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14. ABSTRACT
Triple-negative breast cancer (TNBC) is an overly aggressive breast cancer subtype that disproportionately affects African American women. TNBC is characterized by a lack of expression of the estrogen and progesterone receptors as well as the absence of HER2 amplification. TP53 mutations are the only genetic correlate with poor clinical prognosis in this subtype. We found that p53 mutations in TNBC often coincided with deletion/silencing of the CDKN2A locus that encodes both the ARF and INK4A tumor suppressors. This genetic context was primarily present in African American women with TNBC. Concurrent loss of both p53 and CDKN2A function resulted in massive gains in proliferation and transformation of mouse and human mammary epithelial cells both in vitro and in vivo. These phenotypic tumor gains were the direct result of altered JAK1 and CDK4 activity. JAK1 and CDK4 cooperate to stimulate breast tumor cell proliferation. In our final report, we provide direct evidence that this pathway is altered in TNBC and that targeting this pathway might be a fundamentally plausible avenue for future TNBC treatments.

15. SUBJECT TERMS
Breast cancer, ARF, INK4A, p53, metastasis, CDK4, mammary epithelial cells, patient-derived xenografts

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1. INTRODUCTION

Triple-negative breast cancer (TNBC) is an overly aggressive breast cancer subtype that disproportionately affects African American women. Triple-negative breast cancer is characterized by a lack of expression of the estrogen and progesterone receptors as well as the absence of HER2 amplification/mutation. TP53 mutations are the only genetic correlate with poor clinical prognosis in this subtype. In our preliminary studies, we found that p53 mutations in TNBC often coincided with deletion/silencing of the CDKN2A locus that encodes both the ARF and INK4A tumor suppressors. Surprisingly, this genetic context was primarily present in African American women with TNBC. Concurrent loss of both p53 and CDKN2A function resulted in massive gains in proliferation and transformation of mouse and human mammary epithelial cells both in vitro and in vivo. These phenotypic tumor gains were the direct result of altered JAK1 (through loss of p53 and ARF) and CDK4 (through INK4A loss) activity. In this genetic context, JAK1 and CDK4 cooperate to stimulate breast tumor cell proliferation. For this reason, identifying these key growth-driving kinases is paramount to discovering novel combinatorial therapies for TNBC.

2. KEYWORDS

Breast cancer, ARF, INK4A, p53, metastasis, CDK4, mammary epithelial cells, patient-derived xenografts

3. ACCOMPLISHMENTS

Major Goals of the Project

There were four major goals for this project: 1) Establish a spontaneous TNBC mouse model; 2) Analyze 525 primary TNBC and 30 PDX TNBC tumors; 3) Determine whether TNBC cells are sensitive to JAK1 and CDK4 inhibition; and 4) Determine whether TNBC tumors in mice are sensitive to JAK1 and CDK4 inhibitors.

Goals Accomplished

MAJOR TASK 1: ESTABLISH A SPONTANEOUS TNBC MOUSE MODEL

Subtask 1: Generate TP53$^{fl/fl}$/CDKN2A$^{fl/fl}$/K14-Cre and TP53$^{fl/fl}$/K14-Cre mice. First, we were able to generate both colonies of mice for these studies and confirm each genotype through PCR analysis of isolated mouse DNA. Mice were housed in two separate colonies and followed for mammary tumor formation over 14 months. SUBTASK COMPLETE.

Subtask 2: Harvest mammary tumors from mice. We followed two separate colonies of *TP53$^{fl/fl}$/CDKN2A$^{fl/fl}$/K14-Cre* and *TP53$^{fl/fl}$/K14-Cre* mice for 14 months to allow for mammary gland tumor formation over time. Mice were euthanized based on the conditions set forth in our ACURO approved protocol. We observed a significant increase in tumor formation and death in mice where both p53 and ARF were deleted (*TP53$^{fl/fl}$/CDKN2A$^{fl/fl}$/K14-Cre*) (Figure 1A). Moreover, the double-deleted mice exhibited significant amounts of metastasis to the lung (Figure 1B). Upon visualization the *TP53$^{fl/fl}$/CDKN2A$^{fl/fl}$/K14-Cre* mice also displayed enormous tumor local invasion through the mammary glands (Figure 1C left, yellow lines) compared to *TP53$^{fl/fl}$/K14-Cre* mice (Figure 1C right, yellow lines). SUBTASK COMPLETE.

