AWARD NUMBER: W81XWH-19-1-0503

TITLE: Therapeutic strategies to disrupt Cx26-FAK-NANOG complex to attenuate cancer stem cell self-renewal and triple negative breast cancer progression

PRINCIPAL INVESTIGATOR: Ofer Reizes, Ph.D.

CONTRACTING ORGANIZATION: Cleveland Clinic

REPORT DATE: August 2020

TYPE OF REPORT: Annual

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

REPORT DOCUMENTATION PAGE		*Form Approved OMB No. 0704-0188*
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. **PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.**		
1. REPORT DATE **August 2020**	**2. REPORT TYPE** Annual Report	**3. DATES COVERED** **08/01/2019 - 07/31/2020**
4. TITLE AND SUBTITLE Therapeutic strategies to disrupt Cx26-FAK-NANOG complex to attenuate cancer attenuate cancer stem cell self-renewal and triple negative breast cancer progression		**5a. CONTRACT NUMBER** **5b. GRANT NUMBER** **W81XWH-19-1-0503** **5c. PROGRAM ELEMENT NUMBER**
6. AUTHOR(S) Ofer Reizes, PhD Justin Lathia, PhD Emily Esakov, PhD E-Mail:reizeso@ccf.org		**5d. PROJECT NUMBER** **5e. TASK NUMBER** **5f. WORK UNIT NUMBER**
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Cleveland Clinic Lerner Research Institute		**8. PERFORMING ORGANIZATION REPORT NUMBER**
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Development Command Fort Detrick, Maryland 21702-5012		**10. SPONSOR/MONITOR'S ACRONYM(S)** **11. SPONSOR/MONITOR'S NUMBER(S)**
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited		
13. SUPPLEMENTARY NOTES		

14. ABSTRACT

Triple-negative breast cancer is the most aggressive breast cancer subtype and is resistant to therapies. Our objective is to neutralize cancer stem cells, which are thought to underlie resistance to chemotherapeutics, as well as recurrence and metastasis. In parallel, we seek to minimize collateral damage to normal non-cancer cells. We identified that the protein connexin 26 (Cx26) is necessary and sufficient for the survival of cancer stem cells in triple-negative breast cancer models. While Cx26 was previously proposed to be a tumor suppressor, epidemiological studies suggest otherwise, as patients with high Cx26 had a poorer prognosis. Our studies indicate that Cx26 promotes cancer stem cell survival by forming a protein complex with the transcription factor NANOG, a master regulator of cancer stem cell function, and focal adhesion kinase in triple-negative breast cancer but not in other breast cancers. Our objective is to prevent this complex from forming and thereby inhibit cancer stem cell survival and growth. We will develop a therapeutic strategy to target complex formation that will be tested in pre-clinical models.

15. SUBJECT TERMS

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

Standard Form 298 (Rev. 8-98) Prescribed by ANSI Std. Z39.18

TABLE OF CONTENTS

INTRODUCTION. Triple-negative breast cancer (TNBC), the most aggressive breast cancer subtype, is associated with high rates of recurrence and metastasis as there are no clinically targeted therapies[1-5]. Toxic chemotherapeutic agents are the primary treatment regimen, highlighting the need for new targeted therapies. TNBC contains self-renewing, therapeutically resistant cancer stem cells (CSCs) that are responsible for tumor progression and metastasis[6-9]. The molecular circuitry that underlies stem cell pluripotency includes key transcription factors that are essential self-renewal signaling nodes and are highly expressed in TNBC[10, 11]. To effectively target CSCs, it is essential to disrupt these signaling networks. However, as CSCs are maintained by pluripotency transcription factors, direct targeting remains a critical barrier. We developed a reporter system based on the expression of the promoter of NANOG, a pluripotency transcription factor, to enable rapid and robust assays for studying and disrupting CSC signaling nodes[12]. In collaboration with Dr. Justin Lathia and his lab, we focused on the connexin family of proteins, which we found to be essential for TNBC CSC self-renewal despite a previously hypothesized tumor-suppressor function for some subunits[13, 14]. In a recently published report[15], we found that connexin 26 (Cx26) was elevated in TNBC compared with normal mammary tissue and enriched in CSCs compared with their non-CSC progeny in TNBC cell lines and patient-derived xenograft models. In functional studies, we demonstrated that Cx26 was necessary and sufficient for CSC maintenance and regulated NANOG protein stability. In TNBC, Cx26 localized to an intracellular membrane-bound vesicle in complex with the pluripotency transcription factor NANOG and focal adhesion kinase (FAK). **Hypothesis:** Based on published and preliminary data, **we hypothesize that self-renewal and tumor growth can be reduced by disrupting the Cx26/NANOG/FAK complex.**

KEYWORDS: Cancer stem cells, gap junction, connexin-26, triple negative breast cancer

ACCOMPLISHMENTS:

