K-Ras–Activated Cells Can Develop into Lung Tumors When Runx3-Mediated Tumor Suppressor Pathways Are Abrogated

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https://doi.org/10.14348/molcells.2020.0182
www.molcells.org

K-RAS is frequently mutated in human lung adenocarcinomas (ADCs), and the p53 pathway plays a central role in cellular defense against oncogenic K-RAS mutation. However, in mouse lung cancer models, oncogenic K-Ras mutation alone can induce ADCs without p53 mutation, and loss of p53 does not have a significant impact on early K-Ras–induced lung tumorigenesis. These results raise the question of how K-Ras–activated cells evade oncogene surveillance mechanisms and develop into lung ADCs. RUNX3 plays a key role at the restriction (R)-point, which governs multiple tumor suppressor pathways including the p14ARF–p53 pathway. In this study, we found that K-Ras activation in a very limited number of cells, alone or in combination with p53 inactivation, failed to induce any pathologic lesions for up to 1 year. By contrast, when Runx3 was inactivated and K-Ras was activated by the same targeting method, lung ADCs and other tumors were rapidly induced. In a urethane-induced mouse lung tumor model that recapitulates the features of K-RAS–driven human lung tumors, Runx3 was inactivated in both adenomas (ADs) and ADCs, whereas K-Ras was activated only in ADCs. Together, these results demonstrate that the R-point–associated oncogene surveillance mechanism is abrogated by Runx3 inactivation in AD cells and these cells cannot defend against K-Ras activation, resulting in the transition from AD to ADC. Therefore, K-Ras–activated lung epithelial cells do not evade oncogene surveillance mechanisms: instead, they are selected if they occur in AD cells in which Runx3 has been inactivated.

Keywords: cancer initiation, K-Ras, lung cancer, p53, Runx3

INTRODUCTION

Lung adenocarcinoma (ADC) is the most frequent subtype of lung cancer. Most lung ADCs develop through stepwise progression from atypical adenomatous hyperplasia (AAH) to bronchio-alveolar carcinoma (BAC), and ultimately to multiple types of invasive ADCs (Subramanian and Govindan, 2008; Wistuba and Gazdar, 2006). Human AAH and BAC are considered to be equivalent to mouse lung adenoma (AD). Approximately 25% of human lung ADC cases harbor activating mutations in the K-RAS gene. Although K-RAS signaling has been intensely studied, no drugs have yet been approved to treat K-RAS–mutant cancers: this is primarily because inhibition of K-RAS, which is important for normal cellular function, would be extremely toxic to patients (Drosten et al., 2018). Therefore, identification of effective tumor
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suppressors of K-RAS-dependent lung cancer would provide new strategies for cancer treatment. RUNX3 is inactivated by epigenetic alterations in most cases of human lung ADs (AAH and BAC) (Lee et al., 2010; 2013) and K-RAS-activated lung ADCs (Lee et al., 2013). Runx3 mice spontaneously develop a variety of tumors in old age, most frequently lung ADs (Lee et al., 2010), and disruption of Runx3 in mouse lung leads to AD formation (Lee et al., 2013). The cellular decision regarding whether to undergo proliferation or death is made at the restriction (R)-point, which is disrupted in nearly all tumors (Weinberg, 2014). RUNX3 plays a key role at the R-point which governs the p14ARF-p53 pathway (Chi et al., 2017; Lee and Rae, 2020; Lee et al., 2019a; 2019b). Early after mitogenic stimulation, RUNX3 transactivates R-point-associated genes, which include p14ARF (p19ARF in mouse, hereafter ARF) by recruiting activator complexes to its target loci. When the RAS-MEK pathway is downregulated, RUNX3 suppresses the target genes by recruiting repressor complexes. The cell then passes through the R-point to S phase. If the RAS-MEK pathway is not downregulated, expression of the R-point-associated genes are maintained and the cell cannot pass through the R-point. Therefore, the RUNX3 → R-point → ARF → p53 pathway constitutes a surveillance mechanism against oncogenic RAS (Lee et al., 2019a). Indeed, inactivation of p53 or Runx3 similarly accelerates malignant progression of oncogenic K-Ras-dependent mouse lung tumors (DuPage et al., 2009; Lee et al., 2013).

