Functional cooperation of RKTG with p53 in tumorigenesis and epithelial-mesenchymal transition

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Abstract

RKTG is a potential tumor suppressor gene due to its negative roles in regulating Ras/Raf/MEK/ERK pathway and GPCR Gβγ-subunit signaling. Interestingly, RKTG-deficient mice are free of tumors, although they are prone to form skin cancer upon 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 could stimulate hyperphosphorylation of AKT and GSK3β, 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 ~25% of RKTG-nullizygous and p53-heterozygous mice within 7 months of age. Hyperplasia and epithelial-mesenchymal transition (EMT) were observed in the tumor-overlaying epidermis, in which loss of heterozygosity 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 GSK3β phosphorylation, together with increased accumulation of β-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 of p53 in tumorigenesis, but also suggest that p53 has a EMT checkpoint function and the loss of this function can combine with loss of RKTG to drive EMT and tumor progression.
Introduction

Raf kinase trapping to Golgi (RKTG) or PAQR3 of the Progesterone and AdipoQ Receptor (PAQR) family has been demonstrated as a Golgi-localized membrane protein and participates in the spatial regulation of Ras/Raf/MEK/ERK and G protein-coupled receptor (GPCR) signaling pathways by sequestrating Raf kinase and Gβγ subunit into the Golgi apparatus respectively (1-4). For example, EGF-stimulated EKR phosphorylation is negatively regulated by RKTG (1), and LPA-stimulated and Gβγ 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 Gβγ signaling pathways, it is postulated that RKTG possesses a tumor suppressor function. Such 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 4-5 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 (RB) and p53 led to spontaneous squamous cell carcinoma (13).

Epithelial-mesenchymal transition (EMT) is defined as a process that 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 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 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 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 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. Information of plasmid construction and materials is provided in Supplemental Information.

Cell culture, cell transfection, lentivirus with RKTG and p53 shRNA, confocal microscopy, and cell-based assays

MEF cells, A431 and HepG2 cells were grown in DMEM 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 (Shanghai, China). Cell transfection was performed as previously described (4). Lentivirus with RKTG shRNA was generated as previously described (4, 6) Lentivirus with p53 shRNA was from GenePharma (Shanghai, China). An annealed small interfering RNA (siRNA) cassette with a targeting sequence of GAAACCACTGGATGGAATA for p53 selected from four different target sequences was inserted into the PGPU6/GFP/Neo vector. PRPK siRNA was from GenePharma with a sequence of GCTGAACATTGTGCTCATA selected from three different target sequences.
The methods for cell fixation, immunostaining and confocal analyses were described previously (1). The detailed information of assays for cell senescence, apoptosis, proliferation, dissociation is provided in Supplemental Information.

Antibodies, immunoblotting and immunoprecipitation

The information of antibodies is provided in Supplemental Information. The protocols for immunoblotting and immunoprecipitation have been described previously (4).

Histological analysis, immunohistochemistry and isolation of and primary keratinocytes

Hematoxylin and eosin (HE) staining and immunohistochemistry (IHC) were performed 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 with 3–5 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 polymerase chain reaction (RT-PCR)

The cells were lysed in TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Total RNA was purified and reverse-transcribed according to the manufacturer’s instruction (TaKaRa, Shanghai, China). Real-time quantitative PCR was conducted with ABI Prism 7500 sequence detection system following the manufacturer's recommendations (Applied Biosystems, Foster City, CA, USA). The sequence of primers is provided in the Supplemental Information.
Statistics

