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14. ABSTRACT

The p53 transactivation domain 1 (TAD1) plays a critical role in inducing p53 mediated cell-cycle arrest and apoptosis in response to acute DNA damage caused by irradiation. During radiation therapy of cancers, this p53-induced apoptosis triggers various deleterious pathological side effects in normal tissues. Interestingly, recent studies from our laboratory have demonstrated that p53 TAD1 is completely dispensable for tumor suppression in diverse mouse cancer models. We hypothesize that specific inhibition of p53 TAD1 should ameliorate the p53-associated pathologies occurring in response to acute DNA damage, while keeping p53-mediated tumor suppression intact, thus allowing improvement in the therapeutic index of radiation therapy in cancer. Importantly, because the majority of cancers, such as advanced prostate cancers, have inactivated the p53 pathway, such inhibitors should not compromise the efficacy of treating tumors. We propose to perform high-throughput chemical library screens to identify specific inhibitor of p53 TAD1 that may be administered as adjuvants of chemotherapy and radiotherapy in the context of prostate cancer.

15. SUBJECT TERMS
p53 inhibitor, p53 transactivation mutants

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Table of Contents

Page

Introduction

The p53 protein plays a pivotal role in suppressing tumorigenesis, as evidenced by its inactivation in over half of human cancers. Through its ability to function as a transcriptional regulator, p53 has the capacity to respond to a variety of genotoxic stress signals and induce cell-cycle arrest, apoptosis and cellular senescence to curb neoplastic growth (*1, 2*). Aside from its beneficial tumor suppressive capability, p53 is also a critical mediator of DNA damage signals and this property provokes it to induce deleterious, pathological side effects associated with genotoxic stress-inducing radio- and chemotherapies, particularly in the radiosensitive tissues. The active role of p53 in provoking the detrimental side effects of cancer therapy has opened up the possibility of developing specific p53 inhibitors that can be used as radiotherapy and chemotherapy adjuvants to reduce these side effects. It would be ideal to identify p53 inhibitors that could suppress p53-associated pathologies without perturbing p53 tumor suppressor function. Previously, we established that p53 transactivation domain 1 (TAD1) is critical for inducing cell cycle arrest and apoptosis in response to acute DNA damage but is dispensable for p53-mediated tumor suppression in various mouse cancer models (*3, 4*). Based on these observations, we hypothesize that inhibition of p53 TAD1 will selectively inhibit p53-dependent, radiation-induced cell death and subsequent normal tissue damage during radiation and chemotherapy of p53-negative cancers. Towards this end, we propose to perform a high-throughput chemical library screen to identify p53 TAD1 inhibitors by generating and using a mouse embryonic fibroblast reporter system that expresses dual color luciferase or dual fluorescent reporters under the control of p53 TAD1-dependent and independent target gene regulatory elements.

Keywords

p53 inhibitor, p53 transactivation mutants, p53 transcriptional activation domain inhibitor

Overall Project Summary

Specific Aim 1: To generate mouse embryonic fibroblasts (MEFs) stably expressing p53-inducible fluorescence reporters for screening

Our major goal is to identify p53 transactivation domain 1-specific inhibitors by generating and subjecting reporter-expressing MEFs to a chemical library screen. As described in last year's progress report, we plan to generate reporter-expressing cells expressing two reporters, one under the governance of p53 responsive regulatory sequences of a p53 TAD1-dependent gene, *Cdkn1a* (*p21*), and one under the control of a TAD1-independent gene, *Crip2*. We are generating cell lines with color luciferase, fluorescent, and firefly luciferase reporters to ensure the highest chance of ultimate success with the screen. After testing different approaches and consulting with individuals with great expertise

in small molecule screening, we have settled on the optimal strategy to generate the cell lines. Here we present an update on the construction and validation of these reporter cell lines as well the steps we have taken to initiate the screen.