However, in mouse lung tumor models, oncogenic K-Ras mutation alone can induce ADCs without p53 mutation, and loss of p53 does not have a significant impact on early K-Ras-induced lung tumorigenesis (Feldser et al., 2010; Junttila et al., 2010; Muzumdar et al., 2016). Therefore, it remains unclear how K-Ras-activated cells evade oncogene surveillance mechanisms. In this study, we analyzed genetically engineered and carcinogen-induced mouse models, and obtained evidence demonstrating that K-Ras-activated cells are selected only if the activating K-Ras mutation arises in AD cells in which Runx3 is inactivated.

MATERIALS AND METHODS

Experimental model and subject details

Mice
Runx3<sup>BAC</sup> (Jax 008773), p53<sup>BAC</sup> (Jax 008462), K-Ras<sup>Sc-G12D</sup> (Jax 008179), Rosa26R-Tomato (Jax 007914), Cre<sup>CreERT</sup> (Jax 004682), and Cre<sup>CreERT2</sup> (Jax 008463) mice were obtained from Jackson Laboratory (USA). Unless stated otherwise, all mice analyzed had mixed genetic backgrounds and were age-matched (6-8 weeks old). Sample size was determined based on our experience and previous experiments. No data were excluded from the analysis. Animal experiments described were performed with at least three independent replicates, and representative results are shown in the figures. All animal studies were randomized into control or treated groups; in general, all animals housed in the same cage were in the same treatment group. For analysis of tumor samples, identities were blinded from histopathological assessment. All animals were housed in specific pathogen-free facilities and monitored daily. Animal studies were approved by the Institutional Animal Care Committee of Chungbuk National University (CBNUA-1208-18-02).

Method details

Adenovirus delivery

Adenovirus carrying Cre recombinase (Ad-Cre) was purchased from Vector Biolabs (USA). Each mouse was treated with 2.5 × 10<sup>7</sup> Ad-Cre viral genome copies diluted in warm 50 µl sterile MEM. After treatment, mice were placed on a warm pad until they woke up.

Chemical delivery

To determine the order of Runx3 inactivation and K-Ras activation during urethane-induced development of lung ADC, 6-week-old wild-type (WT) mice were injected intraperitoneally with urethane (ethyldimethylamine: Sigma, USA) (500 mg/kg body weight).

Hematoxylin and eosin staining

H&E staining was conducted according to a standard protocol. Briefly, slides were rehydrated by ethanol, xylene, and water to remove the paraffin. The nuclei were stained with hematoxylin (#3309; DAKO, USA) for 3 min, and the cytoplasm was stained with eosin (HT110280; Sigma) for 30 s. Slides were mounted with Permount (SP15-500; Thermo Fisher Scientific, USA) after the dehybridization and clearing steps.

Histology and immunohistochemistry

For histological analysis, lungs were inflated with 4% paraformaldehyde or formalin (3.7% formaldehyde) and fixed for 36 h. Fixed paraffin sections were rehydrated, subjected to antigen retrieval, and blocked in Tris-buffered saline (0.1% Triton X-100 containing 1% bovine serum albumin) or protein-free blocking solution (DAKO), and sequentially incubated with specific primary antibodies and biotinylated (DAKO) or Alexa Fluor-conjugated secondary antibodies (Invitrogen, USA). Images were produced with equivalent parameters using the ZEN Light Edition software (Carl Zeiss).

