Uncoupling Cancer Mutations Reveals Critical Timing of p53 Loss in Sarcomagenesis

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Abstract

It is well accepted that cancer develops following the sequential accumulation of multiple alterations, but how the temporal order of events affects tumor initiation and/or progression remains largely unknown. Here, we describe a mouse model that allows for temporally distinct cancer mutations. By integrating a Flp-inducible allele of K-ras\(^{G12D}\), with established methods for Cre-mediated p53 deletion, we were able to separately control the mutation of these commonly associated cancer genes in vitro and in vivo. We show that delaying p53 deletion relative to K-ras\(^{G12D}\) activation reduced tumor burden in a mouse model of soft-tissue sarcoma, suggesting that p53 strongly inhibits very early steps of transformation in the muscle. Furthermore, using in vivo RNA interference, we implicate the p53 target gene p21 as a critical mediator in this process, highlighting cell-cycle arrest as an extremely potent tumor suppressor mechanism.

Introduction

Tumorigenesis is a multistep process driven by the accumulation of both genetic and epigenetic alterations in oncogenes and tumor suppressor genes (1). These individual changes occur in a sequential manner and are thought to drive distinct steps in the progression of normal cells to full malignancy (2). Although it is generally believed that the actions of these mutations combine to effect full transformation, it remains unclear how the order of events impacts this process.

Although genetically engineered mice have been instrumental in modeling and experimentally validating various attributes of human cancer, most current models that utilize multiple cancer-associated mutations are not designed to carry out sequential mutagenesis. As a result, one cannot directly test the importance of mutation timing or order. For example, although the use of multiple alleles controlled by the conditional Cre-LoxP site-specific recombinase (SSR) system allows for the introduction of several cancer relevant mutations, these mutations occur simultaneously at the time of tumor initiation (3). Additional SSR modalities, such as those used in Saccharomyces cerevisae—derived Flp-Frt, exist but have been used in a more limited fashion in mouse models (4, 5). By combining different SSR systems within the same model, one could achieve spatiotemporal control of distinct genetic events provided the recombinases are independently regulated.

Through its ability to respond to various forms of cellular stress by inducing cell-cycle arrest or apoptosis, p53 plays a central role in tumor suppression (6). Despite being one of the most thoroughly studied tumor suppressor genes, rather little is known about the precise stage(s) of tumorigenesis at which p53 exerts its functions. In many human cancers, such as those of the lung, colon, and pancreas, p53 alterations have been documented in more advanced stages of tumor development, suggesting that p53 constrains progression of established tumors (7–9). In contrast, the early onset of several cancer types in patients with Li–Fraumeni syndrome, who inherit a germ line mutation in p53, argues for a potential role of this tumor suppressor in inhibiting the early stages of transformation in some cell types (10). Although these studies establish that p53 mutation correlates with different stages of tumor development, whether these differences underlie specific temporal requirements for p53 loss in distinct tissues remains to be determined.

In addition to mutational data from human tumors, numerous mouse models have documented broad tumor-suppressive roles for p53 in numerous contexts (11). Both germ line mutant and Cre-LoxP conditional alleles of p53 have been used to promote tumor formation in multiple tissues. However, most of these studies have not been able to pinpoint a specific requirement for p53 mutation as a function of tumor progression. Here we describe a system for sequentially mutating the commonly associated cancer genes K-ras and p53. By combining an Flp-inducible allele of oncogenic K-ras with an already well-established Cre-regulated p53 deletion allele (12), we were able to separately regulate these mutations.

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mutations, both in cells and in mice. We show, using this dual SSR strategy, a clear effect of p53-mediated tumor suppression in the earliest stages of soft-tissue sarcoma (STS) formation.

