

AWARD NUMBER: W81XWH-21-1-0523

TITLE: The Role of SOX2 in Barrett's Esophagus Development and Progression to Esophageal Adenocarcinoma

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CONTRACTING ORGANIZATION: Baylor College of Medicine, Houston, TX

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14. ABSTRACT Barrett's esophagus (BE) is a precancerous condition defined as replacement of the normal esophageal squamous epithelium with metaplastic columnar intestinal epithelium caused by chronic gastroesophageal reflux disease. Importantly, Barrett's esophagus confers the strongest predisposition to developing esophageal adenocarcinoma. I hypothesize that the dorsal foregut transcription factor, SOX2, functions to maintain foregut squamous epithelial identity, and its loss is a critical step during Barrett's esophagus development and the progression to esophageal adenocarcinoma. I am investigating the molecular effects of SOX2 expression changes on Barrett's esophagus through a series of complementary experiments involving a novel foregut-specific inducible Sox2 knockout mouse model and a wholly novel biobank of human Barrett's esophagus derived organoids. Together, these experiments will elucidate novel molecular pathways involved in BE maintenance and may reveal novel therapeutic avenues to treat BE and prevent esophageal cancer. During the past year, I have continued to be productive by obtaining a Pilot/Feasibility Program Award through the Texas Medical Center DDC; giving presentations at FASEB GI Tract XX (oral presentation), 2022 DDRCC Directors Meeting, Wash U DDRCC GI Research Conference (Invited Speaker), CPTAC Steering Committee Meeting (Invited Speaker), AACR Annual Meeting 2023, Eastern Alliance DDRCC Exchange Program at Johns Hopkins DDRCC (Invited Speaker), and the Texas Medical Center DDC Annual Frontiers in Digestive Diseases Symposium; publishing a manuscript (PMID 36994101); and applying for an NIH PA-20-203 NIDDK Mentored Clinical Scientist Research Career Development Award (K08).					
15. SUBJECT TERMS None listed.					
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TABLE OF CONTENTS

	<u>Page</u>
1. Introduction	4
2. Keywords	4
3. Accomplishments	4
4. Impact	14
5. Changes/Problems	14
6. Products	15
7. Participants & Other Collaborating Organizations	16
8. Special Reporting Requirements	17
9. Appendices	18

1. INTRODUCTION:

Esophageal adenocarcinoma (EA) is a deadly and rapidly increasing cancer seen in the US with poor survival and a lack of novel treatments that reflect our current lack of knowledge regarding this complex disease. Barrett's esophagus (BE) is the precancerous lesion of the esophagus defined as metaplastic conversion of the normal esophagus to an intestinal phenotype in response to chronic acid and bile gastric refluxate injury. In this proposal, I investigate the role of the critical homeostatic esophageal transcription factor, SOX2 in the development of BE and progression to EA using novel genetic mouse models and human organoids. I hypothesize that SOX2 functions to maintain foregut squamous epithelial identity and to inhibit columnar and intestinal specification of the esophageal mucosa, and its loss is a critical step during BE development and EA progression. In Aim 1, I seek to characterize the effects of SOX2 loss on esophageal homeostasis and during injury. The second Aim is to determine the effects of forced expression of SOX2 on the progression of BE and EA.

2. **KEYWORDS:** Esophageal adenocarcinoma, Barrett's esophagus, intestinal metaplasia, SOX2, transcription factor, developmental reprogramming, human patient derived organoids, genetic mouse models

3. ACCOMPLISHMENTS:

- **What were the major goals of the project?**

Specific Aim 1: Characterize the effects of SOX2 loss on esophageal homeostasis and during injury.

Major Task 1: Characterize the in vivo effects of SOX2 loss in the esophagus and forestomach at homeostasis and injury.

Subtask 1: IACUC regulatory protocol approval for animal studies.

Target Date: 9/2021

-100% complete

Subtask 2: Examine the effects of Sox2 loss at 6 weeks, 3 and 6 months.

Target Date: 10/2022

-100% complete

Subtask 3: Determine the effects of Sox2 loss upon DOC injury at 6, 9, and 12 months.

Target Date: 3/2024

-75% complete

Milestone: Identify histologic and transcriptional effects of Sox2 loss in vivo in the adult murine esophagus and foregut at homeostasis and upon injury.

Target Date: 3/2024

-75% complete

Major Task 2: Characterize the in vitro effects of SOX2 loss in esophageal organoids.

Subtask 1: IRB regulatory protocol approval for research involving human cells.

Target Date: 9/2021

-100% complete

Subtask 2: Design SOX2 CRISPR guide RNAs, RNP preparation, and validation in collaboration with the BCM Cell Based Assay Screening Service Core.

Target Date: 9/2021

-100% complete

Subtask 3: Generate stable SOX2 knockout organoid cell lines from CRISPR-Cas9 RNPs developed in task 2.2.

Target Date: 3/2022

-0% complete

Subtask 4: Examine the effects of SOX2 loss in esophageal organoids.

Target Date: 7/2022

-80% complete

Milestone Achieved: Identify histologic and transcriptional effects of Sox2 loss at homeostasis and injury; publication of 1 peer reviewed paper.

Target Date: 3/2024

-50% complete PMID 36994101

Specific Aim 2: Determine the effects of forced expression of SOX2 on the progression of BE and EA.

Major Task 3: Characterize the effects of SOX2 overexpression in human BE and EA organoids.

Subtask 1: Assess for the effects of SOX2 overexpression in human BE organoids.

Target Date: 3/2022

-50% complete

Subtask 2: Assess for the effects of SOX2 overexpression in human EA organoids.

Target Date: 11/2022

-50% complete

Milestone Achieved: Identify histologic and transcriptional effects of SOX2 overexpression in BE and EA.

Target Date: 3/2023

-90% complete

Major Task 4: High throughput screen for compounds and drugs that induce SOX2 expression.

Subtask 1: Generate stable BE organoid cell lines that express a luciferase SOX2 promoter plasmid.

Target Date: 1/2022

-100% complete

Subtask 2: High throughput screen with 480 compound ICCB Known Bioactives library (Enzo) prepared by the BCM Center for Drug Discovery

Target Date: 1/2023

-50% complete

Milestone Achieved: Characterization of effects of SOX2 overexpression in BE and EA; publication of 1 peer reviewed paper.

Target Date: 7/2023

-50% complete PMID 36994101

- What was accomplished under these goals?

Specific Aim 1: Characterize the effects of SOX2 loss on esophageal homeostasis and during injury.

Major Task 1: Characterize the in vivo effects of SOX2 loss in the esophagus and forestomach at homeostasis and injury.

Subtask 2: Examine the effects of SOX2 loss at 6 weeks, 3 and 6 months.

Target Date: 10/2022

I have completed assessment of the loss of Sox2 at various time points (6 weeks, 3 and 6 months). And histologically, the mice (*Krt5^{CreER/+}; Sox2^{flox/flox}; ROSA26^{LSL TdTomato/+}*) exhibit

similar phenotypes of altered squamous epithelial homeostasis compared to control mice (*Krt5^{+/+}; Sox2^{flx/flx}; ROSA26^{LSL}TdTomato^{+/+}*). Specifically, I have shown decreased mature squamous esophageal markers (Keratin 13, Keratin 14, and Involucrin) and increased immature markers (Ki-67). Further detailed transcriptional analysis using microarray data obtained from 4 *Krt5^{CreER}+/+*; *Sox2^{flx/flx}; ROSA26^{LSL}TdTomato^{+/+}* and 3 control mice further confirmed and explained these changes. Using gene set enrichment analysis (GSEA), I found no significantly decreased Hallmark Pathways with SOX2 loss, **Fig 1A**. However, there were many significantly increased pathways including P53_PATHWAY, MTORC1_SIGNALING, OXIDATIVE_PHOSPHORYLATION, MYC_TARGETS, and HYPOXIA, **Fig 1A**. These damage response pathways are consistent with the histologic changes. In addition, GSEA Cell Signatures analysis found many “Gastric Corpus” signatures decreased and many “Fetal Squamous” signatures increased with SOX2 loss, **Fig 1B**. The overall top down-regulated genes are gastric corpus related genes and the top up-regulated genes are squamous epithelium damage response genes, **Fig 1C**.

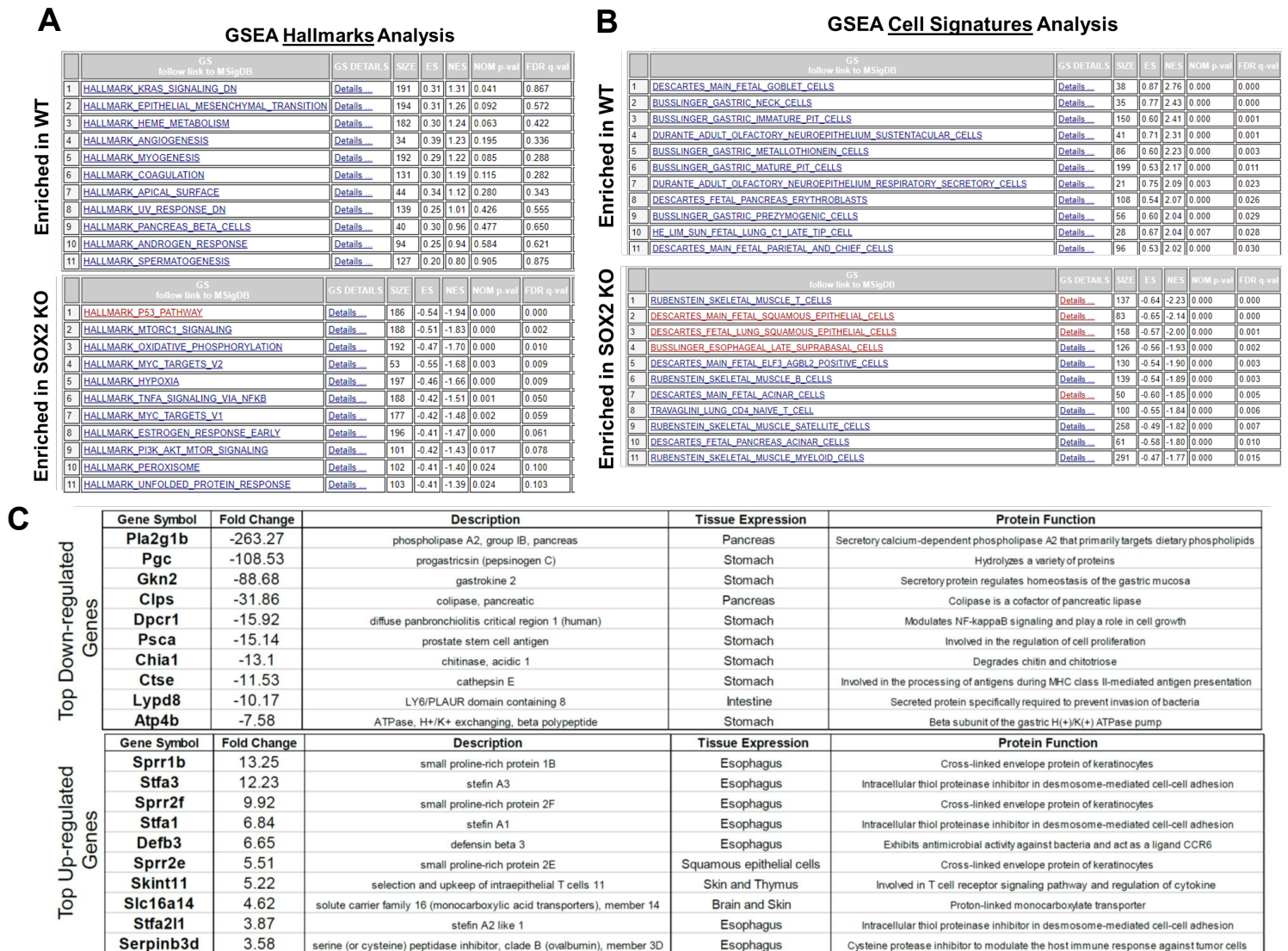


Figure 1. Transcriptional changes due to Sox2 loss. A) GSEA analysis with top Hallmark pathways increased and decreased with Sox2 loss. B) GSEA analysis with top Cell Signature pathways increased and decreased with Sox2 loss. C) Overall Top Down-regulated and Up-regulated genes with Sox2 loss.

Given these interesting transcriptional changes, I have proceeded to validate many of the Hallmark pathway changes and individual gene expression changes upon Sox2 loss, **Fig 2**.

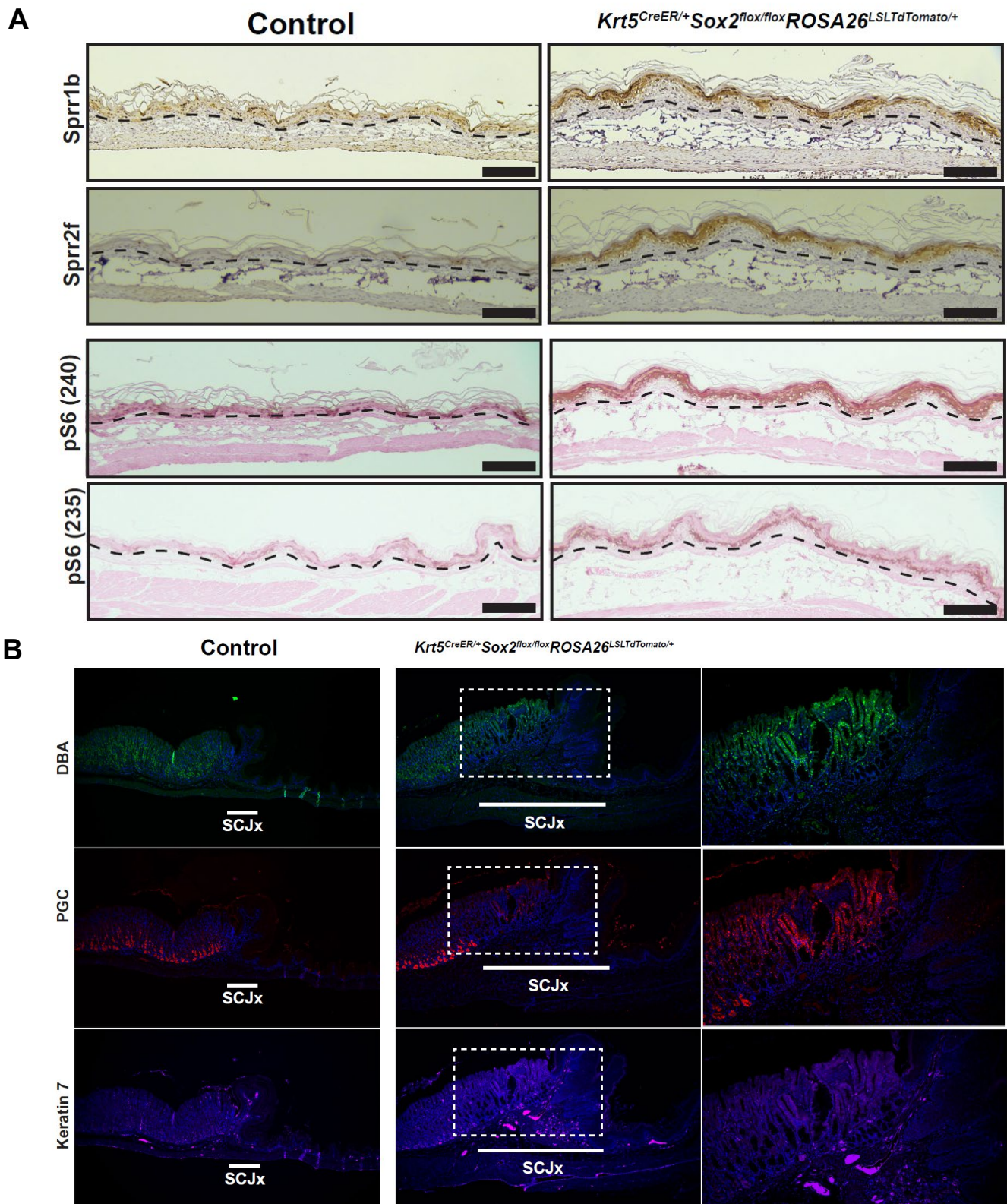


Figure 2. Confirmational histologic changes for *Krt5^{CreER/+}; Sox2^{flx/flx}; ROSA26^{LSLTdTomato/+}* mice compared to control *Krt5^{+/+}; Sox2^{flx/flx}; ROSA26^{LSLTdTomato/+}* mice. A) IHC staining for Sprr1b, Sprr2f, and mTOR pathway (pS6 240 and pS6 235). B) Immunofluorescence staining of DBA (Parietal cell marker, green), PGC (Chief cell marker, red), and Keratin 7 (purple). Squamocolumnar junction (SCJx) detailed.

Specifically, I have shown increase in damage response genes, *Sprr1b* and *Sprr2f*, by IHC, **Fig 2A**. These are proteins important for ROS detoxification during tissue injury (PMID 11279051). In addition, I have also found increase in mTORC pathway signaling (pS6 240 and pS6 235) by IHC, **Fig 2A**. Finally, by immunofluorescence, I found that the squamocolumnar junction is expanded with loss of SOX2, **Fig 2B**, explaining the decrease in gastric corpus genes found via transcriptomic analysis. Future experiments are focused on analysis for increased ROS production using DHE and NHE staining and alterations in p53 pathway.

