AWARD NUMBER: W81XWH-17-1-0410

TITLE: The Role of p53 Synthetic Lethality in Increased Chemosensitivity to DNA-Damaging Agents Conferred by the Exercise Myokine Irisin

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REPORT DATE: Oct 2018

TYPE OF REPORT: Annual

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
OCT 2018	Annual	15 SEP 2017 - 14 SEP 2018

4. TITLE AND SUBTITLE
The Role of p53 Synthetic Lethality in Increased Chemosensitivity to DNA-Damaging Agents Conferred by the Exercise Myokine Irisin

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-17-1-0410

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of New Mexico
Albuquerque, NM 87131- 0001

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

The goal of this project is to address the overarching challenge to revolutionize treatment regimens by replacing them with ones that are more effective and less toxic. Specifically, we are testing the idea that the exercise myokine Irisin can synergize with DNA damaging chemotherapeutics to induce cytotoxicity with less toxic concentrations of the chemotherapeutic. In year 1 we encountered technical challenges relating to the bioactivity of Irisin purchased from commercial sources. This challenge has been overcome by including a robust Irisin bioassay. Nevertheless, Irisin/chemotherapeutic combination therapy regimens used in vitro against the sensitive cell line MDA-MB-231 have yielded some significant yet modest decreases in cell viability, for reasons that we have yet to solve. We have therefore identified additional pathways impacted by Irisin that may play a critical role in attenuating breast cancer progression, including tumor cell migration and inflammation. We are therefore poised to make significant progress in year 2, after overcoming technical and experimental challenges in year 1.

15. SUBJECT TERMS

Breast cancer, irisin, combination therapy, p53 mutations, cytotoxicity, cell viability, migration, inflammation

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	11	19b. TELEPHONE NUMBER *(include area code)*

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1. Introduction

The goal of this project is to address the overarching challenge to revolutionize treatment regimens by replacing them with ones that are more effective and less toxic. Specifically, we are testing the idea that the exercise myokine Irisin can synergize with DNA damaging chemotherapeutics to induce cytotoxicity with less toxic concentrations of the chemotherapeutic. In year 1 we encountered some technical challenges relating to the bioactivity of Irisin purchased from commercial sources. This challenge has been overcome by including a robust Irisin bioassay. Nevertheless, Irisin/chemotherapeutic combination therapy regimens used in vitro against the sensitive cell line MDA-MB-231 have yielded some significant yet modest decreases in cell viability, for reasons that we have yet to solve. As a result, our plan for year 1, to assess the role of p53 mutations in Irisin-dependent cytotoxicity, have been delayed. In the meantime, we have identified additional pathways impacted by Irisin that may play a critical role in attenuating breast cancer progression, including tumor cell migration and inflammation. These bioactivities are being explored using in vitro assays. Our in vivo assays are progressing as planned, based on the approved IACUC protocol, and we will assess additional post-mortem outcomes based on these alternate pathways. Therefore, we are poised to make significant progress in year 2, after overcoming technical and experimental challenges.

2. Keywords

Breast cancer, irisin, combination therapy, p53 mutations, cytotoxicity, cell viability, migration, inflammation

3. Accomplishments

- **Major goals of the project -** For year 1 of the project, as stated in the SOW, the goals were to characterize the synergistic effects of irisin in conjunction with DNA damaging chemotherapeutics on breast cancer cell lines, with respect to proliferation, cytotoxicity, and apoptosis. In addition, we planned to characterize the ATR/Chk and DNA damage/cell cycle/mitotic catastrophe assays. Finally, in year 1 our goals were to obtain IACUC and ACURO approval, transduce cell lines for fluorescent and bioluminescent detection, and establish MD-MBA-231-xenografted NSG mice.

- **Accomplishments -** Once the PI transfer was approved and funding began, we obtained IACUC and ACURO approval for the animal experiments. In addition, we created transduced MDA-MB-231 cell lines for use in both in vitro and in vivo experiments. We then embarked on efforts to assess the ability of Irisin to synergize with chemotherapeutic drugs and decrease proliferation, increase cytotoxicity, and to determine the mechanism(s) for these effects. However, we encountered technical difficulty and for a period of time we were unable to see any irisin synergy with Doxorubicin or 5-Fluorouracil in co-treatment assays using the sensitive cell line, MDA-MB-231. We considered the possibility that commercial recombinant Irisin, a peptide, available from several sources including Caymen Chemical, R & D Systems, AdipoGen, and Phoenix Pharmaceuticals, could be labile, and therefore may lose bioactivity. Therefore, we identified a robust bioassay from the literature, based on the fact that 3T3-L1 preadipocyte cells can be induced to differentiate into adipocytes. In the presence of irisin, lipid deposition is retarded, and this effect can be easily quantitated by measuring binding of a lipophilic dye, Oil

Red O (1). This assay represents an in vitro model of Irisin-dependent transformation of white adipose tissue to brown adipose tissue due to its ability to upregulate gene products associated with lipolysis (1).

