Suppression of the p53-Dependent Replicative Senescence Response by Lysophosphatidic Acid Signaling

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Abstract

Lysophosphatidic acid (LPA) is a lipid mediator of a large number of biological processes, including wound healing, brain development, vascular remodeling, and tumor progression. Its role in tumor progression is probably linked to its ability to induce cell proliferation, migration, and survival. In particular, the ascites of ovarian cancers is rich in LPA and has been implicated in growth and invasion of ovarian tumor cells. LPA binds to specific G protein–coupled receptors and thereby activates multiple signal transduction pathways, including those initiated by the small GTPases Ras, Rho, and Rac. We report here a genetic screen with retroviral cDNA expression libraries to identify genes that allow bypass of the p53-dependent replicative senescence response in mouse neuronal cells, conditionally immortalized by a temperature-sensitive mutant of SV40 large T antigen. Using this approach, we identified the LPA receptor type 2 (LPA2) and the Rho-specific guanine nucleotide exchange factor Dbs as potent inducers of senescence bypass. Enhanced expression of LPA2 or Dbs also results in senescence bypass in primary mouse embryo fibroblasts in the presence of wild-type p53, in a Rho GTPase–dependent manner. Our results reveal a novel and unexpected link between LPA signaling and the p53 tumor-suppressive pathway. (Mol Cancer Res 2008;6(9):1452–60)

Introduction

Tumor suppressor p53 is often found mutated in cancer and plays a central role in protection against tumorigenesis (1, 2). p53 is a transcription factor that is stabilized and activated by cellular stress, for example in response to DNA damage or oncogene activation (3). The regulation of p53 itself is complex and involves transcriptional, translational, as well as posttranslational modifications (4–6). p53 acts through the induction of a wide variety of target genes (4, 7, 8), which can result in diverse cellular responses, including apoptosis, cell cycle arrest, or senescence (1, 6). Cells deficient for p53 function will continue to proliferate in the presence of genotoxic stress and thereby accumulate DNA damage (9). To date, much has been learned about p53 function through study of connective tissue cells like mouse embryo fibroblasts (MEF; refs. 10, 11). When cultured, primary fibroblasts enter into replicative senescence gradually over time, as a result of the stress endured by tissue-culture conditions (3, 10, 12, 13). This leads to triggering of tumor-suppressive p19ARF–p53 signaling, resulting in the induction of antiproliferative p53 targets, such as PAI-1 and p21CIP1, and a cell cycle arrest (14–17). Both in human tumors and in mouse models, it has recently been confirmed that in vivo senescence is also a bona fide tumor-suppressive mechanism and functions as a first barrier to oncogenic transformation (18–21). In tissue culture, cells may escape senescence by loss of p53 function or its upstream activator p19ARF, or by loss of the retinoblastoma family of proteins pRb, p107, and p130 (15, 22–24), because tumor-suppressive pRb proteins are downstream mediators of p53 in senescence induction via regulating E2F activity (25, 26). Loss of p19ARF or p53 function can occur through various mechanisms, including mutation, epigenetic gene silencing, repression of p19ARF gene expression (e.g., by TBX2 or TBX3), functional inactivation of p53 (e.g., by viral oncoproteins such as SV40 LT or HPV E6), or by an override downstream in the face of proper tumor-suppressive p19ARF, p53 signaling (e.g., loss of p53-targets PAI-1 or p21CIP1; refs. 4, 14–16, 23, 27–29).

Cell cycle progression requires growth factor–dependent RAS and phosphatidylinositol 3-kinase–protein kinase B signaling to the retinoblastoma family of proteins (17, 30). Mitogenic signaling increases cyclin-dependent kinase (CDK) activity, leading to inactivation by phosphorylation of the pRb family proteins (15, 17). As a result, derepression of the E2F family of proteins advances G1 cell cycle progression (25, 26, 31). Thus, mitogenic signaling by activation of CDKs cross-reacts with inhibition of the kinases by p16INK4A and p19ARF, p53 signaling on the level of regulation of the pRb G1 cell cycle restriction point.

