 or LacZ (infection control). As shown in Figure 1B, the proliferation ratios of both cells were significantly reduced by infection with Ad-TSC-22. We next examined whether TSC-22 could induce cell cycle arrest and apoptosis in HeLa cells. We found that the G0/G1 population was dramatically increased among Ad-TSC-22-infected cells compared to that in Ad-LacZ-infected cells within 48 h after infection. Furthermore, most Ad-TSC-22-infected cells underwent apoptosis in the late stage (Figure 1C). We next assessed the chromosomal-DNA fragmentation to observe the TSC-22-induced apoptosis in HeLa and Caski cells. As shown in Figure 1D, chromosomal DNA from Ad-TSC-22-infected HeLa and Caski cells showed very high level of fragmentation. Besides, p21 (cell cycle inhibitor) and PUMA (apoptosis inducer) expression levels were markedly enhanced in Ad-TSC-22 infected HeLa and Caski cell (Figure 1D right panel). These effects were more significant in chronically HPV18-infected HeLa cell. p53 and TSC22 level in HeLa cells were barely detected compared to caski cells (Figure 1D right panel). We speculate that their different expressions are caused by the different serotype of HPV. These results indicate that over-expression of TSC-22 induced high levels of cell death. Our results strongly suggest that TSC-22 plays a pivotal role in cervical tumor cell growth and death.

TSC-22 Binds to p53 in a Yeast Two-hybrid Assay

As shown by our data, TSC-22 contributes to the inhibition of cancer cell growth and cell death. This was consistent with previous studies demonstrating that TSC-22 is a potential tumor suppressor [8–11,13,23–25]. In order to elucidate the mechanisms underlying this function, we screened for TSC-22-binding proteins using a yeast two-hybrid assay. Through this, p53 was identified as a TSC-22-binding protein (Figure 2A, left panel). The interaction between TSC-22 and p53 was thus demonstrated in vitro by both cell growth and a β-galactosidase assay (Figure 2A, right panel).

Determination of the Effects of TSC-22 on p53

To better understand the effects of TSC-22 on p53, HeLa and Caski cells were infected with Ad-TSC-22 or LacZ for increasing periods of time. Interestingly, endogenous p53 levels were significantly increased by transfection of Ad-TSC-22 in a time-dependent fashion (Figure 2B). The enhancement of p53 expression was more significant in HeLa cells than in Caski cells. In order to observe the enhancement of p53 target gene activity by the expression of TSC-22, Flag-tagged TSC-22 was introduced into HeLa cells. p53 protein and its target genes, including p21 and puma, were clearly induced by Flag-TSC-22 expression (Figure 2C). Conversely, knocking-down TSC-22 in HeLa cells reduced the protein levels of p53 and PUMA (Figure 2D). However, the level of p53 mRNA was not affected by knock-down and over-expression of TSC-22 (Figure 2C and 2D, bottom panel).

To determine whether p53 activity is regulated by TSC-22 expression in HeLa cells, we assessed the p53RE (responsive element)-driven promoter activity with TSC-22 expression and knock-down of TSC-22 in HeLa cells. A promoter harboring a p53RE was activated by TSC-22 expression in a dose-dependent manner. On the other hand, the promoter activity was decreased by TSC-22 knock-down. These data suggest that p53-mediated transcriptional activity is regulated by TSC-22 (Figure 2E). To evaluate whether decreases in PUMA and p21 are caused by direct regulation of p53 by TSC-22, Flag-tagged TSC-22 plasmid was introduced into HCT116 p53+/+ and p53−/− cells. Enhanced expression of PUMA was observed in p53−/− but not p53+/− cells (Figure 2F). This result suggests that the regulation of PUMA and p21 by TSC22 is dependent on p53. To further explore the enhancement of p53 by TSC-22, we assessed p53 stability in Ad-TSC-22 or Ad-LacZ infected HeLa cells after cycloheximide treatment. As shown in Figure 2G, Ad-TSC-22 greatly enhanced and stabilized endogenous p53 levels. These results suggest that TSC-22 promotes the tumor suppressor function of p53 by enhancing the stability of p53 protein.
Figure 2. TSC-22 induces p53 expression. (A) TSC-22 and p53 cDNA constructs were cotransformed into EGY48 yeast cells to test for protein–protein interaction within the yeast two-hybrid system. Transformants were assayed for their ability to grow on medium lacking leucine (left) and for β-galactoside expression (right). (B) HeLa cells and Caski cells were infected with Ad-TSC-22 for the indicated times. Protein levels were analyzed by Western blotting (WB) and qRT-PCR.

(B) HeLa and Caski cells were infected with Ad-LacZ or Ad-TSC-22 for the indicated times. Protein levels were analyzed by Western blotting (WB) and qRT-PCR.

