Functional Cooperation of RKTG with p53 in Tumorigenesis and Epithelial–Mesenchymal Transition

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Abstract

Raf kinase trapping to Golgi (RKTG) is a potential tumor suppressor gene due to its negative roles in regulating Ras/Raf/MEK/ERK (extracellular signal–regulated kinase) pathway and GPCR (G protein–coupled receptor) G\(\beta\)\(\gamma\) subunit signaling. Interestingly, RKTG-deficient mice are free of tumors, although they are prone to form skin cancer on carcinogen administration. On the other hand, p53 is a well-characterized tumor suppressor gene and p53 heterozygous mice develop sarcoma and other tumors starting from 12 months of age. In RKTG-null mouse embryonic fibroblasts, lypophosphatidic acid (LPA), but not EGF (epidermal growth factor), could stimulate hyperphosphorylation of AKT and GSK3\(\beta\), accompanied by increases in phosphorylation of p53 at Ser15 and accumulation of p53, as well as its target genes p21 and p16. Spontaneous skin cancer–like tumors were detected in about 25% of RKTG nullizygous and p53 heterozygous mice within 7 months of age. Hyperplasia and epithelial–mesenchymal transition (EMT) were observed in the tumor-overlying epidermis, in which LOH of p53 occurred and EMT features emerged. In p53-mutated A431 epithelial carcinoma cells, knockdown of RKTG led to enhancement of LPA-stimulated AKT and GSK3\(\beta\) phosphorylation, together with increased accumulation of \(\beta\)-catenin and appearance of EMT features that were antagonized by p53 overexpression. In HepG2 epithelial cells, LPA-stimulated AKT phosphorylation and EMT features reached maximum when both RKTG and p53 were simultaneously silenced. In summary, these results not only indicate that RKTG has an in vivo tumor suppressor function to cooperate with p53 in tumorigenesis but also suggest that p53 has an EMT checkpoint function and the loss of this function can combine with loss of RKTG to drive EMT and tumor progression. Cancer Res; 71(8); 2959–68. ©2011 AACR.

Introduction

Raf kinase trapping to Golgi (RKTG) or PAQR3 of the progesterone and adipoQ receptor (PAQR) family has been shown as a Golgi-localized membrane protein and participates in the spatial regulation of Ras/Raf/MEK/ERK (extracellular signal–regulated kinase) and G protein–coupled receptor (GPCR) signaling pathways by sequestrating Raf kinase and G\(\beta\)\(\gamma\) subunit into the Golgi apparatus, respectively (1–4). For example, epidermal growth factor (EGF)–stimulated ERK phosphorylation is negatively regulated by RKTG (1), and lypophosphatidic acid (LPA)–stimulated and G\(\beta\)\(\gamma\) subunit–mediated AKT phosphorylation is negatively modulated by RKTG (4). RKTG-deficient mice are viable and display no obvious developmental and phenotypic defects (1, 5). Due to the negative regulatory role of RKTG in regulating Ras/Raf/MEK/ERK and G\(\beta\)\(\gamma\) signaling pathways, it is postulated that RKTG possesses a tumor suppressor function. This hypothesis is supported by the finding that chemical-induced skin cancer is accelerated in RKTG-null mice (5). Furthermore, RKTG has been found to negatively regulate angiogenesis by affecting an autocrine VEGF function, and downregulation of RKTG is frequently found in human clear cell renal cell carcinoma (6).