Among the most abundant serum mitogens is the phospholipid lysophosphatidic acid (LPA). LPA is produced after tissue injury and has been implicated in platelet activation and it promotes the proliferation, migration, and survival of many cell types, both normal and malignant (for review, see refs. 32–34). LPA serves as a ligand for at least five distinct G protein–coupled receptors (LPA1–LPA5), of which...
LPA₁, LPA₂, and LPA₃ are the most closely related [formerly known as endothelial differentiation gene (Edg) 2, Edg4, and Edg7, respectively; ref. 35]. More recently, a new nomenclature has been proposed for these receptors, LPAR1-3, but this is not yet widely used in the field (36). Increased LPA levels and aberrant LPA receptor expression have been linked to tumor progression (34). Receptor activation by LPA results in the activation of various effectors, including phosphatidylinositol 3-kinase and the small GTPases Ras, RhoA, and Rac, with RhoA activation being particularly prominent (33-39). Rho activation is mediated by receptor-linked Gα₁₂/₁₃ protein signaling to guanine-nucleotide exchange factors (GEF), which induce translocation of RhoA to the membrane by inducing a GTP-bound state (40, 41). The GEFs of Rho family GTPases share the Dbl-homology (DH) as a common sequence motif (42). There is a multitude of GEFs for Rho, including Dbs (Dbl’s big sister, also known through its rat orthologue Ost and more recently also referred to as MCF2L; refs. 40, 43). Dbs is capable of transforming NIH3T3 cells (fibroblasts that are deficient for tumor-suppressive p19ARF-p53 signaling due to the absence of the p16INK4A/p19ARF locus) in a RhoA-dependent manner (44, 45). Rho family proteins and their GEFs regulate the actin cytoskeleton and cell adhesion, and thus are involved in, among others, stress fiber formation, cell motility, and cell morphology (41, 46). Their activity is intimately connected to cancer development (40, 47).

We used here an unbiased cDNA library screen to identify genes that, when overexpressed, induce a bypass of p19ARF-p53–dependent senescence. We describe here an unexpected interaction between LPA receptor signaling and tumor-suppressive p53 activity.

Results
To identify cDNAs whose encoded proteins can induce bypass of a p53-dependent senescence arrest, we performed a gain-of-function genetic screen in mouse striatal ST.Hdh(Q111) cells. These mouse neuronal cells harbor a temperature-sensitive mutant of the SV40 Large T (LT) oncogene, which conditionally immortalizes the cells at 32°C, but undergo a robust and uniform senescence arrest after a shift to 39°C, due to inactivation of the LT antigen. A more detailed description of these cells has been published previously (29, 48). We infected the cells at 32°C with retroviral cDNA expression libraries derived from either a polycytemia vera cell line (PCV; Osler-Vaquez disease—a chronic myeloproliferative disorder) or whole human brain and tested for senescence bypass following temperature shift of the infected cells to 39°C. We observed no colonies in the green fluorescent protein (GFP) virus–infected populations used as a control (data not shown). Infection with the cDNA expression libraries yielded one colony from the PCV library and two independent colonies from the whole human brain library. After the expansion of the proliferating senescence bypassing cells, we isolated the integrated cDNA inserts by PCR and subsequently identified their nature by sequencing (Fig. 1A).