(C) HeLa cells were transfected with Flag-TSC22 or vector only. Protein levels were analyzed by Western blotting (WB).

(D) HeLa cells were transfected with Sh-Con or Sh-TSC22. Protein levels were analyzed by Western blotting (WB) and qRT-PCR.

(E) HeLa cells were transfected with different concentrations of Flag-TSC22. Luciferase activity was measured.

(F) HCT116 cells were transfected with Flag-TSC22 or vector only. Protein levels were analyzed by Western blotting (WB).

(G) HeLa cells were infected with Ad-LacZ or Ad-TSC22 for the indicated times. Protein levels were analyzed by Western blotting (WB) and qRT-PCR.
TSC-22 Binds to p53 in vivo

To determine whether TSC-22 interacts with p53 in mammalian cells, we transfected H1299 human lung non-small cell carcinoma cells with the Flag-TSC-22 expression plasmid and *p53* expression plasmid, and then conducted co-immunoprecipitation and Western blot assays. We found that TSC-22 specifically co-immunoprecipitated with p53 in cells expressing both Flag-TSC22 and p53, but not in cells expressing either protein alone (Figure 3A). Conversely, p53 specifically co-immunoprecipitated with TSC-22 with the anti-Flag antibody (Figure 3B), suggesting an interaction between TSC-22 and p53. Next, we tried to confirm the endogenous interaction between p53 and TSC-22. Since we could not purchase an appropriate TSC-22 antibody to use in a co-immunoprecipitation experiment, this interaction was confirmed by reciprocal co-immunoprecipitation with endogenous p53 and exogenously expressed Flag-tagged TSC-22 in HEK293 cells which express high levels of p53 (Figures 3C and D). The results suggest that TSC-22 directly interacts with p53.

TSC-22 Binds to p53 in the Region Including Amino Acids 100 to 200

To further define the region essential for binding TSC-22 to p53, we generated several p53 deletion mutants (Figure 4A). Expression plasmids of p53 and TSC-22 were transfected into H1299 cells together or alone. As shown in Figures 4A and B, TSC-22 was able to bind to p53 several partial deletion mutants including p531–300, p53101–393, and p531–200. However, further deleting a portion of the internal region of the DNA binding domain, such as p531–100, p53101–200, p53100–300, and p53100–200, abolished p53-TSC-22 binding (Figures 4A and B). These results indicate that the region including amino acids 100–200, which is a part of the p53 DNA-binding domain, is required for binding TSC-22. Subsequently, to identify the p53 binding region of TSC-22, Flag-tagged truncated TSC-22 mutants that each contained an α-helix were expressed with p53 as shown in Figure 4C. In co-immunoprecipitation experiments using an anti-p53 antibody (DO-1), TSC-22aa1–154 was co-immunoprecipitated with p53, but TSC-22aa1–110 and TSC-22aa1–200 were not. These data suggest that p53 binds amino acids 110 to 154 of TSC-22. Unfortunately, we could not further confirm the detailed interaction of p53-TSC-22 because TSC-22aa1–154 was not expressed in our experiment (Figure 4C). Taken together, our data demonstrate that TSC-22 and p53 interact at specific domains in each protein.

TSC22 Inhibits HDM2 and E6–mediated p53 Poly-ubiquitination

To determine whether enhancement of p53 stability by TSC-22 is due to inhibition of p53 ubiquitination, H1299 cells were transfected with HDM2, p53, and TSC-22 expression plasmids, and treated with the proteasomal inhibitor MG132 for 6 h in order to conduct in vivo ubiquitination assays. As shown in Figure 5A, p53 was highly ubiquitinated with the expression of HDM2. However, further HDM2-mediated p53 ubiquitination was significantly inhibited by the expression of TSC-22 (Figure 5A). We next wanted to determine whether the inhibition of HDM2-mediated p53 ubiquitination by TSC-22 is caused by interruption of the interaction between HDM2 and p53. Therefore, we introduced p53, HDM2, and TSC22 expression plasmids into H1299 cells as shown in Figure 5B. p53 was then immunoprecipitated from the cell extracts with the DO-1 antibody. Interestingly, this experiment showed that p53 was simultaneously bound to HDM2 and TSC-22. In addition, the p53-HDM2 interaction was not interrupted by expression of TSC-22. These data suggest that TSC-22 can protect p53 from HDM2-mediated ubiquitination by directly binding to p53 in a region separate from the HDM2 binding site.

