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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

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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

NONE LISTED

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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 updated June 2022

    - **What was accomplished under these goals?**

- Major activities accomplished during this reporting period include:
    - We submitted our manuscript detailing our pre-clinical development of peptides based on Cx26 structure. The manuscript was well received but there are some revisions that we are currently making. This report details the revisions and additional data that are in progress.
- Specific objectives accomplished include: manuscript under revision
- Significant results:

Specific Aim 1:

Immunohistochemical analysis of patient specimen (Major task 4, subtask 1). Part of this work entails assessing the Cx26/NANOG/FAK complex in formalin fixed paraffin embedded (FFPE) tissues via immunohistochemistry to assess the expression of each component of the complex in well annotated invasive breast carcinomas. Archival FFPE tissues from the Department of Anatomic Pathology were identified to include breast carcinomas that represent the major molecular subtypes (luminal A, luminal B, basal-like/TNBC and HER2 positive) as previously determined by immunohistochemistry (estrogen receptor, progesterone receptor, HER2 with reflex HER2 FISH as needed and Ki67). Cx26, NANOG and FAK antibody clones were worked up independently on appropriate FFPE control tissues as suggested from the Human Protein Atlas and antibody manufacturer suggestions (see previous report). As we were not able get the three antibodies to perform staining on same IHC slide, we analyzed the findings on adjacent sections. This does not allow us to perform co-staining of the complex within the same cells. We report here the analysis of breast cancer subtypes (**Table 1**). The analysis indicates that in 50% of TNBC tumor specimens Cx26 and Nanog are localized in the nucleus of 2-5% of the cells. This observation is consistent with our published studies in a smaller cohort of human specimens. The observation that only 2-5% of cells contained nuclear Cx26 supports our proposal that the complex and impact on tumorigenesis is specific to CSCs. No nuclear localization of Cx26, Nanog, or FAK was identified in HR+/HER2-, HR+/HER2+, and HR-/HER2+. The lack of expression in nonTNBC cancers is likewise consistent with the specificity of the Cx26/FAK/NANOG complex in TNBC.

	HR+/HER2 - (n=12)	HR+/HER2+ (n=4)	HR-/HER2+ (n=5)	TNBC (n=12)
Nanog	9/12 with weak or moderate intensity cytoplasmic staining in 10-90% of tumor 3/12 with no staining	3/4 with weak intensity cytoplasmic staining in 20-90% of tumor 1/4 with no staining	5/5 with weak to strong intensity cytoplasmic staining in 40-90% of tumor	3/12 with intense nuclear staining in 2-5% of tumor 3/12 with intense nuclear staining in 2-5% of tumor and moderate intensity cytoplasmic staining in 20-50% of tumor 6/12 with no staining
Connexin 26	12/12 with moderate to strong intensity membranous and/or cytoplasmic staining in 80-95% of tumor	4/4 with weak to strong membranous and cytoplasmic or membranous staining in 60-90% of tumor	5/5 with weak to strong intensity membranous and cytoplasmic staining in 90-95% of tumor	7/12 with moderate to strong intensity cytoplasmic and weak to moderate intensity membranous staining in 90% of tumor 5/12 with moderate to strong intensity nuclear staining in 2-10% of tumor and moderate to strong intensity cytoplasmic and membranous staining in 90% of tumor
FAK	10/12 with weak to strong intensity cytoplasmic staining in 5-90% of tumor 2/12 with no staining	2/2 with weak intensity cytoplasmic staining in 70-90% of tumor 2/2 with no staining	5/5 with weak to moderate intensity cytoplasmic staining in 5-90% of tumor	4/12 with weak to strong intensity cytoplasmic staining in 5-90% of tumor 8/12 with no staining

Table 1. Immunohistochemical analysis of Cx26, FAK, and NANOG in breast cancer subtypes.