The inserts we isolated were full-length cDNAs of G protein–coupled receptor LPA₂ (Edg4) from the PCV library and two independent 5′ truncated versions of the GEF Dbs. To
confirm that the isolated cDNAs were indeed responsible for the observed bypass of senescence in the original screen, we used the isolated and cloned cDNAs from the screen in a second-round assay. We infected ST.Hdh\textsuperscript{Q111} cells with the pure virus encoding either LPA\textsubscript{2} or Dbs at 32°C and shifted them to 39°C. In a colony formation assay, we observed that the cDNAs potently induced a senescence bypass compared with a nonfunctional GFP control (Fig. 1B). Moreover, they showed distinct phenotypes when compared with GFP control-infected cells at 39°C (Fig. 1C). In case of LPA\textsubscript{2} overexpression, there is rounding of ST.Hdh\textsuperscript{Q111} cells, which has previously also been observed in other cells of neuronal origin (33, 49, 50). In case of Dbs, we noticed distinct protrusions at the edge of cells when grown at low density and a transformed-like phenotype when grown at high density (Fig. 1C). When we tested the full-length Dbs cDNA, we also observed efficient induction of senescence bypass (data not shown). We therefore conclude that both LPA\textsubscript{2} and Dbs are able to induce bypass of a p53-dependent senescence arrest in mouse striatal ST.Hdh\textsuperscript{Q111} cells.

As LPA\textsubscript{2} is a member of a family of LPA receptors (35, 51), we tested whether other family members could also induce a senescence bypass in ST.Hdh\textsuperscript{Q111} cells. We infected ST.Hdh\textsuperscript{Q111} cells at 32°C with retroviral vectors harboring the full-length cDNAs for LPA\textsubscript{1}, LPA\textsubscript{2}, and LPA\textsubscript{3}; shifted them to 39°C; and tested for senescence bypass in a colony formation assay. We found that—when compared with a nonfunctional GFP control—both LPA\textsubscript{1} and LPA\textsubscript{2} were able to induce a senescence bypass, whereas overexpression of LPA\textsubscript{3} was not (Fig. 2A), although all three cDNAs were expressed approximately equally (Fig. 2B). When we assayed the parental ST.Hdh\textsuperscript{Q111} cells for expression of LPA\textsubscript{1}, LPA\textsubscript{2}, or LPA\textsubscript{3} by reverse transcription-PCR, we found that these cells naturally express LPA\textsubscript{1} and LPA\textsubscript{2} but not LPA\textsubscript{3} (Fig. 2B).

Because we observed that ectopic expression of LPA\textsubscript{2} receptor allows senescence bypass, we next asked whether triggering LPA receptor signaling by the addition of excess ligand could provoke the same response. LPA is a major constituent of serum and is present at concentrations around 1 μm/L. This suggests that the amount of LPA present in serum is sufficient to saturate endogenous LPA receptors and that addition of even more LPA would not increase signaling and allow senescence bypass. We tested this in a colony formation assay in ST.Hdh\textsuperscript{Q111} cells at 39°C following addition of various concentrations of LPA. We observed that besides a change in cell morphology (data not shown), there was no rescue of senescence up to addition of 5 μm/L of LPA to the culture medium. As these supraphysiologic LPA levels should induce maximal receptor activation, we conclude that the rate-limiting event for senescence bypass in ST.Hdh\textsuperscript{Q111} cells is the number of LPA receptors.

The cell system used to identify LPA\textsubscript{2} and Dbs is artificial in that the temperature shift induces a sudden reactivation of p53 and Rb function in the cells. We therefore asked whether we could reproduce the senescence bypass activity of LPA\textsubscript{2} and Dbs in primary MEFs. Quantitative PCR analysis revealed that MEFs express both LPA\textsubscript{1} and LPA\textsubscript{2}, but not LPA\textsubscript{3}, with LPA\textsubscript{1} expression level being 5 to 10 times higher than LPA\textsubscript{2}. We infected two independent populations of late-passage MEFs with either LPA\textsubscript{2} or Dbs retroviral vectors and followed proliferation of these cells over time by making a growth curve. A nonfunctional GFP was used as a negative control, whereas a retroviral short hairpin RNA against p53 (p53\textsuperscript{kd}) was used as a positive control for immortalization (52). We found that both LPA\textsubscript{2} or Dbs overexpression immortalized primary MEFs, albeit at a lower efficiency compared with p53\textsuperscript{kd} (Fig. 3A and B). These results indicate that LPA\textsubscript{2} and Dbs are also sufficient for induction of a senescence bypass in primary mouse fibroblasts.