Additional experiments determining the direct transcriptional targets of *Sox2* are pending. The use of squamous organoids generated from *Krt5^{CreER/+}; Sox2^{flox/flox}; ROSA26^{LSLTdTomato/+}* and control *Krt5^{+/+}; Sox2^{flox/flox}; ROSA26^{LSLTdTomato/+}* mice that reflect *in vivo* histologic and transcriptomic changes are being performed with cleavage under targets and release using nuclease (CUT&RUN) assays that allow assaying for transcription factor binding with improved resolution and decreased background compared to ChIP-seq (PMID 29651053). Optimal cell isolation and permeabilization conditions have been determined, **Fig 3**.

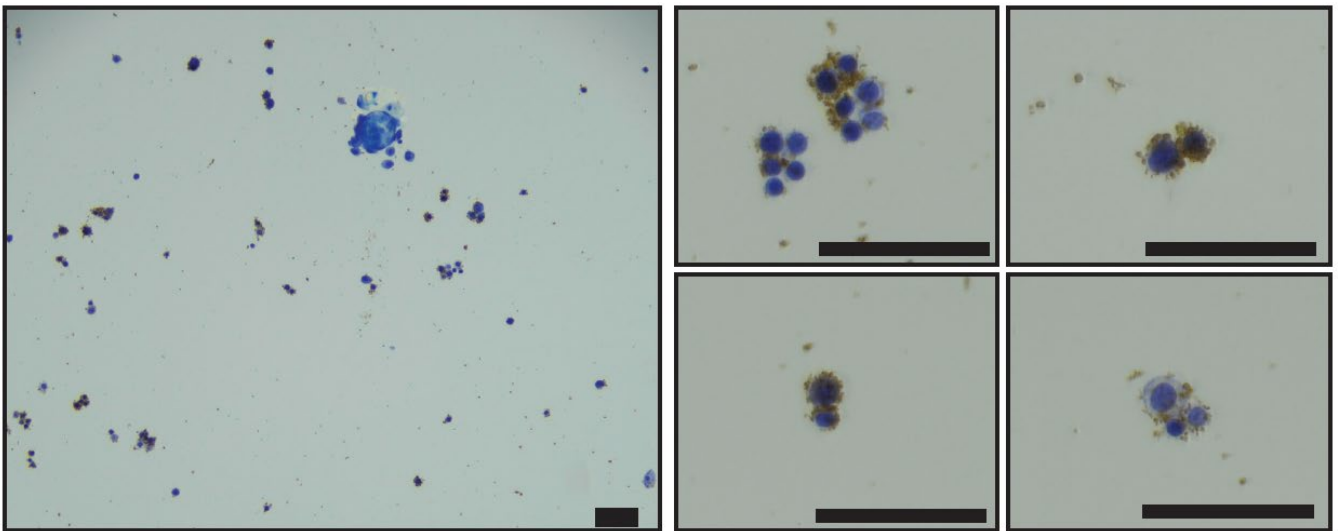


Figure 3. Optimization of squamous organoids for CUT&RUN. Squamous organoids were digested into single cell suspension, bound to concanavalin A beads, and permeabilized. Trypan blue staining show permeabilized squamous cells (blue) bound to concanavalin A beads (brown).

Subtask 3: Determine the effects of *Sox2* loss upon DOC injury at 6, 9, and 12 months.
Target Date: 3/2024

I have treated additional *Krt5^{CreER/+}; Sox2^{flox/flox}; ROSA26^{LSLTdTomato/+}* and control *Krt5^{+/+}; Sox2^{flox/flox}; ROSA26^{LSLTdTomato/+}* mice with 0.3% DOC injury for 6 and 12 months. The *Krt5^{CreER/+}; Sox2^{flox/flox}; ROSA26^{LSLTdTomato/+}* mice were sensitive to DOC injury resulting in increased mortality, **Fig 4**.

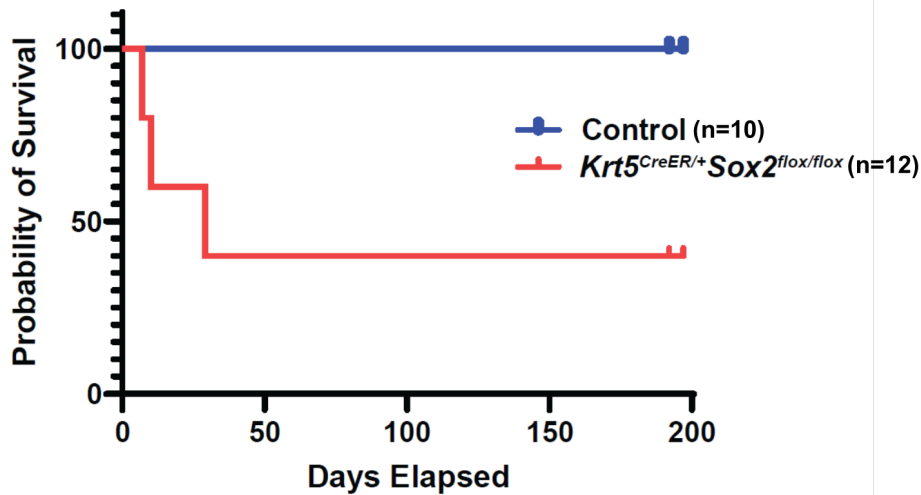


Figure 4. Kaplan-Meier survival analysis for *Krt5*^{CreER/+}; *Sox2*^{flox/flox}; *ROSA26*^{LSLTdTomato/+} (12 mice) and control *Krt5*^{+/+}; *Sox2*^{flox/flox}; *ROSA26*^{LSLTdTomato/+} (10 mice) mice with 0.3% DOC.

Krt5^{CreER/+}; *Sox2*^{flox/flox}; *ROSA26*^{LSLTdTomato/+} mice exposed to bile acid that have survived to 6 months show expansion of mucus containing columnar glands at the squamocolumnar junction, **Fig 5A**. Further histologic analysis of these expanded glands show that they express mCherry (lineage tracing marker indicating a squamous Keratin 5 origin) and the intestinal transcription factor CDX2, **Fig 5B**.

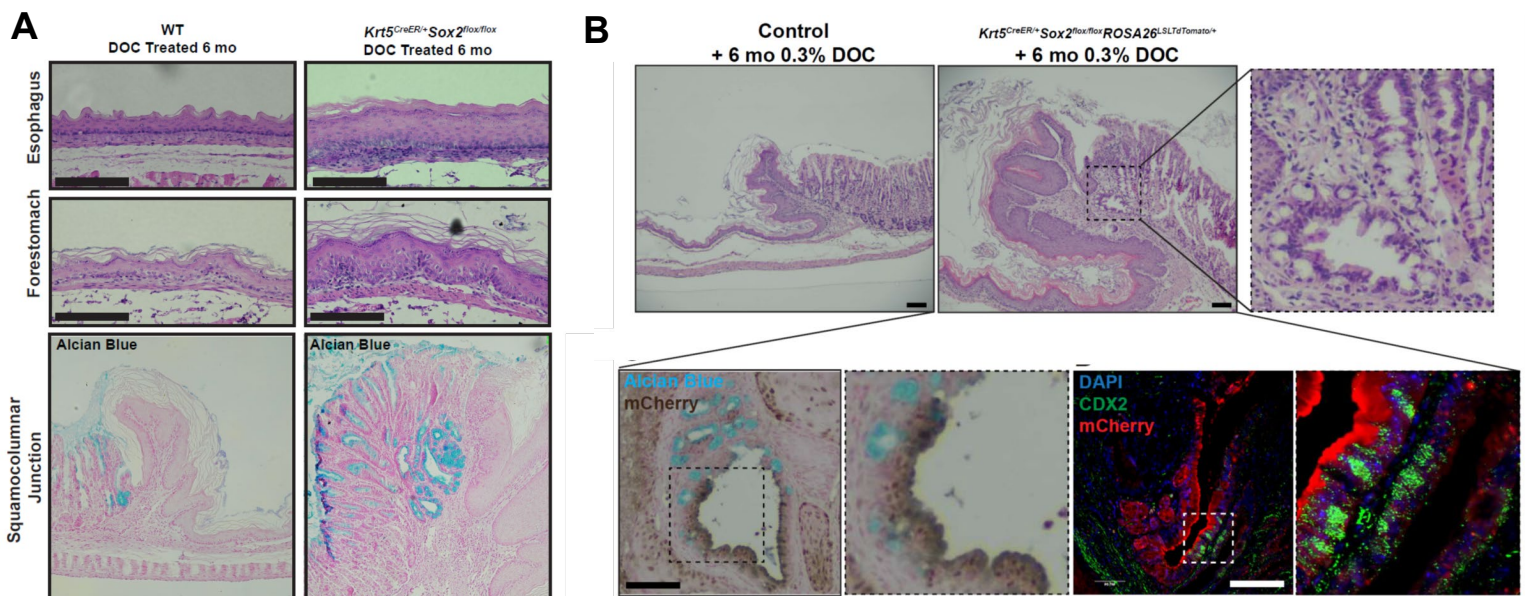


Figure 6. Histologic analysis of Control (WT) and *Krt5*^{CreER/+}; *Sox2*^{flox/flox}; *ROSA26*^{LSLTdTomato/+} mice treated with 0.3% DOC at 6 months. A) H&E and Alcian Blue staining (blue) showing expansion of columnar glands at squamocolumnar junction. B) Co-staining of Alcian Blue (blue) and mCherry (brown) and DAPI (blue), CDX2 (green), and mCherry (red) show squamous origins of expanded intestinal columnar glands.

Additional studies characterizing Control and *Krt5*^{CreER/+}; *Sox2*^{flox/flox}; *ROSA26*^{LSLTdTomato/+} mice treated with 0.3% DOC at 6 and 12 months are pending.

Major Task 2: Characterize the in vitro effects of SOX2 loss in esophageal organoids.

Subtask 4: Examine the effects of SOX2 loss in esophageal organoids.
Target Date: 7/2022

I have generated murine esophageal/forestomach organoids from Control and *Krt5^{CreER/+}; Sox2^{flox/flox}; ROSA26^{LSLTDTomato/+}* mice, I have been able to assess the effects of SOX2 in esophageal organoids. I have performed Affymetrix GeneChip microarray and histologic analyses on these organoids as previously discussed. Cleavage under targets and release using nuclease (CUT&RUN) assays that have now been optimized, **Fig 3**, will be performed to assess for direct transcriptional targets of Sox2.

Specific Aim 2: Determine the effects of forced expression of SOX2 on the progression of BE and EA.

Major Task 3: Characterize the effects of SOX2 overexpression in human BE and EA organoids.

Subtask 1: Assess for the effects of SOX2 overexpression in human BE organoids.
Target Date: 3/2022

Subtask 2: Assess for the effects of SOX2 overexpression in human EA organoids.
Target Date: 11/2022

To accomplish these tasks, I have established a biobank of human BE organoids with varying levels of SOX2 expression. In addition, as previously detailed, this biobank includes *TP53* mutated dysplastic (early esophageal adenocarcinoma) organoids as well. To further elucidate the expression of SOX2 at single-cell resolution, I have performed single-cell RNA sequencing on 4 human BE organoid lines (WU014, WU002, WU010 the *TP53* dysplastic line, and WU012), **Fig 7**.

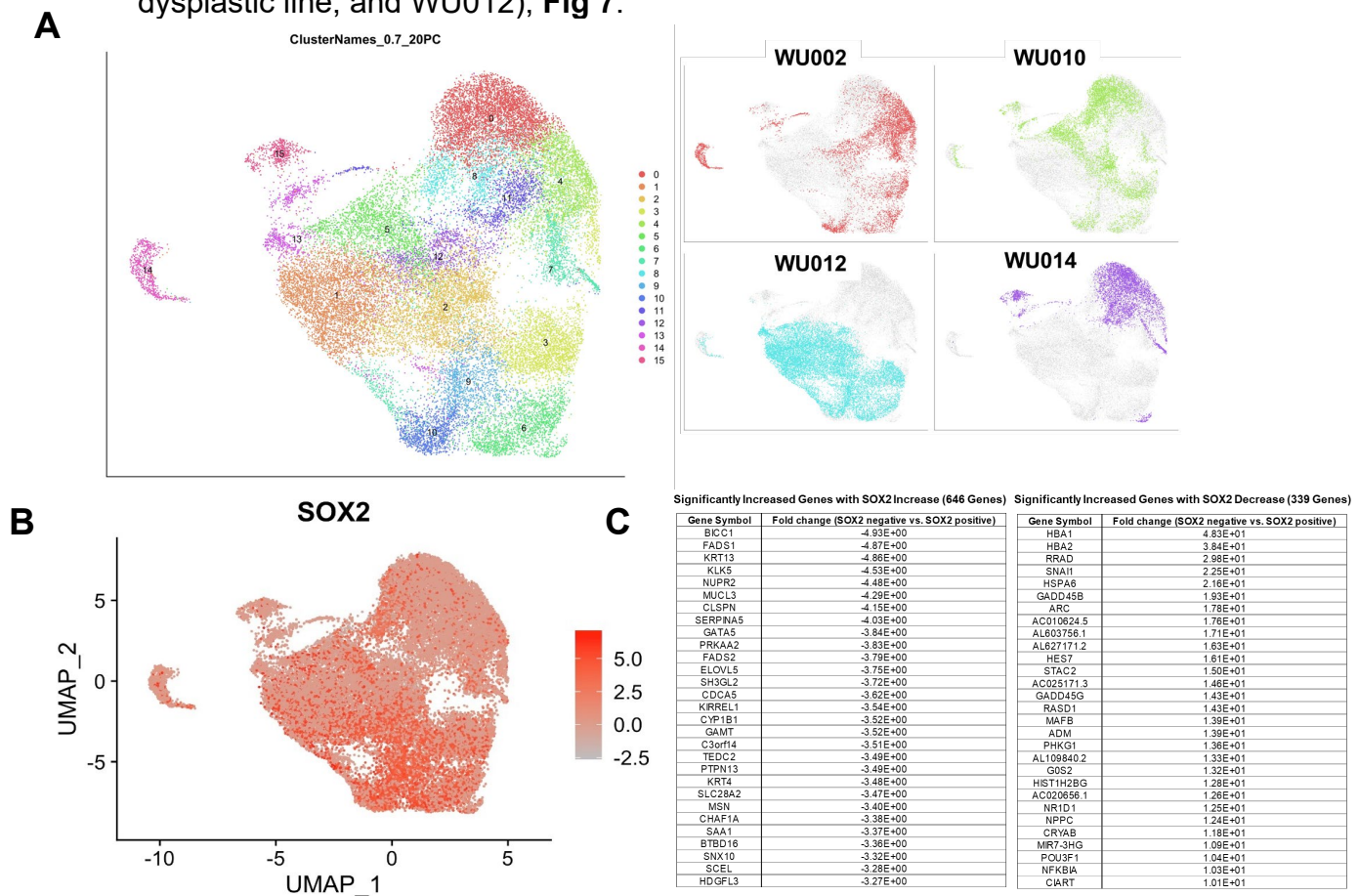


Figure 7. Single-cell RNA sequencing of human BE organoid lines. A) UMAP showing clusters and distribution of WU014 (purple), WU002 (red), WU010 (green), and WU012 (blue) cells. B) SOX2 expressing cells shown in red. C) Genes significantly increased in SOX2 expressing cells (left columns) and significantly decreased in SOX2 expressing cells (right columns).

I have subsequently overexpressed SOX2-eGFP in my WU014 and WU010 (*TP53* dysplastic BE) organoid lines using lentiviral transduction, **Fig 8**. Stable expressing lines have been established and longterm consequences of SOX2 overexpression are currently be assessed.

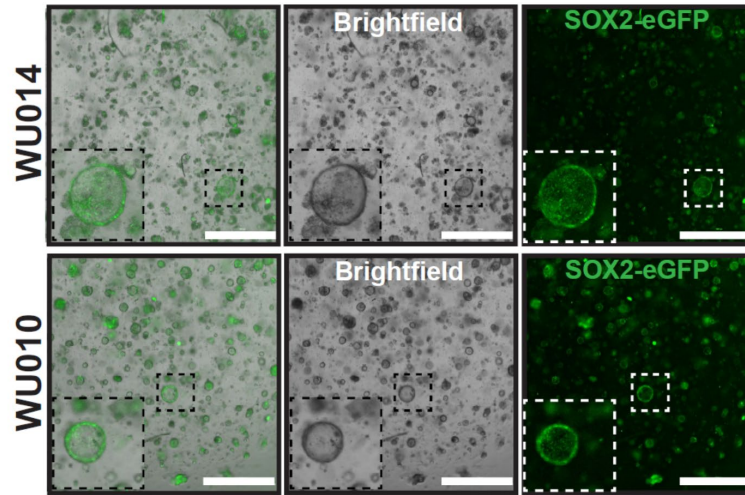


Figure 8. Establishment of SOX2-eGFP overexpression human BE organoids. Live fluorescent imaging showing abundant SOX2-eGFP expressing cells.

To assess for the direct transcriptional targets of Sox2 in BE and dysplastic BE, I have optimized cleavage under targets and release using nuclease (CUT&RUN) assays for BE organoids and successfully performed CUT&RUN for the transcription factor, GATA4, and the histone marker H3K27me3, **Fig 9**.