Task 1a. Construct cell lines stably expressing p53 reporters, to use in chemical library screening

Summary of changes to subtasks

1. Make MEFs of various genotypes: As described in the last progress report, we have made immortal Arf null MEFs for generating the reporter cell lines, to allow us to propagate the cells more efficiently.
2. Make retroviral constructs: We have significantly upgraded our strategy to generate the reporter cell lines. Instead of using retroviral constructs, we have harnessed the latest genome editing tools of the CRISPR/Cas9 (Clustered Regulatory Interspaced Short Palindromic Repeats) system to generate immortalized MEF reporter lines that stably express reporters under the control of the endogenous genes. The CRISPR/Cas9 system, which was recently developed, represents a superior system to the retroviral one because we can introduce the reporter into the endogenous locus in a homogeneous fashion in all cells. This contrasts with the retroviral approach, where integration of the reporters is random in each cell, leading to the generation of a population of cells carrying different retroviral reporter constructs. Thus, even though using the CRISPR/Cas9 system is inherently a longer process, the use of this approach represents a superior strategy that would ensure a higher probability of success in the project.
3. Generate cell lines retrovirally infected with constructs: As mentioned above, we are using CRISPR/Cas9 system to generate the cell lines instead of the archaic retroviral strategies.

A more detailed description of our progress for Task 1a is included below.

Generation of reporter lines in Arf-/- immortalized MEFs.

As described in our previous annual report, we are working toward generating a dual-luciferase (click-beetle Green and Red luciferases) reporter system that would facilitate the identification of p53 TAD1 inhibitors through a single-step luciferase assay-based high-throughput screen of small molecule libraries. As an alternative strategy, we are also constructing a dual-fluorescent reporter system (TdTomato and EGFP) that could additionally be utilized to perform image-based screens.

To initiate the construction of the dual-luciferase and dual-fluorescent reporter lines, we first generated the p21-Green Luciferase and p21-tdTomato reporter cell lines. As described in the previous report, we harnessed the genome editing strategies of Transcription activator-like effector nucleases (TALENs) and the

more recently developed CRISPR/Cas9 system that enable homology-directed repair at a locus of interest. To target the p21 locus with a promoterless click-beetle luciferase or tdTomato reporter, we constructed four pairs of TALENs, 3 CRISPRs and a series of targeting vectors as described in the previous report. We also optimized a nucleofection protocol for introducing these plasmids into Arf null immortalized MEFs. In this report, we describe the successful generation of p21 and Crip2 reporter lines that we achieved over the past year.

First, to determine whether TALENs or CRISPRs were more suitable for inducing homologous recombination at the p21 locus, in the past year we compared their targeting efficiencies by assessing the expression of tdTomato reporter in cells nucleofected with the p21-tdTomato targeting vector together with either p21 TALEN pairs or p21 CRISPR expression plasmids. We found the p21 CRISPRs to be significantly more effective in inducing homologous recombination at the p21 locus compared to the TALENs (data not shown). Hence, we chose the CRISPR/Cas9 system as a preferred method for any subsequent gene targeting experiments.

To generate the p21-Green Luciferase and p21-tdTomato reporter lines, we targeted the p21 locus using p21 locus-specific CRISPR guide RNAs (CRISPRs) and a donor DNA cassette containing either the Green Luciferase or the tdTomato gene replacing the first expressed exon of p21 (Exon 2). The donor cassette also included a Puromycin gene for the purpose of selection of recombinants **(Figure 1A)**. We designed the CRISPRs using the CRISPR design tool from http://crispr.mit.edu/. Three p21 CRISPRs were identified using this tool **(Figures 1B)** and all were tested for their targeting efficiency by assessing integration of the tdTomato reporter (data not shown). p21 CRISPR 3 demonstrated the highest targeting efficiency, as determined by FACS analysis of the p21- tdTomato line.

In the past year, we also optimized a low oxygen cell culture protocol for single cell cloning of the reporter MEF lines. To obtain a clonal population of p21-Green Luciferase line, cells were plated at single cell density in 96-well plates and grown for approximately 3 weeks. This allows us to sub-clone specific cell lines to isolate the correctly targeted population as assessed by Southern blotting (**Figure 2**). Proper integration of each reporter into the cell lines has been verified by Southern blotting analysis of the 5' and 3' ends of the locus of interest.

We have also generated Crip2-Red Luciferase and Crip2-tdTomato lines with Crip2 CRISPRs and donor cassettes containing either Red Luciferase or tdTomato genes using an approach similar to p21 targeting described above (**Figure 3**). We generated a separate Crip2-Red Luciferase reporter line instead of immediately retargeting the p21-Green Luc line to successfully separate the red and green luminescent signals on a luminometer by performing filter corrections to factor out the tail of the green luminescent signal from a red luminescent measurement. Reading green luminescence from a red luciferase

only-expressing line and vice-versa allows for determination of the corrected amount of luminescence.