In contrast to RKTG, p53 is a well-characterized tumor suppressor that is implicated in the regulation of many fundamental cellular activities including cell-cycle control, apoptosis, senescence, and DNA damage repair (7, 8). Almost half human cancers harbor p53 gene mutations or deletion, indicating that loss of p53 function is a critical event in tumorigenesis (9). About half of p53 homozygous deletion mice developed tumors by 4 to 5 months of age with lymphoma being the predominant type (10–12). On the other hand, p53 heterozygous deletion mice start to form tumors by 12 months of age, with sarcoma being the most common type. It is well known that p53 cooperates with other genetic alterations to promote cancer formation. For instance,
somatic inactivation of retinoblastoma and p53 led to spontaneous squamous cell carcinoma (13). Epithelial–mesenchymal transition (EMT) is defined as a process by which a polarized epithelial cell switches to a mesenchymal cell phenotype through multiple biochemical changes, allowing cells to migrate away from the epithelial layer, and is implicated in the formation of invasive and metastatic tumor cells during tumor progression (14, 15). This process involves the acquisition of mesenchymal markers, such as vimentin and fibronectin, together with loss of epithelial cell adhesion molecules such as E-cadherin (16). Recently, cumulative evidence has indicated that the phosphoinositide 3-kinase (PI3K)/AKT signaling pathway plays a role in EMT (17, 18). Hyperactivation of AKT happens frequently in human cancers and is associated with tumor metastasis (19). Constitutively active AKT has been reported to possess a capability to reduce cell–cell adhesion, increase cell motility, and enhance invasiveness in human squamous cell carcinoma lines (20). On the other hand, disruptions of both AKT1 and AKT2 decrease the metastasis of tumor cells in the liver (21). Recently, a series of studies also suggest that p53 is implicated in EMT during tumor progression (22–24). However, whether p53 functionally interacts with the PI3K/AKT pathway in EMT remains largely unclear at present.

Material and Methods

Mouse studies, plasmid construction, and materials

RKTG-null mice (in C57BL/6j X 129Sv genetic background) were generated and identified as previously described (1). The p53 disrupted mice (in C57BL/6j genetic background) were bought from The Jackson Laboratory (12). All animal procedures and protocols were approved by the Institutional Animal Care and Use Committee of the Institute for Nutritional Sciences, Chinese Academy of Sciences. Details of plasmid construction and materials are provided in Supplementary Information.

Cell culture, cell transfection, lentivirus with RKTG and p53 short hairpin RNA, confocal microscopy, and cell-based assays

Mouse embryonic fibroblast (MEF) cells, A431, and HepG2 cells were grown in DMEM (Dulbecco’s modified Eagle’s medium) containing 10% FBS. The method of MEF isolation from wild-type or RKTG-deleted mouse embryos was described previously (1). A431 and HepG2 cells were purchased from and tested by the Bank/Stem Cell Bank, Shanghai Institute for Biological Sciences, Chinese Academy of Sciences. Cell transfection was carried out as previously described (4). Lentivirus with RKTG short hairpin RNA (shRNA) was generated as previously described (4, 6). Lentivirus with p53 shRNA was from GenePharma. An annealed short interfering RNA (siRNA) cassette with a targeting sequence of GAAACCACTGGATGGAGAATA for p53 selected from 4 different target sequences was inserted into the pGPe6/GFP/Neo vector. The p53-related protein kinase (PRPK) siRNA was from GenePharma with a sequence of GCTGAAACATGGTGCCTCA for p53 selected from 3 different target sequences. The methods for cell fixation, immunostaining, and confocal analyses were described previously (1). The details of assays for cell senescence, apoptosis, proliferation, and dissociation are provided in Supplementary Information.

Antibodies, immunoblotting, and immunoprecipitation

The details of antibodies are provided in Supplementary Information. The protocols for immunoblotting and immunoprecipitation have been described previously (4).

Histologic analysis, immunohistochemistry, and isolation of primary keratinocytes

Hematoxylin and eosin (H&E) staining and immunohistochemistry (IHC) were carried out as previously described (5). Skin samples were placed with epidermal side down, and the loose connective tissue was scraped away. The skin was then cut into smaller pieces of 3 to 5 mm in width and placed in dispase solution overnight at 4°C. The epidermis was then peeled off and used in the experiments (25). Mouse keratinocytes were isolated as previously described (5).