Subtask 3: Immunostain tumors. We harvested resulting mammary tumors from both genotypes of mice and fixed them in FFPE. These were then processed onto slides and immunostained with antibodies recognizing mouse ADAR1. We showed that tumors arising in *TP53$^{fl/fl}$/K14-Cre* mice did not exhibit elevated ADAR1 protein expression (Figure 2, far left

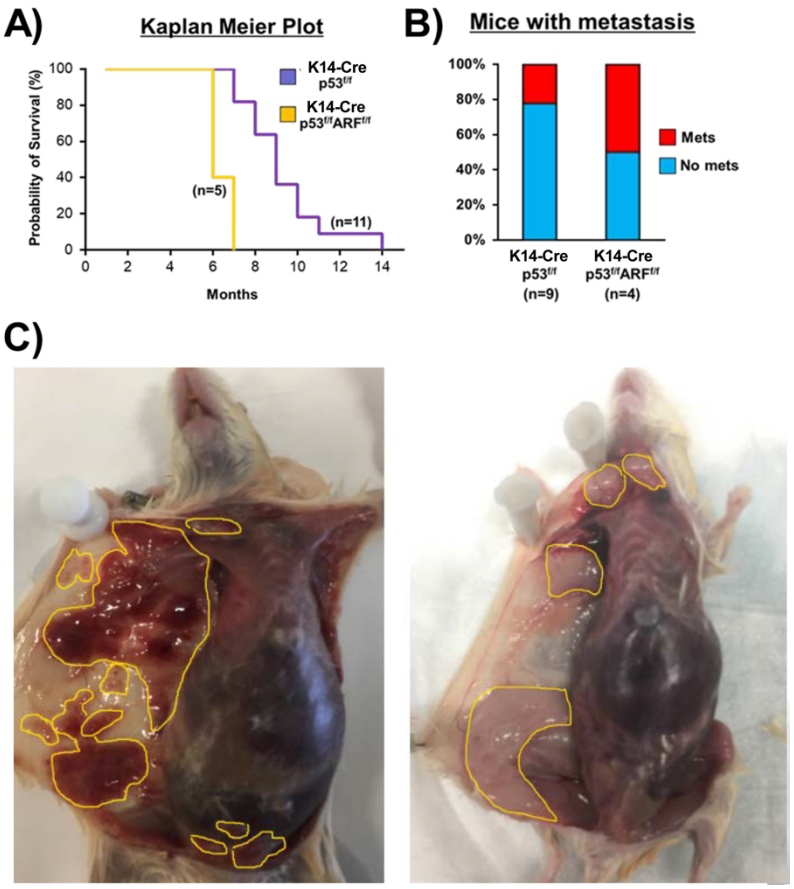

Figure 1. Spontaneous TNBC mouse model.

tumor) while those tumors extracted from *TP53$^{fl/fl}$/CDKN2A$^{fl/fl}$/K14-Cre* mice displayed elevated ADAR1 expression (Figure 2). SUBTASK COMPLETE.

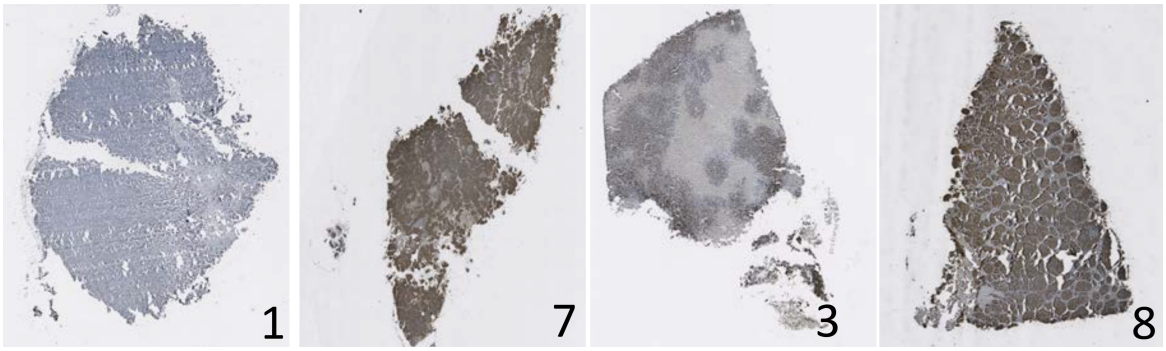

Figure 2. ADAR1 immunostained mammary tumors.