5-Antp disrupts Cx26 and FAK stability in HCC70 cells (Major Task 7 Do the cell-penetrating Cx26 blocking peptide alter CSC function in vitro?). To test whether the Cx26 disrupting peptide can impact CSC function, we assessed expression and localization of Cx26 and FAK. The studies were performed in HCC70 with a focus on validation of studies in MDA-MB-231 cells. These studies were also requested by the reviewers. HCC70 cells were incubated with peptide or vehicle control and after incubation for 5 days, cells were harvested and fractionated for membrane, cytoplasmic, and nuclear compartments. Cx26 was depleted from the membranes and reduced by 25% in the cytoplasm (**Fig. 2A**). In parallel, FAK localization was diminished by >70% in the membrane and nucleus (**Fig. 2B**). The findings support our proposal that the 5-Antp Cx26 peptide can disrupt CSCs.

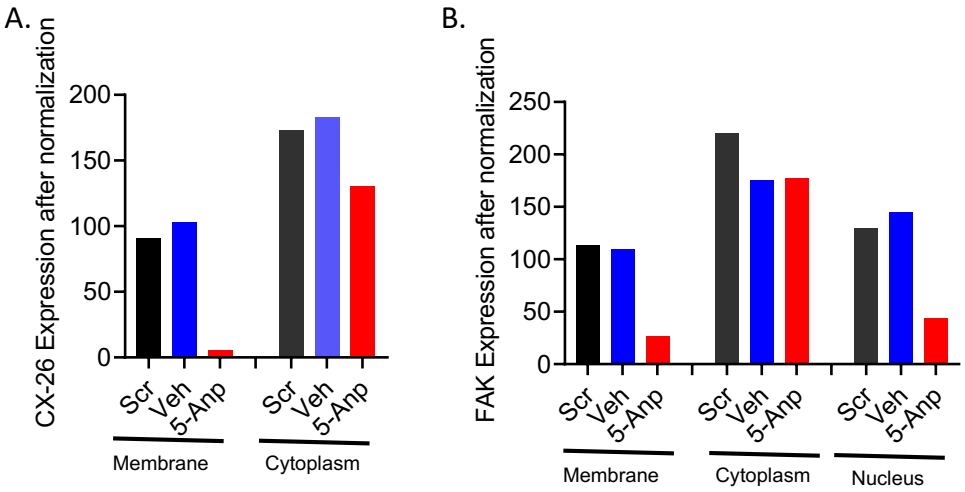

Fig. 2. Cx26 specific peptide inhibits Cx26 and FAK expression and localization in the membrane. HCC70 cells were incubated with Scrambled (Scr, 10 μM), Vehicle (Veh, 1%DMSO in PBS), or 5-Antennapedia (5-Anp. 10 μM) for 5 days. Cells were harvested and fractionated for membrane, cytoplasm, and nuclear compartments, resolved on SDS-PAGE, transferred to immobilon membranes and blotted with antibodies for Cx26 and FAK. Na/K ATPase, Tubulin, and Lamin AC were used as controls for membrane, cytoplasmic, and nuclear fractions. Blots were quantified using Image quant. Analysis was repeated 2 times.

5-Antp inhibits CSCs in HCC70 cells (Major Task 7 Subtask 3: Test ability of cell-penetrating peptide to alter CSC marker expression, proliferation, survival, self-renewal, and migration/invasion). We next assessed the impact of the 5-Antp Cx26 peptide on CSC self-renewal. We used HCC70 cells that were either unsorted or sorted for high and low GFP reflecting the level of NANOG expression. In previous studies, we found that level of NANOG expression directly correlated with the frequency of CSCs in the culture. We determined that regardless of level of NANOG, the Cx26 5Antp peptide significantly reduced CSC frequency based on limiting dilution analysis (**Fig. 3**). The findings support the utility of the Cx26 peptide in disrupting cancer cell growth. We determined a similar level of inhibition using the Incucyte cell proliferation assay system. Collectively, our findings support a role for the 5-Antp peptide in suppressing TNBC growth. Our finding further indicate that the efficacy is specific to TNBC with no impact on other subtypes of breast cancer.