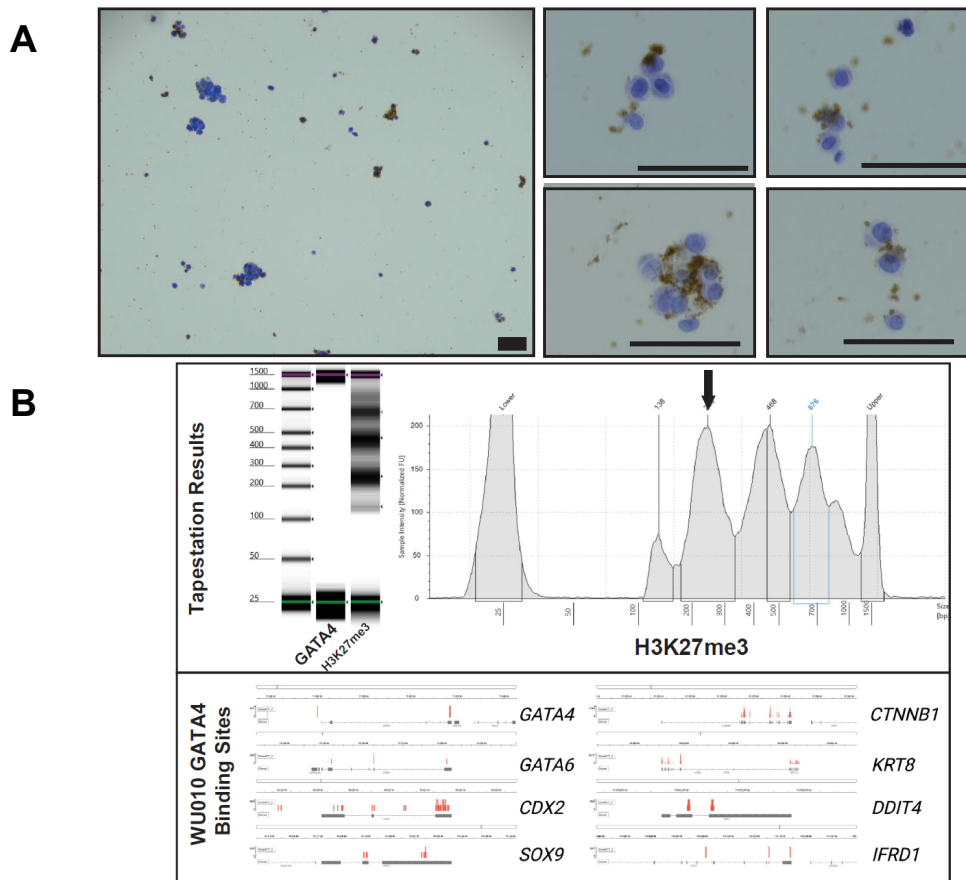


Figure 9. CUT&RUN for BE organoids. A) Trypan blue staining show permeabilized BE organoid cells (blue) bound to concanavalin A beads (brown). B) Agilent TapeStation analysis results showing banding patterns after GATA4 and H3K27me3 (positive control) CUT&RUN of WU010 BE organoids with expected mononucleosome banding pattern

(arrow) for the H3K27me3 positive control. GATA4 binding sites (red) for genes listed after sequencing, alignment, and peak calls.

Major Task 4: High throughput screen for compounds and drugs that induce SOX2 expression.

Subtask 2: High throughput screen with 480 compound ICCB Known Bioactives library (Enzo) prepared by the BCM Center for Drug Discovery.
Target Date: 1/2023

I have established a stable dysplastic BE organoid line that expresses a luciferase SOX2 promoter eGFP plasmid that has been expanded sufficiently for downstream screening and have found a positive control (drug REPSOX at 12.5 uM) that can induce SOX2 expression, **Fig 10**.

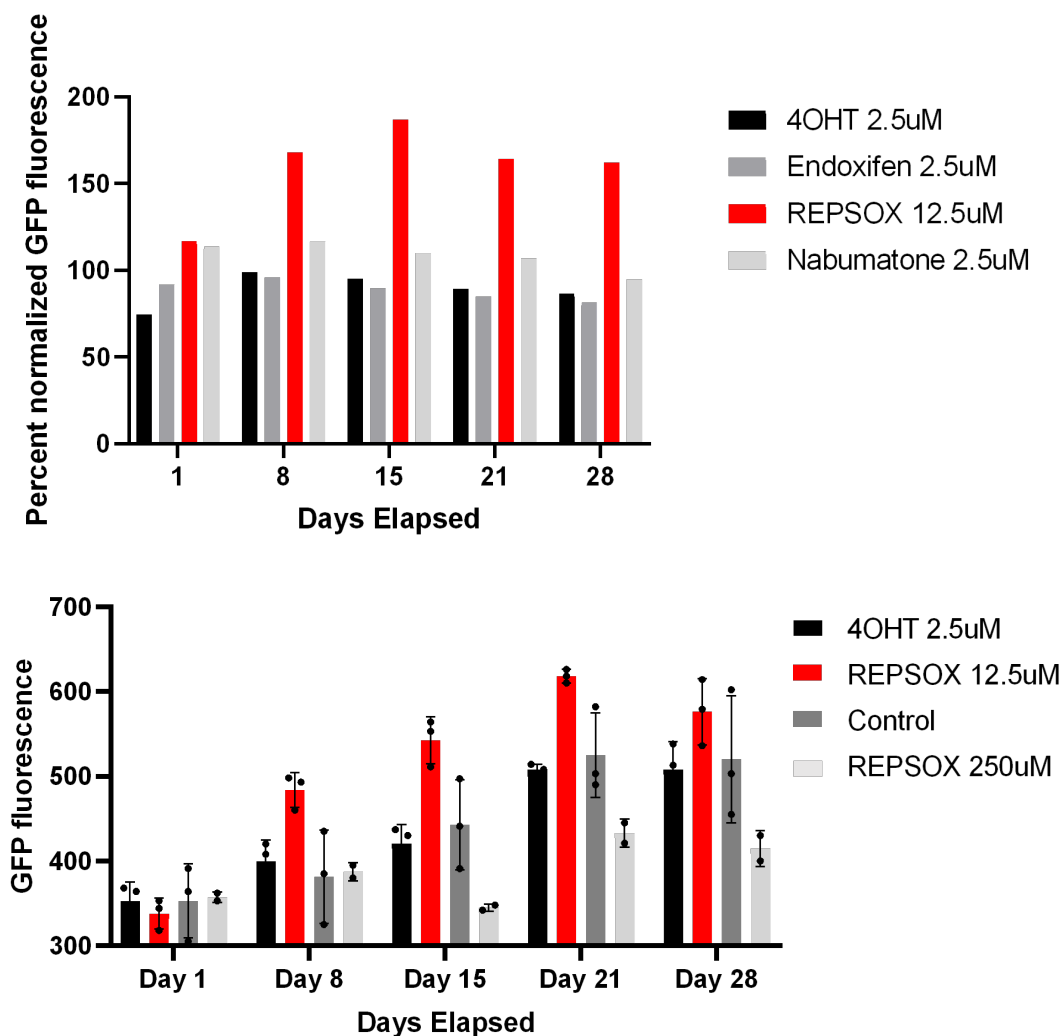


Figure 10. REPSOX induces SOX2 expression in BE organoids. 2 experiments showing the ability of multiple compounds to induce SOX2 expression as measured by GFP fluorescence levels.

Collaborations have been made with the Texas A&M University Institute of Biosciences and Technology to purchase compound/drug screening libraries and to use their live cell plate reader through the BCM Integrated Microscopy Core CellVoyager CV8000 organoid imaging system to proceed with the proposed high throughput screen.

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

The funds provided by this grant along with continued mentorship by Jason Mills have allowed me to develop further expertise and independence in mouse colony management, murine histology, mouse/human organoid culturing techniques, methods for genetic manipulation of organoids, and bioinformatic interpretation of transcriptional (including single-cell RNA sequencing) and genomic data. I have continued to participate in conferences at BCM including weekly Hematology Oncology Grant Rounds, weekly Ben Taub Hospital Multidiscipline GI Tumor Board, weekly Texas Medical Center Digestive Disease Center GI Research Forum, monthly Dan L Duncan Comprehensive Cancer Center Molecular Tumor Board and monthly GI Oncology Clinical Research meetings. In addition, I have been able to present my research and develop collaborations at multiple national/international meetings including FASEB GI Tract XX (oral presentation), 2022 DDRCC Directors Meeting, Wash U DDRCC GI Research Conference (Invited Speaker), CPTAC Steering Committee Meeting (Invited Speaker), AACR Annual Meeting 2023, Eastern Alliance DDRCC Exchange Program at Johns Hopkins DDRCC (Invited Speaker), and the Texas Medical Center DDC Annual Frontiers in Digestive Diseases Symposium. I have also continued to participate in the AGA Abstract Review Committee for the Organoid Models of Gastrointestinal Disorders section for Digestive Disease Week 2023 and have recently been selected to serve as a Councillor for the Cellular & Molecular Gastroenterology (CMG) section of the AGA Institute Council. During the past year, I have also been selected a NIH Clinical Proteomic Tumor Analysis Consortium 4.0 gastric cancer disease expert and leader of the Gastroesophageal Cancer Working Group for the BCM Dan L. Duncan Comprehensive Cancer Center. I have been able to obtain additional funding this year through the Pilot/Feasibility Program Award through the Texas Medical Center DDC. Finally, from the preliminary data generated from this grant, I have recently submitted an NIH PA-20-203 NIDDK Mentored Clinical Scientist Research Career Development Award (K08).

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

The results have been disseminated at multiple national/international meetings including at FASEB GI Tract XX (oral presentation), 2022 DDRCC Directors Meeting, Wash U DDRCC GI Research Conference (Invited Speaker), CPTAC Steering Committee Meeting (Invited Speaker), AACR Annual Meeting 2023, Eastern Alliance DDRCC Exchange Program at Johns Hopkins DDRCC (Invited Speaker), and the Texas Medical Center DDC Annual Frontiers in Digestive Diseases Symposium. Data generated was also published in a manuscript (PMID 36994101) in which I served as the co-corresponding author. I have continued to collaborate actively with investigators including Dr. Michele Battle (Medical College of Wisconsin) and Dr. Rhonda Souza (Baylor University Medical Center at Dallas).

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

I plan to complete my characterization of SOX2 loss in squamous epithelium. Specifically, future experiments are focused on further elucidating pathways increase with SOX2 loss including ROS using DHE and NHE staining and alterations in p53 pathway, **Fig 1A**. Additional experiments will focus on determining the direct transcriptional targets of *Sox2* in the adult squamous epithelium. The use of squamous organoids generated from *Krt5^{CreER/+}; Sox2^{flox/flox}; ROSA26^{LSL TdTomato/+}* and control *Krt5^{+/+}; Sox2^{flox/flox}; ROSA26^{LSL TdTomato/+}* mice that reflect *in vivo* histologic and transcriptomic changes are being used for cleavage under targets and release using nuclease (CUT&RUN) assays that allow assaying for transcription factor binding with improved resolution and decreased background compared to ChIP-seq (PMID 29651053). Optimal cell isolation and permeabilization conditions have been determined, **Fig 3**. I will also perform additional studies characterizing Control and *Krt5^{CreER/+}; Sox2^{flox/flox}; ROSA26^{LSL TdTomato/+}* mice treated with 0.3% DOC at 6 and 12 months

to further elucidate the expanded mucinous columnar glands at the squamocolumnar junction that have intestinal characteristics and are derived from squamous origins, **Fig 6**. I will continue to analyze my human BE organoid data to determine genes that correlate with SOX2 expression in human BE. I also plan to further characterize overexpression of SOX2 in WU014 and WU010 (dysplastic BE organoid line) via transcriptomic and CUT&RUN analyses. Finally, I will pursue my high throughput screen for drugs/compounds that induce SOX2 expression using my now established Luc-pSOX2 human BE organoids.

4. IMPACT:

- **What was the impact on the development of the principal discipline(s) of the project?**

Gastroesophageal cancers are among the most rapidly increasing cancers in the US with poor survival and limited treatment options. As an oncologist, I am committed to not only understanding the basic science of these cancers, but also, taking new discoveries and translating them into much needed treatment improvements. This award has provided critical support for my research to develop improved detection, prevention, and treatment modalities for esophageal cancer. Specifically, my research focuses on a precursor-to-cancer condition called Barrett's esophagus that is defined as the replacement of the normal esophagus lining with an intestinal-like lining in response to long term gastroesophageal reflux disease or "heartburn". I believe that a key factor or gene, SOX2, that is needed to maintain a normal esophagus, and is lost upon development of Barrett's esophagus and during progression to esophageal adenocarcinoma. Thus, a novel way to prevent and treat esophageal adenocarcinoma would be to prevent this loss of SOX2. My studies have elucidated the role of SOX2 in the normal esophagus and during Barrett's esophagus development using new genetically engineered mouse models and human Barrett's esophagus derived organoids or "mini-organs". Together, these experiments will uncover molecular pathways involved in the development of Barrett's esophagus and gastroesophageal cancers and lead to novel therapeutic avenues to treat and prevent these conditions.

- **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

5. CHANGES/PROBLEMS:

- **Changes in approach and reasons for change**

For Specific Aim 1 Major Tasks 1 and 2 and Specific Aim 2 Major Task 3 I will attempt to assess the direct transcriptional targets of the transcription factor, SOX2 in the adult squamous epithelium and in BE. I will use squamous and BE organoids, and cleavage under targets and release using nuclease (CUT&RUN) assays that allow assaying for transcription factor binding with improved resolution and decreased background compared to ChIP-seq (PMID 29651053). I have determined optimal cell isolation and permeabilization conditions, **Fig 3, 9**.

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.

6. PRODUCTS:

○ **Publications, conference papers, and presentations**

▪ **Journal publications.**

Zeng Y, Li QK, Roy S, Mills JC, **Jin RU**. Shared features of metaplasia and the development of adenocarcinoma in the stomach and esophagus. Front Cell Dev Biol. 2023;11:1151790. doi: 10.3389/fcell.2023.1151790. eCollection 2023. PubMed PMID: 36994101; PubMed Central PMCID: PMC10040611. Published.
Acknowledgement of federal support YES

▪ **Books or other non-periodical, one-time publications.**

Nothing to report

▪ **Other publications, conference papers, and presentations.**

Jin RU, Nittolo TM, Wang JS, Li QK, Mills JC. The Role of SOX2 in Barrett's Esophagus Development. Eastern Alliance DDRCC Exchange Program at Johns Hopkins DDRCC. Baltimore, MD (06/2023). Oral Presentation.

Jin RU, Nittolo TM, Wang JS, Li QK, Mills JC. The Role of SOX2 in Barrett's Esophagus Development. AACR Annual Meeting 2023. Orlando, FL (04/2023).

Jin RU, Nittolo TM, Wang JS, Li QK, Mills JC. The Role of SOX2 in Barrett's Esophagus Development. Texas Medical Center DDC Annual Frontiers in Digestive Diseases Symposium. Houston, TX (03/2023)

Jin RU. Gastric Cancer Disease Overview: Unmet Biological and Clinical Questions. CPTAC Steering Committee Meeting. Bethesda, MD (03/2023). Oral Presentation.

Jin RU, Nittolo TM, Wang JS, Li QK, Mills JC. The Role of SOX2 in Barrett's Esophagus and Esophageal Adenocarcinoma. Wash U DDRCC GI Research Conference. St. Louis, MO (02/2023). Oral Presentation.

Jin RU, Nittolo TM, Wang JS, Li QK, Mills JC. The Role of SOX2 in Barrett's Esophagus Development. 2022 DDRCC Directors Meeting. Charleston, SC (10/2022).

Jin RU, Nittolo TM, Wang JS, Li QK, Mills JC. The Role of SOX2 in Barrett's Esophagus Development. FASEB GI Tract XX: Making and Breaking the Gut. Steamboat Springs, CO (08/2022). Oral Presentation.

- **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

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

- **What individuals have worked on the project?**

Name:	Ramon U. Jin, M.D, Ph.D.
-------	--------------------------

Project Role:	Principal Investigator
Researcher Identifier (e.g. ORCID ID):	0000-0001-7805-5800
Nearest person month worked:	7
Contribution to Project:	Ramon U. Jin directs and oversees the research plan including work of Toni Nittolo, and he conducts all proposed experiments.
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Contribution to Project:	Ms. Nittolo is responsible for routine lab procedures like cell culture, genotyping, immunohistochemistry, and mouse colony management.
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- **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**

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- 1. Organization Name:**

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(Full submitted application attached in Appendix).

- 2. Location of Organization:** *(if foreign location list country)*

- 3. Partner's contribution to the project** *(identify one or more)*

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8. SPECIAL REPORTING REQUIREMENTS

- **COLLABORATIVE AWARDS:**



- **QUAD CHARTS:**

9. APPENDICES:



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Shared features of metaplasia and the development of adenocarcinoma in the stomach and esophagus

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Introduction: Plasticity is an inherent property of the normal gastrointestinal tract allowing for appropriate response to injury and healing. However, the aberrancy of adaptable responses is also beginning to be recognized as a driver during cancer development and progression. Gastric and esophageal malignancies remain leading causes of cancer-related death globally as there are limited early disease diagnostic tools and paucity of new effective treatments. Gastric and esophageal adenocarcinomas share intestinal metaplasia as a key precancerous precursor lesion.