As shown in Figure 1, we were able to validate the bioactivity of commercial recombinant Irisin from Phoenix Pharmaceuticals using the 3T3 preadipocyte differentiation assay. Cells were induced to differentiation into adipocytes in the absence or presence of 2 nM or 20 nM Irisin. Cells were then fixed in paraformaldehyde and stained with 0.3% Oil Red O solution. Following staining and washing to remove excess stain, cells were photographed using Phase and fluorescence optics (Fig. 1A). To quantitate lipid accumulation, Oil Red O was extracted with isopropanol, and the eluate was measured by spectrophotometry at 450 nm (Fig. 1B). Addition of recombinant Irisin (Phoenix Pharmaceuticals) to the differentiation media significantly reduced lipid droplet formation in these cells, consistent with published results (1). These results confirmed the bioactivity of that lot of Irisin, and going forward we test each purchased lot to ensure bioactivity using this assay.

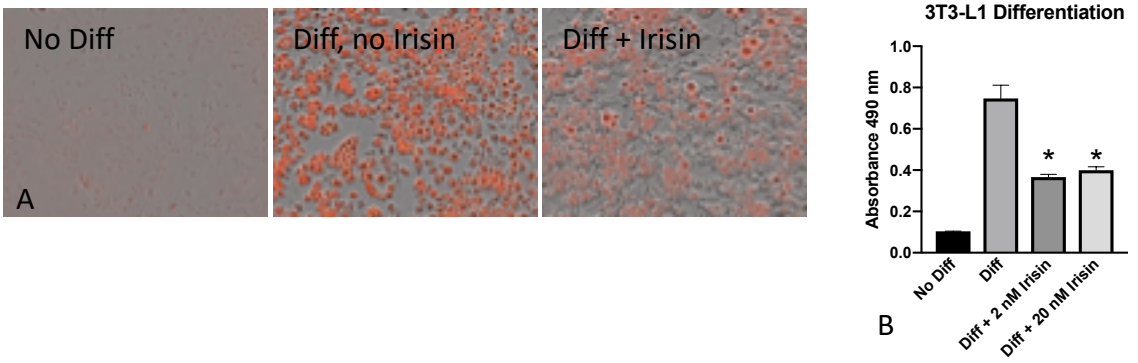

Figure 1: Irisin reduces fat droplet formation. 3T3-L1 adipocytes were cultured in standard media (DMEM, 10% FBS with antibiotics, "No Diff"), or in differentiation media (standard media containing 0.5 mmol/L isobutylmethylxanthine, 0.25 mmol/L dexamethasone, and 10 mg/L insulin, "Diff") with 0, 2 nM, or 20 nM irisin for 48 hours. **A**, Cultures were stained with Oil red O and photographed using a 10X phase contrast lens overlaid with a fluorescent filter (565-605 nm excitation) in an Incucyte® Live Cell Imaging System. **B**, Stain was quantified after isopropanol extraction in a spectrophotometer at 450 nm. Values are expressed as the mean ± SD of 3 experiments. *, $P < 0.0001$.

Using Irisin with confirmed bioactivity, we again tested for synergy with the standard chemotherapeutic drugs Doxorubicin (Dox) and 5-Fluorouracil (5-FU) in the Irisin-sensitive cell line MDA-MB-231. Cell viability was measured using the WST assay, which is based on mitochondrial dehydrogenase cleavage of the tetrazolium salt WST-1 to formazan dye. As shown in Figure 2, 2 nM Irisin led to a modestly reduced cell viability in combination with Dox, compared to Dox alone, which was statistically significant (Fig. 2A). In combination with low concentrations of 5-FU, there was a trend for 2 nM Irisin to reduce viability as well, however due to high variability the differences are not statistically significant (Fig. 2B). These are consistent with those reported in the proposal, but the effects are more modest. We have confirmed the more recent observations, as reported here, using several variations of cell

proliferation, cytotoxicity, and apoptosis assays (not shown). It is not clear why the magnitude of the Irisin:chemotherapeutic is more modest in recent assays, but because of this we have chosen to evaluate other potential molecular pathways suggested by RNAseq results. Although not all studies agree, there is gathering evidence for an anti-neoplastic role for Irisin in a variety of cancers, including the breast (2-6). Those studies have identified a number of anti-neoplastic