LPA\textsubscript{2} is a G protein–coupled receptor that signals to Rho GTPases, and Dbs is a GEF for RhoA and Cdc42. We therefore asked whether constitutive RhoA or Cdc42 activity itself is

\footnote{4 C. Stortelers, L.A. van Meeteren, and W.H. Moolenaar, unpublished results.}
sufficient for senescence bypass. To address this, we used vectors encoding constitutively active mutants of these proteins: RhoAV14 and Cdc42V12. In a colony formation assay in ST.HdhQ111 cells, we found that overexpression of RhoAV14 and Cdc42V12 indeed mediated senescence bypass (Fig. 4A). Furthermore, when tested over a prolonged period of time, we found that enhanced RhoAV14 or Cdc42V12 expression causes a senescence bypass in primary MEFs as well (Fig. 4B and C). We conclude that constitutive RhoA or Cdc42 signaling is sufficient to bypass senescence.

Our results imply that not only LPA2 or Dbs overexpression, but also that of their target GTPases RhoA or Cdc42, is sufficient for senescence bypass in both mouse neuronal and embryo fibroblast cells. This prompted the question of whether the immortalization by LPA2 or Dbs was Rho-GTPase dependent. Furthermore, we also tested whether the immortal cells were resistant to enhanced p53 signaling. Such experiment would help address whether immortalization by LPA2 and Dbs is upstream or downstream of p53. When analyzed in a colony formation assay, we found that, compared with negative control GFP protein–overexpressing cells, overexpression of a dominant negative mutant of RhoA (RhoAN19) completely blocked proliferation in immortalized LPA2 or Dbs MEFs (Fig. 5). The reduction of growth was not due to increased apoptosis (data not shown) and was as effective as the growth inhibition of the immortal RhoAV14 and Cdc42V12 cells. MEFs having p53^fl/fl or pRb^−/−;p107^−−;p130^−− (TKO: retinoblastoma family deficient) MEFs were almost entirely and partially inhibited in their proliferation by RhoAN19, respectively (Fig. 5). Together, these data suggest that not only the LPA2 and Dbs cells but also p53^fl/fl are heavily dependent on RhoA activity for proliferation because these cells are also significantly blocked in proliferation. Loss of the pRb family proteins partially overrides the arrest by RhoAN19, suggesting that these proteins act downstream of RhoA signaling (Fig. 5). We conclude that LPA2- and Dbs-overexpressing cells depend on Rho activity for immortalization. Moreover, we found that not only LPA2- and Dbs- but also RhoAV14- and Cdc42V12-immortalized MEFs were completely refractory to high expression of wild-type p53 (Fig. 5). TKO control MEFs were also not inhibited by excess p53 expression, in support of the notion that pRb proteins are downstream mediators of a p53-dependent arrest (16, 25). Our results suggest that the senescence bypass by LPA2 or Dbs is downstream of p53 but upstream of RhoA.

To further analyze whether the senescence bypass by overexpression of LPA2 or Dbs was indeed downstream of p53, we investigated whether the immortalization took place in the presence of normal p19ARF-p53 signaling. We first asked whether tumor-suppressive p19ARF-p53 signaling was influenced in the immortalized cells because both the ST.HdhQ111 cells and MEFs are dependent on this signaling pathway for induction of the senescence response (29, 49). We analyzed the protein levels of p53 and its downstream target gene p21CIP1 after the addition of the DNA-damaging agent cisplatin. In normal cycling cells, the level and activity of p53 are low. ST.HdhQ111 cells at 32°C exhibited high levels of p53—in accordance with the notion that SV40 LT blocks and stabilizes p53 at this temperature—which is unchanged after cisplatin treatment (Fig. 6A). The p53 stabilization and p21CIP1 induction in the immortalized LPA2 and Dbs cells after DNA damage is comparable with the immortal controls, which have wild-type p53 (NIH3T3 fibroblasts), indicating that p53 is functional in the immortalized cells (Fig. 6A).

