AWARD NUMBER: W81XWH-15-1-0388

TITLE: Viral Infection as a Secondary Driver of Aggressive Breast Cancer Growth and Metastasis

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REPORT DATE: Dec 2018

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
Dec 2018	Final	30 Sep 2015 - 29 Sep 2018

4. TITLE AND SUBTITLE
Viral Infection as a Secondary Driver of Aggressive Breast Cancer

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-15-1-0388

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
In the antiviral setting, type I IFN induction is predominantly triggered through the activation of cytosolic receptors that recognize double-stranded RNA (dsRNA). The dsRNA sensor retinoic acid-inducible gene I (RIG-I) contains a C-terminal DExD/H box RNA helicase domain which interacts with viral dsRNA. Its activation triggers downstream signaling cascades that lead to type I interferon production. In this report, we show that in a p53 deficient setting, upon ARF loss, the canonical type I IFN pathway is activated. In our year 1 report, we demonstrate the critical role of RIG-I in regulating the induction of interferon-beta and its downstream activation of ISG15 expression. Moreover, we show that RIG-I is absolutely required for the enhanced proliferation and cellular transformation properties observed in cells lacking p53 and ARF. This work will be impactful to all breast cancer research trying to understand the relevant pathways that might imbue aggressive properties to tumor cells. This is a significant series of findings due to our ability to identify a novel pathway involved in tumor aggressiveness.

15. SUBJECT TERMS
p53, ARF, metastasis, invasion, RIG-I, interferon-beta

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified	10	19b. TELEPHONE NUMBER *(include area code)*

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1. INTRODUCTION

Metastasis of cancer cells from the breast to distal target organs is the single-most important cause of mortality in women with breast cancer. One of the most challenging aspects of eradicating breast cancer and increasing the survival rates of women with breast cancer is the prevention and/or treatment of metastasis. Arguably, breast cancers that do not metastasize become a far more manageable form of the disease. Recent next generation sequencing studies have confirmed that *TP53* mutations are the most frequent alterations in breast carcinomas, occurring in all subtypes of human breast cancer albeit at varying rates. Moreover, *TP53* mutation can enhance metastasis in basal-like tumors, providing evidence that the p53 tumor suppressor protein acts in part to prevent metastasis. However, loss of p53 alone in the mammary gland is often not sufficient for robust metastasis, suggesting that other regulatory mechanism act in concert with *TP53* mutation to stimulate metastasis. Identifying these key regulatory pathways is paramount to preventing life-threatening metastasis in breast cancer.

2. KEYWORDS

Breast cancer, ARF, p53, Interferon-beta, metastasis, RIG-I, mammary epithelial cells, patient-derived xenografts, invasion

3. ACCOMPLISHMENTS

Major Goals of the Project

There were two major goals for this grant proposal: 1) Determine whether activation of the anti-viral RIG-I pathway was required and/or sufficient for breast cancer invasion and metastasis, and 2) determine whether a direct viral infection or type I interferon release stimulated breast cancer invasion and metastasis.

Goals Accomplished

MAJOR TASK 1: SUBTASK 1: The two most upstream components of the antiviral immune response are the RNA helicases retinoic acid-inducible gene-I (RIG-I) and melanoma differentiation-associated gene 5 (MDA5). They act as double-stranded RNA (dsRNA) sensors, and once bound to dsRNA undergo conformational changes that trigger K-63 mediated ubiquitination through the E3 ligase TRIM25. This activation triggers a downstream cascade ending in the induction of an IFN-mediated immune response. To determine at what stage ARF regulates the type 1 interferon pathway we first looked at protein levels of RIG-I, MDA5, and TRIM25 in dp53R-shScr (deleted p53 /RasV12 transformed) and dp53R-shARF mouse mammary epithelial cells (MMECs). RIG-I, MDA5, and TRIM25 are all induced upon ARF knockdown (Figure 1A). Knock down of RIG-I with two shRNA hairpins returned RIG-I to basal expression levels and resulted in tremendous decreases in ISG15 expression (2-10-fold) in the setting of ARF loss (Figure 1B).