- **What were the major goals of the project?**
    - **Specific Aim 1** will test the hypothesis that the Cx26/NANOG/FAK complex is essential for NANOG stability, activation, and maintenance of self-renewal.
    - **Specific Aim 2** will test the hypothesis that disrupting the integrity of the Cx26/NANOG/FAK complex attenuates self-renewal and tumor growth.
    - See Document 1_SOW
    - **What was accomplished under these goals?**
- Major activities accomplished during this reporting period include:
    - Generation of Antp-tagged Cx26 peptides, binding affinity analysis of generated peptides by SPR, preliminary in vivo studies of peptide efficacy in TNBC tumor progression
- Specific objectives accomplished include: Nothing to Report

- Significant results:

Specific Aim 1: Upon initiation of this part of the project, we discovered successful antibodies against NANOG, Cx26, and FAK for western blot and co-immunoprecipitation studies were no longer commercially available. Our efforts in this aim have focused on screening multiple antibodies for these studies (Major Task 1, Subtask 1). We are also working to validate antibodies to assess complex member expression in tissue microarray samples (**Major task 4, Subtask 1**). In collaboration with Dr. Downs-Kelly, we validated Cx26 and Cx43 (positive control) in luminal breast cancer tissue (**Figure 1**). Cx26 in luminal breast cancer localizes to the cell surface as we showed in our Nature Communications publication.

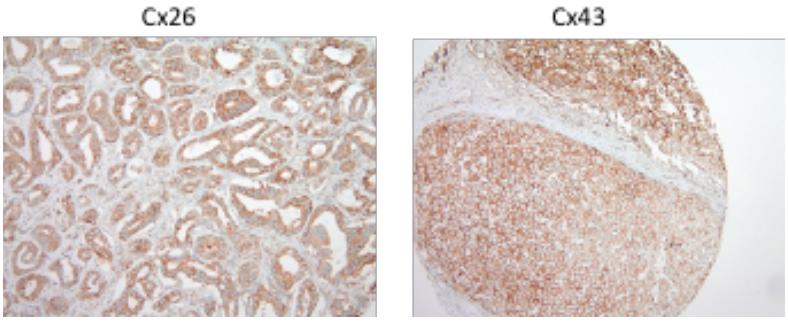


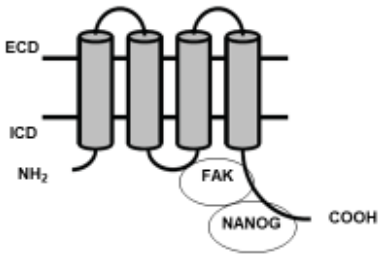
Figure 1. Immunostaining of Cx26 in luminal breast cancer exhibits cells surface distribution. Cx26 and Cx43 (positive control) antibody screening for IHC staining of tissue microarray breast cancer samples.

Specific Aim 2: We have devoted our effort to identification of therapeutically active peptides. Antp-tagged Cx26 peptides have been generated and binding affinity was confirmed by surface plasmon resonance to both FAK and NANOG proteins (Major task 5, Subtask 1 and Major task 6, Subtask 1 and 2), (**Figure 2**). We determined that the cytoplasmic tail had high affinity to Cx26 with a dissociation constant of 1 x 10^{-7} M (**Fig. 2A** and modeled in **Fig. 2B)**. We next developed cell penetrating peptides by addition of Antennapedia (Antp). We confirmed that Antp does not interact with either FAK or NANOG and addition of Antp to the cytosolic tail peptide of Cx26 retained strong affinity to NANOG (**Fig. 2 C**). In contrast, a scrambled cytosolic tail peptide attached to Antp showed low affinity to NANOG (**Fig. 2C**). we next confirmed that cell-penetrating peptides were confirmed to penetrate the cell membrane by flow cytometry and IF staining (**Figure 3A, B**). Notably, the Antp-cytosolic tail modulates NANOG regulated genes in TNBC based on real time PCR (Major task 7, Subtask 1 and 2) (**Figure 3C**).

A.

Analyte	Ligand	Dissociation Constant (KD)
Cytosolic loop	FAK	1.13^{-5} M
Cytosolic tail	FAK	1.49^{-5} M
Cytosolic tail	NANOG	1.7^{-7} M
FAK	NANOG	> 1.00 M

B.



C.

Analyte	Ligand	Dissociation constant (KD)
Antennapedia (Antp) RQIKIWFQNRRMKWKK	FAK	>1M
	NANOG	>1M
Antp- Cytosolic Tail (RQIKIWFQNRRMKWKK-RYCSGKSKKPV)	FAK	1.9^{-5} M
	NANOG	2.0^{-7} M
Antp- 216-220 (RQIKIWFQNRRMKWKK-RYCSG)	FAK	4.0^{-7} M
	NANOG	5.0^{-9} M
Antp- scrambled Cytosolic tail (RQIKIWFQNRRMKWKK-KVCLKDSGKTT)	FAK	>1M
	NANOG	>1M

Figure 2. Cytosolic tail of Cx26 interacts with NANOG and FAK. (A) Table denoting dissociation constants for interactions between Cx26 cytosolic tail and loop and NANOG and FAK proteins. (B) Cartoon depiction of the Cx26/NANOG/ FAK complex binding as identified by SPR. (C) Table showing the Antp tag does not bind to FAK or NANOG alone and does not alter original binding constants of the cytosolic tail. The scrambled Antp-tagged cytosolic does not bind to NANOG and FAK.