Statistical analysis was performed 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 (Figure 1A, lanes 7 and 8). We also analyzed the phosphorylation level of GSK3β, a substrate of AKT, and found the RKTG deletion could also enhance LPA-stimulated GSK3β phosphorylation at Ser9 (Figure 1A). In addition, LPA treatment led to marked stimulation of p53 phosphorylation at Ser15 in RKTG-deleted MEFs (Figure 1A), consistent with previous report demonstrating that AKT is able to activate p53-related protein kinase (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 upon LPA treatment in RKTG-null MEFs (Figure 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 (Figure 1B). Deletion of RKTG could not induce p53 phosphorylation at Ser15, nor increase the protein levels of p53, p16 and p21 in the presence of EGF (Figure 1B). We also found that sphingosine-1-phosphate, another ligand that can act on GPCR and stimulate AKT signaling (28), had similar effect as LPA on AKT phosphorylation and p53/p21
accumulation (SFigure 1). Furthermore, we found that LPA-stimulated AKT activation was enhanced in RKTG-null primary keratinocytes, associated with an increase in p53 and p21 accumulation (SFigure 2). In addition, inhibition of PI3K/AKT pathway by wortmannin, but not inhibition of Ras/Raf/MEK/ERK pathway by PD98059, could completely abrogate LPA-stimulated induction of p53, p21 and p16 under in RKTG-null MEFs (Figure 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 Gβγ 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 (Figure 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 (SFigure 3), indicating that the observed p53 accumulation in LPA-treated RKTG-null MEFs is resulted from an increase in p53 phosphorylation but not due to nuclear localization of MDM2. The activation of p53 pathway was associated with an increased senescence phenotype demonstrated as a significantly increased percentage of β-galactosidase-positive cells (Figure 1E). However, deletion of RKTG had no further effect to alter LPA-mediated changes of cell proliferation and apoptosis (SFigure 4).

Concomitant deletion of RKTG and p53 in mice leads to development of spontaneous skin cancer-like tumors and epidermal EMT in early age
Our result from studies with MEFs implied that there exists 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 three different genotypes of RKTG deletion (RKTG+/+, RKTG+/- and RKTG-/-). While p53+/- RKTG+/+ mice had no detectable tumor at 10 months of age, deletion of either one or two copies of RKTG gene led to development of spontaneous skin cancer-like tumors (Figure 2A and 2B). About 20% of p53+/- RKTG+/- mice and 25% of p53+/- RKTG-/- mice developed skin cancer-like tumors within 10 months age. Furthermore, about 5% of p53+/- RKTG+/- mice and 25% of p53+/- RKTG-/- mice developed such tumors within 7 months of age (Figure 2A). Collectively, these results indicate that RKTG deletion could markedly accelerate formation of spontaneous skin cancer-like tumors in p53+/- background.

We next analyzed the histology of the tumors formed in the p53 and RKTG double mutant mice. The normal mice skin is composed of epidermis with a single layer of cuboid epithelial cells and dermis mainly filled with collective tissue on top of subcutaneous adipose tissue (Figure 2C, left panels). In the skin overlaying 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 (Figure 2C, middle panels). In the interior of the epidermis, the spindle-like epithelial cells infiltrated into the underlying tissue with an apparent loss of basement membrane (Figure 2C, arrows). The tumor mass contained spindle-like cells with platelike hyperplasia structure and heteromorphic nuclei (Figure 2C, right panels). The hyperproliferative feature of the tumors were
reflected by strong Ki67 staining within cells in both the epidermis overlaying the tumor and the tumor interior (Figure 2D). Interestingly, the spindle-like epithelial cells within the border area between epidermis and dermis were extremely positive in Ki67 staining (Figure 2D, arrows). The tumors were mostly negative for CD117 and αSMA (SFigure 5), markers for gastrointestinal stromal tumors and smooth muscle cells. However, some regions of epidermis overlaying the tumor and tumor interior were positive for cytokeratin 14 and keratin 10 (SFigure 5), markers of skin epithelial cells.