DNA exon-sequencing analysis

Standard exome-capture libraries were generated from 1 µg input DNA using the Agilent SureSelect Target Enrichment protocol for Illumina paired-end sequencing library (ver. B.3, June 2015). Probe sets were SureSelect Human All-Exon V6 or SureSelect Mouse All-Exon (Agilent, USA). DNA was quantitated using PicoGreen, and DNA quality was assessed by agarose gel electrophoresis. One microgram of DNA from each cell line was diluted in EB buffer and sheared to a target peak size of 150 to 200 bp using the Covaris LE220 focused ultrasonicator (Covaris, USA). The 8-microTUBE Strips were loaded into the tube holder of the ultrasonicator, and the
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Fig. 1. Runx3 inactivation, but not p53 inactivation, enables immediate proliferation of K-Ras-activated lung epithelial cells. (A and B) KPT and KRT mice were targeted with Ad-Cre (2.5 × 10^7 PFU/mouse, as described by DuPage et al., 2009), and Tomato-positive lung epithelial cells were assessed after 10 days. Clusters of Tomato-positive cells were detected 10 days after infection only in KRT mouse lungs. Enlarged images of the boxed regions are shown (lower panels). (C) Schematic representation of the structures of K-Ras<sup>LoxP-STOP-LoxP-G12D</sup> (K-Ras*), Runx3<sup>flox</sup>, p53<sup>flox</sup>, and Cre<sub>ERT1</sub> (Cre<sub>ERT1</sub>) alleles. Cre recombinase activates K-Ras by removal of a knocked-in STOP transcriptional cassette from the K-Ras<sup>LoxP-STOP-LoxP-G12D</sup> allele and inactivates the p53<sup>flox</sup> and Runx3<sup>flox</sup> alleles by deletion of exons. Survival curves of KR<sub>ERT1</sub>, R-Cre<sub>ERT1</sub>, K-Cre<sub>ERT1</sub>, KP-Cre<sub>ERT1</sub>, and KR-Cre<sub>ERT1</sub> mice in the absence of tamoxifen are shown. The median survival of KR-Cre<sub>ERT1</sub> mice was 48 days. No mice of other genotypes died within 50 weeks after birth (the duration of the experiment). (D) Hematoxylin/eosin (HE) staining of the lungs of K-Cre<sub>ERT1</sub>, KP-Cre<sub>ERT1</sub>, and R-Cre<sub>ERT1</sub> mice (6 months after birth). A magnified image of the boxed region is shown on the right. Adenomatous lesions in a R-Cre<sub>ERT1</sub> lung are indicated by dotted circles. HE staining of the lung tumors of KR-Cre<sub>ERT1</sub> mice (2 and 8 weeks after birth).
DNA was sheared using the following settings: mode, frequency sweeping: duty cycle, 10%; intensity, 5; cycles per burst, 200; duration, 60 s × 6 cycles; temperature, 4°C to 7°C. The fragmented DNA was repaired, an ‘A’ was ligated to the 3’ end, and Agilent adapters were ligated to the fragments. Once ligation was assessed, the adapter ligated product was amplified by polymerase chain reaction (PCR). The final purified product was quantified using TapeStation DNA ScreenTape D1000 (Agilent). For exome capture, 250 ng of DNA library was mixed with hybridization buffers, blocking mixes, RNase block, and 5 µl of SureSelect All-Exon capture library according to the standard Agilent SureSelect Target Enrichment protocol. Hybridization to the capture baits was conducted for 24 h at 65°C in a PCR machine, with the thermal cycle lid heated to 105°C. The captured DNA was washed and amplified. The final purified product was quantified by qPCR according to the qPCR Quantification Protocol Guide (KAPA Library Quantification kits for Illumina Sequencing platforms) and quantified again using the TapeStation DNA ScreenTape D1000 (Agilent). Finally, the DNA was sequenced on the HiSeq 2500 platform (Illumina, USA).

RESULTS

Runx3 inactivation, but not p53 inactivation, enables immediate proliferation of K-Ras-activated lung epithelial cells

To determine whether the oncogenic K-RAS → RUNX3 → ARF → p53 pathway suppresses proliferation of K-Ras-activated lung epithelial cells in vivo, we crossed Rosa26R-Tomato (Tomato+) mice with strains harboring Runx3^{tm/ERT1};tm/ERT2 (K-Ras*) to obtain Tomato+;tm/ERT1 (K-Ras*), yielding K-Ras+;p53^{tm/ERT1}/^ERT2 Tomato+ (KPT) and K-Ras+;Runx3^{tm/ERT1}/^ERT2 Tomato+ (KRT) mice. The mice were targeted by Cre-expressing adenovirus infection (Ad-Cre, 2.5 × 10⁶ pfu/mouse; DuPage et al., 2009), and the Tomato-positive cells were traced 10 days after infection. The results revealed that most of the p53-inactivated and K-Ras-activated cells remained as single cells, i.e., they had not divided (Fig. 1A). By contrast, most of the Runx3-inactivated and K-Ras-activated cells had formed clusters (Fig. 1B). These results indicate that Runx3 inactivation, but not p53 inactivation, enables immediate proliferation of K-Ras-activated lung epithelial cells.