Methods: Here, we utilize an upper GI tract patient-derived tissue microarray that encompasses the sequential development of cancer from normal tissues to illustrate the expression of a set of metaplastic markers.

Results: We report that in contrast to gastric intestinal metaplasia, which has traits of both incomplete and complete intestinal metaplasia, Barrett's esophagus (i.e., esophageal intestinal metaplasia) demonstrates hallmarks of incomplete intestinal metaplasia. Specifically, this prevalent incomplete intestinal metaplasia seen in Barrett's esophagus manifests as concurrent development and expression of both gastric and intestinal traits. Additionally, many gastric and esophageal cancers display a loss of or a decrease in these characteristic differentiated cell properties, demonstrating the plasticity of molecular pathways associated with the development of these cancers.

Discussion: Further understanding of the commonalities and differences governing the development of upper GI tract intestinal metaplasias and their progression to cancer will lead to improved diagnostic and therapeutic avenues.

KEYWORDS

plasticity, paligenosis, Barrett's esophagus, intestinal metaplasia, pseudopyloric metaplasia, SPEM

Introduction

Cancers of the stomach and esophagus are major causes of worldwide cancer-related morbidity and mortality with an estimated 1.7 million new cases each year resulting directly in over 1.3 million cancer-related deaths (Sung et al., 2021). In the United States, there were over 48,000 new cases of stomach and esophageal cancers with 27,000 deaths in 2022 (Siegel et al., 2022). In the developed world, adenocarcinoma histologic subtypes predominate and

carry dismal 5-year survival rates of only 20%–25% (Rubenstein and Shaheen, 2015; Coleman et al., 2018; Thrift and El-Serag, 2020). While new treatment paradigms are being explored (Zeng and Jin, 2022), current curative treatments remain restricted to a relatively limited arsenal comprised of conventional chemotherapy, chemoradiotherapy, and surgical resection of the tumor (Bonenkamp et al., 1999; van Hagen et al., 2012; Shapiro et al., 2015; Al-Batran et al., 2019). Thus, patients would benefit from an improved understanding of the pathogenesis of these disease leading to improved early disease detection tools, and safer and more efficacious treatments.

In general, with the exception of tumors arising in the proximal region of the stomach (cardia), gastric and esophageal adenocarcinomas have been considered separate entities in terms of precursor lesions, progression to malignancy, and subsequent clinical course of tumors (Ajani et al., 2017; Smyth et al., 2017; Saenz and Mills, 2018; Singh et al., 2021; Souza and Spechler, 2022). However, accumulating data indicate that gastric and esophageal adenocarcinomas may have considerably more in common than previously appreciated. Genomic profiling has revealed distinct molecular subtype similarities. The ‘Singapore-Duke’ study (Lei et al., 2013), the Asian Cancer Research Group (ACRG) study (Cristescu et al., 2015), and The Cancer Genome Atlas (TCGA) studies (Cancer Genome Atlas Research Network, 2014; Cancer Genome Atlas Research Network, 2017) have provided the basis for the molecular classification of gastroesophageal cancers (Zeng and Jin, 2022). The predominant molecular subtype for gastric adenocarcinomas (over 52%) and esophageal adenocarcinomas (over 98%) is the Chromosomal Instability (CIN) subtype (Cancer Genome Atlas Research Network, 2014; Cancer Genome Atlas Research Network, 2017). Importantly, these CIN gastric and esophageal adenocarcinomas arise in a background of metaplasia and share common intestine-like features. The metaplastic precursor lesion to esophageal adenocarcinoma (EAC) is defined by replacement of the normal squamous epithelial lining of the esophagus with metaplastic columnar cells in response to chronic gastric refluxate (Spechler et al., 2010; Spechler and Souza, 2014). Metaplasia in the body of the stomach is the precursor lesion to gastric adenocarcinoma (GA), and arises during chronic atrophic gastritis through epithelial reprogramming to a more distal antral-like state (Goldenring and Mills, 2022). Such pyloric or pseudopyloric metaplasia occurs in response to chronic damage induced predominantly by *Helicobacter pylori* infection (Correa, 1992; Goldenring and Mills, 2022). In both the esophagus and stomach, metaplasia can assume an intestine-like state. In the esophagus, such metaplasia is called Barrett’s esophagus (BE), and in the stomach it is known as gastric intestinal metaplasia (GIM).

Here, we performed an immunohistologic examination to compare metaplasia and oncogenesis in the esophagus and stomach. We used a set of markers normally restricted to specific regions of the gastrointestinal epithelium whose expression is induced in metaplasia. We immunostained a microarray of human tissue exhibiting progressive phases of oncogenesis in the esophagus and stomach using antibodies against: trefoil peptides (TFF2 and TFF3), mucins (MUC2, MUC5AC, and MUC6), and transcription factors (CDX2 and SOX2). Our results indicate a similarity between BE and incomplete GIM. By definition,

incomplete GIM manifests as the concomitant expression of both gastric and intestinal markers, which we also observe in BE. The mixed gastric and intestinal differentiated cell features seen in BE and GIM are apparently decreased or lost during oncogenesis. Our sampling of malignant cells largely showed decreased expression of metaplasia markers, suggesting the plasticity of molecular pathways involved in cancer development. Further elucidation of the similarities we identify here in the processes governing the development of metaplasia and cancer progression in the esophagus and stomach could lead to improved diagnostic and therapeutic avenues.

Materials and methods

Study case collection

Clinical cases included in our study were collected between January 2011 and December 2018 at Johns Hopkins Hospitals with patient informed consent. All tissue samples were obtained by esophagogastroduodenoscopy (EGD) biopsy, surgical resection, and/or endoscopic mucosal resection (EMR) procedures. Relevant clinical and pathological information for study cases was reviewed and included in the study. The dysplasia grade for Barrett’s esophagus cases was determined according to the American College of Gastroenterology (ACG) 2022 guidelines (Shaheen et al., 2022) and the WHO classification of digestive system tumors (Nagtegaal et al., 2020). The pathological stage of resected carcinomas was determined according to the eighth edition of the American Joint Committee on Cancer (AJCC) Cancer Staging Manual (Amin et al., 2017) and the WHO classification of digestive system tumors (Nagtegaal et al., 2020). All pathological diagnoses were determined by pathologists certified by the American Board of Pathology and reviewed by gastroenterology pathologists.

The use of pathology material for the study was approved by the Institutional Review Board of Johns Hopkins Medical Institutions. In addition, all methods performed in the study were in accordance with the relevant guidelines and regulations.

Construction of the tissue microarrays

The tissue microarrays (TMA) were constructed using the above-mentioned clinical biopsy, surgical resection, and/or endoscopic mucosal resection tissue samples. All tissues were fixed in 10% formalin and embedded in paraffin. To verify the original pathology reports, hematoxylin and eosin (H&E) stained sections were re-reviewed by a pathologist certified by the American Board of Pathology (QKL) prior to TMA construction to ensure the accurate representation of the lesional area and matched normal tissue. Furthermore, H&E stained slides of individual cases were reviewed, the best representative slides were selected for each case, and the corresponding tissue blocks were selected for TMA inclusion. TMA cores (at a diameter of 2.0 mm for BE cases, and 1.0 mm for all other cases) were obtained from paraffin blocks and transferred to build the TMA blocks. In our TMAs, 3–5 cores were obtained from single tissue blocks, except in two cases of BE due to

TABLE 1 Summary of primary antibodies.

Antibody	Company	Clone	Clonality	Dilution	Catalog #
TFF2	Sigma-Aldrich	N/A	Rabbit Polyclonal	1:250 (0.1 mg/mL)	HPA036705
TFF3	Sigma-Aldrich	N/A	Rabbit Polyclonal	1:2000 (0.1 mg/mL)	HPA035464
MUC2	Leica	Ccp58	Mouse Monoclonal	Prediluted	PA0155
MUC5AC	Cell Marque	MRQ-19	Mouse Monoclonal	1:2000	292M-94
MUC6	Abcam	MUC6/916	Mouse Monoclonal	1:200	Ab216017
CDX2	Abcam	EPR2764Y	Rabbit Monoclonal	1:2000 (0.866 mg/mL)	Ab76541
SOX2	Santa Cruz	E-4	Mouse Monoclonal	1:100 (200 ug/mL)	Sc-365823

scant biopsy material, in which case two cores were obtained from each block. After the construction of the TMAs, H&E stained TMA slides were re-reviewed by a pathologist (QKL) as well as by a pathologist specializing in esophageal and gastric oncogenesis (JCM) to ensure the pathological diagnosis of individual cores.

Immunohistochemical staining of markers

The immunohistochemical (IHC) assays were performed by the Oncology Tissue Services Core at The Johns Hopkins University School of Medicine. Briefly, the TMA blocks were cut into 4 µm unstained slides. All unstained slides were deparaffinized prior to incubation with primary antibodies. Following dewaxing and rehydration, epitope retrieval was performed using Ventana Ultra CC1 buffer (catalog# 6414575001, Roche Diagnostics) at 96°C for 64 min. All IHC stains were performed on a Ventana Discovery Ultra autostainer (Roche Diagnostics). Primary antibodies were applied at 36°C for 60 min, and then detected using an anti-mouse or anti-rabbit HQ detection system (catalog# 7017936001 or 7017782001, Roche Diagnostics) followed by Chromomax DAB IHC detection kit (catalog # 5266645001, Roche Diagnostics), counterstaining with Mayer's H&E, dehydration, and mounting. For SOX2 and CDX2 IHC stains, the counterstaining step was performed with eosin only. Appropriate positive and negative controls were included in the staining assays. The complete details of primary antibodies are summarized in [Table 1](#).

Evaluation and scoring of IHC staining

The strength of staining for each marker (taking into account membranous, cytoplasmic and nuclear staining patterns) was scored by the pathologist (QKL) using a semi-quantitative four-tier system: score 0 (0% of cells stained, no staining), score 1 (<10% of cells stained, weak and sparse focal staining), score 2 (10%–50% of cells stained, medium and focal staining), or score 3 (>50% of cells stained, strong and diffuse staining) ([Ao et al., 2014](#); [Gniadek et al., 2017](#); [Zhu et al., 2019](#); [Jin et al., 2021](#); [Li et al., 2021](#); [Johnson et al., 2022](#); [Li et al., 2022](#)). Care was taken not to interpret entrapped macrophages or mucinous material as positive staining.

All IHC stained slides were scanned using the Concentric Digital Pathology Platform (Proscia) and stored as digital files.

Each core was considered as an individual data point, as more than one core might be obtained from the same tissue block described in detail above ([Ao et al., 2014](#); [Gniadek et al., 2017](#); [Zhu et al., 2019](#); [Jin et al., 2021](#); [Li et al., 2021](#); [Johnson et al., 2022](#); [Li et al., 2022](#)). Depending on the TMA section, not all cores could be evaluated due to the loss of tissue during processing or sectioning.

Data analyses and statistics

For each phenotype, the average histological score was calculated by averaging all the core staining scores for the corresponding diagnostic phenotype, and results were plotted using GraphPad Prism 9.0. In addition, the relative fraction of tissue cores with each score was analyzed by SPSS 19.0 (IBM) for each phenotype, and results were plotted using GraphPad Prism 9.0. The differential expression of individual markers among phenotypic groups was analyzed by the Fisher's exact test ([Supplementary Tables S1, S2](#)). All tests were two-sided with a *p*-value <0.05 considered statistically significant.

Results

Demographics of the TMA study cases

The tissue microarray patient demographics are summarized in [Table 2](#). Overall, we assessed a total of 40 cases from the esophagus and 12 cases from the stomach. As mentioned above, multiple TMA cores may have been taken from a single case based on the presence or absence of specific diagnoses in the sampled region as determined by our expert pathologists. The patients included in our study were mostly males of the White/Caucasian race/ethnicity with an average age of 65 years old.

In our cohort, we examined the presence of intestinal metaplasia, dysplasia and adenocarcinoma. A total of 38 cases of Barrett's esophagus (15 negative for dysplasia, 13 with low-grade dysplasia, and 10 with high-grade dysplasia) and 6 cases of GIM were analyzed. In addition, 30 EACs and 11 GAs cases were included. A subset of our study cases, including 15 EACs and 11 GAs were treated with curative surgical or endoscopic mucosal resection. At time of resection, five of the EACs had

TABLE 2 Demographics of the study cases.

Characteristic		No.	
Location		Esophagus ^a (n = 40)	Stomach ^a (n = 12)
Sex	Female,n(%)	5(12.5)	2(16.7)
	Male,n(%)	35(87.5)	10(83.3)
Race/Ethnicity	Hispanic or Latino,n(%)	0(0.0)	1(8.3)
	African American,n(%)	1(2.5)	1(8.3)
	Asian,n(%)	0(0.0)	1(8.3)
	White/Caucasian,n(%)	35(87.5)	9(75.0)
	Other ^b ,n(%)	4(10.0)	0(0.0)
Age, years (mean [range])		65.5[50–90]	64.5[49–76]
	≥60 years,n(%)	32(80.0)	8(66.7)
	<60 years,n(%)	8(20.0)	4(33.3)
Procedure	Biopsy,n(%)	22(55.0)	2(16.7)
	Endoscopic Mucosal Resection,n(%)	2(5.0)	0(0.0)
	Surgical Resection,n(%)	16(40.0)	10(83.3)
Diagnosis	Intestinal Metaplasia,n(%)	N/A ^c	6(50.0)
	Adenocarcinoma,n(%)	30(75.0)	11(91.7)
	with Lymph Node Metastasis,n(%)	5(12.5)	5(41.7)
	with Liver Metastasis,n(%)	0(0.0)	2(16.7)
	Barrett's Esophagus		
	with Low-Grade Dysplasia,n(%)	13(32.5)	N/A
	with High-Grade Dysplasia,n(%)	10(25.0)	N/A
	Negative for Dysplasia,n(%)	15(37.5)	N/A
Proton Pump Inhibitors Treatment	Treatment,n(%)	23(57.5)	9(75.0)
	No treatment,n(%)	17(42.5)	3(25.0)

^aIn our study, three patients had both a biopsy and follow-up stomach cancer surgical resection; we considered these cases separately.

^bIncluding Native American or Alaska Native, Native Hawaiian or Pacific Islander, or other.

^cCases of esophageal intestinal metaplasia were classified as Barrett's esophagus.

lymph node metastases, while five and two of the GAs had lymph node and liver metastases, respectively. Many of these resected cancer cases had received neoadjuvant chemoradiation or chemotherapy prior to surgery, including 11 of 15 EACs and 5 of the 11 GAs. Our tissue microarray demonstrates an inclusive clinical representation of normal, metaplasia, and adenocarcinoma cases for the esophagus and stomach based on patient demographics.