Interestingly, the skin overlaying the tumor had signs of EMT. The cells in the normal epidermis of p53+/- RKTG+/+ mice were highly positive for E-cadherin (Figure 2D, left upper panel), a classical epithelial marker. However, a portion of epidermis cells overlaying the tumor of p53+/- RKTG-/- mouse, especially the highly proliferative epidermis cells in the border area between epidermis and dermis, had a loss of E-cadherin expression (Figure 2D, middle upper panel, arrows). Meanwhile, the tumor interior was mostly negative for E-cadherin (Figure 2D, right upper panel). Accordingly, some of the skin cells attained expression of vimentin, a marker for mesenchymal cells (Figure 2D). More intriguingly, the spindle-like epithelial cells in the border area between epidermis and dermis were highly positive in vimentin staining (Figure 2D, middle lower panel, arrows). The tumor interior was also partially positive in vimentin expression (Figure 2D, right lower panel). In summary, these results suggest that the epidermis overlaying the tumor were 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 mesenchymal marker vimentin.
The skin overlaying the tumor in p53+-/- RKTG-/- mouse had a loss of p53 and a gain of EMT

We next carefully analyzed the skin overlaying the tumor in p53+-/- RKTG-/- mouse. 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 two mesenchymal markers Snail and vimentin (Figure 3A, lanes 5 and 6, compared with lanes 3 and 4). These results were in part consistent with the finding in MEFs (Figure 1). On the other hand, heterozygous p53 deletion had no apparent effect on the phosphorylation or expression of these proteins (Figure 3A). Interestingly, the skin overlaying 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 (Figure 3A). Interestingly, β-catenin was also evidently increased in the skin overlaying the tumor (Figure 3A). Taken together, these results indicate that two molecular events had occurred in the skin overlaying the tumor, i.e., loss of heterozygosity (LOH) of p53 and emergence of EMT. LOH of p53 and EMT of the skin were further confirmed by RT-PCR analysis. The mRNA levels of E-cadherin, p53 and p21 were significantly reduced in the skin overlaying the tumor, accompanied by significant increase of vimentin and Snail mRNA levels (Figure 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 (SFigure 6).
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 (Figure 1A), LPA could stimulate AKT phosphorylation and such stimulation was prolonged when RKTG was silenced (Figure 4A). Wortmannin was able to completely abrogate LPA-mediated AKT phosphorylation (Figure 4A), indicating that PI3K/AKT signaling pathway is required for the action of LPA. Consistently, RKTG knockdown could enhance LPA-stimulated GSK3β phosphorylation (Figure 4B). LPA treatment alone induced EMT to some extent as demonstrated by slight reduction of E-cadherin and induction of vimentin upon LPA treatment (Figure 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 (Figure 4B). Interestingly, these RKTG knockdown- and LPA-induced EMT features were abrogated by overexpression of wild-type p53 (Figure 4B). Furthermore, the biochemical changes of EMT induced by LPA treatment and loss of RKTG was completely abrogated by wortmannin (Figure 4C). The EMT-like changes were also blocked by the overexpression of GRK-ct (Figure 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 (SFigure 7). 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 expected, phosphorylation of AKT and GSK3β was enhanced by RKTG knockdown upon LPA treatment (Figure 5A, lane 7). The phosphorylation levels of AKT and GSK3β with simultaneous knockdown of RKTG and p53 had no further changes (Figure 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 (Figure 5A). However, EGF treatment in combination with RKTG knockdown and/or p53 knockdown had no effect on the phosphorylation of GSK3β nor the expression levels of E-cadherin and vimentin (Figure 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 (Figure 5C). It is noteworthy that p21 mRNA level was elevated after RKTG knockdown and LPA treatment (Figure 5C), consistent with the results with MEFs (Figure 1A). We also used immunofluorescence staining to confirm the effect of RKTG and p53 knockdown on the expression of E-cadherin and vimentin (SFigure 8).