The dual-color click beetle luciferase system has been specifically developed and optimized for high-throughput screens. However, we experienced some difficulties with parsing out the red and green luciferase signals in our reporter cell lines using the instrumentation accessible to us. We are therefore also continuing with the development of the dual fluorescent reporter cell lines. We also propose to develop an inferior, yet reliable, strategy of a single-luciferase (Firefly Luciferase) reporter system in the case of insurmountable technical difficulties with the dual reporter systems. This reporter would not allow assessment of two reporters in the same cell but two parallel screens could be constructed in Arf-/- cells with the p21-ff-luc and with the Crip2-ff-luc reporters. Importantly, this reporter system is absolutely certain to work.

In the past year, we have generated the following immortalized MEF reporter cell lines and the others are in progress.

a) p21-GFP-Puro
b) p21-tdTomato-Puro
c) p21-Green Luciferase-Puro
d) Crip2-tdTomato-Puro
e) Crip2-Red Luciferase-Puro

In the past year, we have also generated the following vectors for introducing reporters at the p21 or Crip2 loci, and these are ready for targeting:

a) p21-Firefly Luciferase-loxP-UbC-Puro-loxP
b) Crip2-tdTomato-loxP-UbC-Hygro-loxP
c) Crip2-Red Luciferase-loxP-UbC-Hygro-loxP
d) Crip2-Firefly Luciferase-loxP-UbC-Puro-loxP

Specific Aim 2: To conduct chemical library screen for compounds inhibiting *p21* but not *Bax* fluorescence reporter activity in wild-type MEFs upon DNA damage treatment

As described in last year's progress report, we made a few technical changes to this aim to enhance the probability of success with our screens. To summarize, first, we are using a *Crip2* gene reporter instead of a *Bax* reporter as the p53 TAD1-independent reporter. Second, we are using immortal *Arf* null MEFs instead of wild-type MEFs to construct the reporter cell lines. Finally, in addition to dual color luciferase reporters, we are generating cells expressing dual color fluorescent reporters and firefly luciferase reporter cell lines for our high-throughput screening, to provide potential backup approaches in case of any difficulties. We have also had extensive discussions with the director of the

Stanford High-Throughput Bioscience Center to guide the design of the reporter cell lines to ensure success in the screen. Importantly, the Stanford High-Throughput Bioscience Center (HTBS) has the instrumentation for the dual color luciferase-, the dual fluorescent, and the firefly luciferase reporter-based high-throughput screens, allowing us to use any of these approaches.

In the past year, to prepare for screening, we characterized the p21-Green Luciferase reporter cell line described above to define the optimal number of cells for screening by analyzing the reporter activity upon titrating a mixed population of cells (data not shown). We observed that as a few as 1000 cells was sufficient to detect robust luciferase activity that was responsive to doxorubicin DNA-damage treatment (**Figure 4**). This number of cells is optimal for plating in a 384-well plate format that will be used for screening. We will therefore initiate our screen with this number of cells per well.

Specific Aim 3: To confirm that the candidate compound(s) inhibits p53-dependent acute DNA-damage responses and improves prostate cancer treatment in mouse models

No changes.

Key Research Accomplishments

Nothing to report

Conclusions

Our laboratory has provided the first identification of a mechanism distinguishing p53 action in acute DNA damage responses and in tumor suppression, which we leverage here to identify compounds that could be used to mitigate side effects of prostate cancer therapies without causing risk of additional cancer development. Identification of TAD1 inhibitors could allow protection from radiation-induced toxicity to normal tissue, thereby allowing enhanced doses of radiation to be administered and enhancing prostate cancer treatment. The experiments described above are critical initial steps for performing a screen to identify such TAD1 inhibitors.

Publications, Abstracts and Presentations

Nitin Raj, Colleen Brady, Dadi Jiang, Stephano S Mello, Matthew Porteus & Laura D. Attardi. Identifying p53 Transactivation Domain 1 Inhibitors through CRISPR/Cas9-mediated Gene Editing & High-throughput Screening. Stanford University Cancer Biology Program 37th Annual Conference, Sep 12-14, 2014, Santa Cruz, CA (Poster presentation).