Materials and Methods

Mouse studies

Information on the construction of the $K_{ras}^{pSF-G12D}$ targeting construct and mouse strain, as well as details on additional mouse strains used in this study, are found in Supplementary Material. Lung tumors were generated and processed as previously described (13–15), substituting Ad-Flpo (University of Iowa, Gene Transfer Vector Core) for Ad-Cre. Intramuscular viral infections were done as previously shown (16). For green fluorescent protein (GFP) marking experiments, needles were dipped in India Ink before injection to mark the needle track. Tamoxifen (Sigma) was dissolved in corn oil at 10 mg/mL and dipped in India Ink before injection to mark the needle track. Once masses were visible on the legs, tumors were processed for histology and molecular analysis as described in the following text. For mouse embryonic fibroblasts (MEF) tumor experiments, 1.5 × 10^6 cells were resuspended in 200 μL of PBS and injected s.c. Mice were monitored every few days for tumor formation. Animal studies were approved by the Massachusetts Institute of Technology Committee for Animal Care and conducted in compliance with Animal Welfare Act Regulations and other federal statutes relating to animals and experiments involving animals and adheres to the principles set forth in the 1996 National Research Council Guide for Care and Use of Laboratory Animals (institutional animal welfare assurance number A-3125-01).

Cell culture

Primary MEFs of the indicated genotypes were isolated from E13.5 embryos and propagated in Dulbecco’s modified Eagle’s medium supplemented with 10% IFS, 5 mmol/L glutamine, and 100 U/mL penicillin/100 μg/mL streptomycin. Where applicable, 4-hydroxytamoxifen (Sigma) was added to the media at 100 nmol/L. Lentiviral infections were carried out by directly transferring viral supernatant. For adenosine infection in vitro, Ad-Flpo was added to the media at a multiplicity of infection of 10.

Genomic PCR analysis

DNA was prepared from MEFs or tumors and subjected to standard PCR analysis. PCR primers are given in Supplementary Material.

Protein extraction and immunoblots

Lysates from MEFs and tumors were prepared and immunoblotting was carried out as described previously (17). Antibody information is provided in Supplementary Material. Levels of Ras-GTP were determined with the Ras activation kit (Millipore).

Lentiviral vectors and short hairpin RNA cloning

The lentivirus containing pgkCre and a U6-shRNA has been described previously (17). The GFP-Cre lentivirus (Ubc-GFP, pgk-Cre) was provided by M. DuPage (Koch Institute for Integrative Cancer Research, Massachusetts Institute of Technology, Cambridge, MA). Target sequences for short hairpin RNA (shRNA) knockdown were identified and cloned as previously described (17). shRNA sequences are provided in Supplementary Material.

Lentiviral production

Lentivirus was produced as described previously (18).

Histology and immunohistochemistry

Tissues were fixed in 10% formalin for 6 to 8 hours and further processed for histology as previously described (13). For immunohistochemistry (IHC), paraffin-embedded sections were dewaxed, followed by antigen retrieval in 10 mmol/L citrate buffer (pH 6.0) in a pressure cooker. Slides were quenched in 3% hydrogen peroxide and washed in Tris-buffered saline containing 0.05% Tween 20 (TBST). After blocking in TBST/5% goat serum for 1 hour, the primary antibody (rabbit mAb anti-GFP, #2956; Cell Signaling Technology) was diluted 1:100 in SignalStain Antibody diluent (#8112; Cell Signaling Technology) and incubated on slides overnight at 4°C. Detection was carried out using a biotinylated goat anti-rabbit secondary antibody, followed by the Vectastain ABC kit with diaminobenzadine (DAB; Vector Labs). Slides were counterstained with hematoxylin before coverslipping.