Because cells can also bypass senescence by loss of the retinoblastoma family proteins (pRb, p107, p130) and it has been shown that the tumor-suppressive Rb proteins are downstream of p53 in the senescence induction (25), we investigated whether the activity of E2F, a critical target of Rb family proteins, was altered in the immortalized LPA2 and Dbs-overexpressing cells. We found a strong induction of E2F targets cyclin E, p107, E2F1, and proliferating cell nuclear antigen (PCNA) as judged by Western blotting. Their protein levels were distinctly higher than in other immortalized cells (Fig. 6B). Note that the overexpression of E2F1 itself predominantly induces apoptosis (53), which we only observed with very high expression of LPA2 (data not shown). We suggest that overexpression of LPA2 or Dbs in ST.HdhQ111 cells leads to senescence bypass in the presence of functional p19ARF-p53 signaling and is associated with induction of E2F activity.
MEFs are also dependent on a functional p19ARF-p53 pathway and Rb family function for senescence induction (15). We therefore analyzed in the LPA2-, Dbs-, RhoAV14-, or Cdc42V12-immortalized MEFs whether there was active p19ARF-p53 signaling, as this could further suggest the immortalization was indeed downstream of p53 function. No evidence for loss of p19ARF in the immortalized cells was found (Fig. 6C). Compared with p53kd MEFs, which have high p19ARF levels because p53 represses p19ARF expression (6), there was p19ARF expression in the LPA2-, Dbs-, RhoAV14-, or Cdc42V12-overexpressing MEFs, equal to the levels seen in the control infected young and senescent cells (Fig. 6C). To measure p53 activity, we further analyzed by Western blotting the protein levels of p53 and its target p21CIP1 in the immortalized cells after the addition of DNA-damaging agent cisplatin. Compared with young and senescent cells, there was stabilization of p53 and induction of p21CIP1 in the LPA2-, Dbs-, RhoAV14-, and Cdc42V12-overexpressing MEFs, indicating that p53 remained fully functional in these cells (Fig. 6C).

We next investigated whether E2F activity was also enhanced in MEFs immortalized by LPA2, Dbs, RhoAV14, and Cdc42V12. When we analyzed the protein levels of E2F target activation by Western blotting, we again observed induction of p107, E2F1, and PCNA when compared with noncycling senescent cells (Fig. 6D). p107 and E2F1 were induced when compared with young cycling cells but equal to levels in cycling p53kd cells, whereas PCNA levels were comparable between all cycling MEFs (Fig. 6D). Apparently, there are differences in specific E2F target activation in the immortalization of MEFs or ST. HdhQ111 cells by LPA2 or Dbs overexpression (see also Fig. 6A).

It was recently reported that LPA signaling can lead to down-regulation of p53 function by reduction of the nuclear fraction of the protein (54). We therefore investigated the function and localization of p53 in the immortalized LPA2 MEFs. Young wild-type MEFs or proliferating and immortalized p53−/− or LPA2 MEFs were treated with the DNA damaging agent cisplatin, which leads to activation of p53. Figure 6E shows that p53 was exclusively nuclear in all MEFs and was stabilized and activated in both wild-type and LPA2 MEFs, in contrast to p53−/− cells. In accordance, there was induction of p53 target p21CIP1 in these cisplatin-treated cells (Fig. 6E). Quantitative real-time PCR analysis of the same cells showed induction of another bona fide p53 target, PAI-1 (55), in both young and immortalized LPA2 MEFs (Fig. 6F). The high PAI-1 levels in untreated LPA2 MEFs compared with untreated wild-type MEFs reflect the p53-dependent induction of PAI-1 in post-senescent cells (16). It further supports the notion that p53 function is unaltered in these cells. In contrast, there was no PAI-1 induction in p53−/− control MEFs (Fig. 6F). Apparently, p53 localization and function is not altered in immortalized LPA2 MEFs. We suggest that, in line with our findings in striatal ST. HdhQ111 cells, LPA2, Dbs, RhoAV14, and Cdc42V12 immortalize MEFs downstream of functional p19ARF-p53 pathway signaling possibly via induction of E2F activity.