RNA isolation and real-time reverse transcriptase PCR

The cells were lysed in TRIzol reagent (Invitrogen). Total RNA was purified and reverse transcribed according to the manufacturer’s instruction (TaKaRa). Real-time quantitative RT-PCR was then conducted with ABI Prism 7500 sequence detection system following the manufacturer’s recommendations (Applied Biosystems). The sequence of primers is provided in the Supplementary Information.

Statistics

Statistical analysis was conducted using the Student’s t test.

Results

Loss of RKTG induces accumulation of p53 and p21 via PI3K/AKT signaling pathway in MEFs

RKTG has been shown to inhibit GPCR Gβγ signaling such as Gβγ-mediated activation of AKT and GRK by sequestering Gβγ subunit to the Golgi apparatus (4). Consistent with our previous report (4), LPA-stimulated AKT phosphorylation at Ser473 was profoundly increased in RKTG−/− MEFs (Fig. 1A, lanes 7 and 8). We also analyzed the phosphorylation level of GSK3β, a substrate of AKT, and found that RKTG deletion could also enhance LPA-stimulated GSK3β phosphorylation at Ser9 (Fig. 1A). In addition, LPA treatment led to marked stimulation of p53 phosphorylation at Ser15 in RKTG-deleted MEFs (Fig. 1A), consistent with a previous report showing that AKT is able to activate PRPK, which in turn induces p53 phosphorylation at Ser15 (26). Phosphorylation of p53 at Ser15 is associated with an increased stabilization and transactivation activity (27). Accordingly, the level of p53 as well as its transcriptional targets p21 and p16 were significantly increased on LPA treatment in RKTG-null MEFs (Fig. 1A).

On the other hand, RKTG has been shown to inhibit Ras/Raf/MEK/ERK signaling through interacting with Raf-1 (1). However, EGF-stimulated AKT and GSK3β phosphorylation was not altered by RKTG deletion (Fig. 1B). Deletion of RKTG could neither induce p53 phosphorylation at Ser15, nor could it increase the protein levels of p53, p16, and p21 in the...
presence of EGF (Fig. 1B). We also found that sphingosine-1-phosphate, another ligand that can act on GPCR and stimulate AKT signaling (28), had an effect that was similar to that of LPA on AKT phosphorylation and p53/p21 accumulation (Supplementary Fig. S1). Furthermore, we found that LPA-stimulated AKT activation was enhanced in RKTG-null primary keratinocytes, associated with an increase in p53 and p21 accumulation (Supplementary Fig. S2). In addition, inhibition of the PI3K/AKT pathway by wortmannin, but not that of the Ras/Raf/MEK/ERK pathway by PD98059, could completely abrogate LPA-stimulated induction of p53, p21, and p16 in RKTG-null MEFs (Fig. 1C). Collectively, these data indicate that GPCR ligand–induced AKT activation in RKTG-null MEFs, due to the abrogation of RKTG-mediated negative regulation on Gbg signaling downstream of GPCR (4), is associated with activation of p53 pathway.

We examined whether the stability of p53 was altered by RKTG deletion. The half-lives of p53 and p21 proteins were significantly prolonged in RKTG-deficient MEFs (Fig. 1D, lanes 10 to 12). It was previously reported that AKT activation is able to promote the degradation of p53 by increasing nuclear localization of MDM2 (29). However, we found that nucleic MDM2 was not altered by LPA treatment or RKTG deletion (Supplementary Fig. S3), indicating that the observed p53 accumulation in LPA-treated RKTG-null MEFs results from an increase in p53 phosphorylation but not as a result of nuclear localization of MDM2. The activation of p53 pathway was associated with an increased senescence phenotype shown as a significantly increased percentage of β-galactosidase (β-gal)-positive cells (Fig. 1E). However, deletion of RKTG had no further effect on alteration of LPA-mediated changes of cell proliferation and apoptosis (Supplementary Fig. S4).