We next attempted to show that TSC-22 can protect p53 from E6-mediated ubiquitination because the E6 and HDM2 binding domains of p53 overlap. p53 was highly ubiquitinated with the expression of E6 in H1299 cells (Figure 5C, Lane 3); however, further expression of TSC-22 clearly blocked E6-mediated p53 ubiquitination (Figure 5C, Lane 4). We next used HeLa cells that constitutively expressed E6 to examine TSC-22-mediated p53 stability under physiological conditions. Ubiquitination of p53 was greatly increased after MG132 treatment in the cells. In contrast, this ubiquitination clearly disappeared upon over-expression of TSC-22 (Figure 5 D). p53 ubiquitination was rescued by the expression of shRNA specific for TSC-22 in *HeLa* cells in the absence of MG132 (Figure 5E, Lane 2). However, the difference in the level of p53 ubiquitination was not significant between *sh-con* and *sh-TSC-22* cells in the presence of MG132. We further explored the effect of TSC-22 on p53 ubiquitination by the expression of both HDM2 and E6. As shown in Figure 6A, the level of p53 ubiquitination was dramatically induced by both HDM2 and E6. However both of HDM2 and E6-mediated p53 ubiquitination was tightly interrupted by TSC-22 expression (Figure 6A). In addition, we tested whether TSC-22-p53 interaction is essential to inhibit p53 ubiquitination. As shown in Figure 6B, C-terminal deletion mutant TSC-22aa1–154 which does not interact with p53 (Figure 4C), did not inhibit the HDM2 and E6-mediated-p53 ubiquitination (Figure 6B). This result reveal that TSC-22 inhibit the p53 ubiquitination via direct interaction. These data strongly suggest that TSC-22 directly interacts with p53 and blocks the E6 and/or HDM2-mediated p53 ubiquitination, followed by stabilizing the protein of p53.

TSC-22 Suppresses Tumor Growth in Nude Mice

We next determined whether TSC-22 inhibits tumor growth *in vivo*. Exponentially growing *HeLa* cervical cancer cells were injected subcutaneously into immune-deficient BALB/c nude mice. When the tumor volume reached about 100 mm³, 1 × 10⁶ pfu of adenovirus expressing TSC-22 or LacZ were injected into the tumors. After three sequential intra-tumoral injections of adenovirus, the animals (5–7 per group) were monitored for tumor growth.
Figure 3. TSC22 interacts with p53 in vivo. (A–B) TSC22 interacts with ectopic p53. Ectopically expressed TSC-22 interacts with ectopically expressed p53 in H1299 cells. Two μg of Flag-TSC-22 expression vector was cotransfected with Flag-p53 expression vector (A) or a non-tagged p53 expression vector (B) into H1299 cells cultured in 100 mm plates. 48 h after transfection, cell lysates were immunoprecipitated with an anti-p53 (DO-1) (A) or anti-Flag (B) monoclonal antibody. Immunoprecipitates were analyzed by Western blotting using an HRP-conjugated anti-Flag (A) or anti-p53 (FL393) (B) monoclonal antibody. (C–D) TSC-22 interacts with endogenous p53. Ectopically expressed TSC-22 interacts with endogenous p53 in cells. HEK293 cells were transfected with 2 μg of Flag-TSC-22 plasmid for 48 h. Cell lysates were immunoprecipitated with anti-p53 (DO-1) monoclonal antibody, mouse immunoglobulin G (IgG) (C), or an anti-Flag (D) monoclonal antibody. Immunoprecipitates were analyzed with Western blotting using an HRP-conjugated anti-Flag (C) or anti-p53 (FL393) antibody.

doi:10.1371/journal.pone.0042006.g003
Figure 4. Mapping of the binding regions in TSC-22 and p53. (A) Schematic diagram shows the cDNA constructs for the Flag-tagged p53 deletion mutants and full-length p53, and indicates the TSC22 binding domain. (B) H1299 cells were transfected with the indicated plasmids encoding Flag-tagged p53 deletion mutants along with the Flag-TSC-22 plasmid. Cell lysates were immunoprecipitated with the anti-p53 polyclonal
growth. Tumor growth and morphology were analyzed over 30 days. Figure 6C shows that the tumor mass in mice injected with Ad-TSC-22 was remarkably reduced compared to tumors injected with Ad-LacZ or that were untreated. We next investigated the effect of TSC-22 on p53 stabilization in tumor tissues harvested from control and TSC-22 treated mice. TSC-22 transfection was observed to significantly increase the expression level of p53 protein (Figure 6D). Collectively, these results clearly demonstrate that TSC-22 can be a potent tumor suppressor in this animal model.