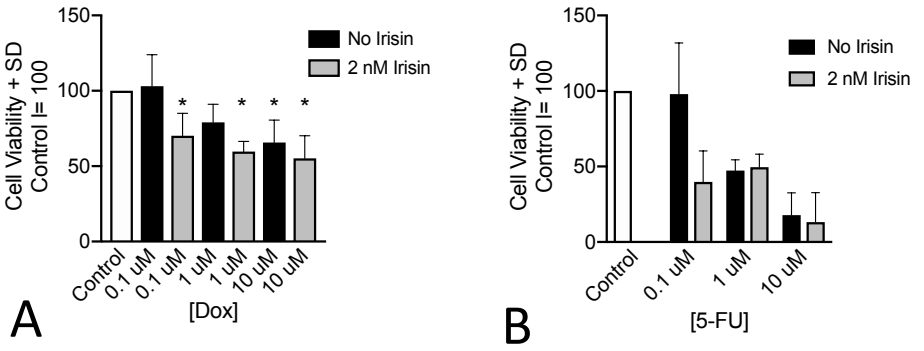

Figure 2. Irisin modestly reduces cell viability in combination with chemotherapeutic drugs. MDA-MB-231 cells were grown in the presence of a range of Dox or 5-FU concentrations, with or without 2 nM recombinant Irisin, for 48 hr. WST reagent was added for 2 hr and fluorescence was measured at 440 nm. **A**, Across a range of Dox concentrations, addition of 2 nM Irisin reduced cell viability compared to Dox alone. B. At 0.1 µM 5-FU, addition of 2 nM Irisin reduced viability, but the results were not statistically significant due to sample variability. No differences were seen with other concentrations of 5-FU with or without 2 nM Irisin. *, $P < 0.0001$.

molecular pathways impacted by Irisin.

As shown in Table 1, among the many gene expression changes identified by RNAseq comparing MDA-MB-231 cells incubated with and without 2 nM Irisin, transforming growth factor β2 (TGFβ2) is implicated in epithelial - mesenchymal transformation (EMT, 7). Irisin treatment significantly reduced TGFβ2 expression, and since EMT is a hallmark of malignancy, we asked if Irisin treatment could have a therapeutic impact. Using a scratch wound assay, we quantitated the ability of MDA-MB-231 cells to migrate into the wound area over time, in the presence or absence of Irisin at 0.1, 1, and 10 nM. As shown in Figure 3, Irisin slowed wound closure at all concentrations, and the differences were statistically significant. It should be noted that as with the cytotoxicity results, Irisin effects were modest.

Table 1		
Gene Name	Fold Change (+Irisin)	Adjusted P Value
TGFβ2	0.13	5.88E-83
TNFAIP8L1	0.43	1.05E-11
TNFRSF21	0.36	6.85E-23
HIF1α	0.26	1.86E-51

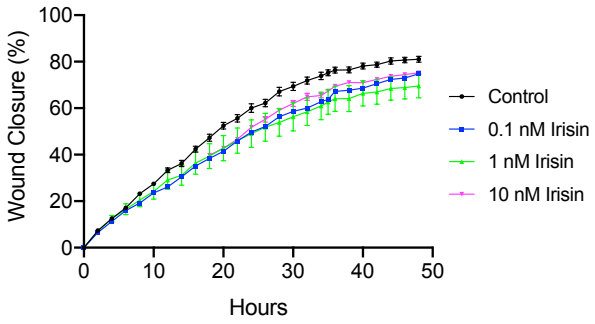

Figure 3. Irisin reduces MDA-MB-231 cell migration. Scratch wound assays were performed using the Incucyte® Live Cell Analysis System, and wound closure was measured over 48 hr in the presence of 0, 0.1 nM, 1 nM, or 10 nM Irisin. All concentrations of Irisin slowed wound closure by a statistically significant margin. $P < 0.0001$.