Results

To create a model for sequential mutagenesis in vivo, we sought to combine Flp-Frt-mediated mutagenesis with already available tools from Cre–LoxP systems. To this end, we first generated a Flp-recombinase–inducible allele of oncogenic $K_{ras}^{pSF-G12D}$, following a very similar strategy used to construct the well-studied Cre-inducible $K_{ras}^{LSL-G12D}$ allele (ref. 19; Fig. 1A and B). To characterize the activity of this allele, we first generated MEFs from $K_{ras}^{pSF-G12D/+}$ embryos. Introduction of an engineered thermostable version of Flp (Flpe; ref. 20) but not Cre led to removal of the stop cassette, as shown by PCR analysis (Fig. 1C). In addition, Flpe expression in $K_{ras}^{pSF-G12D/+}$ MEFs induced high levels of active Ras-GTP, along with appropriate downstream signaling events such as upregulation of cyclin D1 (Fig. 1C).

We next tested the activity of the $K_{ras}^{pSF-G12D}$ allele in vivo, which was of particular importance given the evidence that Flpe is much less efficient than Cre in mammalian systems (21). Although introduction of adenosinoviruses or lentiviruses expressing Cre (Ad-Cre or LV-Cre) into the lungs of K-ras^{LSL-G12D/+} mice results in significant lung tumor formation (13, 14), infection with Ad-Flpo or LV-Flpe failed to generate lung tumors in K-ras^{pSF-G12D} mice (data not shown). However, using a mammalian codon-optimized version of Flp, termed Flpo (21), we were able to initiate numerous lung tumors in K-ras^{pSF-G12D/+} mice with intratracheal instillation of Ad-Flpo and LV-Flpo (Fig. 1D; data not shown). Together, these data indicate that $K_{ras}^{pSF-G12D}$ is the functional equivalent of the $K_{ras}^{LSL-G12D}$ allele.
Given the well-known genetic interaction between \( K\)-ras and \( p53 \) in cellular transformation, we chose to combine mutations in these 2 genes for initial sequential mutagenesis experiments in MEFs. Previous work had shown that primary MEFs expressing endogenous \( K\)-ras\(^{G12D} \) have some characteristics of transformation, but they are not fully transformed and do not form tumors in immunocompromised mice (19). With the ability to separate \( p53 \) from \( K\)-ras mutation in time, we set out to examine the consequences of delayed \( p53 \) deletion to form tumors in immunocompromised mice. Given the recent evidence that innate immune cells can clear \( p53 \)-deficient tumors in mice undergoing \( p53 \)-dependent cell-cycle arrest/senescence (14), we wondered whether \( FK* \) cells failed to form tumors (22), we repeated these experiments using nonobese diabetic/severe combined immune-deficient (NOD/SCID) mice, which could tamoxifen treatment still result in tumor formation. FK*CP cells were introduced into different groups of mice that varied in their tamoxifen treatment schedule: beginning 1 day (group A), 1 week (group B), or 3 weeks (group C) following their introduction into the animal. As expected, the resulting tumors showed complete recombination of both \( p53 \) alleles (Fig. 2B). Importantly, this system showed no leakiness, as mice injected with FK*CP cells and treated with vehicle (corn oil) failed to form tumors (Fig. 2A). In addition, the ability of tamoxifen treatment to promote tumor development depended on complete loss of \( p53 \), as shown by the lack of tumorigenicity of FK*C cells following tamoxifen administration (Fig. 2A).

We next investigated the fate of partially transformed FK*CP cells in vivo by addressing how long after injection could tamoxifen treatment still result in tumor formation. FK*CP cells were introduced into different groups of mice that varied in their tamoxifen treatment schedule: beginning 1 day (group A), 1 week (group B), or 3 weeks (group C) following injection. Strikingly, tumor development was efficient in groups A and B, whereas almost no tumors formed in group C (Fig. 2D). These results suggest that injected FK*CP cells were either removed in a \( p53 \)-dependent manner or became resistant to \( p53 \) loss sometime between 1 and 3 weeks following their introduction into the animal. Given the recent evidence that innate immune cells can clear cells undergoing \( p53 \)-dependent cell-cycle arrest/senescence (22), we wondered whether FK*CP cells failed to form tumors in group C because they were removed in this fashion. Therefore, we repeated these experiments using nonobese diabetic/severe combined immune-deficient (NOD/SCID) mice, which...
are more immunocomprised than the nu/nu strain and deficient in the cell populations implicated in senescent cell clearance. The results of the time-course experiment in NOD/SCID were very similar to those in nu/nu mice (Supplementary Fig. S1). These data suggest that innate immune cell–mediated clearance of senescing cells likely does not explain the failure of the partially transformed FK*CP cells to remain responsive to p53 deletion and form tumors following prolonged times in vivo. In summary, although primary MEFs expressing endogenous levels of oncogenic K-ras proliferate continuously in cell culture conditions, transfer into an in vivo environment leads rapidly to engagement of the p53 pathway and irreversible tumor suppression.