Runx3 inactivation allows initiation of K-Ras-dependent lung cancer

Currently, all available genetically engineered K-Ras–dependent mouse lung cancer models require activation of K-Ras in a large number of cells (10⁵–10⁶ cells/mouse) and a long latency period (Guerra et al., 2003; Tuveson et al., 2004). Under physiological conditions, however, activation of K-Ras does not occur simultaneously in a large number of cells. Therefore, we asked whether activation of K-Ras, alone or in combination with inactivation of p53 or Runx3, in a very small number of cells could induce lung cancer. To target Runx3, p53, and/or K-Ras in a very small number of cells, we crossed Cre^{ERT1} mice with strains harboring Runx3^{tm/ERT1};tm/ERT2, p53^{tm/ERT1};tm/ERT1, and K-Ras+ and obtained various genotype combinations. In these mice, Cre recombine can be activated with tamoxifen to activate K-Ras and inactivate p53 or Runx3 (Fig. 1C). However, due to weak Cre leakage in this model, in the absence of tamoxifen, genes are targeted in very few cells (Kemp et al., 2004), providing a convenient means of targeting a limited number of cells. Therefore, we examined tumor formation and survival in these mice in the absence of tamoxifen.

All K-Ras+;Runx3^{tm/ERT1}/^ERT2 (KR), K-Ras+;Cre^{ERT1} (K-Cre^{ERT1}), K-Ras+;p53^{tm/ERT1}/^ERT2, Cre^{ERT1} (KP-Cre^{ERT1}), and Runx3^{tm/ERT1}/^ERT2 (R-Cre^{ERT1}) mice survived for more than 1 year (Fig. 1C). Pathological analysis revealed that the mice were completely tumor-free (Fig. 1D), whereas R-Cre^{ERT1} mice developed a few small ADs (Fig. 1D). By contrast, all mice with the K-Ras+;Runx3^{tm/ERT1}/^ERT2 (KR-Cre^{ERT1}) genotype developed lung ADCs rapidly, as early as 2 weeks after birth, and died within 12 weeks of birth in the absence of tamoxifen (median survival, 48 days) (Figs. 1C and 1D). These results indicated that while activation of K-Ras alone or in combination with inactivation of p53 in a small number of cells does not result in lung cancer formation, concurrent K-Ras activation and Runx3 inactivation does.

To determine whether the same thing would happen when K-Ras activation and Runx3 inactivation occurred in an even smaller number of cells, we used Cre^{ERT1}, which is much more tightly regulated by tamoxifen than Cre^{ERT2}. These experiments were performed in Rosa26R-Tomato (Tomato+) mice to allow visualization of the targeted cells. Cre^{ERT1} mice and Tomato+ mice were crossed with Runx3^{tm/ERT1}/^ERT2, p53^{tm/ERT1}/^ERT2, and K-Ras+ mice to obtain Tomato+;Cre^{ERT1} (T-Cre^{ERT1}), K-Ras+;Tomato+;Cre^{ERT1} (KT-Cre^{ERT1}), K-Ras+;p53^{tm/ERT1}/^ERT2, Tomato+;Cre^{ERT1} (KP-Cre^{ERT1}), and K-Ras+;Runx3^{tm/ERT1}/^ERT2 (KRT-Cre^{ERT1}) mice. The mice were maintained in the absence of tamoxifen. Six months after birth, a few Tomato-positive cells (average, 20 cells per section) were detected in the lungs of T-Cre^{ERT1} mice (Fig. 2A), confirming that Cre^{ERT1}, like Cre^{ERT2}, is leaky. However, Tomato-positive cells in the lungs of KT-Cre^{ERT1} mice were extremely rare (average, 0.5 cells per section) (Fig. 2A). Simple calculation suggests that only about 2.5% of K-Ras–activated cells survived, and that these cells were in a quiescent state. These results suggest that the majority of K-Ras–alone-activated cells are eliminated through oncogene surveillance mechanisms.