Functional *in vitro* Studies. Peptides were assessed for their impact on proliferation and self-renewal, in TNBC and non-TNBC cell lines (Major task 7, Subtask 3). We did not detect significant changes in cell proliferation with peptide incubation, either over time or dose dependently (**Figure 4 A, B**). As we previously showed the complex impacts CSC

phenotype, we tested the peptides in self-renewal using spheroid assays. We determined specific and significant reduction in spheroid frequency in TNBC cells treated with Antp-Cyto peptide but not in cells treated with either Antp or vehice (**Figure 4 C-E**). In luminal breast cancer cell, MCF7, we did not observe significant inhibition with the Antp-cytoosolic tail peptide (**Fig. 4F**).

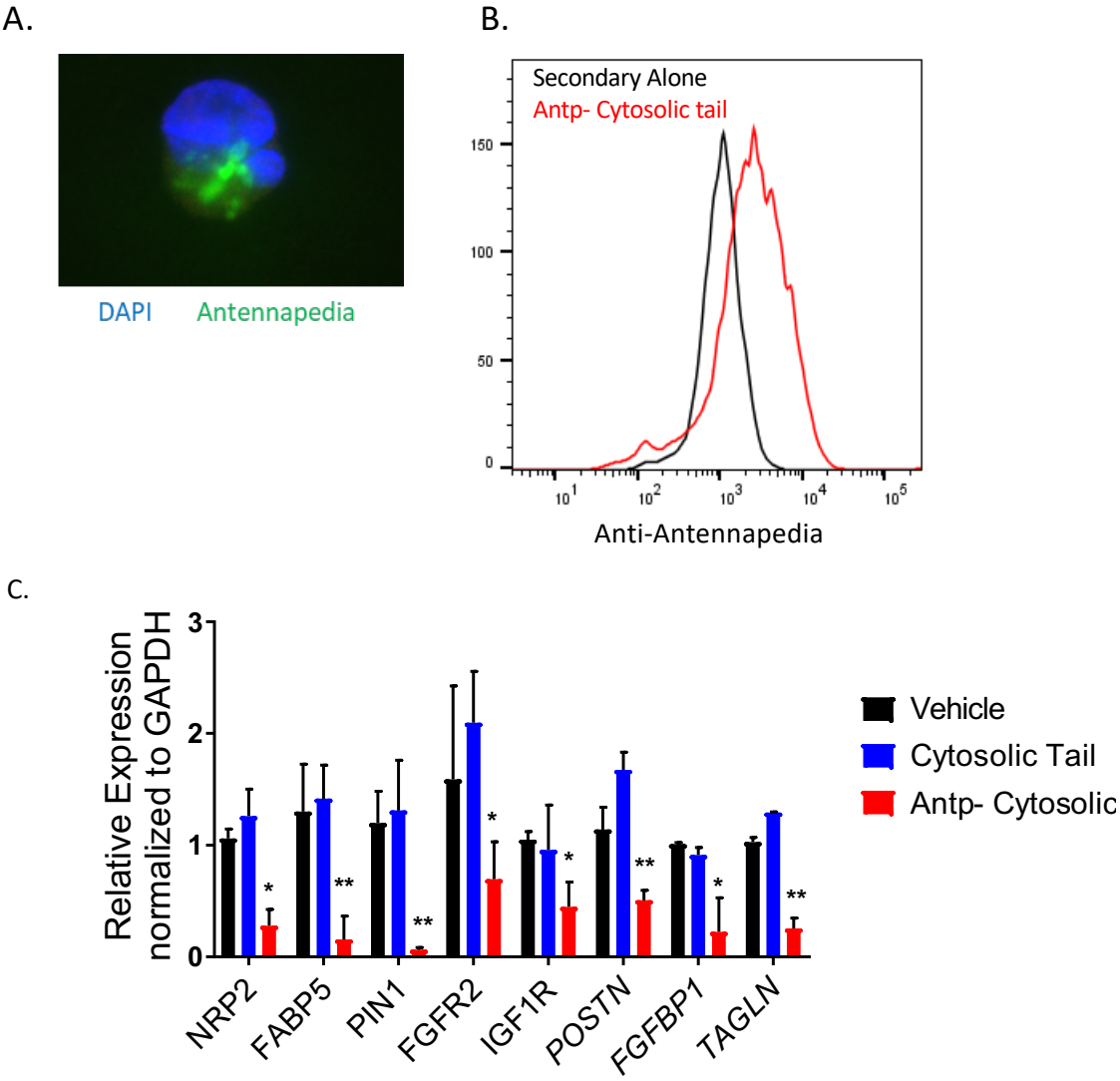


Figure 3. **Antp peptide penetrates TNBC cells and tagged Antp-Cyto Cx26 peptide attenuates NANOG regulated genes.** MDA-MB-231 cells were analyzed by immunofluorescence (A) and intracellular flow cytometry (B) following 10μM Antp-tagged cytosolic tail peptide treatment, showing the ability of the peptide to cross the plasma membrane. (C) Following 7 days treatments with 10μM Antp-tagged cytosolic tail peptide (red), MDA-MB-231 cells had decreased NANOG regulated gene expression by qPCR compared to cells treated with either cytosolic tail peptide alone (blue) or vehicle (black). There is no significant difference in gene expression between vehicle and cytosolic tail alone peptide treated groups. *p<0.05, **p<0.01

Functional *in vivo* Studies. Preliminary *in vivo* studies have been completed utilizing cell penetrating peptides and MDA-MB-231 TNBC cells injected in mice. Mice were flank injected with MDA-MB-231 TNBC cells. Once tumors were detected, Veh, Antp-scrambled Cyto tail, or Antp-Cyto (cell permeable) were injected directly into the tumors daily for 10 days. Tumor growth was monitored weekly for up to 3 weeks (**Fig. 5A**). Tumors in vehicle and scrambled cytoplasmic injected mice grew with no apparent changes in growth kinetics, but cytosolic tail-Antp treated tumors grew at a significantly decreased rate (**Fig. 5B**). Overall Cytosolic Tail-Antp treated mice demonstrated a significant decrease in luciferase signal upon IVIS imaging of tumor burden by total flux (**Figure 5C, D**).