Having validated the sequential mutagenesis system using dual SSR technology, we next applied the system to an endogenous tumor model. Previous work had shown that intramuscular infection of Ade-Cre into the limbs of K-rasLSL-G12D/+; p53 flox/flox animals resulted in efficient sarcomagenesis (16). However, the presence of just one wild-type allele of p53 completely inhibited tumor formation. Furthermore, careful histologic analysis of non–tumor-bearing muscles of K-rasLSL-G12D/+; p53 flox/+ animals several months after Ade-Cre infection failed to identify any microscopic lesions (data not shown). These results suggest that activation of endogenous K-ras G12D in the muscle leads to rapid p53-dependent tumor suppression.

Despite these initial observations, the exact nature of the tumor suppression, and thus the fate of K-ras G12D–expressing cells in the p53 flox/+ background, had not been explored. One possibility was that oncogenic K-ras–positive cells persisted in the muscle but were limited in their proliferative capacity due to p53-dependent growth arrest. In this case, a secondary mutation in the p53 pathway could potentially unleash the oncogenicity of these latent cells. Another possible scenario
Figure 3. Delaying p53 loss relative to \( \text{K-ras}^{G12D} \) activation reduces tumor formation in a mouse model of STS. A, experimental outline of sequential mutagenesis strategy, showing the genotype of the compound mutant mice and the treatment regimen. See text for details. B, bar graph illustrating tumor incidence in mice in which tamoxifen treatment began on day 0 (black bar), day 10 (red bar), or day 21 (green bar) following Ad-Flpo infection. Tam, tamoxifen. Color scheme is same as in (B); Student’s t-test was used for statistical significance. D, PCR analysis of \( \text{K-ras} \) and p53 loci. Tam, tamoxifen.

was that \( \text{K-ras}^{G12D} \) activation resulted in a p53-dependent removal or irreversible arrest of the cells, such that subsequent p53 mutations would be ineffective in promoting full transformation of the initially targeted cells.

To distinguish between these 2 possibilities, we used the sequential mutagenesis strategy. Compound mutant mice of the genotype \( \text{K-ras}^{G12D};\text{p53}^{lox/lox};\text{R26CreER-T2/CreER-T2} \) were generated and separated into 3 groups. All 3 groups received simultaneous intramuscular infection with Ad-Flpo to activate oncogenic K-ras. They were then segregated on the basis of their tamoxifen treatment schedule and consequent p53 deletion. Tamoxifen was administered on the same day of Ad-Flpo infection for group A, or on day 10 postinfection (group B), or on day 21 postinfection (group C; Fig. 3A). If oncogenic K-ras–expressing cells persisted and remained fully competent to respond to p53 loss, then all 3 groups would be expected to form sarcomas efficiently. In contrast, if mutant K-ras cells were removed or rendered refractory to delayed p53 deletion, sarcomagenesis would be inhibited in groups B and C. In fact, sarcoma formation efficiency varied greatly between the 3 groups. Figure 3B shows that although all mice in group A presented with sarcomas, the percentage of affected mice was significantly lower in group B (56%) and group C (40%). Importantly, tumors from all groups displayed the expected recombination patterns at both K-ras\(^{G12D}\) and p53\(^{lox}\) loci (Fig. 3D). Of the sarcomas that did form in group C, some appeared with delayed kinetics compared with group A (Fig. 3C). Taken together, these results suggest that the pool of susceptible K-ras\(^{G12D}\)–expressing cells in the muscle is significantly diminished over time, either in number or in function, by a strong p53 response. Therefore, a mutation in K-ras must be soon followed by disengagement of the p53 pathway for efficient tumorigenesis to occur in the muscle. Alternatively, the inhibition of the p53 pathway would have to occur first in this cell type to render the cells sensitive to subsequent oncogene activation.