A tissue microarray that encompasses sequential formation of esophageal and gastric adenocarcinomas

H&E and corresponding Periodic Acid–Schiff (PAS) staining showed the histological characteristics of normal squamous,

Barrett's esophagus negative for dysplasia (BE-NFD), Barrett's esophagus with low-grade dysplasia (BE-LGD), Barrett's esophagus with high-grade dysplasia (BE-HGD), and EAC from the esophageal cases (Figures 1A, B); and normal gastric corpus, GIM, and GA from the gastric cases (Figures 1C, D). As expected, PAS staining highlighted mucus-containing goblet cells in cores from both BE and GIM samples. In addition, we carefully examined full glands encompassing the top (closest to the lumen of the esophagus or stomach) and the deeper gland areas for BE-NFD and GIM. We did this, as molecular analyses of BE in recent years have highlighted basal, deep glandular mucous cell features in these lesions (Busslinger et al., 2021; Evans et al., 2022). Importantly, we performed all immunohistologic analyses on the same cores using serial sections to demonstrate the relationship of multiple assessed markers on the same tissue areas. From the TMA esophageal and stomach cases, we have also included samples of columnar-lined

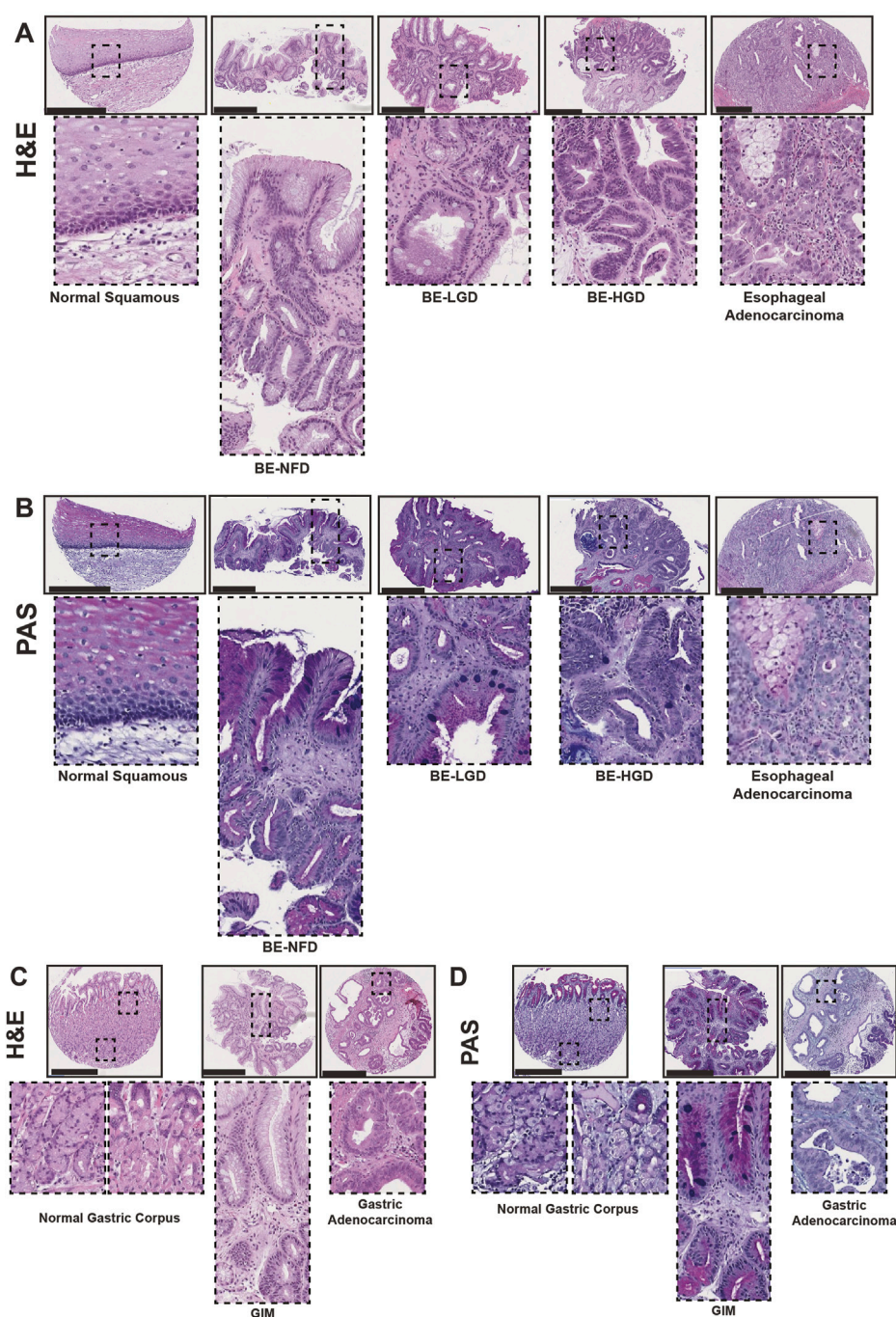


FIGURE 1

Human tissue microarray of normal squamous-BE-EAC and normal gastric gland-GIM-GA progression sequence. (A) Morphology of representative H&E staining from the human tissue microarray of esophageal cases, from left to right: normal squamous, BE-NFD, BE-LGD, BE-HGD, and EAC. (B) Morphology of representative PAS staining from the human tissue microarray of esophageal cases, same sequence as in (A). (C) Morphology of representative H&E staining from the human tissue microarray of gastric cases, from left to right: normal gastric corpus (left inset from base of the glands, right inset from the surface of the glands), GIM, and GA. (D) Morphology of representative PAS staining from the human tissue microarray of gastric cases, in the same sequence as in (C). All scale bars, 500 μ m.

mucosa of the esophagus (DeMeester and DeMeester, 2000) and complete GIM of the stomach (Correa et al., 2010; Goldenring and Mills, 2022) (Supplementary Figures S1, S2). Our TMAs provide a valuable tool to compare and contrast critical features during the development of BE and GIM and their sequential progression to cancers in the esophagus and stomach.

TFF2 and TFF3 expression in normal squamous-BE-EAC and normal gastric gland-GIM-GA progression sequences

Pyloric or pseudopyloric metaplasia is a gastric repair process in response to injury usually caused by *Helicobacter pylori*

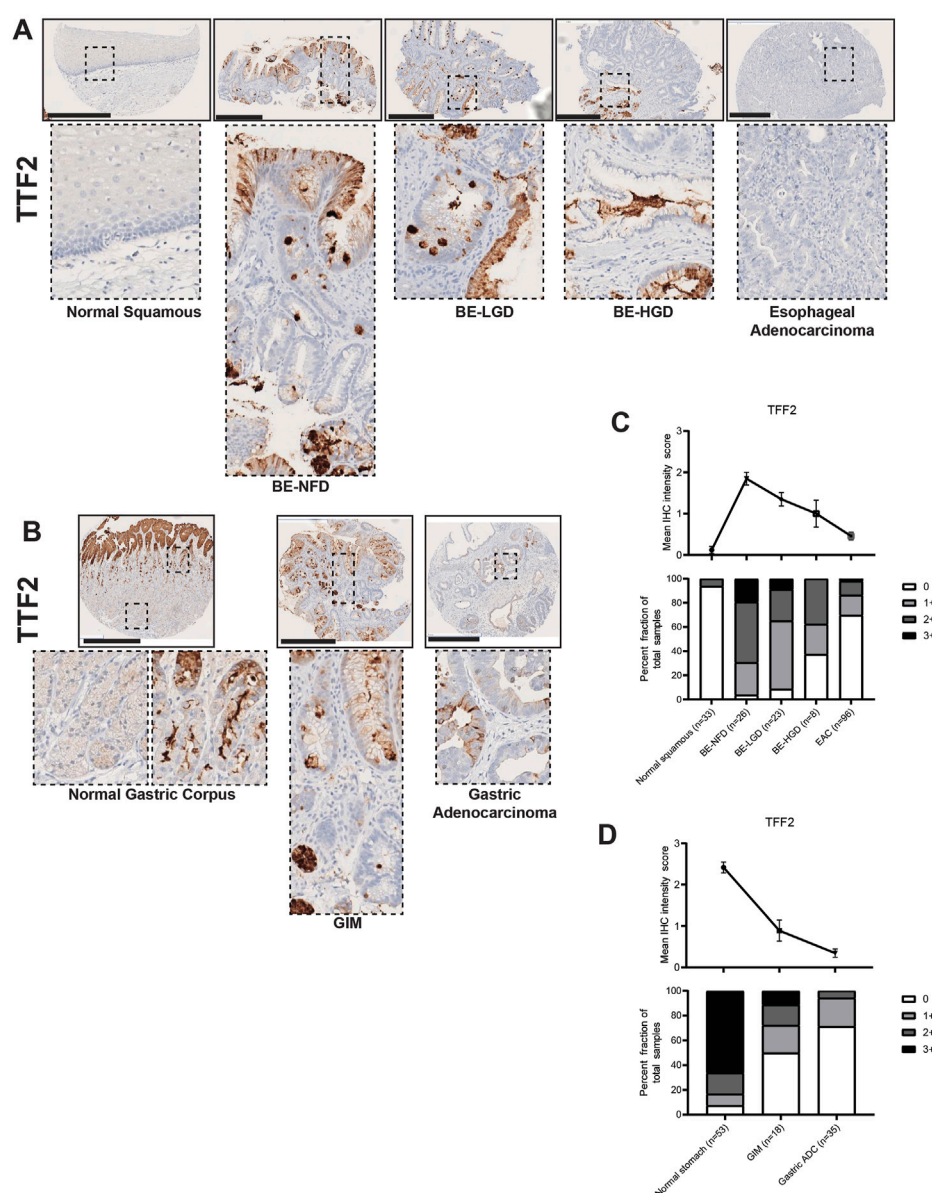


FIGURE 2

TFF2 expression in normal squamous-BE-EAC and normal gastric gland-GIM-GA progression sequence. **(A)** Immunohistochemistry of TFF2 staining in human tissue microarray esophageal cases, from left to right: normal squamous, BE-NFD, BE-LGD, BE-HGD, and EAC. **(B)** Immunohistochemistry of TFF2 staining in human tissue microarray gastric cases, from left to right: normal gastric corpus (left inset from base of the glands, right inset from the surface of the glands), GIM, and GA. All scale bars, 500 μ m. **(C)** Analysis of the human esophageal tissue microarray with normal squamous, BE-NFD, BE-LGD, BE-HGD, and EAC tissue cores stained by immunohistochemistry for TFF2. Top: the TFF2 average IHC intensity score of each esophageal phenotype is plotted. For staining intensity, score 0 (undetectable) to 3 (most intense). Bottom: the TFF2 fraction of esophageal tissue cores with each score is plotted. Each phenotype's total tissue core number is provided at the bottom of each column. **(D)** Analysis of the human gastric tissue microarray with normal, GIM, and GA tissue cores stained by immunohistochemistry for TFF2. Top: the TFF2 average IHC intensity score of each gastric phenotype is plotted. For staining intensity, score 0 (undetectable) to 3 (most intense). Bottom: the TFF2 fraction of gastric tissue cores with each score is plotted. Each phenotype's total tissue core number is provided at the bottom of each column.

infection (Goldenring et al., 2010; Goldenring and Mills, 2022). During this process, the normal gastric glands exhibit loss of parietal cells, and mature chief cells become metaplastic with abnormal expression of the gastric gland neck cell lineage marker, Trefoil Factor 2 (TFF2) (Weis et al., 2013; Saenz and Mills, 2018; Willet et al., 2018). We note that such metaplastic cells are called spasmolytic polypeptide-expressing metaplasia (SPEM) because TFF2 is now the official name for spasmolytic

polypeptide. GIM and gastric cancer are thought to arise from pyloric metaplasia (Goldenring et al., 2010; Graham et al., 2019; Goldenring and Mills, 2022). Also, it should be noted that the lesion "chronic atrophic gastritis" is essentially indistinguishable from pyloric metaplasia as the loss of parietal cells and reprogramming of chief cells are the atrophic changes that give that lesion its name (Goldenring and Mills, 2022; Hsieh et al., 2022).

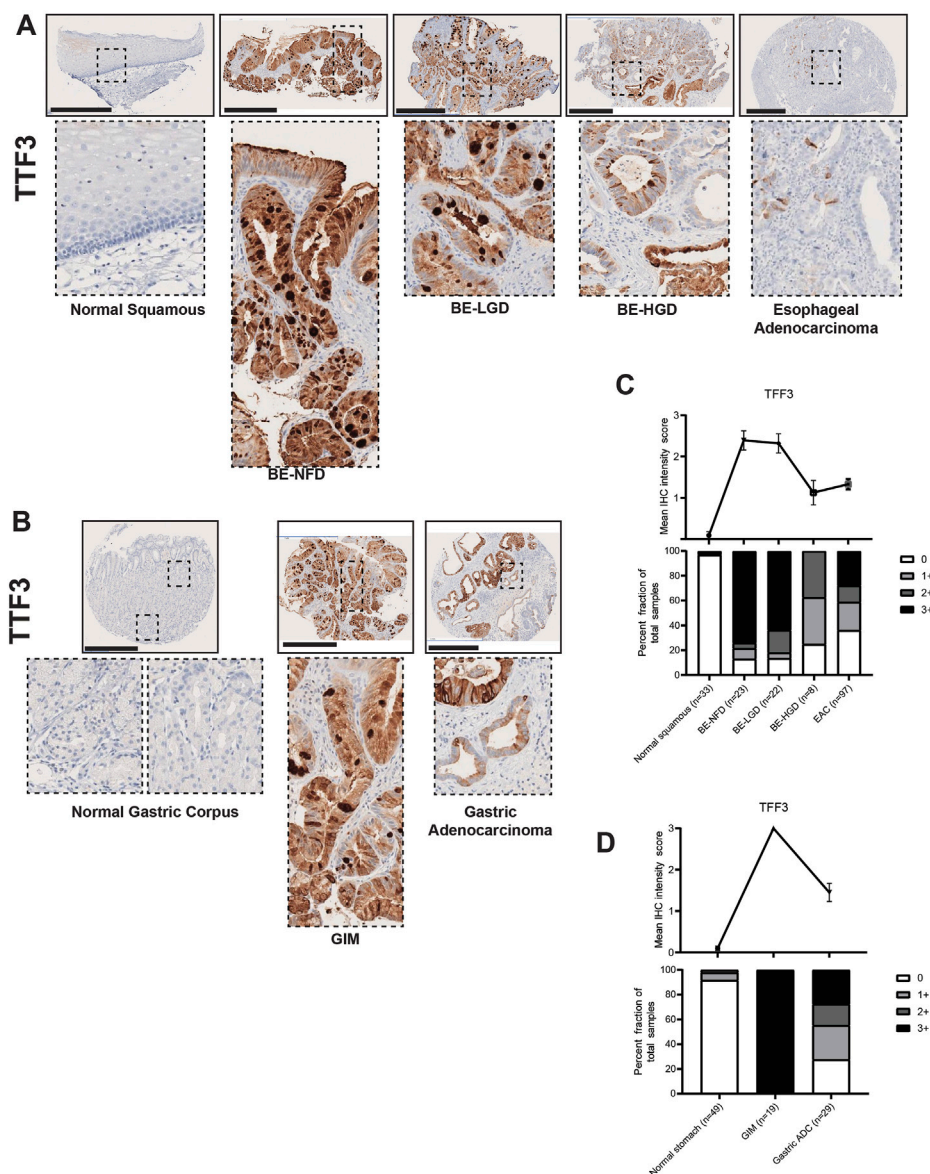


FIGURE 3

TFF3 expression in normal squamous-BE-EAC and normal gastric gland-GIM-GA progression sequence. **(A)** Immunohistochemistry of TFF3 staining in human tissue microarray esophageal cases, in the same sequence as in Figure 2A. **(B)** Immunohistochemistry of TFF3 staining in human tissue microarray gastric cases, in the same sequence as in Figure 2B. All scale bars, 500 μ m. **(C)** Top: the TFF3 average IHC intensity score of each esophageal phenotype is plotted in the same sequence as in Figure 2C. Bottom: the TFF3 fraction of esophageal tissue cores with each score is plotted in the same sequence as in Figure 2C. Each phenotype's total tissue core number is provided at the bottom of each column. **(D)** Top: the TFF3 average IHC intensity score of each esophageal phenotype is plotted in the same sequence as in Figure 2D. Bottom: the TFF3 fraction of esophageal tissue cores with each score is plotted in the same sequence as in Figure 2D. Each phenotype's total tissue core number is provided at the bottom of each column.

In our study, TFF2 was expressed in intestinal metaplasia lesions in both the esophagus and stomach (Figures 2A, B). As expected, TFF2 was not expressed in the normal esophageal squamous epithelium, but its expression was correlated with the development of BE (Figure 2A). In the normal gastric corpus, TFF2 was expressed in mucous neck cells with no expression in mature chief cells at the gland bases (Figure 2B). We also detected TFF2 in foveolar pit cells, which may be an inadvertent cross-reaction against TFF1 as the two antigens are over 60% homologous, and similar pit-cell

TFF2 reactivity has been reported before (Hoffmann, 2015; Hsieh et al., 2022). Given that TFF2 is a gastric marker, it was predictably decreased in GIM (Figure 2B). However, TFF2 was abundantly detected in BE with foci of expression at the surface and more intense positivity in deep glandular cells. Interestingly, TFF2 expression levels decreased with the development of dysplastic BE and EAC (Figure 2C; Supplementary Table S1). There was a similar TFF2 decreasing expression pattern with the development of GA (Figure 2D; Supplementary Table S2).