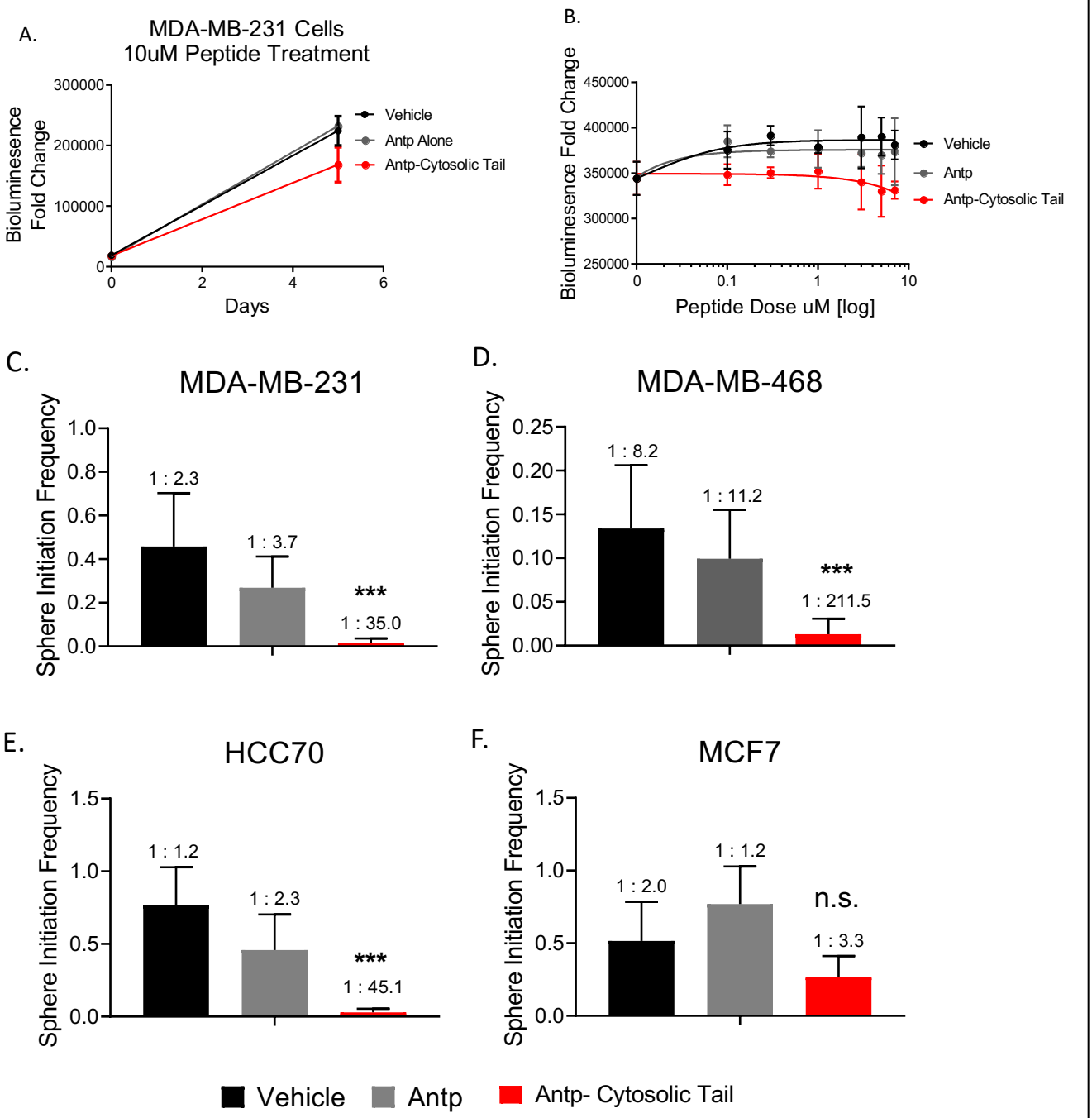


Figure 4. **Cell permeable Cx26 cytosolic peptide inhibits TNBC CSC growth.** CSC phenotype following peptide treatment in vitro. Cell proliferation was not significantly affected by peptide treatment compared to controls over time (A) or by concentration of peptide (B). Tumorsphere initiation frequency is decreased in MDA-MB-231 (C), MDA-MB-468 (D), and HCC70 (E) TNBC cells treated with Antp- cytosolic tail peptide compared to Vehicle or Antp alone controls but is not affected in MCF7 luminal breast cancer cells (F). ***p>0.001