MAJOR TASK 2: ANALYZE 525 PRIMARY TNBC TUMORS AND 30 PDX TNBC TUMORS

Subtask 1: Generate tumor microarray from 525 TNBC samples. This task was performed solely by Dr. Ma. SUBTASK COMPLETE.

Subtask 2: Immunostain tumor microarray. Human TNBC samples were used to generate a 525-core tissue microarray. Tissues were immunostained with antibodies recognizing human ADAR1, p14ARF and p53. We completed the staining of all 525 patient tumor samples and show a single representative slide for each protein stain (Figure 3). SUBTASK COMPLETE.

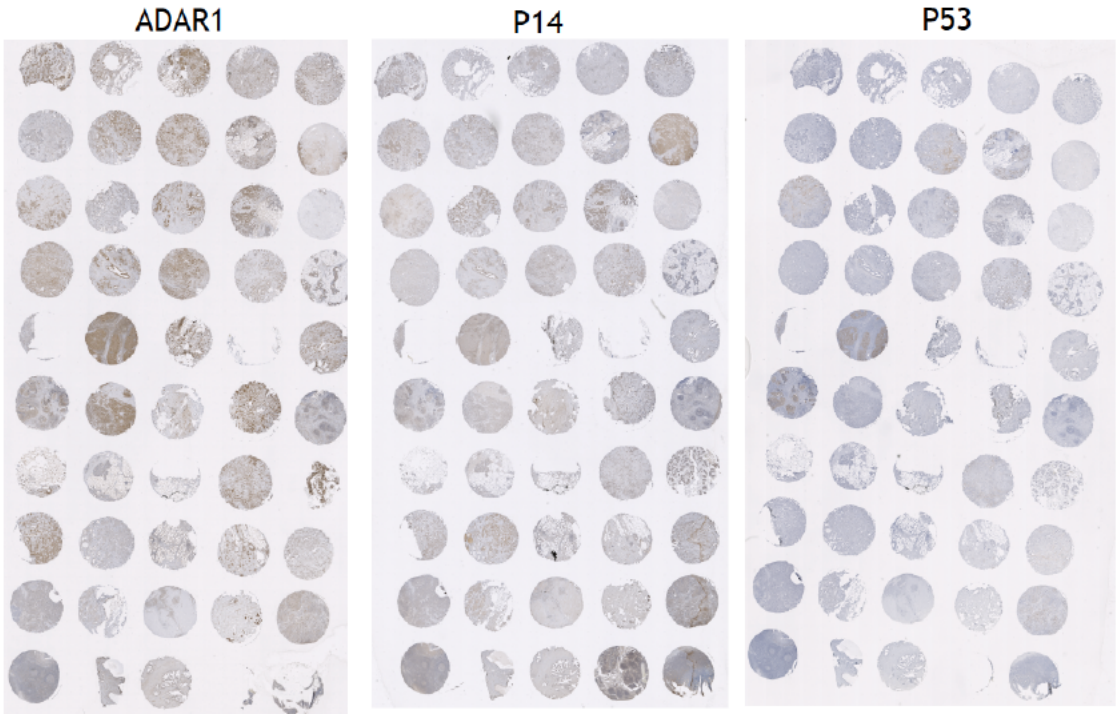

Figure 3. Immunostaining of human TNBC tumors using antibodies recognizing ADAR1, p14ARF and p53.

Subtask 3: Analyze tumor microarray staining. Tumor staining was analyzed by a board-certified breast pathologist to ensure that staining was computed for tumor only and not surrounding stroma. We concluded that 74% of patients exhibited elevated ADAR1 expression, 26% displayed high p14ARF expression, and 58% exhibited high p53 expression. SUBTASK COMPLETE.

Subtask 4: Stain and analyze 30 PDX tumors. We had proposed to analyze 30 PDX tumors for p53, ARF, INK4A, phospho-STAT1 and ISG15 proteins via immunohistochemistry. We have stained and analyzed 29 PDX tumors for p53, ARF and ISG15. As shown in Table 1, we have now double-blinded scoring for ISG15, ADAR1, p14ARF and p53 in all human xenografts. The WashU Human In Mouse (WHIM) xenografts are numbered in the far left column. The relative staining for each protein is provided as a numerical score with '0' being no staining and '3' being the highest level of staining. SUBTASK COMPLETE.