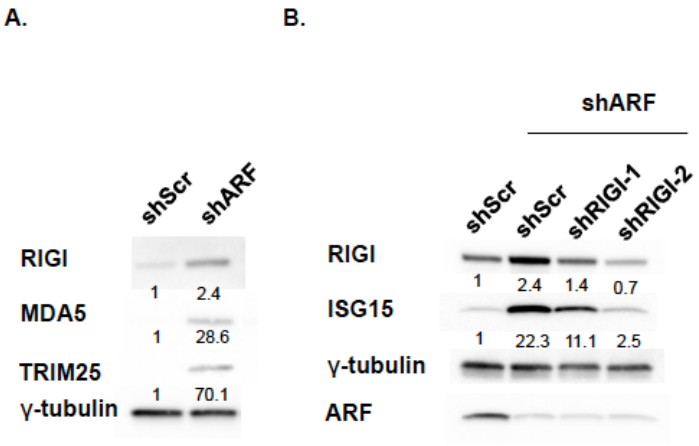


Figure 1. Induction of RIG-I and its role in ISG15 accumulation. **A**. dp53R MECs were transduced with shRNAs targeting scrambled (shScr) or ARF (shARF). Cells were lysed and proteins separated by SDS-PAGE and immunoblotted with the indicated antibodies. Numbers indicate fold-change. **B**. The same MECs were transduced with an addition shScr or shRNA targeting RIG-I (shRIGI-1 and -2). Cells were lysed and proteins separated by SDS-PAGE and immunoblotted with the indicated antibodies. Numbers indicate fold-change.

Loss of ARF in the dp53R cells results in a tremendous gain in IFN-β mRNA expression (Figure 2), demonstrating a role for ARF in tightly suppressing IFN-β production in cells. We knocked down RIG-I expression using two independent short hairpins and found that IFNβ mRNA levels were significantly reduced (Figure 2), indicating that induced RIG-I expression is required for the IFN-β induction following ARF loss.

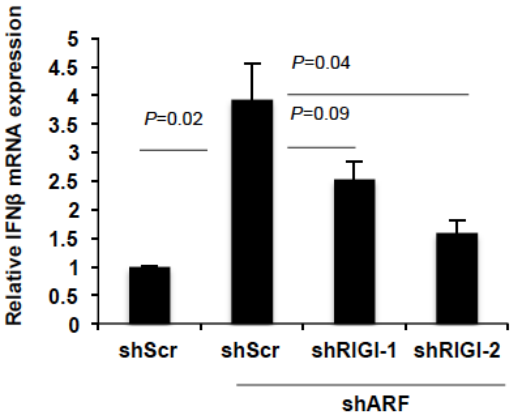


Figure 2. RIG-I expression is required for IFN-β induction in the absence of ARF. Dp53R cells were transduced with either shScr or shARF knockdown constructs. Cells knocked down for ARF were then transduced a second time with shScr or shRIG-I shRNAs. IFN-β mRNA expression was measure by qRT-PCR.

We next sought to determine the requirement for RIG-I in the enhanced proliferation and tumorigenesis of cells lacking ARF. Given our above results that clearly showed a requirement for RIG-I in IFN-b production and downstream signaling to ISG15, we fully expected to see a requirement for RIG-I in proliferation. Indeed, RIG-I levels were critical for increased short-term and long-term proliferation of dp53R-shARF MECs as measured by counting total cell numbers over four days (Figure 3, left panel) and by colony formation (Figure 3, right panel). Together, these findings point to a critical role of RIG-I in mediating the pro-proliferative gains seen in the absence of ARF.