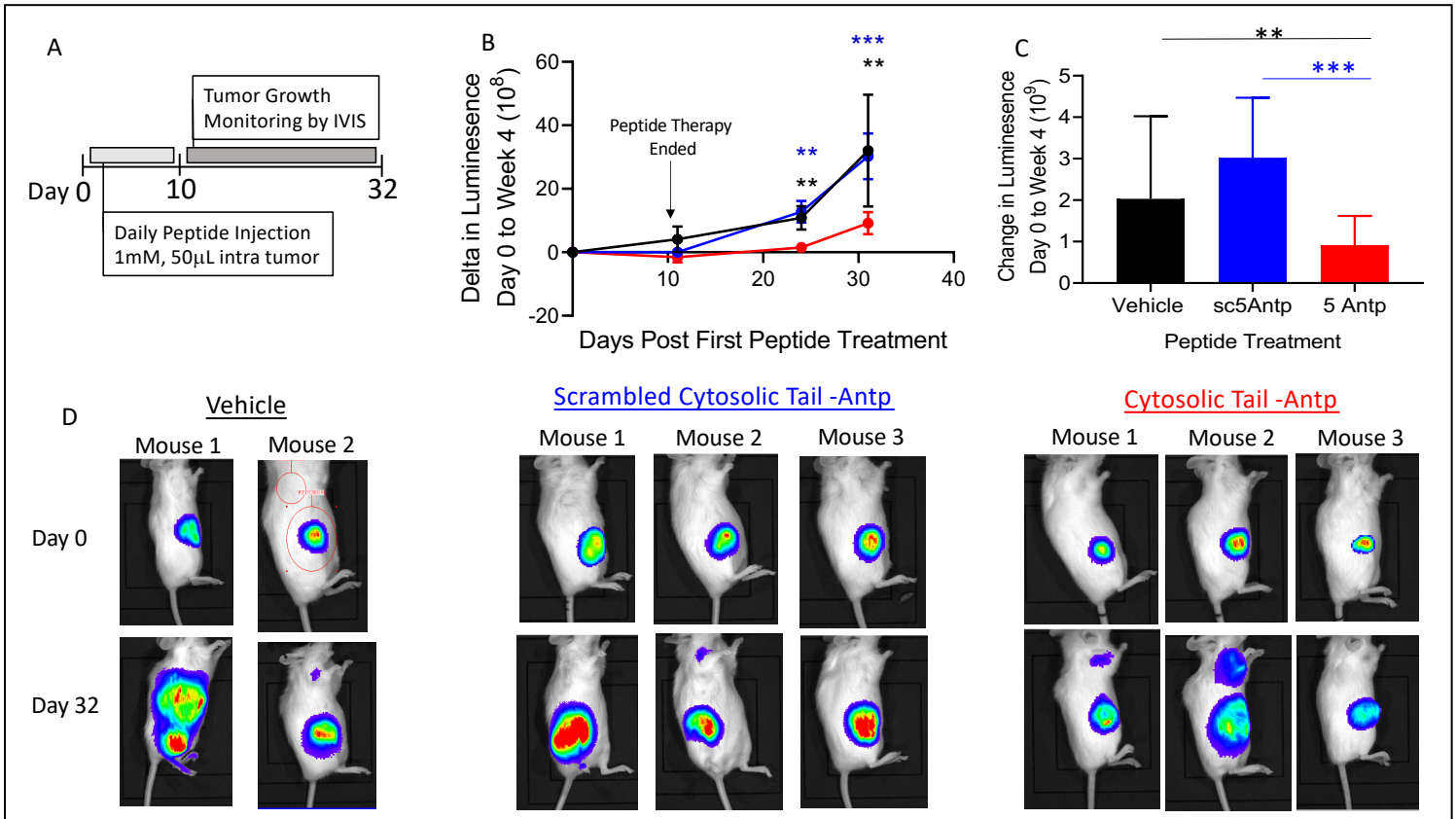


Figure 5. Cytosolic Tail-Antp treated mice exhibited decreased overall TNBC tumor growth. (A) Schematic of mouse study. (B) Tumor progression was monitored via IVIS over time. (C) Change in tumor growth to baseline was measured at endpoint as overall change in luminescence signal (total flux) *p<0.05, **p<0.01, ***p<0.003 (D) Representative IVIS images at day 0 and day 32 for the three cohorts.

- **What opportunities for training and professional development has the project provided?**

  Professional Development:

  Emily Esakov: oral and poster presentation at the International Gap Junction Conference in Victoria Canada as well as poster presentations at the LRI Research Day

- **How were the results disseminated to communities of interest?**

  - Preliminary studies have been discussed at the International Gap Junction Conference as well as locally at the Case Comprehensive Cancer Center.

- **What do you plan to do during the next reporting period to accomplish the goals?**

  - During the next reporting period the team involved in the project will work diligently and efficiently to accomplish the goals and objectives. We will plan experiments according to the SOW and troubleshoot with the help of experienced colleagues in the event any problems arise experimentally.

IMPACT:

- **What was the impact on the development of the principal discipline(s) of the project?**
  - *Nothing to Report*

- **What was the impact on other disciplines?**
  - *Nothing to Report*
- **What was the impact on technology transfer?**
  - *Nothing to Report*
- **What was the impact on society beyond science and technology?**
  - *Nothing to Report*

- **CHANGES/PROBLEMS:**
  - **Changes in approach and reasons for change**
    - Aim 2, Major Task 8, Subtask 1 outlines the use of microcapsules as a vehicle of delivery for the cell penetrating peptides. Upon formulation and successful peptide release studies, micro particles were no longer sterile. Our efforts to identify a mechanism of sterilization are continuing. We continued the proposed studies used the Antp-tagged cell penetrating without nanoparticles and were able to demonstrate efficacy *in vitro*, prompting the *in vivo* studies as well. These data are presented in the accomplishments section. We will continue to pursue the gel carrier as needed following additional *in vivo* testing.
  - **Actual or anticipated problems or delays and actions or plans to resolve them**
    - An unexpected delay occurred due to validated antibodies no longer being manufactured by Santa Cruz. We are in the process of screening multiple alternate antibodies as well as making an anti-Cx26 antibody through our Hybridoma Core facility to be used in the co-immunoprecipitation studies outlined.