Discussion

We have found in a genetic screen that enhanced expression of the LPA2 receptor or the Rho-GEF Dbs induces senescence bypass. Furthermore, we report that constitutive Rho-GTPase family signaling by overexpression of active mutants of RhoA or Cdc42 is also sufficient for senescence bypass. LPA2, Dbs, and Rho GTPases mediate bypass of senescence downstream of tumor-suppressive p19ARF-p53 signaling, because the immortalized cells still express p19ARF and retain normal p53 function. LPA2 or Dbs overexpression results in immortalization in the presence of enhanced E2F activity.

Thus far, most links between enhanced LPA2 expression and cancer are related to the later stages of tumorigenesis. For example, a higher rate of lymphatic invasion and metastasis correlates with LPA2 expression in intestinal-type carcinomas (56), and high LPA2 expression may be involved in thyroid pathogenesis (57). In ovarian cancer, enhanced levels of LPA2 bypass.
are frequent and ascitic fluid from ovarian cancer patients contains elevated levels of LPA (58-60). In addition, autotaxin, the major LPA-producing enzyme, promotes tumor progression in mouse models and is found overexpressed in various human cancers (34, 61-63). We now find that enhanced LPA2 activity is also sufficient to bypass p53-dependent senescence. Our findings are consistent with a model in which enhanced LPA signaling can bypass potent tumor-suppressive p53-dependent signaling to mediate escape from replicative senescence. Murph et al. (54) recently reported that LPA signaling reduced p53 transcriptional activity and nuclear p53 protein abundance in A549 lung carcinoma cells. In contrast, we find that LPA2-immortalized cells retain a functional p53 response following treatment with a DNA-damaging agent (Fig. 6). Furthermore, Murph et al. observed that all three LPA receptors suppress p53 activity, whereas we found that only LPA1 and LPA2 but not LPA3 can bypass p53-dependent senescence. These differences in the effect of LPA signaling on p53 may be related to the differences in genetic background used. In our studies, we used nontransformed cells, whereas HepG2 and A549 cells used by Murph et al. are cancer cell lines. Irrespective of the precise mechanism(s) involved, both studies highlight an unexpected connection between LPA signaling and p53.

There have been reports of intersection of p53 and Rho GTPase functions. For instance, the migratory effect of p53 loss is phosphatidylinositol 3-kinase and Rac dependent in two-dimensional cultures and Rho dependent in three-dimensional cultures (64, 65), although in two-dimensional culture Rho is normally more associated with invasion (47). Rho activity is elevated in p53−/− mouse fibroblasts (66) and Cdc42-dependent filopodia formation is interrupted downstream by p53 (67). Furthermore, in a nonfunctional p19ARF-p53 background, mitogenic activation of Rho GTPases promotes hyperproliferation and transformation (66, 68). Whether immortalization by LPA2 or Dbs is critically dependent on actin reorganization or growth factor signaling downstream of RhoA activity needs to be elucidated. The effects that Rho-GTPases have on cell proliferation are thus far thought to reflect the crucial roles of anchorage- or adhesion-dependent signals. In the immortalized LPA2 or Dbs cells, E2F activity seems increased as judged by the expression of several of its downstream target genes. Consistent with a possible role for increased E2F signaling in the senescence bypass observed here, it has been shown that increased expression of E2F target genes can mediate escape from senescence (25). Furthermore, it has been previously shown that LPA induces the expression of urokinase-type plasminogen activator (uPA) both in vitro and in vivo (69, 70) and this induction of uPA requires p38MAPK activity (71). Interestingly, we have recently shown that ectopic expression of uPA or inhibition of the expression of the antagonist of uPA, PAI-1, mediates bypass of senescence (16). Thus, induction of uPA by LPA signaling may also contribute to the bypass of senescence observed here.