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 upon sphingosine-1-phosphate administration (SFigure 9). In addition, IGF-1, another ligand that can
activate AKT (33), could lead to induction of EMT features after p53 was silenced ((SFigure 10), 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 co-immunoprecipitation assay, we found that only simultaneous knockdown of RTKG and p53 led to reduction of the E-cadherin/β-catenin complex (Figure 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 (Figure 5D). The decrease of E-cadherin/β-catenin complex formation after RTKG and p53 knockdown was abrogated by wortmannin, but not by PD98059 (Figure 5D). Furthermore, AKT inhibitor VIII completely blocked the effects of p53 and RTKG silencing on the formation of E-cadherin/β-catenin complex (SFigure 11), providing additional evidence that PI3K/AKT pathway is implicated in this process.
**Discussion**

In this study, we found that there exists a functional interplay between RKTG and p53 in both physiological and pathophysiological scenarios (Figure 6). Under physiological condition, loss of RKTG enhances GPCR-mediated AKT activation via releasing the inhibitory function of RKTG on G\(\beta\gamma\) signaling (Figure 6A). This is reflected by the finding that in MEFs, mouse skin and epithelial cells, loss or knockdown of RKTG was associated with enhanced AKT phosphorylation as well as the phosphorylation of AKT substrate GSK3\(\beta\) (Figures 1, 3, 4 and 5). Activation of AKT, likely via its regulation on p53-related protein kinase (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 RKTG silencing (SFigure 12). The activation of 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 RKTG 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 RKTG-null mouse.

Under pathophysiological condition as encountered in RKTG 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 (Figure 6B). Oncogenic stress, such as AKT and ERK activation resulted from RKTG 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 RKTG in p53 heterozygous deletion background leads to formation of skin cancer-like tumors in early age of mice (Figure 2). As deletion of one copy of RKTG is sufficient to increase tumor incidence in p53+/− background, our results indicate that RKTG 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 RKTG- 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 was only recently being recognized (22-24, 35). At the animal level, LOH of p53 was accompanied by induction of EMT in hyperplasic mouse skin with concomitant loss of RKTG (Figures 2 and 3). At the cellular levels, concomitant downregulation of both p53 and RKTG heralded maximal induction of EMT features in epithelial cells (Figures 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 (Figure 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.
(Figures 4 and 5). The β-catenin itself is a structural component of cell-cell adhesion and plays a central role in Wnt signaling pathway via regulating Lef/Tcf 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 shut-off 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 overlaying the tumor has transformed into spindle-like cells (Figure 2C, arrows). Meanwhile, these spindle-like cells are highly proliferative (Figure 2D, arrows), negative in E-cadherin and positive in vimentin expression (Figure 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, i.e., EMT itself could drive formation of tumors of mesenchymal features from epithelial cells. In other word, EMT is not only involved in invasiveness and metastasis in late stage of tumorigenesis (15), but 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.
Footnotes

The Supplemental Information contains supplemental Materials and Methods and 12 Supplemental Figures.

Acknowledgements

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Reference


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Figure legend

Figure 1. RKTG-deficiency triggers activation of p53 pathway and senescence in primary mouse embryonic fibroblasts (MEFs)

(A, B) LPA but not EGF induces hyperactivation of AKT, phosphorylation of p53, and accumulation of p53/p21/p16 in RKTG-deleted MEFs. MEFs with or without deletion of RKTG were treated with LPA (10 μM) or EGF (100 ng/ml) for 6 h and the cell lysate was used in immunoblotting with the antibodies as indicated.

(C) LPA-induced p53/p21 accumulation is abrogated by wortmannin. MEFs were treated with wortmannin (100 nM) or PD98059 (50 μM) for 1 h, and then treated with or without LPA (10 μM) for 6 h before immunoblotting.

(D) The half-lives of p53 and p21 are increased by LPA in RKTG-null MEFs. MEFs were treated with cycloheximide (CHX, at 30 μg/ml) for the time as indicated, with or without LPA treatment (10 μM) for 6 h. The cell lysate was used in immunoblotting.

(E) LPA induces senescence in RKTG-deleted MEFs. MEFs were treated with or without LPA (10 μM) for 6 h, and then cultured for 4 d before cell senescence assay. The percentage of β-gal positive cells is shown as mean ± SD with ** indicating p < 0.01 in comparison with the RKTG(+/+) control group.