Concomitant deletion of RKTG and p53 in mice leads to development of spontaneous skin cancer–like tumors and epidermal EMT in early age

Our results from studies with MEFs imply that there may exist a functional interplay between RKTG and p53. To investigate the potential functional interaction between p53 and RKTG in vivo, we intercrossed RKTG heterozygous mice and p53 heterozygous mice to generate RKTG and p53 double knockout mice. We focused our studies on the mice with heterozygous p53 deletion (p53+/−) but with 3 different genotypes of RKTG deletion (RKTG+/+, RKTG+/−, and RKTG−/−). Although p53−/− mice had no detectable tumor at 10 months of age, deletion of either 1 or 2 copies of RKTG
gene led to the development of spontaneous skin cancer–like tumors (Fig. 2A and B). About 20% of p53<sup>+/−</sup> RKTG<sup>+/−</sup> mice and 25% of p53<sup>+/−</sup> RKTG<sup>−/−</sup> mice developed skin cancer–like tumors within 10 months of age. Furthermore, about 5% of p53<sup>+/−</sup> RKTG<sup>+/−</sup> mice and 25% of p53<sup>+/−</sup> RKTG<sup>−/−</sup> mice developed such tumors within 7 months of age (Fig. 2A). Collectively, these results indicate that RKTG deletion could markedly accelerate formation of spontaneous skin cancer–like tumors in p53<sup>+/−</sup> background.

We next analyzed the histology of the tumors formed in the p53 and RKTG double mutant mice. The normal mouse skin is composed of epidermis with a single layer of cuboid epithelial cells and dermis mainly filled with connective tissue on top of subcutaneous adipose tissue (Fig. 2C, left). In the skin

Figure 2. Concomitant deletion of RKTG and p53 induces spontaneous skin cancer–like tumors and epidermal EMT in early age of mouse. A and B, simultaneous deletion of RKTG and p53 leads to development of skin cancer–like tumors in early age of mouse. Twenty mice in each group were monitored and the incidence of spontaneous skin cancer–like tumors in each group within 7 and 10 months of age is shown in (A). Representative images of the mice carrying the tumors at different ages are shown in (B) with arrows indicating the tumors. C, H&E staining of normal skin, skin on top and inside the tumor. The arrows indicate spindle-like cells in the interior of epidermis. D, Ki67 staining of the samples. The arrows mark spindle-like cells in the interior of epidermis with strong Ki67 staining in the interior of epidermis. E, emergence of EMT features in the skin overlying the tumor by immunohistochemical analysis of E-cadherin and vimentin. The arrows indicate the cells within the interior of epidermis with negative staining for E-cadherin and positive staining for vimentin.
overlying the tumor in p53 and RKTG double mutant mice, the thickness of the epidermis was profoundly augmented and the epithelial cells lost the cuboid shape (Fig. 2C, middle). In the interior of the epidermis, the spindle-like epithelial cells infiltrated into the underlying tissue with an apparent loss of basement membrane (Fig. 2C, arrows). The tumor mass contained spindle-like cells with plate-like hyperplasia structure and heteromorphic nuclei (Fig. 2C, right). The hyperproliferative feature of the tumors was reflected by strong Ki67 staining within cells in both the epidermis overlying the tumor and the tumor interior (Fig. 2D). Interestingly, the spindle-like epithelial cells within the border area between epidermis and dermis were extremely positive in Ki67 staining (Fig. 2D, arrows). The tumors were mostly negative for CD117 and αSMA (Supplementary Fig. S5), markers for gastrointestinal stromal tumors and smooth muscle cells. However, some regions of epidermis overlying the tumor and tumor interior were positive for cytokeratin 14 and keratin 10 (Supplementary Fig. S5), markers of skin epithelial cells.