Discussion

In our search to identify genes associated with cervical cancer development, expression pattern analyses following cDNA microarray experiments and RT-PCR revealed that the TSC-22 gene was consistently reduced in tumor tissues (Fig. 1A). Several previous studies reported that TSC-22 is down-regulated in human salivary gland tumors [23], mouse liver tumors [11], and human brain tumors such as astrocytomas [26]. These results suggest that down-regulation of TSC-22 may play a role in the development of cancer in diverse tissues. However, these previous reports did not address the problem of TSC-22 being reduced in cancer cells. A reasonable explanation is that TSC-22 may inhibit cancer cell development. Our results showed that TSC-22 dramatically inhibited cell proliferation and induced cell death when expressed with an adenovirus expression system (Figures 1C and D). These results also showed that over-expression of TSC-22 in human cervical carcinoma cells [10].

Given that TSC-22 is a transcription repressor [3], the role of TSC-22 in tumor suppression has been suggested by Mari et al. [11]. Through experiments using TSC-22 siRNA, this group demonstrated that the DNA damage-inducible gene 43 β (Gadd45β) and putative tumor suppressor 2 (Lzts2) are putative targets of TSC-22. However, the relationships through which these targets play pivotal roles in tumor suppression by TSC-22 were not directly revealed. In our attempt to find a novel function of TSC-22 in tumor suppression, we identified a binding protein in a yeast two-hybrid experiment. The study showed that TSC-22 directly binds to p53 (Figure 2A). We further observed that TSC-22 up-regulated the protein levels of p53 without altering the levels of p53 mRNA. TSC-22 over-expression and knock-down experiments showed that TSC-22 facilitates the function of p53 as a transcriptional activator of target genes that can inhibit tumorigenesis (Figure 2C–G). These results indicated that increased p53 protein levels are associated with increased apoptosis in human gastric carcinoma cells [10].

We also performed quantitative RT-PCR and found the suppressed mRNA level of p53 and TSC-22 and high expression level of E6 mRNA in cervical cancer cell lines and patients’ tissue samples, but we could not find the physical correlation among them (Fig. S1). Although our results could not show the correlation of HPV/E6 and p53 protein expression level in cervical cancer tissues expressing less amount of TSC-22, it is well known that most of cervical cancer is caused by E6 expression after infection of human papillomavirus (HPV), followed by down-regulation of p53 [22]. Taken together, our results imply that TSC-22 can suppress the oncogenic potential of HPV by preventing the degradation of p53 by E6-mediated ubiquitination. However, how TSC-22 inhibits ubiquitination and degradation promoted by HDM2 and E6 remains to be investigated in the future. In an attempt to address the effect of TSC-22 on tumor suppression in vivo, we found that the growth of Hela cells inoculated into nude mice was significantly reduced by TSC-22 adenoviral transfection (Figure 6C). This result suggests that TSC-22 could be targeted for future cancer gene therapies.

In conclusion, our study identified TSC-22 as a novel factor interacting with p53. We also showed that TSC-22 prevents the degradation of p53 protein by HDM2 and E6, which suggests a novel mechanism by which TSC-22 regulates apoptosis and cell proliferation. Our results also revealed a previously unrecognized mechanism underlying the effects of TSC-22 on tumor suppression, and demonstrated that TSC-22 is a possible new target for human cancer therapy.