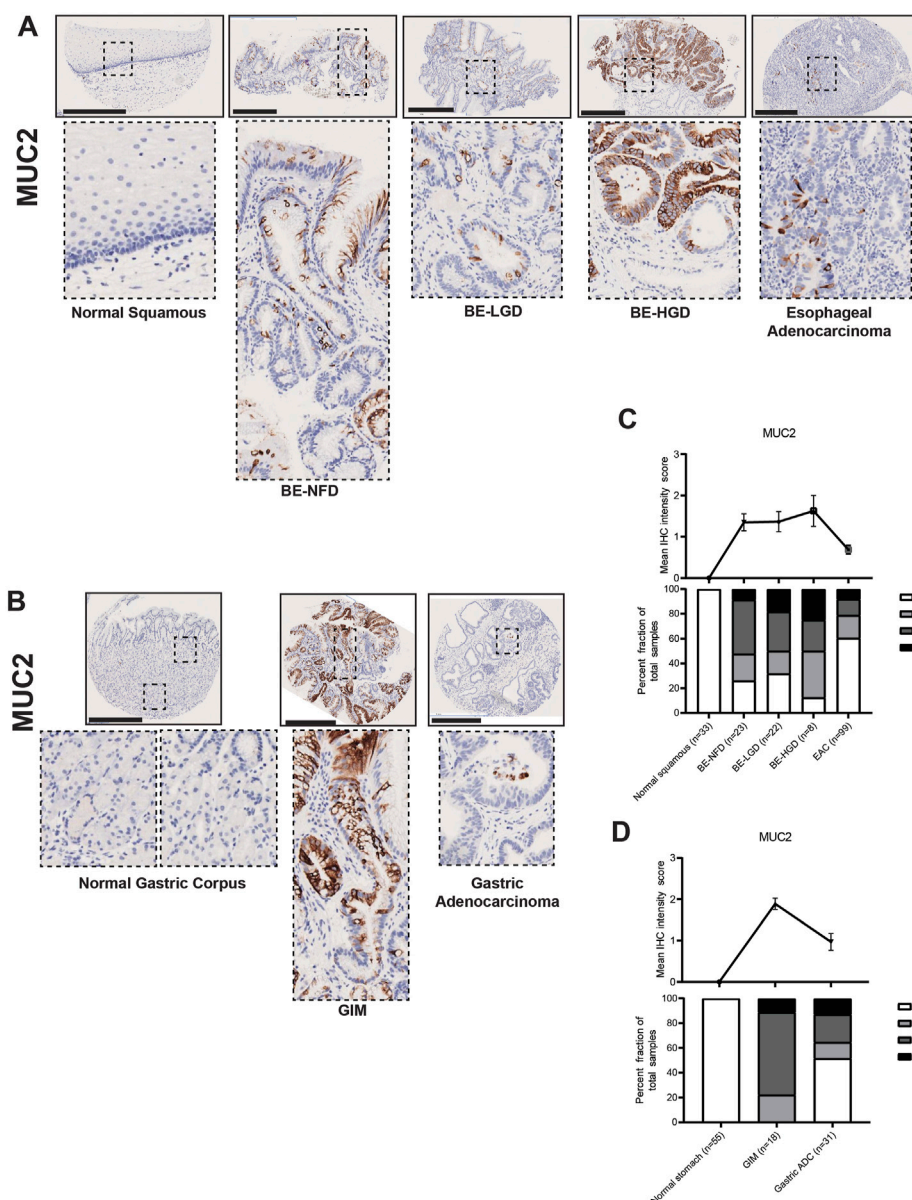
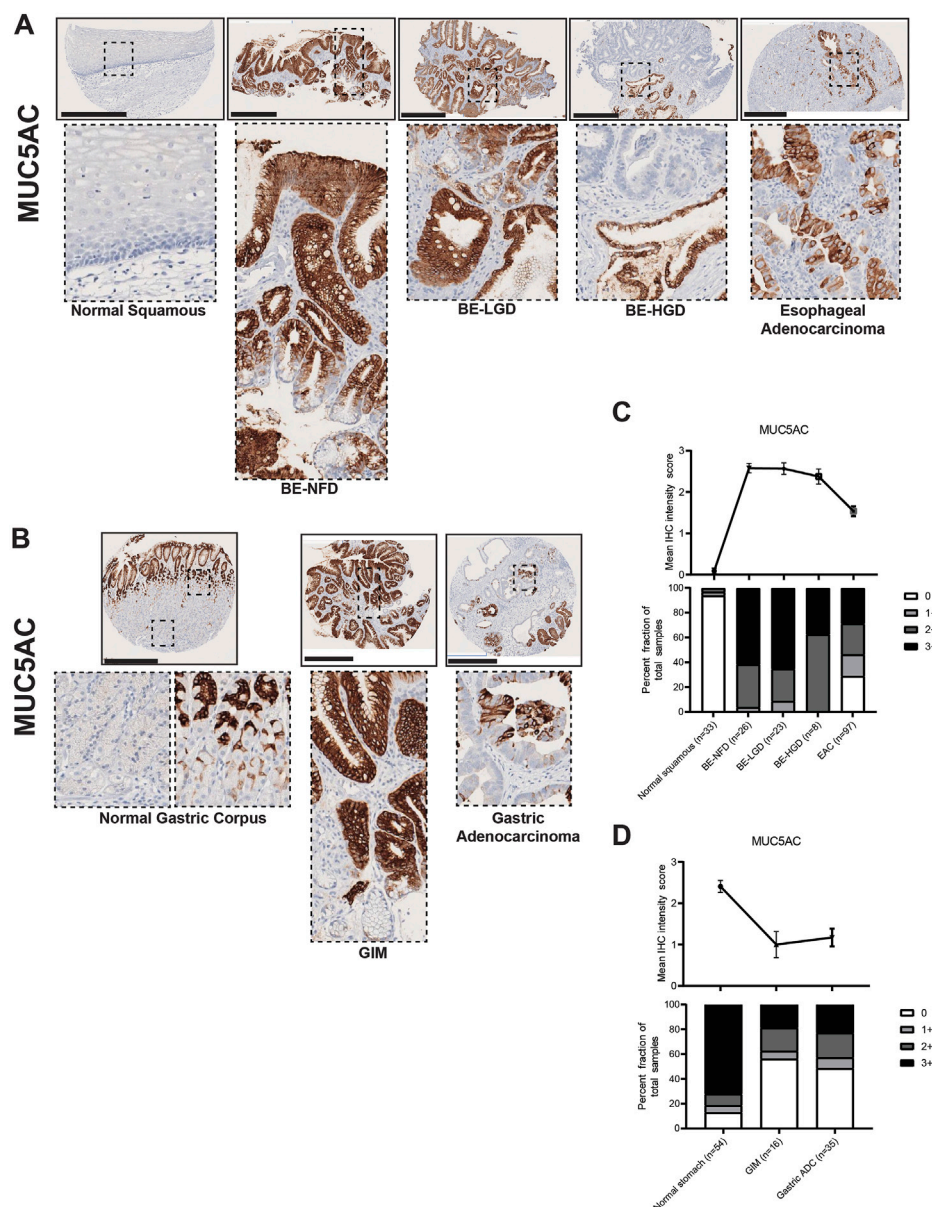


FIGURE 4

MUC2 expression in normal squamous-BE-EAC and normal gastric gland-GIM-GA progression sequence. **(A)** Immunohistochemistry of MUC2 staining in human tissue microarray esophageal cases, in the same sequence as in [Figure 2A](#). **(B)** Immunohistochemistry of MUC2 staining in human tissue microarray gastric cases, in the same sequence as in [Figure 2B](#). All scale bars, 500 μ m. **(C)** Top: the MUC2 average IHC intensity score of each esophageal phenotype is plotted in the same sequence as in [Figure 2C](#). Bottom: the MUC2 fraction of esophageal tissue cores with each score is plotted in the same sequence as in [Figure 2C](#). Each phenotype's total tissue core number is provided at the bottom of each column. **(D)** Top: the MUC2 average IHC intensity score of each esophageal phenotype is plotted in the same sequence as in [Figure 2D](#). Bottom: the MUC2 fraction of esophageal tissue cores with each score is plotted in the same sequence as in [Figure 2D](#). Each phenotype's total tissue core number is provided at the bottom of each column.

Expression of another trefoil factor protein, Trefoil Factor 3 (TFF3), has been shown to be increased in BE ([Lauren, 1965](#); [Lao-Sirieix et al., 2009](#); [Fitzgerald et al., 2014](#); [Lavery et al., 2014](#); [Fitzgerald et al., 2020](#)) and also strongly induced during gastric mucosal injury ([Taupin et al., 2001](#)). Accordingly, our TMA immunohistochemistry results demonstrated that TFF3 was not significantly expressed in the normal esophagus nor in the normal gastric corpus, but its expression was significantly increased with the development of intestinal metaplasia ([Figures](#)

[3A–D](#); [Supplementary Tables S1, S2](#)). Furthermore, compared with TFF2 expression in the IM glands, TFF3 was expressed throughout the entire metaplastic gland in a diffuse distribution ([Figures 3A, B](#)). We also found that the expression level of TFF3 gradually decreased during the BE metaplasia-to-dysplasia to EAC progression ([Figures 3A, C](#); [Supplementary Table S1](#)) and during the GIM to GA progression ([Figures 3B, D](#); [Supplementary Table S2](#)). In our study, the samples of columnar-lined mucosa of the esophagus showed dual expression of TFF2 and TFF3 ([Supplementary](#)

**FIGURE 5**

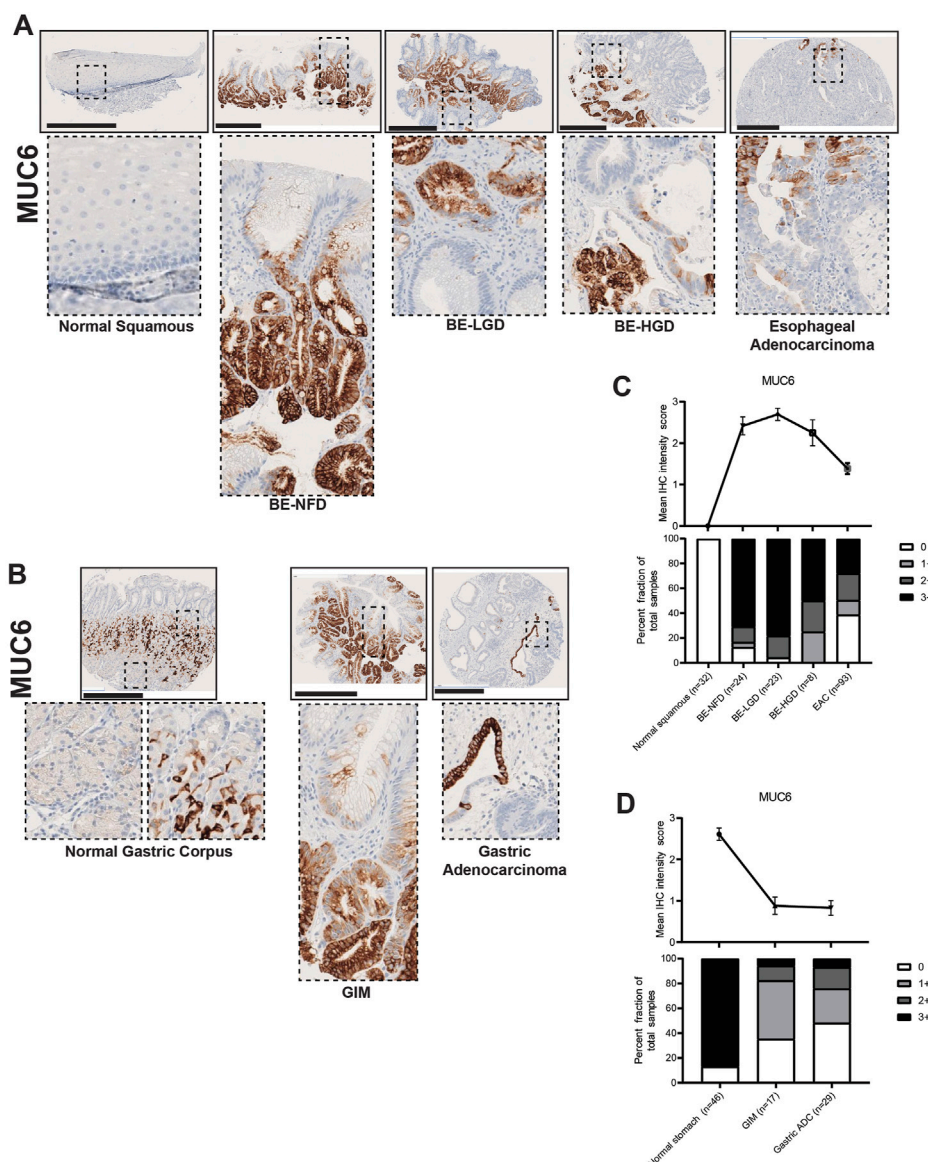
MUC5AC expression in normal squamous-BE-EAC and normal gastric gland-GIM-GA progression sequence. **(A)** Immunohistochemistry of MUC5AC staining in human tissue microarray esophageal cases, in the same sequence as in [Figure 2A](#). **(B)** Immunohistochemistry of MUC5AC staining in human tissue microarray gastric cases, in the same sequence as in [Figure 2B](#). All scale bars, 500 μ m. **(C)** Top: the MUC5AC average IHC intensity score of each esophageal phenotype is plotted in the same sequence as in [Figure 2C](#). Bottom: the MUC5AC fraction of esophageal tissue cores with each score is plotted in the same sequence as in [Figure 2C](#). Each phenotype's total tissue core number is provided at the bottom of each column. **(D)** Top: the MUC5AC average IHC intensity score of each esophageal phenotype is plotted in the same sequence as in [Figure 2D](#). Bottom: the MUC5AC fraction of esophageal tissue cores with each score is plotted in the same sequence as in [Figure 2D](#). Each phenotype's total tissue core number is provided at the bottom of each column.

[Figure S1](#)), while complete GIM showed predominantly singular expression of TFF3 ([Supplementary Figure S2](#)).

In summary, we show that TFF2, which is normally present in the stomach, becomes abundantly expressed in BE, is decreased in expression in GIM, and its expression decreases during gastroesophageal cancer formation. TFF3 is an intestinal marker not expressed normally in the upper GI tract, but is strongly expressed in both BE and GIM, and its expression also decreases during cancer development.

MUC2, MUC5AC, and MUC6 expression in normal squamous-BE-EAC and normal gastric gland-GIM-GA progression sequences

Mucins are glycosylated proteins expressed throughout the GI tract. MUC5AC is normally expressed in the gastric foveolar pit cells, and MUC6 in gastric mucous neck cells, while MUC2 is not expressed in the normal gastric mucosa, but is rather an intestinal

**FIGURE 6**

MUC6 expression in normal squamous-BE-EAC and normal gastric gland-GIM-GA progression sequence. **(A)** Immunohistochemistry of MUC6 staining in human tissue microarray esophageal cases, in the same sequence as in Figure 2A. **(B)** Immunohistochemistry of MUC6 staining in human tissue microarray gastric cases, in the same sequence as in Figure 2B. All scale bars, 500 μ m. **(C)** Top: the MUC6 average IHC intensity score of each esophageal phenotype is plotted in the same sequence as in Figure 2C. Bottom: the MUC6 fraction of esophageal tissue cores with each score is plotted in the same sequence as in Figure 2C. Each phenotype's total tissue core number is provided at the bottom of each column. **(D)** Top: the MUC6 average IHC intensity score of each esophageal phenotype is plotted in the same sequence as in Figure 2D. Bottom: the MUC6 fraction of esophageal tissue cores with each score is plotted in the same sequence as in Figure 2D. Each phenotype's total tissue core number is provided at the bottom of each column.

mucin (Reis et al., 1999). Our TMA IHC results showed that both BE and GIM contained MUC2-positive cells (Figures 4A, B). Compared to normal esophageal squamous and normal gastric corpus cells, MUC2 was highly expressed in intestine-specific goblet cells, which were mainly distributed more towards the luminal surface rather than deep within glands in both BE and GIM (Figures 4A, B). In addition, MUC2 expression decreased during progression to EAC and GA (Figures 4A–D; Supplementary Tables S1, S2).

Our IHC data demonstrated that MUC5AC was expressed by surface foveolar pit cells in the normal gastric corpus, and

mainly distributed in the superficial glandular areas in both BE and GIM (Figures 5A, B). Interestingly, although MUC5AC paralleled the decreasing expression pattern of MUC2 in the BE-NFD, BE-LGD, BE-HGD and EAC progression, MUC5AC expression was decreased in GIM and did not further decrease in GA in our study (Figures 5C, D; Supplementary Tables S1, S2).

In contrast to MUC2 and MUC5AC, MUC6 was mainly expressed in the deeper gland regions of BE and GIM (Figures 6A, B). MUC6 was not expressed in the normal squamous