Figure 2. Concomitant deletion of RKTG and p53 induces spontaneous skin cancer-like tumors and epidermal EMT in early age of mouse

(A, 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) HE staining of normal skin, skin on top of the tumor and tumor interior. The arrows indicate spindle-like cells in the interior of epidermis.

(D) Ki67 staining of the samples. The arrows mark spindle-like cells with strong Ki67 staining in the interior of epidermis.

(E) Emergence of EMT features in the skin overlaying 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.

Figure 3. The skin overlaying the tumor of RKTG/p53 double-deleted mice has a loss of p53 accompanied by emergence of EMT

(A) Mouse skin isolated from mice of different genotype and at the surface of tumors was used in immunoblotting with the antibodies as indicated.

(B) Real-time RT-PCR was performed with the samples as in (A) to determine the mRNA levels of E-cadherin, vimentin, Snail, p53, and p21. The data are calculated from triplicate experiments and shown as mean ± SD. * and ** indicate p < 0.05 or p < 0.01 in comparison with the RKTG(+/+) p53(+/+) group.
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 μM) for different lengths of time. The cells were pretreated with wortmannin (100 nM) for 1 h 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 μM) for 4 h as indicated. After 24 h 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.

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 μM) for 6 h. After 24 h culture, the cell lysate was used in immunoblotting.

(B) EGF can not induce EMT after knockdown of RKTG and p53. A similar experiment as in (A) was performed 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 ** indicate $p < 0.05$ and $p < 0.01$ between the experimental group and the control group without transfection nor LPA treatment. ^^ indicates $p < 0.01$ between the last two 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 μM) for 6 h. 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 nM) or PD98059 (50 μM) for 1 h before LPA stimulation as indicated.

**Figure 6. Models of functional cooperation of RKTG with p53 in physiology and tumorigenesis**

(A) A model to depict the functional interplay between RKTG and p53 under physiological condition.

(B) A model to illustrate the effect of losing both p53 and RKTG on EMT and tumorigenesis. Maximal EMT only occurs when AKT is activated by loss of RKTG and EMT checkpoint function is disrupted by loss of p53.
Figure 2

A

B

C

D

E

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Figure 4

A

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>shRKTG</th>
<th>Wortmannin</th>
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<tbody>
<tr>
<td>p-AKT(Ser473)</td>
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</tr>
<tr>
<td>total AKT</td>
<td></td>
<td></td>
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</tbody>
</table>

LPA (min): 0 5 15 30 0 5 15 30 0 5 15 30

B

1 2 3 4 5 6 7 8

- p-AKT(Ser473)
- total AKT
- p-GSK3β(Ser9)
- total GSK3β
- E-cadherin
- Vimentin
- β-catenin
- p53
- Tubulin

LPA: - - - - + + + +
RKTG-shRNA: - - + + - - + +
p53: - + - + - + - +

C

1 2 3 4 5 6

- p-AKT(Ser473)
- total AKT
- E-cadherin
- Vimentin (short exposure)
- Vimentin (long exposure)
- Tubulin

LPA: - + - + + + +
RKTG-shRNA: - - + + + + +
Wortmannin: - - - - + -
Figure 6

A

<table>
<thead>
<tr>
<th>GPCR (e.g. by LPA)</th>
<th>RTK (e.g. by EGF)</th>
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<tbody>
<tr>
<td>Gβγ</td>
<td>RKTG</td>
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<td>Raf</td>
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<tr>
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<td>ERK</td>
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<tr>
<td></td>
<td>p53 phosphorylation and activation</td>
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<td>p16, p21</td>
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<td>Cell senescence</td>
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<td>Cell proliferation</td>
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B

<table>
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<tr>
<th>RKTG loss</th>
<th>p53 loss</th>
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<tbody>
<tr>
<td>ERK</td>
<td>AKT</td>
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<tr>
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<td>Cell cycle checkpoint</td>
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<td>Senescence checkpoint</td>
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<td>EMT checkpoint</td>
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<tr>
<td>Tumor</td>
<td>EMT</td>
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