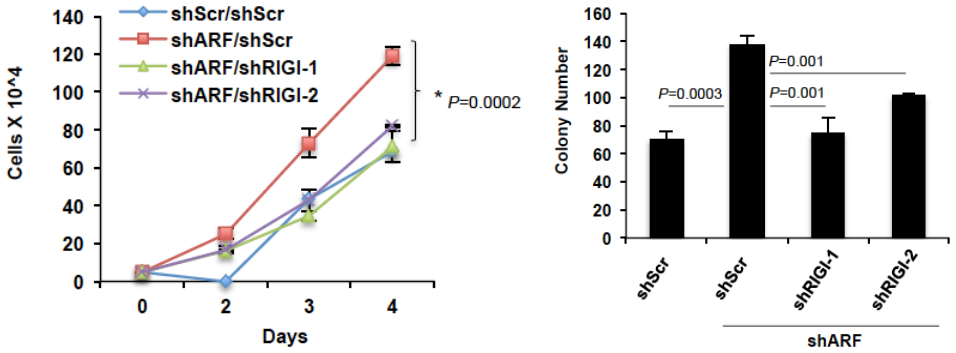


Figure 3. RIG-I expression is required for short- and long-term proliferation. Left panel: dp53R cells were first transduced with shScr or shARF knockdown constructs and then transduced a second time with either shScr or shRIG-I constructs. Cells were then plated (50,000) and counted every day for four consecutive days. Right panel: these same cells were plated at a low density (3,000 cells per 100mm dish) and cultured for 14 days. Resulting cell colonies were stained with Giemsa stain and counted.

We next sought to determine the impact of RIG-I on cellular transformation. Dp53R cells readily form colonies in soft agar and this transformed phenotype is extremely enhanced by knockdown of endogenous ARF (Figure 4). However, knockdown of RIG-I reverts these transformed cells back that observed in the parental dp53R cells that maintain high ARF levels. RIG-I knockdown reduced the transformative properties of dp53R-shARF MEFs significantly as seen in the severe reduction of soft agar colony numbers and sizes (Figures 4), indicating a significant role for RIG-I in promoting enhanced cellular transformation in cells lacking p53 and ARF.

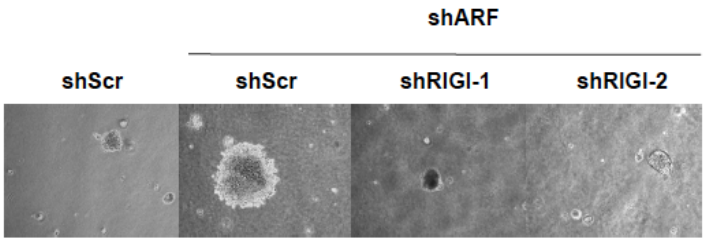


Figure 4. RIG-I expression is required for the transformation of ARF-deficient cells. dp53R cells were first transduced with shScr or shARF knockdown constructs and then transduced a second time with either shScr or shRIG-I constructs. Resulting cells were plated in semi-solid soft agar media. Colonies were visualized and counted 14 days later.

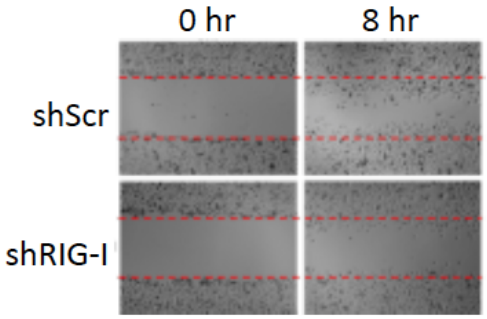


Figure 5. RIG-I expression is required for BT549 cell invasion. BT549 cells were first transduced with shScr or shRIG-I knockdown constructs and then plated Resulting confluent cells were then subjected to a large scratch (dotted red line) and allowed to fill this gap for 8 additional hours.

MAJOR TASK 1: SUBTASK 2:

We next established an in vitro assay typically used to determine the motility of cells. BT549 cells were seeded onto plates and infected with lentiviruses encoding shScramble (shScr) or shRIG-I aimed at reducing endogenous RIG-I expression. These cells were then plated ($1x10^5$) onto 6 well plates and confluency was reached 48 hours later. I small pipet tip was used to induce a wide (100 micron) scrape across the monolayer of cells. This resulted in the effective removal of cultured BT549 cells from this region and they were subsequently aspirated from the media. The remaining cells were cultured in fully supplemented media for 8 additional hours and allowed to move into the gap created by the scratch. As shown in Figure 5, control BT549 cells rapidly began to fill the open space while those cells expressing the shRIG-I construct were significantly slower in this regard. This suggests that BT549 cells require RIG-I expression to mediate cell motility and invasion into open space.