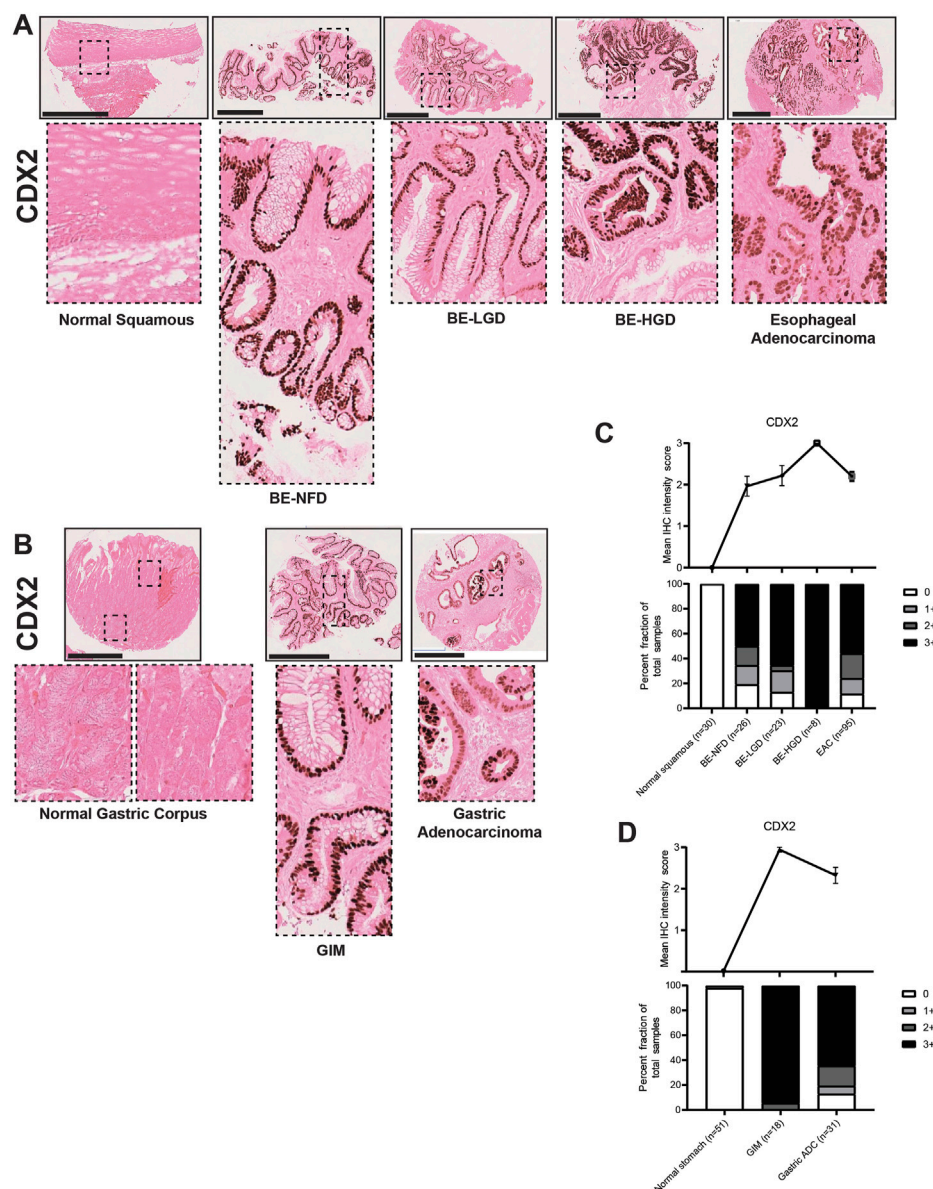
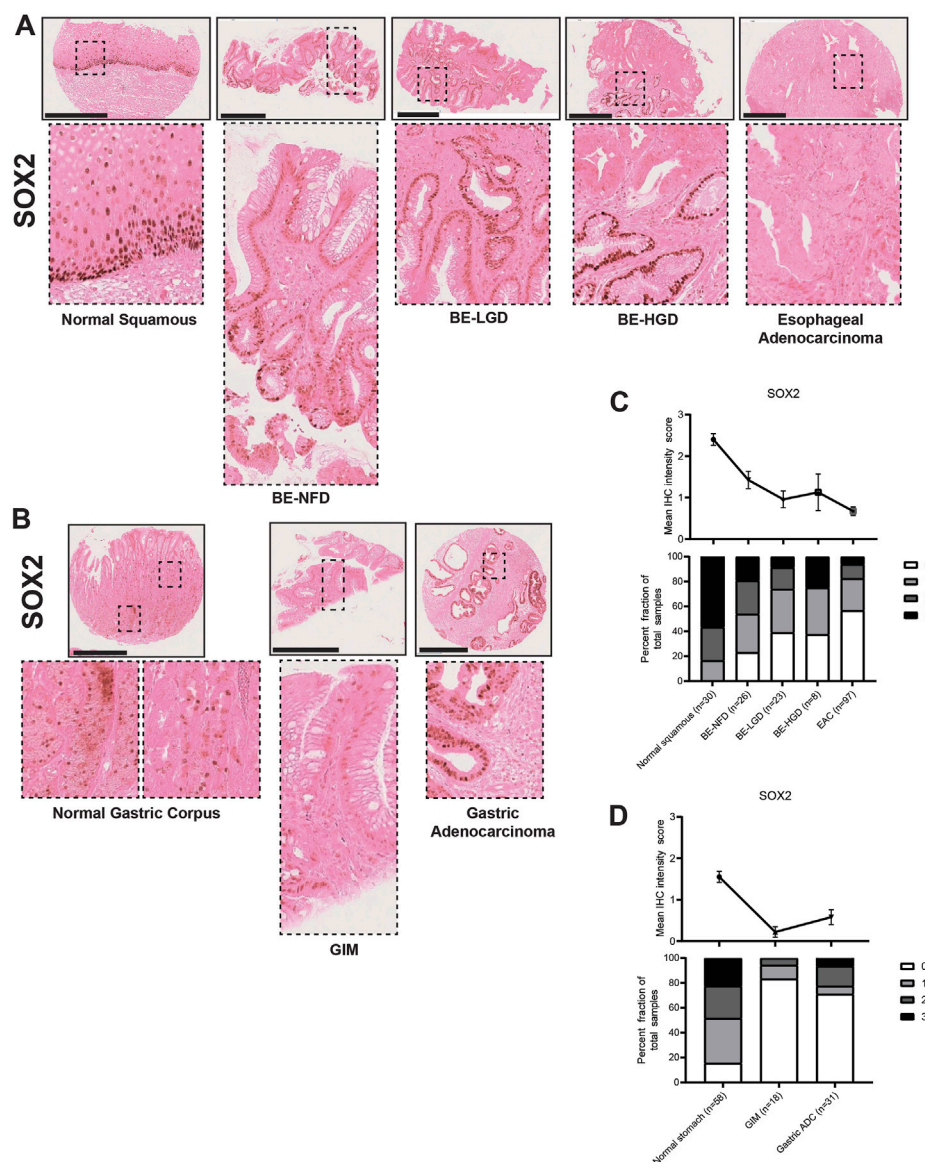


FIGURE 7

CDX2 expression in normal squamous-BE-EAC and normal gastric gland-GIM-GA progression sequence. **(A)** Immunohistochemistry of CDX2 staining in human tissue microarray esophageal cases, in the same sequence as in Figure 2A. **(B)** Immunohistochemistry of CDX2 staining in human tissue microarray gastric cases, in the same sequence as in Figure 2B. All scale bars, 500 μ m. **(C)** Top: the CDX2 average IHC intensity score of each esophageal phenotype is plotted in the same sequence as in Figure 2C. Bottom: the CDX2 fraction of esophageal tissue cores with each score is plotted in the same sequence as in Figure 2C. Each phenotype's total tissue core number is provided at the bottom of each column. **(D)** Top: the CDX2 average IHC intensity score of each esophageal phenotype is plotted in the same sequence as in Figure 2D. Bottom: the CDX2 fraction of esophageal tissue cores with each score is plotted in the same sequence as in Figure 2D. Each phenotype's total tissue core number is provided at the bottom of each column.

epithelium of the esophagus but was expressed in the mucus neck cells of the gastric corpus (Figures 6A, B). Akin to MUC5AC, MUC6 expression decreased with cancer formation in the esophagus but not in the stomach (Figures 6C, D; Supplementary Tables S1, S2). In addition, samples of columnar-lined mucosa of the esophagus showed focal expression of MUC5AC and diffuse expression of MUC6 (Supplementary Figure S1). Complete GIM showed a predominantly singular expression of MUC2 (Supplementary Figure S2).

Here, we show that MUC2, an intestinal marker not expressed in the esophagus or stomach, is strongly expressed in both BE and GIM, and its expression decreases during cancer development. MUC5AC and MUC6, which are normal mucins expressed in the stomach, become aberrantly expressed in the esophagus in nearly all BE lesions. MUC5AC and MUC6 expression is decreased in GIM indicating that many of these lesions (i.e., complete GIM) may lose gastric characteristics. MUC5AC and MUC6 expression decreases during esophageal

**FIGURE 8**

SOX2 expression in normal squamous-BE-EAC and normal gastric gland-GIM-GA progression sequence. (A) Immunohistochemistry of SOX2 staining in human tissue microarray esophageal cases, in the same sequence as in Figure 2A. (B) Immunohistochemistry of SOX2 staining in human tissue microarray gastric cases, in the same sequence as in Figure 2B. All scale bars, 500 μ m. (C) Top: the SOX2 average IHC intensity score of each esophageal phenotype is plotted in the same sequence as in Figure 2C. Bottom: the SOX2 fraction of esophageal tissue cores with each score is plotted in the same sequence as in Figure 2C. Each phenotype's total tissue core number is provided at the bottom of each column. (D) Top: the SOX2 average IHC intensity score of each esophageal phenotype is plotted in the same sequence as in Figure 2D. Bottom: the SOX2 fraction of esophageal tissue cores with each score is plotted in the same sequence as in Figure 2D. Each phenotype's total tissue core number is provided at the bottom of each column.

adenocarcinoma formation, but not gastric adenocarcinoma progression.

CDX2 and SOX2 expression in normal squamous-BE-EAC and normal gastric gland-GIM-GA progression sequences

CDX2 and SOX2 are key transcription factors involved in gut development (McGrath and Wells, 2015; Miao et al., 2020; Ma et al.,

2022) with SOX2 involved in foregut development (Que et al., 2007) and CDX2 functioning in intestinal development (Silberg et al., 2000). In our study, CDX2, as expected, had no expression in the normal esophageal squamous epithelium or the normal gastric corpus (Figures 7A, B). However, CDX2 became dramatically expressed in BE, and its expression was increased with the development of dysplasia; CDX2 expression was downregulated but still maintained at high levels in EAC (Figures 7A–C; Supplementary Table S1). Similar to the esophagus, nuclear CDX2 expression was significantly increased in GIM cells, while

downregulated and kept at high levels in GA (Figures 7B, D; Supplementary Table S2).

In the esophagus, SOX2 expression levels were gradually downregulated during normal esophageal squamous, BE metaplasia, dysplasia to EAC progression sequences (Figures 8A, C; Supplementary Table S1). In the stomach, SOX2 was expressed in the normal gastric glands in a predominantly chief cell distribution and decreased in GIM and GA (Figures 8B, D; Supplementary Table S2). The CDX2 and SOX2 expression demonstrated a converse pattern in the metaplasia-dysplasia-adenocarcinoma progression sequence in both esophageal and gastric cases. Interestingly, the samples of columnar-lined mucosa of the esophagus showed aberrant expression of CDX2 and decreased SOX2 despite the absence of intestinal goblet cells (Supplementary Figure S1), and complete GIM showed expression of CDX2 with no detectable SOX2 (Supplementary Figure S2). Our findings show an increase in CDX2 expression and a decrease in SOX2 expression in both normal squamous-BE-EAC and normal gastric gland-GIM-GA progression sequences.

Taken together, our TMA IHC observations hint that BE and pure incomplete GIM are similar and consist of both gastric and intestinal features. But randomly sampled GIM lesions show a mixture between complete and incomplete types as exhibited by a decrease in gastric markers including TFF2, MUC5AC, MUC6, and SOX2. In addition, during esophageal adenocarcinoma progression there is an apparent de-differentiation with loss of both gastric and intestinal characteristics. However, during gastric cancer formation, there is a preferential loss of intestinal markers with maintained expression of gastric markers.

Discussion

Despite the molecular similarities between EAC and GA (Cancer Genome Atlas Research Network, 2014; Cancer Genome Atlas Research Network, 2017; Zeng and Jin, 2022), there have been few immunohistological studies that have compared these diseases or their precursor metaplastic lesions. The few previous studies directly comparing metaplasias in the stomach and esophagus have focused on the differences between these lesions (El-Zimaity and Graham, 2001; Glickman et al., 2001; Piazuelo et al., 2004) rather than the similarities (Spechler et al., 2017). A key strength of our study has been the focus on similarities and differences between BE and GIM by recognizing and highlighting complete and incomplete intestinal metaplasia. In addition, we have also broadened our analysis to include dysplastic and neoplastic lesions of the stomach and esophagus.

To this end, we have analyzed the similarities and differences involved in the formation of EAC and GA using a unique patient tissue microarray of the upper GI tract and a panel of histologic markers including trefoil peptides (TFF2 and TFF3), mucins (MUC2, MUC5AC, and MUC6), and transcription factors (CDX2 and SOX2). Several key findings have emerged from our study. First, we found that most cases of BE in our case series resemble incomplete GIM (Correa et al., 2010; Goldenring and Mills, 2022). Specifically, BE manifests as co-expression of the trefoil factors TFF2 and TFF3, as well as co-expression of MUC2, MUC5AC, and MUC6 (Figures 2–6). In BE, there is also gain of

the intestinal transcription factor, CDX2, with concomitant loss of the foregut transcription factor, SOX2 (Figures 7, 8). In contrast, complete GIM is characterized by TFF3 and MUC2 expression without co-expression of TFF2, MUC5AC, or MUC6 (Supplementary Figure S1).

The fact that intestinal metaplasia is heterogenous has been established in the stomach (Correa et al., 2010; Goldenring and Mills, 2022). Using morphology and mucin expression patterns, GIM can be further divided into three distinct types (Filipe et al., 1994). Complete or type I GIM expresses intestinal goblet cell sialomucins reflecting a “pure” intestinal metaplasia (Supplementary Figure S1). Incomplete or type III GIM expresses sulfomucins, and type II (also termed incomplete) expresses both types of mucins reflecting lesions with mixed gastric and intestinal traits (Figure 1D). While these same intestinal metaplasia classifications have not been applied to the esophagus, there has been emerging recognition that BE harbors mixed gastric (specifically more pyloric gland characteristics) and intestinal traits. The mechanism by which this occurs is still unclear; studies have suggested a transdifferentiation process originating from the native squamous epithelium (Huo et al., 2010; Minacapelli et al., 2017), the submucosal glands (Leedham et al., 2008; Owen et al., 2018), or the gastric cardia (Quante et al., 2012; Nowicki-Osuch et al., 2021). We argue that the incomplete intestinal metaplasia seen in BE hints towards the potential origin of these glands initially as normal, oxyntic gastric-type mucosa that undergoes sequential reprogramming events to take on first pyloric, then intestinal characteristics (Goldenring et al., 2010; Jin and Mills, 2018; Goldenring and Mills, 2022).

The clinical implications of these findings are important. Pathologists in the United States diagnose BE largely on the presence of intestinal goblet cells (Naini et al., 2016; Shaheen et al., 2022). However, more “intestinalized” BE lesions have an unclear higher risk of cancer progression (Bhat et al., 2011; Srivastava et al., 2015; Shaheen et al., 2022). In addition, despite a similar risk of cancer development compared to BE (de Vries et al., 2008; Hvid-Jensen et al., 2011; Song et al., 2015), GIM lacks endoscopic cancer screening guidelines in the United States (Gawron et al., 2020; Gupta et al., 2020). Importantly, the risk of gastric cancer development differs between complete and incomplete GIM with incomplete GIM possessing a significantly higher risk of cancer progression (Gonzalez et al., 2016; Gawron et al., 2020). In accordance, emerging clinical practice guidelines have suggested the adoption of surveillance for incomplete GIM (Gawron et al., 2020; Gupta et al., 2020). Thus, the presence of both gastric and intestinal features (i.e., lineage plasticity), may be a driver of cancer progression in the esophagus and stomach. As such, the ability to differentiate intestinal phenotypic differences shown in our study through differential PAS staining patterns, TFF2/TFF3 expression patterns, and MUC2/MUC5AC/MUC6 staining distribution in BE and GIM will be an important clinical management consideration in the future to determine cancer risk.

In our study, we demonstrate a loss of gastric and intestinal markers with the development of EAC and GA. Interestingly, we find that “differentiated” cell features found in the normal stomach and intestines, and in BE and GIM, are often decreased or lost after cancer development. Specifically, during normal esophageal squamous, BE metaplasia, dysplasia, and EAC progression there

is a progressive increase in TFF2, TFF3, MUC2, MUC5AC, MUC6, CDX2 with the development of intestinal metaplasia. Then upon further progression to dysplasia and EAC, there is a subsequent decrease in these markers (Figures 2–7). In contrast, during normal gastric gland to GIM progression, there is an increase in intestinal metaplasia markers, TFF3, MUC2, and CDX2, with a decrease in markers normally expressed in gastric cell lineages, TFF2, MUC5AC, and MUC6 (Figures 2–7). With progression from GIM to GA, there is a decrease in TFF2, TFF3, MUC2, and CDX2 with maintained expression of MUC5AC, MUC6, and SOX2 (Figures 2–7).

This retained gastric marker expression in gastric cancer is consistent with certain GAs maintaining a “gastric” differentiation phenotype (Wakatsuki et al., 2008; Boltin and Niv, 2013). While our methods of characterizing EAC and GA have become more sophisticated through the large scale “omics” studies (Cancer Genome Atlas Research Network, 2014; Cancer Genome Atlas Research Network, 2017; Zeng and Jin, 2022), the significance of this differentiation state flux and plasticity has largely been ignored for solid tumors both as a means to develop cancer and a mechanism for cancer treatment resistance (Saenz and Mills, 2018; Jin and Mills, 2019; Zeng and Jin, 2022). In fact, the most persistent, informal histological description of CIN-type tumors in the stomach is misleadingly “intestinal”. While most of these tumors do exhibit intestinal characteristics not normally seen in the stomach, this should not mislead scientists and clinicians into ignoring that the tumors are highly heterogeneous with both intestinal and gastric characteristics that vary from region to region and from patient to patient. Leveraging such plasticity inherent in cancer cells may be an efficacious and safe means to treat cancers in the future (de The, 2018; Enane et al., 2018).

In summary, our study demonstrates the important similarities and differences that occur during the development of intestinal metaplasia and its progression to cancer in the upper GI tract. We show the plasticity that exists during this process as a mix of gastric and intestinal differentiation traits. Our findings also have potential clinical impact for the understanding the molecular bases of EAC and GA. Continued study, especially multi-omic unbiased analyses of the mechanisms underlying the plasticity of the upper GI tract will lead to improved early cancer risk stratification, safer more efficacious oncologic treatments, and perhaps methods to prevent or reverse the development of these metaplasias and dysplasias.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Materials, further inquiries can be directed to the corresponding authors.