Materials and Methods

Patient’s Samples

Cervical cancer tissues were obtained from patients of the Department of Obstetrics and Gynecology, Samsung Medical Center, Seoul, Korea. All tissue samples were prepared during surgery and stored at −70°C. Before RNA extraction, a part of each tissue sample was sliced by paraffin section and examined by
Figure 5. TSC22 inhibits HDM2- and E6-mediated p53 ubiquitination. (A) TSC22 inhibits HDM2-mediated p53 ubiquitination. H1299 cells were transfected with the indicated plasmids. The transfected cells were treated with MG132 (20 μM) for 5 h before harvest. Cell lysates were immunoprecipitated with an anti-HA antibody. Ubiquitinated p53 was detected by Western blotting with an anti-p53 antibody (DO-1). Ubiquitinated p53 is indicated as Ub(n)-p53 (upper panel). The expression of total p53, HDM2, Flag-TSC-22 and HA-Ub proteins are shown in the lower panels. (B) TSC-22 does not interrupt interaction between p53 and HDM2. H1299 cells were transfected with Flag-p53 along with Flag-TSC-22 or a Flag-mock vector in the presence of an HDM2 expression vector. Cell lysates were immunoprecipitated with the anti-p53 (DO-1) antibody followed by Western blotting using the indicated antibodies. Lysate (5%) was analyzed by Western blotting using indicated antibodies (lower panel). (C) TSC-22 inhibits E6-
mediated p53 ubiquitination. H1299 cells were transfected with the indicated plasmids in presence of an HA-Ub expression vector. 48 h after transfection, the transfected cells were treated with MG132 (20 μM) for 5 h before they were harvested. Cell lysates were immunoprecipitated with an anti-HA antibody. Ubiquitinated p53 was detected by Western blotting with the anti-p53 antibody (DO-1). Ubiquitinated p53 is indicated as Ub(n)-p53 (upper panel). The expression of total p53, Myc-E6, Flag-TSC-22, and HA-Ub proteins are shown in the lower panels. (D) TSC-22 disrupts ubiquitination of p53 in HeLa cells. HeLa cells were cotransfected with Flag-TSC-22 or a Flag-mock vector and HA-Ub expression vector. 48 h after transfection, cells were treated with 20 μM MG132 for 5 h prior to harvesting. Cell lysates were immunoprecipitated with an anti-HA antibody. Ubiquitinated p53 was detected by Western blotting with an anti-p53 antibody (DO-1). Ubiquitinated p53 is indicated as Ub(n)-p53 (upper panel). The expression of total p53, Myc-E6, Flag-TSC-22, and HA-Ub proteins are shown in the lower panels. (D) TSC-22 disrupts ubiquitination of p53 in HeLa cells. HeLa cells were cotransfected with Flag-TSC-22 or a Flag-mock vector and HA-Ub expression vector. 48 h after transfection, cells were treated with 20 μM MG132 for 5 h prior to harvesting. Cell lysates were immunoprecipitated with an anti-HA antibody. Ubiquitinated p53 was detected by Western blotting with an anti-p53 antibody (DO-1). (E) Stable TSC-22 knock-down or control HeLa cells were treated with 20 μM MG132 for 5 h prior to harvesting. Ubiquitination of p53 was analyzed as described above.

doi:10.1371/journal.pone.0042006.g005

hematoxylin and eosin staining. Tissue samples containing more than 50% tumor cells were selected.

Cell Lines and Mice

Immune-deficient BALB/c nude mice were purchased from Orient Bio (Gyeonggi, Republic of Korea). Human 293, H1299, HeLa, Caski, HT3, HCT116 p53+/− and HCT116 p53−/− cell were cultured in recommended medium supplemented with 10% (V/V) fetal bovine serum (FBS), TSC22 knock-down HeLa cell was established by previous established method [18, 35]. In brief, TSC-shRNA-expressing recombinant plasmid (sh-TSC) was constructed by manipulation of the pSilencer3.1 vector (Ambion) with the ds-oligonucleotides: forward; GATCCGTTGATCTAGGAGTTACCATCTCTTGAATGG-3′. After transfection of recombinant plasmid into HeLa cell, TSC22 knock-down HeLa cells were isolated by culture supplemented with 1 μg/ml of puromycin.

DNA transfections were carried out using lipofectamine2000 (invitrogen), or Fugene HD (Roche), each according to the manufacturer’s instructions.

Real-time and Semi-quantitative RT-PCR Analysis

Total RNA were obtained by extracting tissues in TRizol reagent (Invitrogen) according to the manufacturer’s instructions. First strand cDNA was prepared from total RNA and oligo dT using iScript cDNA synthesis kit (Bio-Rad, USA). Real-time PCR was conducted with a Mini Opticon System (Bio-Rad) and SYBR Green (Bio-Rad). Specific primers used for real-time PCR were shown in Table 1. DNA transfections were carried out using lipofectamine2000 (invitrogen), or Fugene HD (Roche), each according to the manufacturer’s instructions.

Ethics Statement

All animal studies were approved by the Animal Care and Use Committee of Samsung Medical Center.

Luciferase Assay

Luciferase assay were performed as described previously with minor modification [18]. Briefly, different amount of Flag-TSC22 plasmid was co-transfected with 200 ng of p53RE-conjugated luciferase reporter plasmid (pGL3, Promega) together with control pCMV-lacZ plasmid into the HeLa cells for 48 h. The luciferase activity was measured and normalized (to β-galactosidase) using Bright-Glo Luciferase Assay system (Promega) and the Genios Luminometer (TECAN, Austria). Data are represented by error bar ± SEM.