WHIM	ISG15	ADAR1	P14	P53
2	2	3	0	1
3	3	3	0	1
4	3	1	2	1
5	3	1	1	0
6	3	1	2	1
10	1	2	0	1
12	2	2	0	2
13	1	2	0	3
14	1	2	0	3
17	2	1	1	2
21	1	2	2	3
25	2	1	1	3
29	2	1	2	2
30	3	2	1	1
31	2	3	1	3
34	2	3	1	3
36	1	3	0	3
41	2	3	2	3
46	2	2	1	1
48	2	3	1	2
50	2	2	1	1
52	2	2	1	1
53	0	1	1	2
54	2	1	1	0
55	1	0	1	1
65	2	2	1	0
68	1	1	2	1
69	3	1	1	0
71	2	2	2	2
Total Positive	21	18	9	14
Percent Postive	72.4138	62.069	31.0345	48.2759

Table 1. Immunostaining of PDX TNBC tumors using antibodies recognizing ISG15, ADAR1, p14ARF and p53.

MAJOR TASK 3: DETERMINE WHETHER TNBC CELLS ARE SENSITIVE TO JAK1 AND CDK4 INHIBITION

Subtask 1: Establish in vitro proliferation assays for established breast cancer cell lines. We cultured four established human breast cancer cell lines that we received commercially from ATCC: HCC1806, DT549, MDA-MB-231, and MDA-MB-157. Cells were initially cultured in varying amounts of fetal bovine serum without any supplements. Proliferation was measured as an increase in total cell number by counting subsequent cells on each day after the initial plating. Our results show that 10% serum is optimal for proliferation and that the

HCC1806 cells have the most rapid proliferation rate of all four established cell lines (Figure 4). SUBTASK COMPLETE.

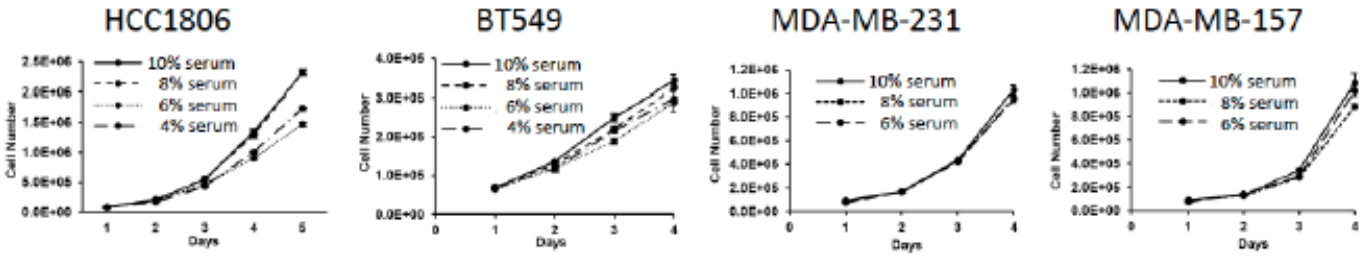

Figure 4. Establishing the proliferation rates of established human breast cancer cell lines.

Subtask 2: Treat cell lines with drugs and measure proliferation and apoptosis. We proposed to treat established breast cancer cell lines with JAK1 and CDK4 inhibitors alone or in combination. We have begun these experiments. We plated equal numbers of four established human breast cancer cell lines and treated each cell line with varying concentrations of the selective JAK1 inhibitor, itacitinib. Cells remained in drug for three days and remaining cells on each plate were counted. Using this method, we were able to establish an IC50 for each cell line for itacitinib treatment (Figure 5). While the IC50 varies, the concentrations are all quite similar. We decided to use concentrations of Palbociclib that have been previously used by other groups for the treatment of breast cancer cell lines. SUBTASK COMPLETE.