We have identified additional intriguing Irisin-dependent gene expression changes that suggest that Irisin could be acting to reduce inflammation and cellular metabolism pathways (Table 1). These include significant reduction in the expression of Tumor Necrosis Factor Alpha Induced Protein Like 1 (TNFAIP8L1), which contributes to inflammation after upregulation by TNFα (8), and TNF Receptor Superfamily Member 21 (TNFRSF21), which contributes to inflammation after upregulation by NF-κB (9). In addition, Hypoxia-Inducible Factor Alpha (HIF1α), which has been linked to elevated cellular metabolism in response to low oxygen, and inflammation (10), is significantly down-regulated.

Collectively our results thus far lead us to suggest that Irisin has the potential to act as an anti-neoplastic agent in breast cancer through multiple pathways, including cellular proliferation/survival, migration, and inflammation. Inflammation is increasingly being identified as a critical factor in cancer progression, including in the breast (11). Intriguingly, obesity is associated with both an inflammatory state, and with increased breast cancer risk (12). Irisin may emerge as one possible link between these outcomes, since irisin reduces adipocyte deposition as well as its proposed role in attenuating cancer progression (2). Inflammation in particular is optimally studied with in vivo models, and our in vivo assays are in progress. Although there are no results to report at this time, we can assess a variety of endpoints beyond those that were proposed, including inflammation, invasion, etc., without modifying our animal protocol, because these endpoints are all post-mortem. We will seek approval for any modifications to the use of animals that may result from these observations.

As a result of technical challenges with Irisin bioactivity and the unexpectedly modest synergistic effects of Irisin with chemotherapeutics on MDA-MB-231 cell cytotoxicity in recent assays, we have not met several goals for year 1 as specified in the SOW. We reasoned that more impactful outcomes could be achieved by evaluating other potential molecular pathways suggested by RNAseq results, and we are confident that these pursuits will yield highly significant results in year 2.

- References cited in Section 3, Accomplishments

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- **Training and professional development opportunities -** Although the project is not specifically intended to provide training opportunities, we have been fortunate to host an undergraduate scholar of the Minority Access to Research Careers (MARC) Program, Ms. Shania Sanchez. The MARC Program is an NIH-funded program to provide underrepresented minority (URM) undergraduate students training in STEM fields, to increase students' competitiveness for graduate programs, and to increase URM representation in STEM fields such as biomedical science. Ms. Sanchez's stipend is supported by the MARC Program, and she devotes approximately 15 hr/week to research in our lab. Ms. Sanchez is mentored by myself and the Research Specialist supported by this award, Ms. Rachel Earley. Ms. Sanchez's efforts are

integral to the project, and she will be co-author on manuscripts generated by these efforts in year 2.

- **Dissemination of results -** These results have been disseminated at a UNM-based undergraduate meeting of the MARC program in August 2018, and at the Society for Advancement of Chicano and Native Americans in Science (SACNAS) annual meeting in October, 2018, by Ms. Shania Sanchez. These venues exposed members of groups underrepresented in biomedical science

Plans during the next reporting period to accomplish the project's goals – Based on the unexpectedly modest synergistic effects of Irisin with chemotherapeutics on MDA-MB-231 cell cytotoxicity in recent assays, we have not met several goals for year 1 as specified in the SOW. To produce more impactful outcomes in year 2, we are evaluating other potential molecular pathways suggested by RNAseq results as described above in accomplishments. These include Irisin-dependent attenuation of tumor cell migration (and as a corollary, tumor cell invasion), and attenuation of inflammation pathways. We are pursuing these angles using in vitro assays, and with the in vivo xenograft assays that are underway, we will evaluate these additional outcomes first by examining post-mortem endpoints. We expect to find significant changes relating to inflammation and cell migration/invasion, which can be used to redesign significant follow-up in vivo studies, which will be submitted for approval by our IACUC and by the DOD team before any changes to animal procedures are undertaken. Based on our preliminary observations we are confident that these pursuits will yield highly significant results in year 2.

4. Impact

- **Impact on the development of the principal discipline of the project –** We believe that the progression of this project has the potential to identify a novel role for irisin in breast cancer progression, beyond that which was stipulated in the proposal. These potentially include attenuation of tumor cell migration and inflammation. This will be rigorously tested in year 2.

- **Impact on other disciplines -** Nothing to report for this project period.