MAJOR TASK 1: SUBTASK 3:

In order to evaluate the ability of RIG-I to induce breast cancer cell invasion and motility in vivo, we generated MMECs that were devoid of p53 expression and thus immortal. These cells were then infected with lentiviruses encoding wild type RIG-I to drive its overexpression. Subsequently, control or RIG-I overexpressing cells were injected into the mammary fat pads of syngeneic host mice. Once the tumor reached 1 cm, it was excised and animals were monitored for an additional three weeks. Euthanized animals were subjected to analysis of distal organs including the lungs and liver. As shown in Figure 6, mice receiving control *p53*-null cells did not result in any detectable metastasis to the lung or liver. However, RIG-I overexpressing cells were quite capable of seeding these distant organs, resulting in significant metastatic burden in these animals.

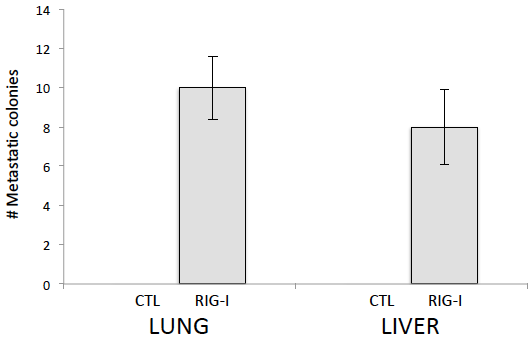


Figure 6. RIG-I expression drives metastasis. P53-null MMECs were first transduced with control (CTL) or RIG-I overexpression constructs and then injected into the mammary fat pads of syngeneic mice. Primary tumors were excised and distant organs analyzed three weeks later for signs of metastatic cell colonies.

MAJOR TASK 2: SUBTASK 1: We next established an in vivo model of metastasis using 4T1 cells. For these experiments, we injected $1x10^6$ 4T1 cells into the mammary fat pad of isogenic Balb/c mice. The parental 4T1 cells express significant amounts of RIG-I and IFN-beta. We also generated an additional 4T1 cell line where we successfully knocked down endogenous expression of RIG-I (Figure 7). These cells, 4T1-RIGkd (knocked down) were also injected into mammary fat pads of recipient mice. The injected mice were followed for 8 weeks and euthanized to analyze breast tumor cell metastasis. We utilized gross pathology to observe metastasis in this model system and report that 4T1 cells readily form large metastatic lesions in the colons of recipient mice while none were observed in 4T1-RIGkd recipient animals (Figure 8).

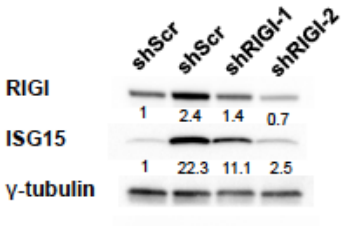
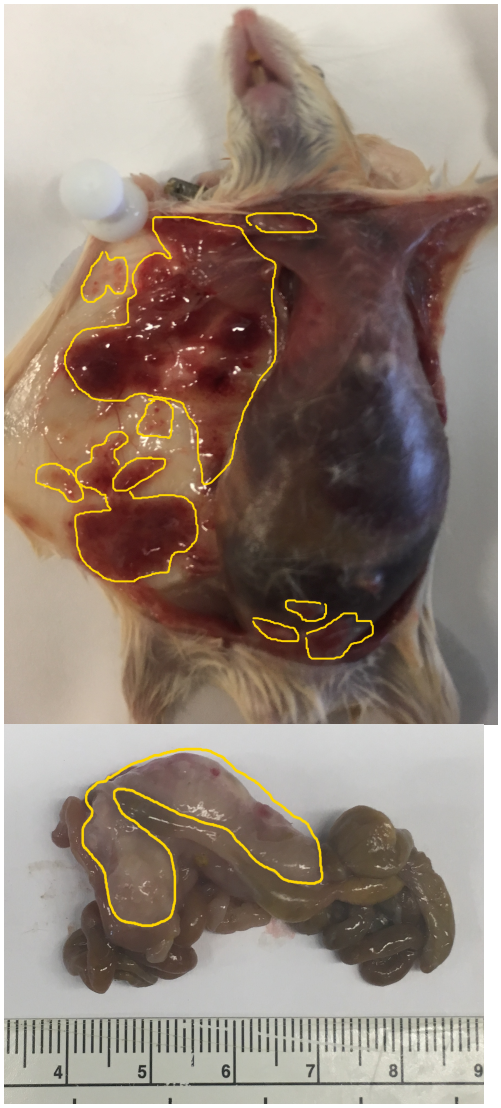