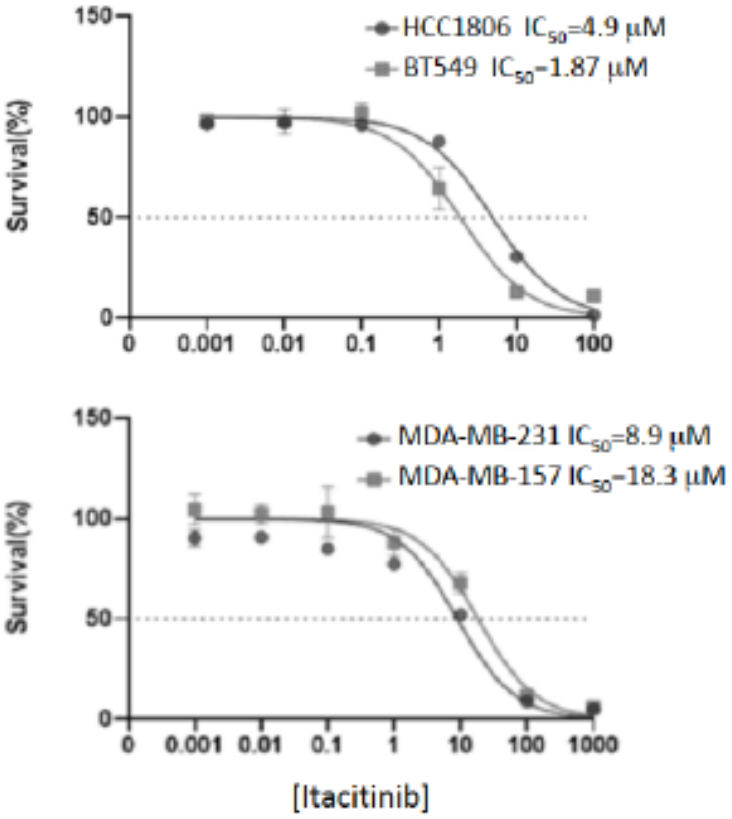

Figure 5. Treatment of breast cancer cell lines with JAK1 inhibitor.

We next cultured three established human breast cancer cell lines that we received commercially from ATCC: HCC1806, SKBR3, and MDA-MB-231. Cells were initially cultured in complete media and then treated with the indicated amounts of drug. We had to switch to Ruxolitinib due to Covid-19 restrictions on our original supplier for Itacitinib. Proliferation was measured as an increase in total cell number by counting subsequent cells 48 hours after the initial treatment. Our results show that the two TNBC cell lines, HCC1806 and MDA-MB-231, are sensitive to both Ruxolitinib (JAK1 inhibitor) and Palbociclib (CDK4 inhibitor), while the HER2+ cell line, SKBR3, is largely resistant (Figure 6). Moreover, the proliferation of the HCC1806 TNBC cell line appears to

be synergistically inhibited by the combination of Ruxolitinib and Palbociclib treatment (Figure 6). SUBTASK COMPLETE.