Similar to what we observed with Cre^{ERT1} mice, KPT-Cre^{ERT1} mice (n = 5) were completely tumor-free 6 months after birth (Fig. 2B). By contrast, all KPT-Cre^{ERT1} mice (n = 5) developed lung ADCs (Fig. 2B). In the lungs of KPT-Cre^{ERT1} mice, we detected clusters of Tomato-positive cells, but these cells formed a normal alveolar architecture and did not exhibit any dysplastic features (Fig. 2C). These results suggest that p53 inactivation enables K-Ras–activated cells to survive and weakly proliferate, but not to develop into tumors. By contrast, Tomato-positive cells in KRT-Cre^{ERT1} mouse lungs formed tumors (Fig. 2D), indicating that Runx3 inactivation enables K-Ras–activated cells not only to survive and proliferate, but also to develop into tumors. These results suggest that p53 inactivation abrogates the oncogene surveillance mechanism only partly, whereas Runx3 inactivation does so completely.

Overall, these findings indicate that K-Ras activation in a
limited number of cells, either alone or in combination with p53 inactivation, does not induce lung cancer, whereas the combination of K-Ras activation and Runx3 inactivation immediately induces lung cancer. These results suggest that Runx3 functions as a gatekeeper against K-Ras-dependent lung tumorigenesis.

**The gatekeeper activity of Runx3 against K-Ras activation also operates in other tissues**

Leaky activation of Cre<sup>tm/ERT2</sup> occurs in a wide range of tissues (Kemp et al., 2004). Indeed, tumors in KR-Cre<sup>ERT2</sup> mice were not limited to the lungs. Although lung ADCs developed in all KR-Cre<sup>ERT2</sup> mice, other types of tumors also developed. In particular, six of twenty KR-Cre<sup>ERT2</sup> mice developed skin cancers in the anus (Fig. 3A), six had a grossly enlarged spleen with massive lymphoid hyperplasia (Fig. 3B), and seven had an enlarged thymus with thymic lymphoma (Fig. 3B). None of these tumors were detected in KP-Cre<sup>ERT2</sup> mice. Notably, thymic lymphoma cells infiltrated into the lung along the broncho-vascular bundle in some KRT-Cre<sup>ERT2</sup> mice (Fig. 3C). The targeting of K-Ras<sup>*</sup> and Runx3<sup>fl/fl</sup> by leaky activation of Cre<sup>tm/ERT1</sup> or Cre<sup>tm/ERT2</sup> in the tumors was confirmed by genotyping (Fig. 3D). These results imply that the proposed gatekeeper activity of Runx3 against K-Ras ac-
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Lung ADs develop via inactivation of Runx3 and progress into ADCs via K-Ras activation
Mouse lung tumors induced by urethane, a tobacco carcinogen, recapitulate the natural history of smoking-associated, K-RAS–driven human lung ADCs (Westcott et al., 2015). The mice develop slow-growing ADs about 20 weeks after urethane treatment and fast-growing ADCs about 58 weeks later (Fig. 4A). We previously showed that Runx3 expression is downregulated in nearly all urethane-induced lung ADs and ADCs, mainly due to hypermethylation of the Runx3 CpG island (Lee et al., 2010). In this study, we detected K-Ras path-