Interestingly, the skin overlying the tumor had signs of EMT. The cells in the normal epidermis of p53+/− RKTG+/+ mice were highly positive for E-cadherin (Fig. 2D, left top), a classical epithelial marker. However, a portion of epidermis cells overlying the tumor of p53+/− RKTG+/− mice, especially the highly proliferative epidermis cells in the border area between epidermis and dermis, had a loss of E-cadherin expression (Fig. 2D, middle top, arrows). Meanwhile, the tumor interior was mostly negative for E-cadherin (Fig. 2D, right top). Accordingly, some of the skin cells attained expression of vimentin, a marker for mesenchymal cells (Fig. 2D). More intriguingly, the spindle-like epithelial cells in the border area between epidermis and dermis were highly positive in vimentin staining (Fig. 2D, middle bottom, arrows). The tumor interior was also partially positive in vimentin expression (Fig. 2D, right bottom). In summary, these results suggest that the epidermis overlying the tumor was highly proliferative and had signs of EMT, especially in the interior region of the epidermis. On the other hand, the tumor interior had...
mixed properties in terms of proliferative index, expression of keratinocyte markers, and expression of the mesenchymal marker vimentin.

The skin overlying the tumor in p53<sup>−/−</sup> RKTG<sup>−/−</sup> mice had a loss of p53 and a gain of EMT

We next carefully analyzed the skin overlying the tumor in p53<sup>−/−</sup> RKTG<sup>−/−</sup> mice. In normal skin, loss of RKTG was associated with increases in phosphorylation of AKT, GSK3β, ERK, and p53, together with elevated expression of p53 and p21 and a slight increase of 2 mesenchymal markers Snail and vimentin (Fig. 3A, lanes 5 and 6, compared with lanes 3 and 4). These results were, in part, consistent with the finding in MEFs (Fig. 1). On the other hand, heterozygous p53 deletion had no apparent effect on the phosphorylation or expression of these proteins (Fig. 3A). Interestingly, the skin overlying the tumor had a loss of p53 expression, a loss of p21 expression, a markedly decreased expression of epithelial marker E-cadherin, and a robustly increased expression of Snail and vimentin (Fig. 3A). Interestingly, β-catenin was also evidently increased in the skin overlying the tumor (Fig. 3A). Taken together, these results indicate that two molecular events had occurred in the skin overlying the tumor, that is, LOH of p53 and emergence of EMT. LOH of p53 and EMT of the skin were further confirmed by real-time RT-PCR analysis. The mRNA levels of E-cadherin, p53, and p21 were significantly reduced in the skin overlying the tumor, accompanied by significant increase of vimentin and Snail mRNA levels (Fig. 3B). In addition, by immunohistochemical analysis, p53 protein was lost in many regions of the epidermis adjacent to the tumor as well as in the tumor interior (Supplementary Fig. S6).

A cooperative role between RKTG and p53 in regulating EMT in A431 epithelial cells

Our results using the mouse models suggest that loss of RKTG could cooperate with loss of p53 to induce EMT in mouse skin adjacent to the tumors. We further analyzed the functional interaction between RKTG and p53 in human A431 squamous carcinoma cells in which p53 is mutated and inactivated (30). Consistent with our previous results with MEFs (Fig. 1A), LPA could stimulate AKT phosphorylation and such stimulation was prolonged when RKTG was silenced (Fig. 4A). Wortmannin was able to completely abrogate LPA-mediated AKT phosphorylation (Fig. 4A), indicating that PI3K/AKT signaling pathway is required for the action of LPA. Consistently, RKTG knockdown could enhance LPA-stimulated GSK3β phosphorylation (Fig. 4B). LPA treatment alone induced EMT to some extent as shown by slight reduction of E-cadherin and induction of vimentin on LPA treatment (Fig. 4B). Intriguingly, knockdown of RKTG profoundly induced EMT in the presence of LPA, shown as robust reduction of E-cadherin and marked elevation of vimentin and β-catenin (Fig. 4B). Interestingly, these RKTG-knockdown–induced and LPA-induced EMT features were abrogated by overexpression of wild-type p53 (Fig. 4B). Furthermore, the biochemical changes of EMT induced by LPA treatment and loss of RKTG was completely abrogated by wortmannin (Fig. 4C). The EMT-like changes were also blocked by the overexpression of GRK-ct (Fig. 4C, lane 6), which is the C-terminal fragment of GRK2 that can bind with GPCR Gβγ subunit and abrogates Gβγ-activated AKT phosphorylation (31). In addition, RKTG shRNA could enhance cell dissociation accompanied with acquisition of fibroblast-like morphology after LPA treatment (Supplementary Fig. S7). These data, therefore, indicate that loss of RKTG can cooperate with loss of function of p53 to induce EMT in A431 cells.