Inventions, Patents and Licenses

Nothing to report

Reportable Outcomes

Nothing to report

Other Achievements

Nothing to report

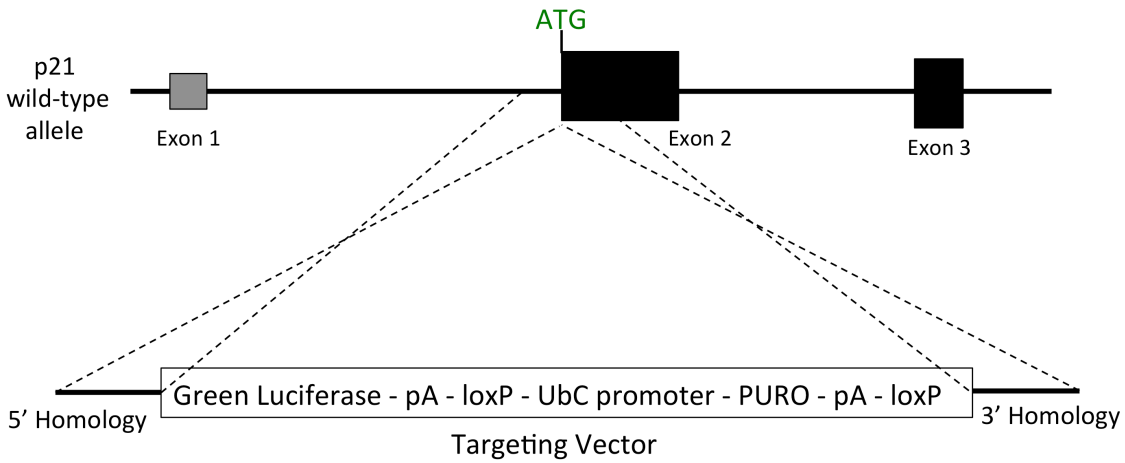
References

1. K. H. Vousden, C. Prives, Blinded by the Light: The Growing Complexity of p53. *Cell* **137**, 413 (May 1, 2009).
2. K. T. Bieging, S. S. Mello, L. D. Attardi, Unravelling mechanisms of p53-mediated tumour suppression. *Nature reviews. Cancer* **14**, 359 (May, 2014).
3. C. A. Brady *et al.*, Distinct p53 transcriptional programs dictate acute DNA-damage responses and tumor suppression. *Cell* **145**, 571 (May 13, 2011).
4. D. Jiang *et al.*, Full p53 transcriptional activation potential is dispensable for tumor suppression in diverse lineages. *Proceedings of the National Academy of Sciences of the United States of America* **108**, 17123 (Oct 11, 2011).

Appendices

Figure 1

A



B

```
                                Target sequence + PAM
p21 CRISPR guide 1: 5'- GATGTCCGACCTGTTCCGCA CAG -3'
p21 CRISPR guide 2: 5'- CTAGGCTCCGAACGCGCTCC CAG -3'
p21 CRISPR guide 3: 5'- CCGTGATTGCGATGCGCTCA TGG -3'
```

Fig.1 (A) Targeting scheme used to generate p21-Green Luciferase-Puro MEF line. Dotted gray lines indicate the targeted sites for homologous recombination around the p21 translation start site in Exon 2 **(B)** p21 CRISPR guide RNA sequences identified by the MIT CRISPR design tool.