**Fig. 3.** KR-CreERT1 mice develop lymphomas and skin cancers, as well as lung ADs/ADCs. (A) Gross and microscopic images of a skin cancer that developed in a KR-CreERT1 mouse. (B) Gross and microscopic images of the spleen and thymus of WT and KR-CreERT1 mice. Histological analysis revealed that the cortex-medulla boundary in the enlarged thymuses was obscure and filled with undifferentiated T-cells, indicating thymic lymphoma. (C) HE-stained gross images and magnified microscopic images of thymus and lung of KRT-CreERT2 mice (6 months after birth). Lung-invading tumor cells were morphologically identical to thymic lymphoma cells, indicating that the lung-invading tumors originated from a thymic lymphoma. (D) Targeting of the K-Ras* and Runx3flo alleles by leaky activation of CreERT1 in tumors was verified by genomic PCR. L, lung ADC; Th, thymus; Sp, spleen; Sk, skin cancer. Targeting of the K-Ras* and Runx3flo alleles by leaky activation of CreERT2 in tumors was verified by genomic PCR. M, DNA size marker; L, lung ADC; Th, thymus; LT, lung infiltrated thymic lymphoma.
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**Fig. 4. Sequential molecular events associated with the development of lung AD and progression to ADC.** (A) Mice (FVB strain) were intraperitoneally injected with urethane at a dose of 500 mg/kg body weight. Growth of cancer in mouse lungs vs. time after urethane injection is shown. Five mice were used for each time point. Ave, average diameter (mm) of cancer; SD, standard deviation. (B) Mouse lung ADs that developed 20 weeks (20W) after urethane injection and lung ADCs that developed 58 weeks (58W) after injection were analyzed by immunohistochemical (IHC) staining with anti-phospho-Erk (p-Erk) and anti-Runx3 antibodies. Runx3 expression was markedly downregulated in both ADs and ADCs relative to adjacent normal regions. By contrast, Erk was activated only in ADCs. Nor, normal. (C) WT mouse lung and mouse lung ADs developed at 20 weeks (20W, diameter < 1 mm), relatively small lung ADCs (58W-S, diameter ≒ 2 mm) and relatively large lung ADCs (58W-L, diameter ≒ 3 mm) developed at 58 weeks, and large lung ADCs (68W-L, diameter ≒ 6 mm) developed at 68 weeks after urethane injection were obtained. Four ADs or ADCs from each group were analyzed by whole-exon sequencing. Among the known major oncogenes and tumor suppressors involved in lung cancer, only K-Ras mutations were detected. Amino acid changes in K-Ras and the ratio of mutation signal to total signal are shown. d, diameter; ratio, ratio of oncogenic K-Ras–mutated allele relative to total K-Ras. Bar diagram demonstrating average ratios of K-Ras mutation in each group of ADs/ADCs. P = P value. (D) Schematic representation of the molecular events associated with the development of lung ADs and progression into ADCs. Runx3↓ and K-Ras↑ indicate Runx3 inactivation and K-Ras activation, respectively. Human AAH and BAC correspond to mouse lung ADs.
way activation (Erk phosphorylation) in almost all ADCs (19 out of 20), but not in any ADs (n = 20) in which Runx3 was inactivated (Fig. 4B). Consistent with this, whole-exon sequencing revealed that oncogenic K-Ras mutation was present in all ADCs but in no ADs (Fig. 4C, Supplementary Data 1). No mutations within exons of other known oncogenes and tumor suppressors involved in lung cancer were detected in ADs or ADCs (Supplementary Data 1). Notably, the proportion of oncogenic K-Ras–mutated alleles (representing the proportion of K-Ras–mutated cells within the tumor) increased with cancer growth (Fig. 4C). These results, together with our previous observations that Runx3 deletion in mouse lung results in development of AD (Lee et al., 2013), suggest that lung ADs develop via inactivation of Runx3, and that AD cells that acquire oncogenic K-Ras mutation selectively proliferate and form ADCs (Fig. 4D).

DISCUSSION

Although K-RAS mutation is the most frequently detected oncogenic mutation in human cancers, to date no drugs have been approved to treat K-RAS–mutant cancers. Recently, oncogenic K-RAS–specific inhibitors have been developed and are currently in clinical trials (Canon et al., 2019). However, more recent reports show that shortly after treatment with these inhibitors, some cancer cells bypass the effect of oncogenic K-Ras inhibition and resume proliferation (Xue et al., 2020). In addition, even when a cancer is effectively regressed by knockdown of oncogenic K-Ras in a mouse lung cancer model, the cancer recurs rapidly, not because the gene knockdown was unsuccessful but because other oncogenes were activated (Shao et al., 2014). These results suggest that existing strategies for inhibiting oncogenic K-RAS have a limited ability to achieve a durable response in cancer treatment. Given that normal mice do not suffer from tumors as frequently as tumor-regressed mice, the rapid recurrence phenomenon observed in cancer-regressed mice (Xue et al., 2020) implies that a defense mechanism is abrogated in K-Ras–activated lung tumors. However, loss of p53 does not have a significant impact on early K-Ras–induced lung tumorigenesis (Feldser et al., 2010). These observations imply the existence of an effective defense mechanism against oncogenic K-Ras signaling, and that this mechanism is disrupted by a hidden molecular event in K-Ras–dependent lung cancers. Therefore, to achieve a complete and durable response, it will be necessary to identify the hidden defense mechanism against K-Ras–dependent lung cancers.