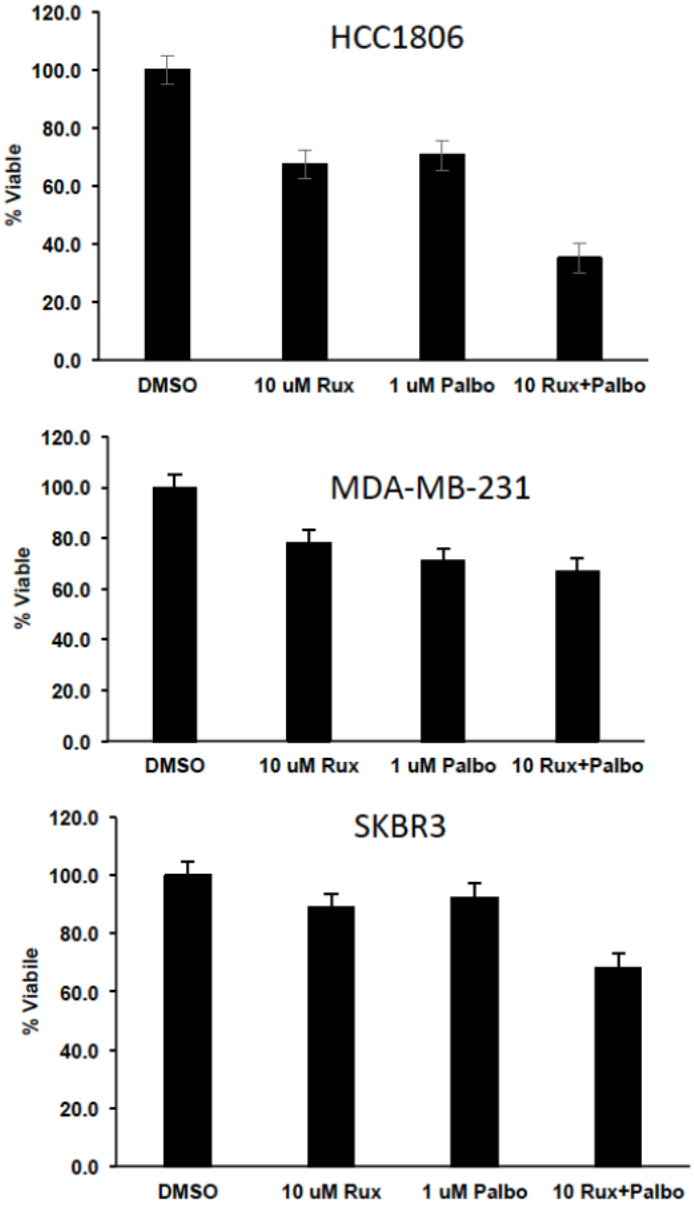

Figure 6. Treatment of breast cancer cell lines with CDK4 and JAK1 inhibitors.

MAJOR TASK 4: DETERMINE WHETHER TNBC TUMORS IN MICE ARE SENSITIVE TO JAK1 AND CDK4 INHIBITORS

Subtask 1: Treat TP53$^{fl/fl}$/CDKN2A$^{fl/fl}$/K14Cre mice with JAK1 and CDK4 inhibitors. We began the treatment of animals with inhibitors at 8 weeks of age. Mice were monitored for mammary tumor growth (using calipers to measure tumor size up to 2cm) over a span of 22 weeks. We did not observe any palpable mammary tumors in any of the mice where we had deleted p53 and ARF and had treated with palbociclib alone or in combination with Ruxolitinib. However, all the mice treated with vehicle or Ruxolitinib alone developed tumors by 22 weeks. SUBTASK COMPLETE.

Subtask 2: Treat PDX mice with JAK1 and CDK4 inhibitors. For PDX treatments, fresh xenografts were allowed to establish up to 1cm in size prior to dosing. PDX mice were monitored 4-6 weeks for mammary tumor growth (using calipers to measure tumor size up to 2cm). Similar to the results from the spontaneous mouse model above, PDX tumor treated with either Palbociclib alone (Figure 7, middle column) or in combination with Ruxolitinib (Figure 7, far right column) resulted in a significant attenuation of tumor size compared to vehicle (Figure 7, left column). SUBTASK COMPLETE.

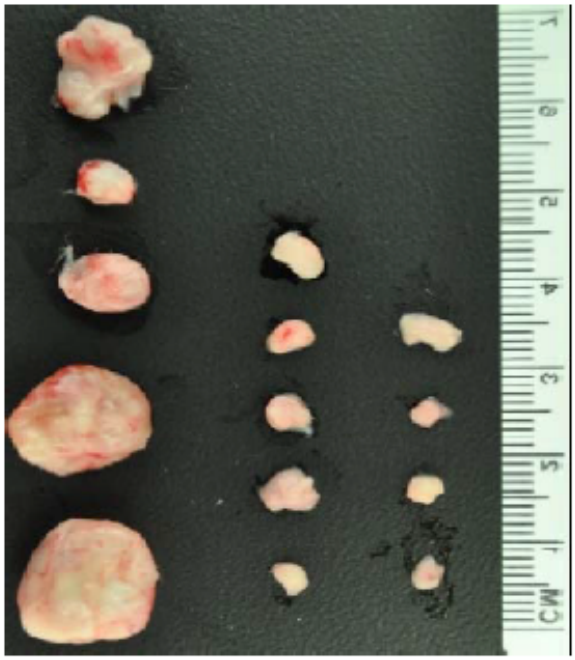

Figure 7. Treatment of five PDX TNBC tumors with CDK4 and JAK1 inhibitors.

Subtask 3: Analyze tumors from treated mice by immunohistochemistry. Due to Covid-19 protocols established at Washington University that severely limited our amount of time in the animal facilities and laboratory, we were unable to complete this subtask. SUBTASK INCOMPLETE.