Figure 7. Knock down of RIG-I in 4T1 cells. Using two separate small hairpins, we successfully knocked down endogenous RIG-I expression in 4T1 cells. This also resulted in a significant decrease in ISG15 expression that is downstream of RIG-I. Fold change is indicated below each immunoblot.



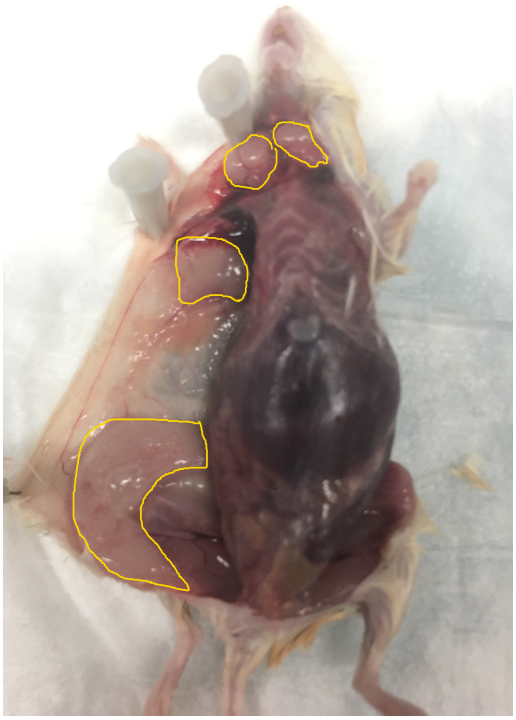


Figure 8. Metastasis of 4T1 cells requires RIG-I expression. Left upper panel, parental 4T1 cells were injected into a single mammary fat pad and monitored for 8 weeks. Animals were euthanized and analyzed for distal metastasis. Metastasis was apparent throughout the animal (outlined in yellow). Left bottom panel, additional metastatic lesions were observed as a large mass in the colon of 4T1 recipient mice. Right upper panel, 4T1-RIGkd cells injected into multiple mammary fat pads (outlined in yellow) grew but did not result in any obvious metastasis.

MAJOR TASK 2: SUBTASK 2: We infected three PDX cell lines with CMV titers to determine whether this viral infection would result in an induction of RIG-I expression and subsequent IFN-beta secretion. However, these experiments did not lead to any positive results. None of the three cell lines that were infected with CMV expressed any significant changes in either RIG-I expression or IFN-beta production. They also did not exhibit any changes in cellular motility or transformation properties. These results suggest that a simple infection is not sufficient to induce the oncogenic RIG-I pathway in the absence of functional p53. However, we were able to determine the overall affect of IFN-beta treatment on cell growth and proliferation. The addition of 2U IFN-beta to the culture medium of p53-null cells resulted in a dramatic increase in ISG15 and concomitant increases in cell proliferation and transformation (Figure 9). Treatment with 1000U of IFN-beta resulted in an even greater increase in ISG15, but rather displayed a decrease in proliferation and transformation. 1000U of IFN-beta is typically what has been observed during a viral infection. These data point to a threshold effect of IFN-beta production with a small amount (2U) mimicking the gains in proliferation and transformation seen with ARF loss and a large amount of IFN-beta (1000U) mimicking an inhibition of growth in response to a viral infection. This most likely also explains why we were unable to replicate our proliferative gains with CMV infection; CMV infection increased IFN-beta to a level that was inhibitory to cell proliferation.