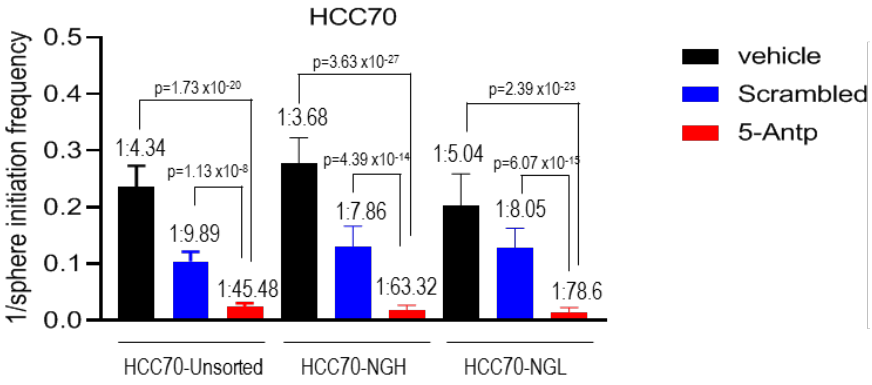

Fig. 3 Cx26 disrupting peptide leads to suppression of CSCs. HCC70 were either left unsorted or sorted for Nanog high (NGH) or Nanog low (NGL). HCC70 cells were treated for 48 hours with vehicle (1% DMSO in PBS), scrambled, or 5-Antp peptide at 10 μM. Cells were collected and plated 1, 5, 10, 20, and 100 cells per well in DMEM supplemented with B27 necessary for CSC analysis. Plates were incubated at 37C for 2 weeks followed by counting spheroids and extreme limiting dilution analysis (https://bioinf.wehi.edu.au/software/elda/). Data are representative of an analysis repeated at least 4 times.

Our effort continued to focus on preclinical validation. One of the critics by the reviewers was a request for additional validation in a second TNBC model. Our first set of data focused on the MDA-MB-231 TNBC model and in our repetition, we focused on HCC70. The findings are summarized in **Fig. 4**. We determined that Cx26 5-Antp improved overall survival (**Fig. 4A and B**) and inhibited tumor growth (**Fig. 4C**) when injected intratumorally compared to scrambled peptide control. While the data with the HCC70 exhibits a similar trend as observed with the MDA-MB-231, the efficacy is not statistically significant. We propose the limited efficacy is due to the kinetics of tumor growth and insufficient number of mice. The studies are currently being repeated in a larger cohort of mice.

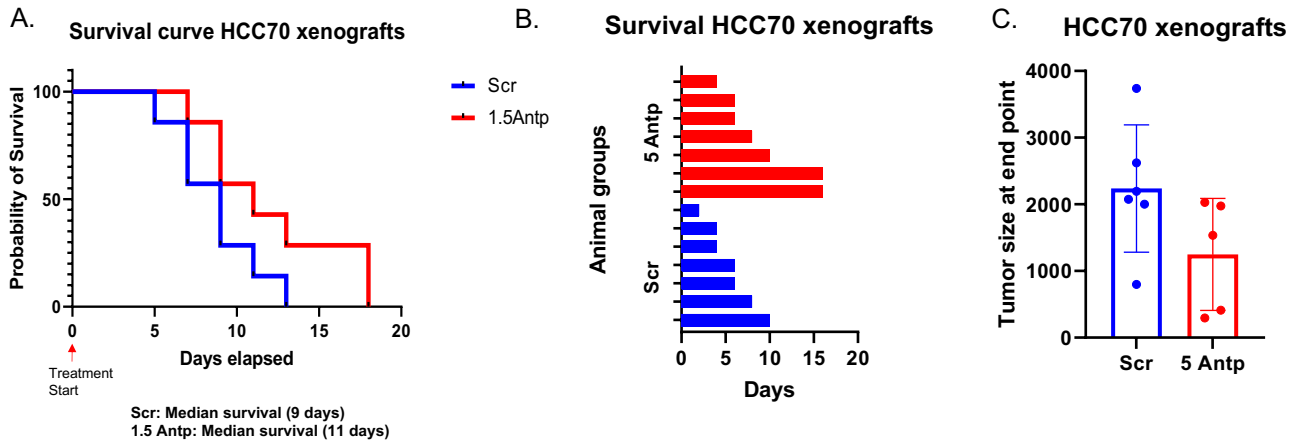

Fig. 4. Cx26 5-Antp improves overall survival and reduces tumor burden in HCC70 TNBC tumor model. HCC70 cells were injected in subq in immune deficient NSG mice (14 total mice). Tumor implantation and growth were monitored via palpation and in vivo imaging system (IVIS). Once tumors were detected, 5 Antp or Scr Antp peptides were injected intratumorally every other day for 20 days. Tumors were monitored weekly including analysis of overall body composition score to determine endpoint. **A.** Kaplan-Meier Analysis of mouse survival post-treatment with every other day intra-tumoral injection of peptide. **B.** Representative data showing individual mouse survival during treatment. **C.** Tumor size of Scrambled and 5-Antp tumors based on IVIS analysis prior to necropsy.