    - We did experience some limited delays during the COVID-19 shuttering of the research institute. The presented study was allowed to complete but we put on hold a second study that is currently being reinitiated. We utilized the time to draft the manuscript on these studies.
  - **Changes that had a significant impact on expenditures**
    - *Nothing to report*
  - **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**
    - *Nothing to report*
  - **Significant changes in use or care of human subjects :** Nothing to Report
  - **Significant changes in use or care of vertebrate animals:** Nothing to Report
  - **Significant changes in use of biohazards and/or select agents:** Nothing to Report

- **PRODUCTS:**
  - **Publications, conference papers, and presentations**
    - **Journal publications.** Nothing to Report. We have a draft of a manuscript in preparation based on data highlighted in the report.
    - **Books or other non-periodical, one-time publications.** Nothing to Report
    - **Other publications, conference papers, and presentations.** As indicated above, Preliminary studies have been discussed at the International Gap Junction Conference as well as locally at the Case Comprehensive Cancer Center.
      - **Website(s) or other Internet site(s)** Nothing to Report
      - **Technologies or techniques** Nothing to Report
      - **Inventions, patent applications, and/or licenses** Nothing to report
  - **Other Products** Nothing to Report

- **PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS**
  - **What individuals have worked on the project?**

Name:	*Ofer Reizes, PhD*
Project Role:	*PI*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*1.8*
Contribution to Project:	Dr. Reizes is project lead and meets weekly with the project team to review progress.
Funding Support:	No Change
Name:	*Justin Lathia, PhD*
Project Role:	Co-I
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	1

Contribution to Project:	Dr. Lathia provides insights on project related to connexins.
Funding Support:	No change
Name:	*Emily Esakov, PhD*
Project Role:	Post-doctoral Fellow
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	6
Contribution to Project:	Dr. Esakov works to complete all project aims through the development of research studies and data analysis as shown in SOW.
Funding Support:	
Name:	Erin Mulkearns-Hubert
Project Role:	Post-doctoral fellow
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	6
Contribution to Project:	Dr. Mulkearns-Hubert provides valuable insight and helps with co-immunoprecipitation studies and data analysis as shown in SOW.
Funding Support:	No change
Name:	Lexie Trestan
Project Role:	Technician
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	12
Contribution to Project:	Ms. Trestan provides support for all animal studies completed.
Funding Support:	No change