In summary, we describe here a connection between LPA signaling and p53-dependent cell cycle progression. Our findings suggest that LPA2 and Rho activity may not only be involved in tumor progression but may also contribute to tumor initiation by regulating p53-dependent senescence.

**Materials and Methods**

**Antibodies and Vectors**

For Western blotting, antibodies against cyclin E (M20), p107 (C18), E2F1 (C20), PCNA (PC10), and CDK4 (C22) were from Santa Cruz Biotechnology; anti-p53 (Ab7) was from Oncogene Research Products; and HSP90 was from Cell Signaling. pLZRS-LPA1 and pLZRS-LPA3 have been described (72). Control infections were done with GFP vectors.

**Cell Culture, Transfection, and Retroviral Infection**

Mouse ST.HdhQ111 striatal cells, MEFs, and Phoenix cells were cultured in DMEM (Life Technologies) supplemented with 8% heat-inactivated fetal bovine serum (Perbo), 2 mmol/L L-glutamine, and penicillin/streptomycin (Life Technologies). Transfections were done with the calcium phosphate precipitation technique. Retroviral supernatants were produced by

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**Figure 5.** Senescence bypass by LPA2 and Dbs is Rho dependent. Colony formation assay of various depicted immortalized MEFs infected with dominant negative retroviral cDNA constructs for RhoA (RhoAN19), p53, or negative control GFP. Growth was in the absence of selection and plates were stained after 1 wk.
transfection of Phoenix packaging cells. Viral supernatants were filtered through a 45-A Millex HA filter (Millipore), and infections were done in the presence of 4 μg/mL polybrene (Sigma). Drug selections in ST. Hdh Q111 or MEFs were done with 1 μg/mL puromycin. ST. Hdh Q111 mouse striatum cells express a mutant version of the huntingtin protein with an expanded polyglutamine repeat from a knock-in ST. Hdh Q111 allele and a temperature-sensitive mutant of the SV40 T antigen, introduced by retroviral transduction. ST. Hdh Q111 cells were cultured at 32°C and were shifted to the nonpermissive temperature of 39°C when indicated.

Retroviral Library Screen

High-titer retroviral library supernatant derived from human whole brain (Clontech) or PCV cDNA libraries were used to infect 2 × 10⁶ ST. Hdh Q111 cells. Twenty-four hours after infection, cells were plated at a density of 0.8 × 10⁵ per 10-cm dish and after 48 h, the cells were shifted to the nonpermissive temperature of 39°C. Colonies appeared only in the cDNA library–infected populations. These colonies were picked and expanded at 39°C. Retroviral cDNA inserts were PCR-amplified using specific retroviral primers, recloned into pLIB, and identified by sequencing. To analyze whether the recloned