Training Opportunities
Nothing to Report

Result Disseminated to the Community
I participated this past year in disseminating our initial findings to three independent groups of large donors to the American Cancer Society, including the CEO of the ACS. These donors visited my laboratory at Washington University where I discussed the research in this grant proposal and how our results were moving the field of breast cancer research forward. We engaged in a question-and-answer session where the donors queried me on the clinical impact of this work. I anticipate doing this laboratory tour again next year and have already been asked by the American Cancer Society to do so. I also was the keynote speaker at the American Cancer Society Strides Against Breast Cancer event. I was also able to present our findings to both the Dean of the medical school and his council as well as the Alumni Council of the medical school. We presented our initial findings at the San Antonio Breast Cancer Conference and the Cancer Biology Training Consortium in 2019.

4. IMPACT

Impact on Principal Discipline
Our current work will be incredibly impactful for those studying breast cancer aggression in vitro and in vivo. We have uncovered a novel pathway underlying the ability of breast cancer epithelial cells to proliferate at a high rate and readily form transformed colonies in soft agar. These are all hallmarks of aggressive tumors. We completed our studies by treating tumors in vivo with inhibitors of CDK4 and JAK1, hoping to underscore the importance of this pathway in tumor aggressiveness and metastasis.

Impact on Other Disciplines
Nothing to Report

Impact on Technology Transfer
Nothing to Report

Impact on Society

We have disseminated the data and ideals from this grant proposal to several groups in the St. Louis community. They were encouraged by our progress and excited about the future clinical impact our work might provide.

5. CHANGES/PROBLEMS

Changes in Approach

Nothing to Report

Anticipated Problems or Delays

Due to COVID-19, the lab was operating at 20% capacity from May 15, 2020-July 15, 2020, then 50% capacity from July 16, 2020-September 30, 2020, 80% capacity from October 1, 2020-February 28, 2021, and finally 100% capacity after March 1, 2021. This caused an extreme delay in animal experiments that were proposed in Major Task 4. As such, we initiated but were unable to complete Major Task 4, Subtask 3.

Changes in Human, Animal, Biohazards and/or Selective Agents

Nothing to Report

6. PRODUCTS

Publications, Conference Papers and Presentations

The work was presented at the following conferences:

- Dean's Council, Washington University (Oral Presentation)
- Alumni Council, Washington University (Oral Presentation)
- San Antonio Breast Cancer Meeting (Poster Presentation)
- Cancer Biology Training Consortium, Baltimore, MD (Oral Presentation)

Kung CP, Cottrell KA, Ryu S, Bramel ER, Kladney RD, Bross EA, Freeman EC, Sabloak T, Maggi LB, **Weber JD**. Evaluating the therapeutic potential of ADAR1 inhibition for triple-negative breast cancer. *Oncogene.* 2021 Jan 40: 189-202. PMCID: PMC7796950.

Internet Sites

Nothing to Report

Technologies or Techniques

Nothing to Report

Inventions, Patents and/or Licenses

Nothing to Report

7. PARTICIPANTS

Individuals That Have Worked on Project

Name:	Jason D. Weber
Project Role:	PI
Nearest person month worked:	1.2
Contribution to Project:	Dr. Weber served as the mentor for Dr. Kung in planning all experiments and overseeing the final data analysis.
Funding Support:	NIH R01CA190986

Name:	Shunqiang Li
Project Role:	Co-Investigator
Nearest person month worked:	0.6
Contribution to Project:	Dr. Li is growing all of the patient-derived tumors for both specific aims
Funding Support:	None

Name:	Pat Kung
Project Role:	Staff Scientist
Nearest person month worked:	12
Contribution to Project:	Dr. Kung was responsible for all of the mouse breeding (Task 1) in vitro assays in year 2 (Major Task 3) and the immunohistochemistry (Major Task 2).
Funding Support:	None

Changes in Active Other Support for PD/PI

Restoring functional ARF tumor suppression in non-small cell lung carcinoma with peptide mimics
Principal Investigator: Weber
Agency: DOD
Type: Lung Cancer Concept Award (GRANT13136080)
Period: June 1, 2021 – May 31, 2022
Effort: 0.60 person months (Calendar)
Country: USA
Annual Direct Costs:
Specific Aims:
Aim 1. To restore ARF tumor suppression in non-small cell lung carcinoma.

Other Organizations Involved as Partners

Nothing to Report