To confirm the existence of this defense mechanism and identify the critical genes involved, we need to understand why tumor development in animal models requires activation of oncogenes in a large number of cells. For example, K-Ras–dependent mouse models develop lung cancer several months after K-Ras is targeted in a large number of cells (10^6–10^7 cells/mouse) (Guerra et al., 2003; Tuveson et al., 2004). Therefore, cancer development must be a very rare event within the large population of K-Ras–activated cells. To explain this, we can consider two possibilities. First, the cells that originate the tumors may be very rare, making the probability of targeting K-Ras in any given origin cell very low (single-step model, assuming the absence of a defense mechanism). Second, the cells of origin of tumors may be relatively abundant, but K-Ras activation alone cannot induce cancer unless the defense mechanism is abrogated (two-step model, assuming the existence of the defense mechanism).

In this study, we observed the following: (1) A combination of Runx3 inactivation and K-Ras activation in a very limited number of cells immediately induced lung ADCs, whereas K-Ras activation alone or in combination with p53 inactivation did not. (2) In the carcinogen-induced mouse lung cancer model, Runx3 inactivation occurred earlier than K-Ras activation. Our results strongly support the second possibility described above, which assumes the existence of the defense mechanism: the majority of lung ADs are initiated by Runx3 inactivation, and AD cells, in which R-point-associated oncogene surveillance mechanism is abrogated, cannot defend against K-Ras activation, resulting in a transition from ADs to ADC.

The Arf-p53 pathway is the primary defense against oncogenic Ras in primary cells (Serrano et al., 1997). Why, then, is only limited tumor suppressor activity of p53 observed in mouse lung tumor models (Feldser et al., 2010)? In our interpretation, the results do not imply a limitation of p53 tumor suppressor activity, but instead suggest that p53-independent tumor suppressive mechanisms exist in the lung: hence, if lung tumorigenesis is suppressed by multiple pathways, loss of p53 can be compensated by other pathways. In this regard, it is worth emphasizing that the R-point, which is disrupted in nearly all tumors, governs multiple tumor suppression pathways (Weinberg, 2014). When a death decision is made at the R-point, the cell defends against tumorigenesis by regulating not only intracellular programs (cell cycle, apoptosis, and metabolism) but also extracellular programs (inflammatory and immune responses) (Lee et al., 2019a; Samarakkody et al., 2020; Seo and Taniuchi, 2020). Because Runx3 functions as a decision-maker of the R-point, inactivation of Runx3 abrogates the entire R-point-associated defense programs (Lee et al., 2019a). By contrast, p53 functions as an executor of the R-point decision, and inactivation of the gene abrogates only intracellular programs. The critical roles of Runx3 in R-point regulation may explain how Runx3 functions as a potent gatekeeper against oncogenic K-Ras.

Targeted therapies that inhibit activated oncogenes have yielded clinical responses but eventually lead to cancer recurrence with secondary oncogene activation in almost all malignancies (Janne et al., 2009; Podsypanina et al., 2008). Therefore, it would be of great therapeutic value to understand why cancers recur after the failure of therapies that target activated oncogenes. Our results explain why cancers recur: inhibition of an activated oncogene causes the cancer to regress, but the regressed cells remain cancer-prone because their oncogene surveillance mechanisms are inactivated.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

ACKNOWLEDGMENTS

S-C Bae is supported by a Creative Research Grant (2014R1A3A2030690) through the National Research Foun-
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