The rapid and robust tumor suppression observed in the sequential mutagenesis experiments prompted us to further characterize the activation and function of the p53 pathway in the muscle. The kinetics of the p53 response suggested that this pathway is strongly activated very soon after oncogenic K-ras expression. Previous studies have shown that removal of p19\(^{Arf}\), an upstream activator of p53, could substitute for p53 loss in sarcomagenesis (16). Therefore, we monitored p53 pathway activation with the \( \text{Arf}^{GFP} \) allele, a p19\(^{Arf}\)-specific GFP reporter that also functionally inactivates this tumor suppressor (23). Importantly, established sarcomas derived from intramuscular Ad-Cre infection of \( \text{K-ras}^{G12D}/\text{+} \): \( \text{Arf}^{GFP/GFP} \) animals displayed high GFP levels (Fig. 4A). To determine whether this activation occurs shortly after \( \text{K-ras}^{G12D} \) expression, we assayed for reporter induction 6 days post–Ad-Cre infection in \( \text{K-ras}^{G12D}/\text{+} : \text{Arf}^{GFP/GFP} \) and \( \text{K-ras}^{G12D}: \text{Arf}^{GFP/GFP} \) mice. Interestingly, we observed small areas of intense GFP staining in an oncogenic K-ras–dependent manner (Fig. 4A), consistent with the notion that the p19\(^{Arf}\)-p53 pathway is rapidly induced in the muscle following oncogenic stress.

We next sought to determine the tumor-suppressive mechanism downstream of p53. Through its ability to transcriptionally upregulate a number of different genes under various stress conditions, p53 can induce distinct cell fates, such as apoptosis, cell-cycle arrest, or senescence (24).
In this system, intramuscular Lenti-Cre infection could be used to introduce a shRNA targeting both p19Arf and p16Ink4a into muscle sections (ii and iii) from intramuscular Ad-Cre infection of K-rasLSL-G12D/+ animals. The arrow in (ii) points to darkly staining cells, shown at higher magnification in (iii). Scale bars, 100 μm.

Previously, we showed that deletion of Bak and Bax, two critical mediators of the intrinsic pathway of apoptosis, could not substitute for p53 loss during K-rasG12D-induced sarcomagenesis (16). This suggested that p53 does not function through the induction of apoptosis in this setting.

To address whether p53 acts through cell-cycle arrest and/or senescence, we focused on the cyclin-dependent kinase inhibitor p21, which has been shown to be critical for the G1 cell-cycle arrest elicited by p53 in a number of settings (25–27). For these studies, we used our recently reported RNA interference–mediated gene silencing method, which involves a lentivirus-based system for in vivo expression of Cre and a shRNA in the context of immunocompromised (Rag2–/–) animals (29). In this system, intramuscular Lenti-Cre infection of K-rasLSL-G12D/+;ArfGFP/+/Rag2–/– animals failed to generate sarcomas owing to strong p19Arf–/–p53 pathway activation. However, a shRNA targeting both p19Arf and p16Ink4a could block this tumor suppression and generate sarcomas. To determine whether p21 is an important component of p53-dependent tumor suppression in the muscle, we introduced a shRNA targeting p21 into the bifunctional lentiviral vector and infected K-rasLSL-G12D/+;ArfGFP/+/Rag2–/– mice. Interestingly, knockdown of p21 could promote sarcoma formation (Fig. 4B). Molecular analysis confirmed significant knockdown of p21 in the resulting tumors (Fig. 4C). In addition, PCR analysis of tumor DNA indicated retention of the wild-type allele of p19Arf, suggesting no additional selective pressure for p53 pathway inactivation during tumor formation (data not shown). Together, these data indicate that p21 is required for the strong tumor suppressor activity of p53 in the muscle following expression of oncogenic K-ras. This suggests that the primary function of p53 in this context is the induction cell-cycle arrest, and possibly, senescence.