Concomitant knockdown of RKTG and p53 promotes EMT in HepG2 epithelial cells

We next analyzed the cooperative effect of RKTG and p53 on EMT using another epithelial cell model, human hepatoma–derived HepG2 cells that harbor wild-type p53 (32). As

Figure 4. Knockdown of RKTG cooperates with p53 inactivation to induce EMT in A431 cells. A, LPA-induced AKT phosphorylation is enhanced by RKTG shRNA. A431 cells were transiently transfected with FG12 lentiviral vector (control) and FG12 containing RKTG shRNA, followed by LPA treatment (10 μmol/L) for different lengths of time. The cells were pretreated with wortmannin (100 nmol/L) for 1 hour as indicated. The cell lysate was used in immunoblotting. B, RKTG-knockdown-induced EMT is abrogated by overexpression of wild-type p53. A431 cells transfected with RKTG shRNA and wild-type p53 were treated with LPA (10 μmol/L) for 4 hours as indicated. After 24 hours of culture, the cell lysate was used in immunoblotting. C, RKTG knockdown–induced EMT is blocked by wortmannin and GRK-ct. A431 cells were transfected with RKTG shRNA and GRK-ct as indicated, treated with LPA and wortmannin as in (A), and then used in immunoblotting.
expected, phosphorylation of AKT and GSK3β was enhanced by RKTG knockdown on LPA treatment (Fig. 5A, lane 7). The phosphorylation levels of AKT and GSK3β with simultaneous knockdown of RKTG and p53 had no further changes (Fig. 5A, lane 8), indicating that loss of p53 had no additional effect on AKT activation. However, simultaneous knockdown of RKTG and p53 could induce maximal reduction of E-cadherin and maximal elevation of vimentin (Fig. 5A). However, EGF treatment in combination with RKTG knockdown and/or p53 knockdown had no effect on the phosphorylation of GSK3β or on the expression levels of E-cadherin and vimentin (Fig. 5B). At the mRNA level, simultaneous knockdown of RKTG and p53 could also induce maximal reduction of E-cadherin and maximal stimulation of vimentin and Snail expressions (Fig. 5C). It is noteworthy that p21 mRNA level was elevated after RKTG knockdown and LPA treatment (Fig. 5C), consistent with the results with MEFs (Fig. 1A). We also used immunofluorescence staining to confirm the

Figure 5. Concomitant knockdown of RKTG and p53 promotes EMT in HepG2 epithelial cells. A, effect of RKTG and/or p53 knockdown on AKT phosphorylation and EMT at the protein level. HepG2 cells transfected with RKTG shRNA and p53 shRNA as indicated were treated with or without LPA (10 μmol/L) for 6 hours. After 24 hours of culture, the cell lysate was used in immunoblotting. B, EGF cannot induce EMT after knockdown of RKTG and p53. A similar experiment as in (A) was carried out except for using EGF treatment (100 ng/mL). C, effect of RKTG and p53 knockdown on gene expression at the mRNA level. HepG2 cells as in (A) were used in RNA isolation and real-time RT-PCR. The data are shown as mean ± SD. * and **, P < 0.05 and P < 0.01, respectively, between the experimental group and the control group without transfection or LPA treatment. ^^^, P < 0.01 between the last 2 groups. D, simultaneous knockdown of RKTG and p53 reduces E-cadherin/β-catenin complex formation. HepG2 cells were infected with the lentivirus as indicated, followed by LPA treatment (10 μmol/L) for 6 hours. Total cell lysate was subjected to immunoblotting (IB) and immunoprecipitation (IP) using antibodies as indicated. The nuclear extract was also used in IB. Rabbit IgG was used as a negative control for IP. The cells were pretreated with wortmannin (100 nmol/L) or PD98059 (50 μmol/L) for 1 hour before LPA stimulation as indicated.
effect of RKTG and p53 knockdown on the expression of E-cadherin and vimentin (Supplementary Fig. S8).