- **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**
  - Nothing to report
- **What other organizations were involved as partners?**
  - Nothing to report

STATEMENT OF WORK – 10/31/2018
PROPOSED START DATE September 1, 2019

Site 1: Cleveland Clinic
Lerner Research Institute
PI: Reizes

Specific Aim 1. Test the hypothesis that the formation of the Cx26/NANOG/FAK complex is essential for NANOG stability leading the activation of functions that promote pluripotency.	**Timeline % completed**	**Site 1**
Major Task 1 Is the Cx26/NANOG/FAK complex present in other breast cancer subtypes and across models?		
Subtask 1: Determine Cx26/NANOG/FAK protein complex via co-immunoprecipitation in cell line and PDX models (TNBC, ER+/PR+, HER2+) and control cell lines (mammary epithelial cells, fibroblasts).	1-4 10%	Drs. Reizes, Esakov, and Lathia
Subtask 2: Determine Cx26/NANOG/FAK protein complex via co-immunoprecipitation in CSC models derived from MDA-MB-231 and HCC70 cells.	2-6 0%	Drs. Reizes, Esakov, and Driscoll
Subtask 3: Generate additional CSC models in TNBC, ER+/PR+, and HER2+ cell lines for complex assessment in Subtask 2.	1-9 0%	Drs. Reizes and Esakov
Subtask 4: Assessment of NANOG mutants in complex formation and sequence of events leading to complex formation.	1-6 10%	Drs. Reizes and Esakov
Subtask 5: Assessment of interaction between Cx26, NANOG, and FAK via co-immunoprecipitation assays in primary tumor tissue with pathological characterization.	3-9 0%	Drs. Reizes, Esakov, and Downs-Kelly
Major Task 2 Does Cx26/NANOG/FAK complex disruption alter NANOG stability and function?		
Subtask 1: Test Cx26 mutants in TNBC and nonTNBC models (Table 1) on NANOG transcriptional activity via promoter reporter constructs and ChIP assays with established targets.	3-9 0%	Drs. Reizes, Driscoll, and Esakov
Subtask 2: Test FAK mutants in TNBC and nonTNBC models (Table 1) on NANOG transcriptional activity via promoter reporter constructs and ChIP assays with established targets.	9-18 0%	Drs. Reizes, Driscoll, and Esakov
Subtask 3: Test Cx26 knockdown TNBC and nonTNBC models (Table 1) on NANOG protein stability.	9-18 0%	Drs. Reizes, Driscoll, and Esakov
Subtask 4: Test bound and free NANOG activity using in vitro transcription assays.	9-18 0%	Drs. Reizes, Driscoll, and Esakov
Major Task 3 Does the Cx26/FAK/NANOG ternary complex alter response to chemotherapy?		
Subtask 1: Test conditions in Major Task 2 with Paclitaxel for Cx26/NANOG/FAK protein complex and NANOG function.	9-18 0%	Drs. Reizes, Lathia, Esakov and Mr. Braley

Subtask 2: Analyze tumors for complex disruption based on IP and NANOG stability.	12-20 0%	Lathia, Esakov and Mr. Braley
Major Task 4 Does the Cx26/FAK/NANOG ternary complex inform patient outcome?		
Subtask 1: Assess Cx26, NANOG, and FAK protein expression in test tissue microarray (128 samples) representing multiple breast cancer subtypes.	12-18 10%	Drs. Reizes, Esakov, and Downs-Kelly
Subtask 2: Assess Cx26, NANOG, and FAK protein expression in validation tissue microarray (50 samples) representing multiple breast cancer subtypes.	12-20 0%	Drs. Reizes, Esakov, and Downs-Kelly
Subtask 3: Analyze expression relative to breast cancer subtype and clinical outcome.	15-20 0%	Drs. Reizes, Esakov, and Downs-Kelly
Milestone(s) Achieved: (1) Defining the breast cancer subtype(s) in which the Cx26/NANOG/FAK protein complex is present and correlation to patient prognosis; (2) Evaluation of NANOG function with intact and disrupted Cx26/NANOG/FAK protein complex; (3) Co-author manuscript describing function of NANOG in the context of the Cx26/NANOG/FAK protein complex.		
Deliverables: This aim will elucidate the mechanism by which Cx26 regulates NANOG stability and determine the functional consequence on NANOG transcriptional activity. Furthermore, we will establish the subset of tumors containing the ternary complex for diagnostic and prognostic purposes. This deep biological and mechanistic understanding is necessary for future therapeutic development.		
Specific Aim 2. Test the hypothesis that disrupting the integrity of the Cx26/NANOG/FAK complex attenuates self-renewal and reduces tumor growth.		
Major Task 5 Do the identified Cx26 interacting domains bind NANOG and FAK in breast cancer cells?		
Subtask 1: Generate antennapedia-tagged peptides for co-immunoprecipitation in TNBC and nonTNBC cells (Table 1).	1-9 100%	Drs. Reizes, Lathia, Esakov, and Mr. Braley
Subtask 2: Validate peptide binding to NANOG and FAK in breast cancer cells, PDX models, and CSCs compared to mammary epithelial cells and fibroblasts and assess intracellular localization.	3-9 0%	Drs. Reizes, Lathia, Esakov, and Mr. Braley
Major Task 6 Does the cell-penetrating Cx26 blocking peptide bind to NANOG and FAK?	6-15	
Subtask 1: Synthesize peptides with antennapedia sequence to provide intracellular access.	6-12 100%	Drs. Reizes and Esakov
Subtask 2: Determine binding affinity of peptides to NANOG and FAK via surface plasmon resonance and isothermal calorimetry.	6-15 50%	Drs. Reizes, Lathia, Esakov, and Mr. Braley
Major Task 7 Do the cell-penetrating Cx26 blocking peptide alter CSC function in vitro?		
Subtask 1: Test ability of cell-penetrating peptide to disrupt Cx26/NANOG/FAK complex integrity as outlined in Major Task 1.	12-18 10%	Drs. Reizes, Lathia, and Esakov