As the sequential mutagenesis experiments suggested that initially targeted cells lose the capacity for full transformation even after subsequent p53 deletion, this cell-cycle arrest must result either in clearance of affected cells and/or be relatively stable and possibly irreversible. To gain further...
insight into the fate of K-rasG12D-expressing cells, we pursued a cell marking strategy. Administering lentiviruses encoding Cre and GFP allowed us to mark infected cells by IHC for GFP. As a control, lentiviral infection of K-rasLSL-G12D;Cre;flox/;Rag2/C0 animals efficiently generated sarcomas that stained for GFP (Supplementary Fig. S2). Interestingly, an examination of infected cells before macroscopic tumor formation revealed regions of GFP+ cells that increased in size over time, most likely corresponding to incipient tumor lesions (Supplementary Fig. S2). However, although we could readily detect infected cells in K-rasLSL-G12D;Cre;flox/;Rag2/C0 mice, they never appeared to expand into small lesions, similar to what was seen in K-ras+/+ muscles (Fig. 4D). Importantly, many infected cells were present at time points at which p53 deletion could not efficiently promote sarcomagenesis in the sequential mutagenesis experiments (Figs. 3B and 4D). At present, we have been unable to detect any markers of senescence in these infected cells (data not shown). Although we cannot rule out the possibility that some cells are cleared from the animals, the continued presence of GFP+ cells in these animals might suggest that p53 engages an irreversible cell-cycle arrest to potently suppress transformation in muscle cells.

Discussion

The immediate and robust p53-dependent response in muscle cells harboring an oncogenic Ras mutation might partially explain the low frequency of STSs compared with many other malignancies in humans. Interestingly, the incidence of STSs is dramatically elevated in Li–Fraumeni patients inheriting a germ line mutation of p53 (10), indicating that preexisting deficiencies in p53 function allow for muscle cell transformation, which is otherwise a rare occurrence. Moreover, somatic p53 mutations have been proposed to be relatively early events in other forms of sarcomas (28), further suggesting that muscle cells must overcome an early p53-dependent blockade during tumor initiation. This potent p53-dependent tumor suppression most likely selects for a particular sequence of events in which mutation of p53 occurs very early during spontaneous sarcomagenesis. Because p53 alterations rarely provide an immediate growth advantage in otherwise normal cells, this is not conduutive to efficient tumor initiation.

Several recent reports have analyzed p53 function during the early stages of tumorigenesis, although the identity of the cooperating oncogenic event(s) and its precise temporal relationship to p53 manipulation were unknown. In one study, Christophorou and colleagues showed robust tumor suppression after a small window of p53 activity occurring shortly after irradiation (IR)-induced tumor initiation (29). In a separate study, deleting p53 at different time points after IR revealed that the continued presence of p53 was required to prevent IR-induced tumorigenesis (30). In fact, once p53 was removed, tumor formation occurred with the same kinetics as seen in irradiated p53+/− animals. Apparently, in this context p53 simply blocked the overt transformation of preneoplastic cells in a manner that was completely reversible once p53 was deleted. This is in stark contrast to our observations involving K-rasG12D-expressing muscle cells, where secondary p53 deletion failed to fully recover the tumorigenic capacity of mutated cells. A further understanding of the factors governing potent tumor suppression of p53 in the muscle might guide therapeutic strategies aimed at generating such a strong and irreversible response in more resistant tissues.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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