Similar to the findings with LPA treatment, simultaneous knockdown of RKTG and p53 could lead to maximal repression of E-cadherin and maximal elevation of vimentin on sphingosine-1-phosphate administration (Supplementary Fig. S9). In addition, IGF-1 (insulin-like growth factor), another ligand that can activate AKT (33), could lead to induction of EMT features after p53 was silenced (Supplementary Fig. S10), providing additional evidence that PI3K/AKT signaling is involved in EMT that is enhanced by loss of p53.

We next investigated the association between E-cadherin and β-catenin in HepG2 cells as both proteins can form a complex that is implicated in EMT (3, 34). By a coimmuno-precipitation assay, we found that simultaneous knockdown of RTKG and p53 led to reduction of the E-cadherin/β-catenin complex (Fig. 5D, lane 6). Consistently, concomitant knockdown of RTKG and p53 could reduce E-cadherin expression and elevate β-catenin expression in the cells, together with nuclear accumulation of β-catenin (Fig. 5D). The decrease of E-cadherin/β-catenin complex formation after RTKG and p53 knockdown was abrogated by wortmannin, but not by PD98059 (Fig. 5D). Furthermore, AKT inhibitor VIII completely blocked the effects of p53 and RTKG silencing on the formation of E-cadherin/β-catenin complex (Supplementary Fig. S11), providing additional evidence that the PI3K/AKT pathway is implicated in this process.

Discussion

In this study, we found that there exists a functional interplay between RTKG and p53 in both physiologic and pathophysiologic scenarios (Fig. 6). Under physiologic condition, loss of RTKG enhances GPCR-mediated AKT activation via releasing the inhibitory function of RTKG on Gβγ signaling (Fig. 6A). This is reflected by the finding that in MEFs, mouse skin, and epithelial cells, loss or knockdown of RTKG was associated with enhanced AKT phosphorylation as well as the phosphorylation of AKT substrate GSK3β (Figs. 1 and 3–5). Activation of AKT, likely via its regulation on PRPK (26), induces p53 phosphorylation at Ser15, which in turn increases p53 stabilization and transactivation activity (27). This notion is supported by our finding that PRPK knockdown was able to completely abrogate p53 accumulation induced by LPA treatment and RTKG silencing (Supplementary Fig. S12). The activation of the p53 pathway would elicit an anti-growth signal to the cells executed by cell-cycle arrest and induction of cell senescence. On the other hand, loss of RTKG would impose a pro-growth signal to the cell due to enhancement of AKT and ERK signaling pathways. We hypothesize that such a pro-growth signal is counteracted by the anti-growth signal via AKT-mediated p53 activation, leading to no apparent change in cell proliferation and overall phenotype in RTKG-null mice.

Under pathophysiologic conditions as encountered in RTKG and p53 double knockout mice, however, the intricate balance between the pro-growth signal and anti-growth signal is disrupted due to LOH of p53 (Fig. 6B). Oncogenic stress, such as AKT and ERK activation resulting from RTKG deletion, may impose a selective force to trigger loss of the wild-type copy of p53. The p53 heterozygous deletion mice developed tumors by 12 months of age with sarcoma being the most common tumor type (10–12). However, deletion of RTKG in p53 heterozygous deletion background leads to formation of skin cancer–like tumors in early-age mice (Fig. 2). As deletion of one copy of RTKG is sufficient to increase tumor incidence in p53+/− background, our results indicate that RTKG is a haploinsufficient tumor suppressor gene that can cooperate with loss of p53 to promote tumorigenesis.