Adenovirus and Vector Construction

PCR-amplified, full-length human TSC22 fragment (500 bp) was cloned into pcGRI-TOPO vector. The primers used to generate the full-length were 5′-GGGTGGTTTTTGGCTGCAAT and 3′-TTCAGTTGACGCCAGAAG. The cloning product was confirmed for sequence from both directions. The pCR-TOPO-TSC22 was digested with EcoRI and cloned into the pACMV/p/A EcoRI site. The pAAMCV-TSC22 and adenovirus backbone vector, pJM17 were cotransfected into a packaging cell line, 293 using Flagene HD transfection reagent (Boehringer Mannheim). A replication competent virus (RCV) negative clone was propagated in 293 cells and purified through two rounds of CsCl density gradient centrifugation. The Ad-TSC22 construct was used at 100 M.O.I. for all transfection experiments. AdCMV-lacZ was used as a positive control. Full and partial length cDNAs of human TSC22 and p53 were introduced into pcDNA3-Flag or pcDNA3 (Invitrogen), respectively. Human papillomavirus E6 (type 16) were cloned into pcDNA3-Flag vector. pcDNA3-HDM2 vector and HA-Ubiquitin expression plasmids were pleasan gifted from J.W. Song (SKKU). DNA transfections were carried out using lipofectamine2000 (Invitrogen), or Fugene HD (Roche), each according to the manufacturer’s instructions.

Cell Proliferation Assay

Cells were plated in triplicate at a density of 2 × 105 cells in 6-well plates. Twenty-four hours later, the cells were infected with Ad-TSC22 or Ad-LacZ. Beginning 24 h after infection, cells were counted each day by MTT assay for up to 3 days.

Flow Cytometry and Apoptosis Assay

Flow cytometry analysis was carried out as described previously [18, 36]. In brief, cells infected with Ad-LacZ and Ad-TSC22 were harvested at indicated time point, fixed in 70% ethanol for 1 h, and then stained with a propidium iodide (PI) solution containing RNaseA (Sigma) for 30 min at room temperature in the dark. Samples were then analyzed by FACS Calibur (BD Bioscience) with CellQuest software.

HeLa and Caski cells were plated onto six-chamber slides and infected with the indicated Ad-LacZ or Ad-TSC22. For the
Figure 6. TSC-22 inhibits tumor growth in nude mice. (A) HDM2 and Myc-E6 were transfected with indicated plasmids into H1299 cells. The transfected cells were treated with MG132 (20 μM) for 5 h before harvest. Cell lysates were immunoprecipitated with an anti-HA antibody. Ubiquitinated p53 was detected by Western blotting with an anti-p53 antibody (DO-1). Ubiquitinated p53 is indicated as Ub(n)-p53 (upper panel). The expression of total p53, HDM2, Flag-TSC-22 and HA-Ub proteins are shown in the lower panels. (B) Flag-tagged wild type (WT) or mutant TSC221–110 was co-transfected with the indicated plasmids into H1299 cells. The transfected cells were treated with MG132 (20 μM) for 5 h before harvest. Cell lysates were immunoprecipitated with an anti-HA antibody. Ubiquitinated p53 was detected by Western blotting with an anti-p53 antibody (DO-1). Ubiquitinated p53 is indicated as Ub(n)-p53 (upper panel). The expression of total p53, HDM2, Flag-TSC-22 and HA-Ub proteins are shown in the lower panels.
observation of nuclear apoptotic body, the nuclei were fixed in methanol and stained with 40, 60-diamidino-2-phenylindole (DAPI, Sigma-aldrich) for 15 min and rinsed twice with PBS, then examined with the fluorescence microscope.

**Western Blot and Antibodies**

Samples were separated on 10% polyacrylamide-SDS gels. The gel was transferred overnight to nitrocellulose membrane and blocked in TBS- Tween-20 (0.1% v/v) with dry skimmed milk (5% w/v) (PBSTm) for 1 h at RT. And then western blotting was carried out by using respective antibody and ECL (Amersham). Antibodies against the following proteins were purchased; p53 mAb (DO-1), p53 pAb (FL393), HDM2 pAb, HA pAb (H9), HRP-conjugated Myc Ab, mouse IgG and rabbit IgG were obtained from Santa Cruz Biotechnology. p21waf/cip mAb (DO-1), p53 pAb (FL393), HDM2 pAb, HA pAb (H9), HRP-conjugated Myc Ab, mouse IgG and rabbit IgG were purchased from Cell signaling technology. Flag (or HRP conjugated) mAb (M2) and β-actin mAb were purchased from Sigma –Aldrich.

**In vivo Ubiquitination Assay**

In vivo ubiquitination assay were performed as described [36]. In brief, H1299 cells were transfected with plasmids as described in the legend to figure. The cells were treated with 20 μM of MG132 for 5 h and then harvested at 48 h post-transfection. Ubiquitinated proteins were immunoprecipitated by HA poly clonal antibody. And then ubiquitination of p53 was detected by western blotting by using p53 Antibody (DO-1).