Subtask 2: Test ability of cell-penetrating peptide to disrupt NANOG function as outlined in Major Task 2.	18-24 20%	Drs. Reizes, Lathia, and Esakov
Subtask 3: Test ability of cell-penetrating peptide to alter CSC marker expression, proliferation, survival, self-renewal, and migration/invasion.	21-30 90%	Drs. Reizes, Lathia, and Esakov
Major Task 8 Can the cell-penetrating Cx26 blocking peptide integrated into a gel carrier?		
Subtask 1: Formulate cell-penetrating peptide in a pluronic gel carrier (Pluronic F127, Sigma) or microcapsules in collaboration with the Gourdie laboratory (see letter).	1-6 50%	Drs. Reizes, Lathia, Gourdie, and Esakov
Subtask 2: Evaluate release dynamics of cell-penetrating peptide.	1-6 100%	Drs. Reizes, Lathia, Gourdie, and Esakov
Subtask 3: Validate function of cell-penetrating peptide on Cx26/NANOG/FAK complex as outlined Major Task 1.	7-12 10%	Drs. Reizes, Lathia, Gourdie, and Esakov
Major Task 9 Does the cell-penetrating Cx26 blocking peptide alter TNBC tumor growth in vivo?	1-36	
Subtask 1: Test ability of cell-penetrating peptide to alter tumor growth and metastatic activity in established xenografts from TNBC cell lines and PDX models. **In vivo calculation:** We have utilized references within the Guide for the Care and Use of Laboratory Animals from the National Research Council to estimate the minimal number of animals necessary to achieve statistical significance. The sample size is determined based on the following calculation: $N = 2[(u_a + u_b)\, s/d]^2$ where: N = group size, $u_a = 1.96$ ($p<0.05$), $u_b = 1.282$ (beta error=0.1), s = standard error, d = difference between the groups. Based on the data we obtained to date, standard error for tumor size is approximately 200 mm^3. To detect a >40% difference in tumor size, **we need 10 mice per group to achieve appropriate power for the study**. This group size will be used for all tumor growth studies. **To optimize usage of mice, we will only test 2 TNBC cell sources (1 cell line and PDX line) for in vivo studies before moving on to other breast cancer subtypes (ex. PR+/ER+ and HER2+ as outlined in Major Task 10).** Tumor growth: 2 TNBC cell sources x 6 experimental conditions x 10 mice per group= 120 mice Metastatic activity: 2 TNBC cell sources x 6 experimental conditions x 10 mice per group= 120 mice	1-12 50%	Drs. Reizes, Lathia, and Esakov
Subtask 2: Test ability of cell-penetrating peptide in combination with Paclitaxel to alter tumor growth in established TNBC xenografts from cell lines and PDX models. We will perturb established tumors using 2 TNBC cell sources with 4 cell-penetrating peptide complex conditions alone and in combination with Paclitaxel. 2 TNBC sources x 4 complex conditions x 2 paclitaxel concentrations x 10 mice per group = 160 mice **Total: 400 mice for Task**	24-36 0%	Drs. Reizes, Lathia, and Esakov

Table 1. Cx26 c-terminal cytoplasmic peptides

Cytosolic tail	
Cytosolic tail-Antp	
Antp-	
Scrambled Cytosolic tail	
Mutant peptides:	
Peptide name	Peptide Sequence
xR216	XYCSGKSKKPV
xV226	RYCSGKSKKPX
xRY	XXCSGKSKKPV
xPV	RYCSGKSKKXX
216-220	RYCSGXXXXXX
221-226	XXXXXKSKKPV
A216-220	RYCSGAAAAAA
A221-226	AAAAAKSKKPV