One of the important findings in our study is that both RTKG- and p53-mediated pathways play functional roles in EMT. Although the diverse functions of p53 in a majority of biological processes have been extensively characterized, its function in EMT has only recently been recognized (22–24, 35). At the animal level, LOH of p53 was accompanied by induction of EMT in hyperplastic mouse skin with concomitant loss of RTKG (Figs. 2 and 3). At the cellular levels, concomitant downregulation of both p53 and RTKG heralded maximal induction of EMT features in epithelial cells (Figs. 4 and 5). These results, therefore, would further strengthen a previously underappreciated function of p53 in EMT. We propose that...
p53 has an EMT checkpoint function that prevents the tumor cells from attaining EMT (Fig. 6B). Loss of p53, as commonly seen in many malignant tumors, would deprive the cells of EMT checkpoint, allowing other signals such as AKT activation to induce EMT.

We speculate that β-catenin may play a critical role in executing EMT under the condition of concomitant activation of AKT (as induced by loss of RKTG in this study) and loss of p53. In epithelial cells, maximal accumulation of β-catenin only occurred when both RKTG and p53 were simultaneously downregulated, accompanied by maximal induction of EMT features (Figs. 4 and 5). The β-catenin itself is a structural component of cell–cell adhesion and plays a central role in the Wnt signaling pathway via regulating LEF/TCF (lymphoid enhancer factor/T-cell factor) transcription factors. In many cancers, β-catenin is highly accumulated due to dysregulation of components involved in β-catenin degradation. It is known that β-catenin plays a critical role in EMT as the released β-catenin is able to be translocated into the nucleus in which it activates transcription of a series of target genes that promote tumor cell invasion (36). How could only simultaneous loss/knockdown of RKTG and p53 lead to maximal accumulation of β-catenin in epithelial cells? This phenomenon can be explained by the finding that wild-type p53 is involved in the degradation of β-catenin (37–39). In addition, p53 could increase the rate of β-catenin phosphorylation by GSK3β (39). Loss/knockdown of RKTG may render the cells with hyperactivation of PI3K/AKT signaling and GSK3β phosphorylation. GSK3β phosphorylation leads to its inactivation that subsequently results in decreased degradation of β-catenin. We propose that inactivation of GSK3β may synergize with LOH of p53 to result in maximal shutoff of β-catenin degradation, leading to maximal induction of EMT.

At present, we are not sure about the cell origin of the spontaneous tumors formed in the RKTG and p53 double deficient mice. Interestingly, we found that the epidermal cells in the depths of skin overlying the tumor has transformed into spindle-like (Fig. 2C, arrows). Meanwhile, these spindle-like cells are highly proliferative (Fig. 2D, arrows), negative in E-cadherin and positive in vimentin expression (Fig. 2E, arrows).

We speculate that these spindle-like skin cells have undertaken EMT to become highly invasive and proliferative. In theory, these cells could migrate into the dermis and form sarcoma-like tumors. Under such consideration, the spontaneous tumors observed in the RKTG and p53 double deletion mice could be originated from the skin epithelial cells as a result of EMT, due to the loss of EMT checkpoint after LOH of p53. Although this hypothesis still needs careful elucidation in the future, it uncovers a potential new mechanism of tumor formation, that is, EMT itself could drive formation of tumors of mesenchymal features from epithelial cells. In other words, EMT is not only involved in invasiveness and metastasis in late stages of tumorigenesis (15) but is also directly involved in the formation of tumors in the early stage, consistent with a speculation that metastasis is an early event in breast cancer (40).

In summary, our study indicates for the first time that there exists a functional interplay between RKTG and p53 in EMT and tumorigenesis. Through a series of experiments at the cellular and animal levels, we propose that p53 has an EMT checkpoint function and the loss of such function can combine with oncogenic signals such as PI3K/AKT activation caused by loss of RKTG to drive EMT and tumor formation. This hypothesis, although still awaiting substantiation using many other systems, would aid in understanding the complex features of tumorigenesis and broadening our insight in the combat against cancers.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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