**Yeast-two Hybrid Assay**

LexA-human TSC22 fusion protein was constructed and used to screen binding proteins from a human ovary cDNA library (Clontech, Palo Alto, CA). The binding proteins were expressed as pB42 fusion proteins. cDNA encoding full length human p53 were PCR amplified and cloned separately into the EcoRI/XhoI sites of pB42. Experiment of yeast two hybrid assay was previously described [37]. In brief, positive interactions were confirmed by cell growth on leucine-depleted yeast synthetic medium and blue colony formation on 5-bromo-4-chloro-3-indolyl-h-D-galactoside (X-gal, 5 mmol/L)-containing medium. The activity of the colony formation on 5-bromo-4-chloro-3-indolyl-h-D-galactoside and blue coloration were performed by quantitative RT-PCR by using GAPDH protein as reference gene. (TIF)

**Supporting Information**

**Author Contributions**

Conceived and designed the experiments: SHL, CHY. Performed the experiments: CHY SBR STK SK SHL. Analyzed the data: CHY SBR JP SHL. Contributed reagents/materials/analysis tools: ISJ SSK JHL SHL. Wrote the paper: CHY SHL. Most of experiment for revision: SBR SW SK.

**References**

1. Shibamura M, Kuroki T, Nose K (1992) Isolation of a gene encoding a putative leucine zipper structure that is induced by transforming growth factor beta 1 and other growth factors. J Biol Chem 267: 10219-10224.
2. Ahn H, Han T (2011) Regulation of TGF-beta signaling by PKC depends on Tsc-22 inducibility. Mol Cell Biochem online published.
3. Kester HA, Blanchetot C, den Hertog J, van der Saag PT, van der Burg B (1999) Transforming growth factor-beta-stimulated clone-22 is a member of a family of leucine zipper proteins that can homo- and heterodimerize and has transcriptional repressor activity. J Biol Chem 274: 27439-27447.
4. Hashiguchi A, Okahashiy K, Asahima M (2004) Role of TSC-22 during early embryogenesis in Xenopus laevis. Dev Growth Differ 46: 533-544.
5. Dobens LL, Hsu T, Twombly V, Gelbart WM, Raferty LA, et al. (1997) The Drosophila bunched gene is a homolog of the growth factor stimulated mammalian TSC-22 sequence and is required during oogenesis. Mech Dev 65: 197-208.
6. Choi SJ, Moon JH, Ahn YW, Ahn JH, Kim DU, et al. (2005) Tsc-22 enhances TGF-beta signaling by associating with Smad4 and induces erythroid cell differentiation. Mol Cell Biolchem 271: 23-28.
7. Yan X, Zhang J, Pan L, Wang P, Xue H, et al. (2011) TSC-22 promotes transforming growth factor beta-mediated cardiac myofibroblast differentiation by antagonizing Smad7 activity. Mol Cell Biol 31: 3700-3709.
8. Kawamura H, Fujimoto T, Imai Y (2004) TSC-22 (TGF-beta-stimulated clone-22) is a novel molecular target for differentiation-inducing therapy in salivary gland cancer. Curr Cancer Drug Targets 4: 321-329.
9. Kawamura H, Nakashiro K, Uchida D, Hino S, Omotera F, et al. (1998) Induction of TSC-22 by treatment with a new anti-cancer drug, vesnarinone, in a human salivary gland cancer cell. Br J Cancer 77: 71-78.
10. Ohira S, Yanagihara K, Nagata K (1997) Mechanism of apoptotic cell death of human gastric carcinoma cells mediated by transforming growth factor beta. Biochem J 324 (Pt 3): 777-782.
11. Iida M, Anna CH, Gaskin ND, Walker NJ, Devereux TR (2007) The putative tumor suppressor Tsc-22 is downregulated early in chemically induced hepatocarcinogenesis and may be a suppressor of Gadd34. Toxicol Sci 99: 41-50.
12. Shostak KO, Dmitrenko VV, Vudmaska MI, Naidenov VG, Beletsii AV, et al. (2005) Patterns of expression of TSC-22 protein in astrocytic gliomas. Exp Oncol 27: 314–318.

13. Yu J, Ershler M, Yu L, Wei M, Hackanson B, et al. (2009) TSC-22 contributes to hematopoietic precursor cell proliferation and repopulation and is epigenetically silenced in large granular lymphocyte leukemia. Blood 113: 5558–5567.

14. Nakamura M, Kitaura J, Enomoto Y, Lu Y, Nishimura K, et al. (2011) TSC-22 is a negative-feedback regulator of Ras/Raf signaling: Implications for tumorigenesis. Cancer Sci. online published.

15. Lee JH, Rho SB, Park SY, Chun T (2008) Interaction between fortilin and transforming growth factor-beta stimulated clone-22 (TSC-22) prevents apoptosis via the destabilization of TSC-22. FEBS Lett 582: 1210–1216.