- **Impact on technology transfer** - Nothing to report for this project period.

- **Impact on society beyond science and technology** - Nothing to report for this project period.

5. Changes/Problems

- **Changes in approach and reasons for change –** As stated in Section 3, Accomplishments, we have observed more modestly reduced cell viability in with Dox/Irisin combinations against the sensitive cell line MDA-MB-231, for reasons that are still unclear. As a result, we have explored other putative anti-neoplastic Irisin bioactivities suggested by gene expression analysis and the literature. These include attenuation of tumor cell migration and attenuation of inflammation. We will continue to explore variations on cell viability/cytotoxicity assays to determine if the issue is an experimental one that can be resolved by altering the approach.

- **Actual or anticipated problems or delays and actions or plans to resolve them -** As stated in Section 3, Accomplishments, we adapted a robust assay to measure Irisin bioactivity, namely inhibition of lipid droplet formation during differentiation of 3T3-L1 cells, so that we can validate the efficacy of individual Irisin lots. To address the issue of unexpectedly modest Irisin/chemotherapeutic outcomes on cell viability, we have expanded our investigation to pursue other promising anti-neoplastic pathways.

- **Changes that had a significant impact on expenditures -** As a result of the changes in approach, we have not proceeded with the plan to have Horizon Discovery generate the mutated cells. This was budgeted at $61,630 in year 1. Depending on the outcome of the experiments underway, we propose to create any desired mutations by CRISPR mutagenesis in our lab. Co-Investigator Hartley has experience with this technique, as do several colleagues at UNM. We would then propose to allocate a portion of this ~ $60,000 to hiring an additional technician, so as to accelerate the experimental pace in year 2, in order to make up for the delays due to experimental problems we encountered in year 1.

- **Significant changes in use or care of human subjects -** Not applicable.

- **Significant changes in use or care of vertebrate animals -** Our use of vertebrate animals has not changed from the approved IACUC protocol at this time. The in vivo portion of the project is underway as proposed. Depending upon the outcome of the first experiments, if changes are necessary we will seek IACUC approval prior to making any changes in approach, and convey these changes to the USAMRMC for approval.

- **Significant changes in use of biohazards or select agents -** Not applicable.

6. Products

- **Publications, conference papers, and presentations -**

  - **Journal Publications** - Nothing to report during this project period.

  - **Books or other non-periodical, one-time publications** - Nothing to report during this project period.

  - **Other publications, conference papers, and presentations -**

    - Oral presentation - University of New Mexico Minority Access to Research Careers (MARC) Symposium - August 25, 2018. Presented by MARC Scholar Shania Sanchez
    - Poster presentation - 2018 SACNAS National Diversity in STEM Conference, Oct. 11 - 13, 2018, San Antonio, TX. Presented by MARC Scholar Shania Sanchez.
    - Websites - N/A
    - Technologies or techniques - N/A
    - Inventions, patent applications, licenses - N/A
    - Other products - N/A

7. Participants & Other Collaborating Organizations

- **Individuals working on the project -**

Name:	Helen Hathaway
Project Role:	PI
Researcher Identifier:	ORCID ID - 0000-0002-7879-5056
Nearest person month worked:	1
Contribution to Project:	Provide overall oversight and guidance; coordination of work; train and supervise technician and student assistant
Funding Support:	This award

Name:	Laurie Hudson
Project Role:	Co-I
Researcher Identifier:	TBD
Nearest person month worked:	1
Contribution to Project:	provide guidance in experimental design and data interpretation; consult regularly with PI; assist with experimental design, especially for drug combination studies
Funding Support:	This award

Name:	Rachel Earley
Project Role:	Research Specialist
Researcher Identifier:	N/A
Nearest person month worked:	12
Contribution to Project:	Performs experiments, collates data, presents data to PI and Co-I's, provides day-to-day training to student
Funding Support:	This award

Name:	Shania Sanchez
Project Role:	Undergraduate Research Assistant
Researcher Identifier:	N/A
Nearest person month worked:	4.5
Contribution to Project:	Performs in vitro experiments, cell culture
Funding Support:	Minority Access to Research Careers (MARC) Program - T34 GM008751 (NIH)

- **Any change in active other support of PD/PI or senior/key personnel** - Nothing to report for this project period.

- **Other organizations involved as partners** - Nothing to report for this project period.

8. Special Reporting Requirements - N/A

9. **Appendices** – N/A