Overall, our findings support the development of peptide therapeutics to disrupt Cx26/FAK/NANOG for suppression of TNBC. We are repeating the pre-clinical mouse studies using the HCC70 model as the current studies were insufficiently powered to reach statistical significance. Our continuing studies will determine whether combination chemotherapy with standard of care and Cx26 directed peptides will yield a more effective combination to support further development studies that are beyond the current scope of work.

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

Professional Development: Dr. Esakov has departed the lab to take on a clinical project manager position in our cancer institute. The training in managing the proposed studies outlined in the SOW were instrumental in providing Dr. Esakov the experience to take on clinical trial project management.

- **How were the results disseminated to communities of interest?**
  - Nothing to Report
- **What do you plan to do during the next reporting period to accomplish the goals?**

The No Cost Extension (NCE) will allow us to continue to implement the proposed studies from the initial SOW including the combination studies and further elucidation of the impact of Cx26/FAK/NANOG complex in TNBC.

Our studies will focus on Major Task 2 Does Cx26/NANOG/FAK complex disruption alter NANOG stability and function? We will perform the following subtasks:
Subtask 1: Test Cx26 mutants in TNBC and nonTNBC models on NANOG transcriptional activity via promoter reporter constructs and ChIP assays with established targets.

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.
Subtask 3: Test Cx26 knockdown TNBC and nonTNBC models (Table 1) on NANOG protein stability. Subtask 4: Test bound and free NANOG activity using in vitro transcription assays.

A second goal if funds are sufficient will be to perform:
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.
Subtask 2: Analyze tumors for complex disruption based on IP and NANOG stability.

- **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**
    - **Actual or anticipated problems or delays and actions or plans to resolve them**
        - Nothing to report
    - **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.** Manuscript in revision
        - **Books or other non-periodical, one-time publications.** Nothing to Report
        - **Other publications, conference papers, and presentations.** I presented a seminar at the recent International Gap Junction Conference in A Caruna Spain in July 2022. The title of the talk was “Peptide-based targeting Cx26 complex in triple negative breast cancer inhibits cancer stem cells and suppresses tumor growth.
    - **Website(s) or other Internet site(s)** Nothing to Report
    - **Technologies or techniques** 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 monitors progress. As PI, he is responsible for manuscripts and reporting to the DoD.*
Funding Support:	No Change
Name:	*Justin Lathia, PhD*
Project Role:	Co-I
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	1.2
Contribution to Project:	*Dr. Lathia provides insights on project related to connexins*.
Funding Support:	No change
Name:	*Rashmi Bharti, PhD*
Project Role:	Post-doctoral Fellow
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	6
Contribution to Project:	Dr. Bharti works to complete all project aims through the development of research studies and data analysis.
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 gives valuable insight and helps with co-immunoprecipitation studies and data analysis.
Funding Support:	No change
Name:	Sadie Johnson
Project Role:	Technician
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	3
Contribution to Project:	Ms. Johnson provides support for all animal studies completed.
Funding Support:	No change
Name:	Alex Myers
Project Role:	
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	3
Contribution to Project:	Mr. Myers provides support for all bench studies
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, updated 06/06/2021
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 20%	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 10%	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 30%	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 50%	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 20%	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 70%	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 30%	Drs. Reizes, Esakov, and Downs-Kelly
Subtask 3: Analyze expression relative to breast cancer subtype and clinical outcome.	15-20 40%	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 80%	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.	6-15 100%	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 50%	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 50%	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.	21-30 100%	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).	1-6 100%	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.	7-12 100%	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 75%	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 10%	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