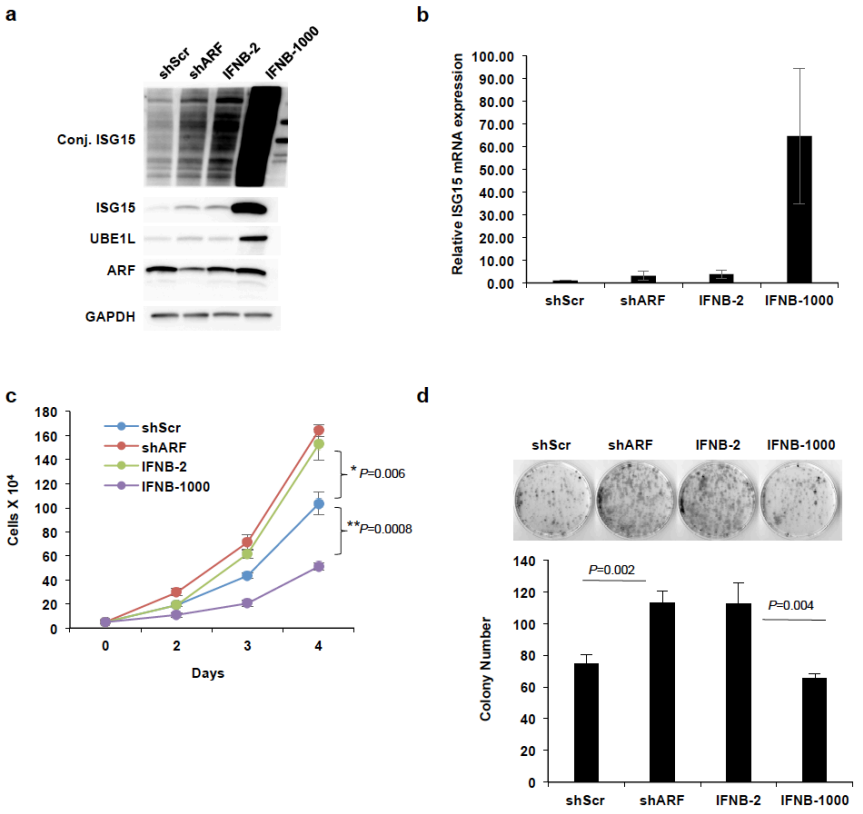


Figure 9. IFN-beta treatment transforms p53 mutant cells. Cells lacking p53 were treated with IFN-beta (2 or 1000U) and a) immunoblotted using antibodies recognizing ISG15, UBE1L, ARF and GAPDH; b) assayed for ISG15 mRNA expression; c) assayed for proliferation of cell number; d) plated for soft agar colony formation.

Training Opportunities
Nothing to Report

Results 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. 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 also was the keynote speaker at the American Cancer Society Strides Against Breast Cancer event.

Plans for Next Reporting Period
Nothing to report.

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.

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 including the American Cancer Society and Bridget's Brigade for breast cancer. 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
Nothing to Report

Changes in Human, Animal Biohazards and/or Selective Agents
Nothing to Report

6. PRODUCTS
Publications, Conference Papers and Presentations
We are currently completing a manuscript for submission using all of the data generated from this grant proposal.

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 Ms. Kuzmicki in planning all experiments and overseeing the final data analysis.
Funding Support:	NIH R01CA190986, NIHR01CA174743, W81XWH-15-1-0528

Name:	Catherine Kuzmicki
Project Role:	Graduate Student
Nearest person month worked:	12
Contribution to Project:	Ms. Kuzmicki performed all of the experiments outlined in specific aim1 & 2 for year 2

Name:	Leonard B. Maggi, Jr.
Project Role:	Research Assistant Professor
Nearest person month worked:	2.4
Contribution to Project:	Dr. Maggi was responsible for making the 4T1-RIGkd cells outlined in aim 2.
Funding Support:	NIH R01CA190986, NIHR01CA174743

Changes in Active Other Support for PD/PI
Nothing to Report

Other Organizations Involved as Partners
Nothing to Report