Figure 2

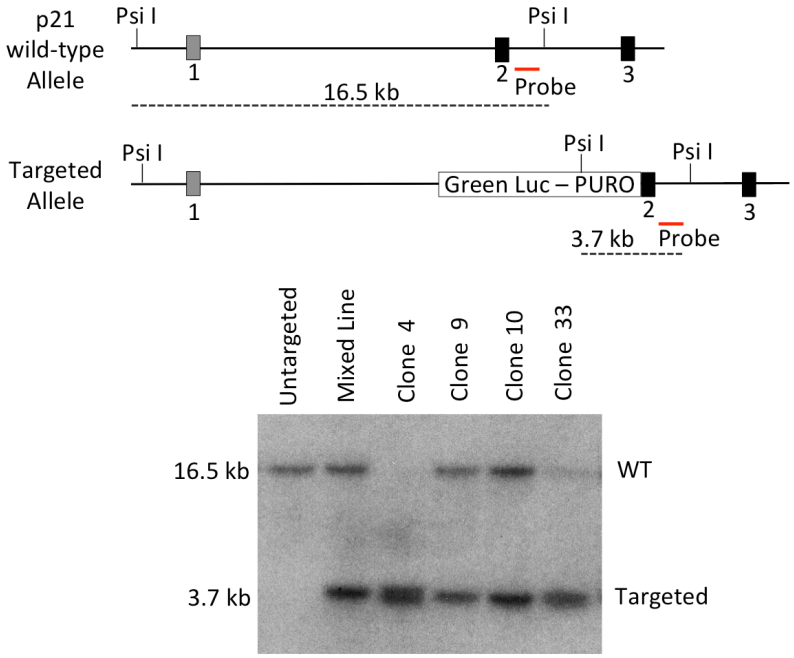
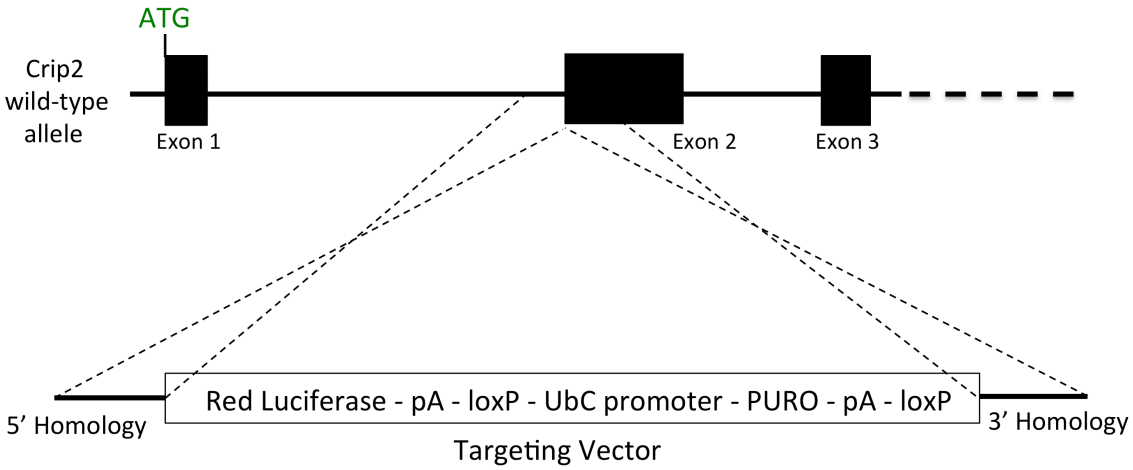


Figure 2. Southern blotting analysis of MEF cell lines expressing Green Luciferase under the control of endogenous p21 promoter. Genomic DNA was digested with PsiI and the Southern blot was probed with the 3' external probe indicated with red bar. Blot shows two correctly targeted heterozygous single cell lines (Clones 9 and 10) and two correctly targeted homozygous single cell lines (Clones 4 and 33) compared with an untargeted cell line shown in Lane 1.

Figure 3

A



B

```
                                  Target sequence + PAM
Crip2 CRISPR guide 1: 5'- ACTTGAGACAGAACTTGTGC CAG -3'
Crip2 CRISPR guide 2: 5'- ACTGGCACAAGTTCTGTCTC AAG -3'
Crip2 CRISPR guide 3: 5'- TGCAACAAGACACTGACCCC CGG -3'
```

Fig.3 (A) Targeting scheme used to generate Crip2-Red Luciferase-Puro MEF line. Dotted gray lines indicate the targeted sites for homologous recombination around the Crip2 Exon 2 **(B)** Crip2 CRISPR guide RNA sequences identified by the MIT CRISPR design tool.

Figure 4

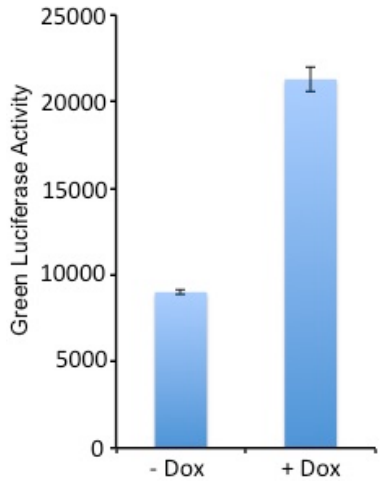


Figure 4. Analysis of CRISPR-generated MEFs with *p21-Green luciferase* reporter. Robust reporter activity was detected from one thousand cells in a microwell plate and this activity was induced by doxorubicin (Dox) treatment.