FIGURE 6. Retention of p53 status and enhanced E2F activity in LPA₂- and Dbs-immortalized ST. Hdh Q111 cells and MEFs. A. Western blot analysis of senescent normal and LPA₂- and Dbs-immortalized ST. Hdh Q111 cells for p53 activation and p21(CIP) target induction by cisplatin addition versus cycling NIH3T3 and Ras V12/p53⁰/⁰ cells. CDK4 is a loading control. Proteins were isolated after 10 d at 39°C. LPA₂- and Dbs-immortalized ST. Hdh Q111 cells have normal p53 function. B. Western blot analysis of normal and LPA₂- or Dbs-immortalized ST. Hdh Q111 cells for E2F targets cyclin E, p107, E2F1, and PCNA versus cyclin NIH3T3 and Ras V12/p53⁰/⁰ cells. CDK4 is a loading control. Proteins were isolated after 10 d at 39°C. There is induction of E2F targets in LPA₂- and Dbs-immortalized ST. Hdh Q111 mouse striatal cells. C. Western blot analysis for p19ARF, p53 activation, and its target p21CIP in young and senescent wild-type MEFs, and LPA₂-, Dbs-, RhoA V14-, or Cdc42 V12-immortalized MEFs after cisplatin addition. p53⁰/⁰ is a positive control for loss of p53 function, whereas CDK4 is a loading control. D. Western blot analysis of various normal and immortalized MEFs for E2F targets p107, E2F1, and PCNA versus cycling p53⁰/⁰ cells. CDK4 is a loading control. There is retention of E2F target activation in LPA₂- and Dbs-immortalized cells. E. Western blot analysis of nuclear (N) and cytoplasmic (C) protein fractions of young wild-type (Wt), or immortal p53-deficient (p53⁻/⁻) or LPA₂-overexpressing MEFs before (−) and after (+) cisplatin treatment. Activation of p53 and its target p21(CIP) was analyzed by probing with the respective antibody. Hsp90 is a cytoplasmic loading control. *, a nuclear-specific background band showing equal loading of nuclear fractions. F. Quantitative real-time PCR analysis of the induction of PAI-1 in young wild type or immortal p53-deficient or LPA₂-overexpressing MEFs before (−) and after (+) cisplatin treatment. GAPDH serves as the control.
cDNAs were responsible for the senescence bypass, a second round with recloned library cDNA was done. In case of LPA2, a full-length cDNA was isolated from the PCV library; in case of Dbs, two independent cDNA fragments missing the first 104 amino acids was isolated from the whole brain library as the LPA was purchased from Avanti Polar Lipids, Inc. p53kd-, three independent experiments are shown. High-purity grade Colony Formation Assays

ST.Hah Q111 cells were infected with cDNA constructs at 32°C. Twenty-four hours after infection, cells were selected and plated at a density of 5 × 10^4 per 10-cm dish, and after another 24 h the cells were shifted to the nonpermissive temperature of 39°C. Cells were stained after 10 days at 39°C. For all colony formations, representative examples of at least three independent experiments are shown. High-purity grade MEFs were infected with GFP, RhoAN19, or p53 virus, and late-passage MEFs were infected with retroviral cDNA constructs, selected, and plated (150,000 MEFs) in a 6-cm dish (time 0 days). Every 4 d, cells were counted and 150,000 cells were replated. Total cell amounts in all growth curves were displayed as cumulative over time. For all growth curves, representative examples of at least three independent experiments are shown.

Growth Curves

Late-passage MEFs were infected with retroviral cDNA constructs, selected, and plated (150,000 MEFs) in a 6-cm dish (time 0 days). Every 4 d, cells were counted and 150,000 cells were replated. Total cell amounts in all growth curves were displayed as cumulative over time. For all growth curves, representative examples of at least three independent experiments are shown.

Real-time PCR

Reverse transcriptase and PCR reaction were done following standard procedures. First-strand cDNA was synthesized with Superscript II reverse transcriptase (Invitrogen) and oligo(dT) primers. LPA1,2,3-specific 20-mers were designed to recognize human LPA receptors, lacking any significant similarity with other LPA receptors.

Cell Culture Images

Images were obtained using a Zeiss Axiovert 25 microscope with A-Plan 10× or LD A-plan 20× objectives on a Canon Powershot G3 14× zoom camera.

Western Blotting

Selected cells were lysed in radioimmunoprecipitation assay buffer [50 mmol/L Tris (pH 8); 150 mmol/L NaCl; 1% NP40; 0.5% DOC; 0.1% SDS]. Forty or 80 μg of protein were separated on 8% to 12% SDS-polyacrylamide gel by electrophoresis and transferred to polyvinylidene difluoride membranes (Millipore). Western blots were probed with the indicated antibodies. For all Western blots, representative examples of at least two independent experiments are shown.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.


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