16. Vogelstein B, Lane D, Levine AJ (2000) Surfing the p53 network. Nature 408: 307–310.

17. Yu J, Wang Z, Kinzler KW, Vogelstein B, Zhang L (2003) PUMA mediates the apoptotic response to p53 in colorectal cancer cells. Proc Natl Acad Sci U S A 100: 1931–1936.

18. Yoon CH, Lee ES, Lim DS, Bae YS (2009) PKR, a p53 target gene, plays a crucial role in the tumor-suppressor function of p53. Proc Natl Acad Sci U S A 106: 7852–7857.

19. Kubbutat MH, Jones SN, Vousden KH (1997) Regulation of p53 stability by Mdm2. Nature 387: 299–303.

20. Haupt Y, Maya R, Kazaz A, Oren M (1997) Mdm2 promotes the rapid degradation of p53. Nature 387: 296–299.

21. Schiefner M, Huihregte JM, Howley PM (1994) Identification of a human ubiquitin-conjugating enzyme that mediates the E6-AP-dependent ubiquitination of p53. Proc Natl Acad Sci U S A 91: 8797–8801.

22. Schiefner M, Huihregte JM, Vierstra RD, Howley PM (1993) The HPV-16 E6 and E6-AP complex functions as a ubiquitin-protein ligase in the ubiquitination of p53. Cell 75: 495–505.

23. Nakashiro K, Kawamata H, Hino S, Uchida D, Miwa Y, et al. (1998) Down-regulation of TSC-22 (transforming growth factor beta-stimulated clone 22) markedly enhances the growth of a human salivary gland cancer cell line in vitro and in vivo. Cancer Res 58: 549–555.

24. Uchida D, Kawamata H, Omotemara F, Miwa Y, Hino S, et al. (2000) Over-expression of TSC-22 (TGF-beta stimulated clone-22) markedly enhances 5-flourouracil-induced apoptosis in a human salivary gland cancer cell line. Lab Invest 80: 955–963.

25. Omotemara F, Uchida D, Hino S, Begum NM, Yoshida H, et al. (2000) In vivo enhancement of chemosensitivity of human salivary gland cancer cells by over-expression of TGF-beta stimulated clone-22. Oncol Rep 7: 737–740.

26. Shostak KO, Dmitrenko VV, Garfulin OM, Rozumenko VB, Khomenko OV, et al. (2003) Down-regulation of putative tumor suppressor gene TSC-22 in human brain tumors. J Surg Oncol 82: 57–64.

27. Pomerantz J, Scherber-Agus N, Liegeois NJ, Silverman A, Alland L, et al. (1998) The Ink4a tumor suppressor gene product, p19Arf, interacts with MDM2 and neutralizes MDM2’s inhibition of p53. Cell 92: 713–723.

28. Zhang Y, Xiaoyang Y, Yarthrong W (1998) ARF promotes MDM2 degradation and stabilizes p53. ARF-INK4a locus deletion impairs both the Rb and p53 tumor suppression pathways. Cell 92: 725–734.

29. Sui G, Affar el B, Shi Y, Brignone C, Wall NR, et al. (2004) Yin Yang 1 is a negative regulator of p53. Cell 117: 859–872.

30. Higashitsuji H, Inok K, Sakurai T, Nago T, Sumitomo Y, et al. (2005) The oncoprotein gankyrin binds to MDM2/HDM2, enhancing ubiquitylation and degradation of p53. Cancer Cell 8: 73–87.

31. Lohrum MA, Ladweg RL, Kubbutat MH, Harlon M, Vousden KH (2003) Regulation of HDM2 activity by the ribosomal protein L11. Cancer Cell 3: 577–587.

32. Tang J, Qa LK, Zhang J, Wang W, Michaelson JS, et al. (2006) Critical role for Ddx5 in regulating Mdm2. Nat Cell Biol 8: 355–362.

33. Coldhae IN, Toso D, Nuciforo P, Senic-Matuglia F, Galmiberti V, et al. (2008) NUMB controls p53 tumour suppressor activity. Nature 451: 76–80.

34. Dai MS, Sun XX, Lu H (2008) Aberrant expression of nucleostemin activates Mdm2 and induces cell cycle arrest via inhibition of Mdm2. Mol Cell Biol 28: 4365–4376.

35. Lee JH, Rho SB, Park SY, Chun T (2008) Interaction between fortilin and transforming growth factor-beta stimulated clone-22 (TSC-22) prevents apoptosis via the destabilization of TSC-22. FEBS Lett 582: 1210–1216.

36. Lee SH, Son MJ, Oh SH, Rho SB, Park K, et al. (2005) Thymosin (beta)10 inhibits angiogenesis and tumor growth by interfering with Ras function. Cancer Res 65: 137–148.