Ethics statement

The studies involving human participants were reviewed and approved by the use of pathology material for the study was

approved by the Institutional Review Board of Johns Hopkins Medical Institutions. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

Author contributions

YZ contributed to the study’s conception and design, data analysis and visualization, and manuscript writing, review and editing. QL contributed to the data collection and analysis, and manuscript writing, review and editing. SR contributed to data collection. JM contributed to study supervision, and manuscript writing, review and editing. RJ contributed to the study’s conception and design, study supervision, and manuscript writing, review and editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fcell.2023.1151790/full#supplementary-material>

SUPPLEMENTARY FIGURE S1

Immunohistochemical analysis of columnar-lined mucosa of the esophagus. (A) H&E and (B) PAS staining showing morphology of columnar-lined mucosa of the esophagus. Immunohistochemistry showing (C) TFF2, (D) TFF3, (E) MUC2, (F) MUC5AC, (G) MUC6, (H) CDX2, and (I) SOX2 staining of columnar-lined mucosa of the esophagus. All scale bars, 500 μ m.

SUPPLEMENTARY FIGURE S2

Immunohistochemical analysis of complete gastric intestinal metaplasia. (A) H&E and (B) PAS staining showing morphology of complete gastric intestinal metaplasia. Immunohistochemistry showing (C) TFF2, (D) TFF3, (E) MUC2,

(F) MUC5AC, (G) MUC6, (H) CDX2, and (I) SOX2 staining of complete gastric intestinal metaplasia. All scale bars, 500 μ m.

SUPPLEMENTARY TABLE S1

The Fisher's exact test *p*-values of IHC marker expression for esophageal cases. *P*-values were calculated by testing for the difference in the mean IHC scores of the indicated markers. Fisher's exact test was used. *P* < 0.05 is considered statistically significant. Comparisons were between normal and lesion phenotype groups, or 2 lesion phenotype groups (BE-NFD: Barrett's esophagus negative for dysplasia, BE-LGD: Barrett's esophagus with low-grade dysplasia, BE-HDG:

Barrett's esophagus with high-grade dysplasia, EAC: esophageal adenocarcinoma).

SUPPLEMENTARY TABLE S2

The Fisher's exact test *p*-values of IHC marker expression for gastric cases. *P*-values were calculated by testing for the difference in the mean IHC scores of the indicated markers. Fisher's exact test was used. *P* < 0.05 is considered statistically significant. Comparisons were between normal and lesion phenotype groups, or 2 lesion phenotype groups (GIM: gastric intestinal metaplasia, GA: gastric adenocarcinoma).

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PILOT/FEASIBILITY AWARDS APPLICATION SUMMARY SHEET

PI Information:

Last name	Jin	First name	Ramon	Degree	MD, PhD
Academic title	Instructor	Department	Internal Medicine/Section of Hematology Oncology	Institution	Baylor College of Medicine
Mail station	MS233	Phone	(216)-544-2529	Fax	(713)-798-7811
Email	ramon.jin@bcm.edu				

Short title of project
(up to 56 characters and spaces) THE ROLE OF SOX2 IN BARRETT'S ESOPHAGUS DEVELOPMENT

Type of application Pilot project

Budget (No indirect costs on these pilot awards)

Supplies	\$ 22000	Small equipments	\$ 0	Personnel	\$ 0
Other	\$ 13000	Total	\$ 35000		

Has the PI of a pilot project application recently been part of a group led by a more advanced investigator?
(If yes, a letter citing independence of the PI for this project must be included.)

Yes

If the PI of the pilot project has not had a previous grant, does the PI have a mentor?
(A letter describing the commitment of the mentor and a mentoring plan must be included.)

Yes

DDC cores to be utilized

Cellular & Molecular Morphology	☒
Functional Genomics and Microbiome	☒
Gastrointestinal Experimental Model Systems (GEMS)	☒
Study Design & Clinical Research	☐

Check applicable boxes

Are human subjects or human tissues/fluids being used?	☐ Yes	☒ No
Are animals or animal tissues/fluids being used?	☒ Yes	☐ No
Are radioisotopes being used?	☐ Yes	☒ No
Are biohazards being used?	☐ Yes	☒ No

Summary: Write a short paragraph, not to exceed the space below, describing major aims of the project and the approach to be used

Barrett's esophagus (BE) is a precancerous condition defined as replacement of the normal esophageal squamous epithelium with metaplastic columnar intestinal epithelium caused by chronic gastroesophageal reflux damage. A thorough elucidation of the development context in which BE develops will uncover novel avenues to treat BE and halt the progression to cancer. SOX2 is a key transcription factor for normal esophageal development and homeostasis. I hypothesize that SOX2 functions to maintain foregut squamous epithelial identity, and its loss is a critical step during Barrett's esophagus development and the progression to esophageal adenocarcinoma. To assay the molecular effects of SOX2 loss in the foregut, I have generated a novel inducible Sox2 knockout mouse model, Krt5CreER/+; Sox2flox/flox; ROSA26LSLTdTomato/+, and have assessed the histologic and transcriptional effects of SOX2 loss on squamous epithelial homeostasis. In Aim 1, I will validate the transcriptional changes due to SOX2 loss in the murine foregut squamous epithelium. Using qPCR and immunohistochemical analyses, I will confirm the top increased and decreased genes upon SOX2 loss in the Krt5CreER/+; Sox2flox/flox; ROSA26LSLTdTomato/+ mice. In Aim 2, I will assay for direct transcriptional targets of SOX2 in the murine foregut squamous epithelium. Using a novel Cleavage Under Targets and Tagmentation (CUT&Tag) assay and tissues/organoids generated from Krt5CreER/+; Sox2flox/flox; ROSA26LSLTdTomato/+ mice, I will determine the direct transcription factor targets of SOX important in the adult murine squamous epithelium. Together, these experiments will elucidate the molecular pathways involved in BE development and reveal new therapeutic avenues to treat BE.

APPLICANT'S SIGNATURE:

DATE: 9-23-2022

ACADEMIC UNIT CHAIR'S SIGNATURE:

DATE: 9/23/22

The name of the program director/principal investigator must be provided at the top of each printed page and each continuation page.

RESEARCH GRANT TABLE OF CONTENTS

	<i>Page Numbers</i>
Face Page	1
Description, Project/Performance Sites, Senior/Key Personnel, Other Significant Contributors, and Human Embryonic Stem Cells	N/A
Table of Contents	2
Detailed Budget for Initial Budget Period	3
Budget for Entire Proposed Period of Support	4
Budgets Pertaining to Consortium/Contractual Arrangements	N/A
Biographical Sketch – Program Director/Principal Investigator (<i>Not to exceed five pages each</i>)	5-8
Other Biographical Sketches (<i>Not to exceed five pages each – See instructions</i>)	
Resources	
Checklist	
Research Plan	11
1. Introduction to Resubmission Application, if applicable, or Introduction to Revision Application, if applicable *	
2. Specific Aims *	11
3. Research Strategy *	14-15
4. Bibliography and References Cited/Progress Report Publication List	16-18
5. Vertebrate Animals	
6. Select Agent Research	
7. Multiple PD/PI Leadership Plan	
8. Consortium/Contractual Arrangements	
9. Letters of Support (e.g., Consultants)	
10. Resource Sharing Plan(s)	
11. Authentication of Key Biological and/or Chemical Resources	
12. PHS Human Subjects and Clinical Trials Information	
Appendix (<i>Two identical CDs.</i>)	☐ Check if Appendix is Included

* Follow the page limits for these sections indicated in the application instructions, unless the Funding Opportunity Announcement specifies otherwise.

**DETAILED BUDGET FOR INITIAL BUDGET PERIOD
DIRECT COSTS ONLY**FROM
01/01/23THROUGH
12/31/24List PERSONNEL (*Applicant organization only*)

Use Cal, Acad, or Summer to Enter Months Devoted to Project

Enter Dollar Amounts Requested (*omit cents*) for Salary Requested and Fringe Benefits

NAME	ROLE ON PROJECT	Cal. Mnths	Acad. Mnths	Summer Mnths	INST.BASE SALARY	SALARY REQUESTED	FRINGE BENEFITS	TOTAL
Ramon Jin	PD/PI	6	6	0	0	0	0	0
SUBTOTALS						0	0	0

CONSULTANT COSTS

0

0

EQUIPMENT (*Itemize*)

0

0

SUPPLIES (*Itemize by category*)

General Lab Supplies (Plastic consumables, pipets, tips, gloves, etc.) \$2,000

Organoid Cell Culture Supplies (Matrigel, conditioned media, growth factors, etc.) \$7,000

Cell Imaging Reagents (Primary and Secondary Antibodies, IHC kits, etc.) \$6,000

Molecular Biology (CUT&Tag reagents, reagents for qRT-PCR, etc.) \$7,000

\$22,000

TRAVEL

0

0

INPATIENT CARE COSTS 0

0

OUTPATIENT CARE COSTS 0

0

ALTERATIONS AND RENOVATIONS (*Itemize by category*)

0

0

OTHER EXPENSES (*Itemize by category*)

DDC Tissue Analysis & Molecular Imaging Core (Tissue processing, slide mounting, histology, etc.)

\$3,000

DDC Gastrointestinal Experimental Model Systems Core (Organoid experiment support, etc.)

\$2,000

DDC Functional Genomics and Microbiome Core (Sequencing support, etc.)

\$8,000

\$13,000

CONSORTIUM/CONTRACTUAL COSTS

DIRECT COSTS

SUBTOTAL DIRECT COSTS FOR INITIAL BUDGET PERIOD (*Item 7a, Face Page*)**\$ \$35,000**

CONSORTIUM/CONTRACTUAL COSTS

FACILITIES AND ADMINISTRATIVE COSTS

TOTAL DIRECT COSTS FOR INITIAL BUDGET PERIOD**\$ \$35,000**

BUDGET FOR ENTIRE PROPOSED PROJECT PERIOD DIRECT COSTS ONLY

BUDGET CATEGORY TOTALS	INITIAL BUDGET PERIOD (from Form Page 4)	2nd ADDITIONAL YEAR OF SUPPORT REQUESTED	3rd ADDITIONAL YEAR OF SUPPORT REQUESTED	4th ADDITIONAL YEAR OF SUPPORT REQUESTED	5th ADDITIONAL YEAR OF SUPPORT REQUESTED
PERSONNEL: <i>Salary and fringe benefits. Applicant organization only.</i>	0	0	0	0	0
CONSULTANT COSTS	0	0	0	0	0
EQUIPMENT	0	0	0	0	0
SUPPLIES	0	0	0	0	0
TRAVEL	0	0	0	0	0
INPATIENT CARE COSTS	0	0	0	0	0
OUTPATIENT CARE COSTS	0	0	0	0	0
ALTERATIONS AND RENOVATIONS	0	0	0	0	0
OTHER EXPENSES	0	0	0	0	0
DIRECT CONSORTIUM/ CONTRACTUAL COSTS	0	0	0	0	0
SUBTOTAL DIRECT COSTS (Sum = Item 8a, Face Page)	35,000	0	0	0	0
F&A CONSORTIUM/ CONTRACTUAL COSTS	0	0	0	0	0
TOTAL DIRECT COSTS	35,000	0	0	0	0
TOTAL DIRECT COSTS FOR ENTIRE PROPOSED PROJECT PERIOD					\$ 35,000

JUSTIFICATION. Follow the budget justification instructions exactly. Use continuation pages as needed.

Personnel:

Ramon Jin, M.D., Ph.D., Principle Investigator. Dr. Ramon Jin is an Instructor in Medicine in the Section of Hematology Oncology at BCM with an interest in with upper GI malignancies with greater than 75% of his time protected for research. He is interested in developing new disease models including genetic mouse models and human organoid systems to study upper GI tract injury response pathways that lead to the development of metaplasias and their progression to cancer. He will direct and oversee the research plan and conduct all proposed experiments. We are not requesting salary and fringes support for Dr. Jin.

Supplies:

General Lab Supplies: Consumable costs include disposable plastics, tissue culture plates, flasks, pipettes, glassware, general buffers and chemicals, and western blotting supplies. (YR1 \$2,000).

Organoid Cell Culture Supplies: This includes media (to be purchased from the Gastrointestinal Experimental Model Systems Core), serum and growth supplements for organoid culture experiments. (YR1 \$7,000).

Cell Imaging Reagents: Immunofluorescent (IF) and Immunohistochemistry (IHC) supplies include primary and secondary antibodies. (YR1 \$6,000).

Molecular Biology: Kits and reagents required for sample preparation for CUT&Tag and qRT-PCR including primers and probes. (YR1 \$7,000).

Other Expenses:

DDC Tissue Analysis & Molecular Imaging Core: Tissue processing, slide mounting, and histology (H&E staining). Each sample will be paraffin embedded and sectioned, with 1 slide per block for H&E, and 10 unstained slides as a routine. Current costs= \$3.00/block, \$5.00/H&E, \$1.75/unstained slide. The PI will also perform some of the sectioning and most of the staining himself. (YR1 \$3,000).

DDC Gastrointestinal Experimental Model Systems Core: Assistance for organoid experiment support. (YR1 \$2,000).

DDC Functional Genomics and Microbiome Core: CUT&Tag sequencing and results analyses will be performed in collaboration with the Functional Genomics and Microbiome Core. (YR1 \$8,000)

BIOGRAPHICAL SKETCH

Provide the following information for the Senior/key personnel and other significant contributors.
Follow this format for each person. **DO NOT EXCEED FIVE PAGES.**

NAME: Jin, Ramon

eRA COMMONS USER NAME (credential, e.g., agency login): RAMON_JIN

POSITION TITLE: Instructor in Medicine

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, include postdoctoral training and residency training if applicable. Add/delete rows as necessary.)*

INSTITUTION AND LOCATION	DEGREE (if applicable)	Completion Date MM/YYYY	FIELD OF STUDY
Case Western Reserve University, Cleveland, OH	BS	05/2006	Biology
Washington University School of Medicine, St. Louis, MO	MD/PhD	05/2015	Medicine and Molecular Cell Biology
Barnes-Jewish Hospital, St. Louis, MO	Internship /Residency	06/2017	Internal Medicine
Washington University School of Medicine, St. Louis, MO	Fellowship	06/2021	Hematology-Oncology

A. Personal Statement

I intend to combine my background in basic science with my clinical experience to explore gastrointestinal cancer from a novel perspective, and to directly translate these discoveries to new therapeutics. My academic training and research experience have provided me with the fundamental tools and background to succeed. As an undergraduate, I developed an interest in cell biology and genetics. During this time, I worked on the role of the gene, *CFTR*, in metabolic and hormonal regulation, and was able to master basic molecular techniques and mouse genetics. As a predoctoral MD/PhD student, my work focused on developmental biology and the transcriptional regulation of cell development. I was able to show that in secretory acinar cells, a single transcription factor, MIST1, directly activates a subset of effector genes. Included in these targets are RAB26 and RAB3D, small protein GTPases that function in vesicle trafficking to allow cells to change shape and function. From this experience, I was able to publish several first author papers, present my work at national meetings, and most importantly expand my knowledgebase in molecular cell biology, bioinformatics, stem cell biology, and developmental biology. My research experience has been complemented by my medical training during medical school, internal medicine residency, and hematology-oncology fellowship. For my postdoctoral training, I have applied my previous experience in genetics, cell biology, and developmental biology to upper gastrointestinal malignancies. Survival in gastric and esophageal cancers is still poor, and surgery and chemoradiotherapy remain the mainstay of treatment. The lack of novel targeted therapies with improved treatment efficacy and less off target toxicities reflects a need for further understanding about the pathogenesis of these diseases. My research mentor, Dr. Jason Mills, is an internationally recognized leader in the field of gastrointestinal development and stem cell biology. He has an extensive record for training postdoctoral fellows and physician scientists. Our proposed research will develop new disease models including genetic mouse models and human organoid systems to study gastroesophageal cancers and their pre-malignant precursor intestinal metaplasia (i.e. Barrett's esophagus) lesions. In addition, I am interested in the injury response of the upper GI tract to chronic inflammation, and the mechanisms by which these processes including autophagy, selective apoptosis, and protein stress response induced carcinogenesis. From the knowledge gained through my benchside research, I plan to initiate translational human upper GI cancer clinical trials to target novel pathways. My work will elucidate basic and therapeutically relevant principles of upper GI cancers from genetic, developmental, and

cell biological perspectives. I believe that the depth and breadth of my basic science training combined with my expanding clinical expertise will allow me to become a leader in the field of gastrointestinal oncology.

Ongoing and recently completed projects that I would like to highlight include:

T32 HL007088

Oh (PI), Role: Postdoctoral Trainee

07/01/2018 -6/30/2021

Pre and Postgraduate Training in Molecular Hematology

U54 CA163060

Mills (PI), Role: Postdoctoral Research Scholar

05/01/2019 – 04/30/2022

Barrett's Esophagus Translational Research Network (BETRNet) Developmental Research Program Award.

P30 DK052574

Jin (PI)

08/01/2020-7/31/2021

Washington University DDRCC Pilot & Feasibility Program

W81XWH-20-PRCRP-CDA

Jin (PI)

07/01/2021-06/30/2024

Department of Defense Peer Reviewed Cancer Research Program Career Development Award

2021YIA-8674301298

Jin (PI)

07/01/2021-06/30/2022

ASCO 2021 Conquer Cancer Young Investigator Award

Highlighted Citations:

1. Zeng Y, **Jin RU**. Molecular pathogenesis, targeted therapies, and future perspectives for gastric cancer. *Semin Cancer Biol.* 2021 Dec 18:S1044-579X(21)00297-2. doi: 10.1016/j.semcancer.2021.12.004. Epub ahead of print. PMID: 34933124.
2. **Jin RU**, Brown JW, Li QK, Bayguinov PO, Wang JS, Mills JC. Tropism of Severe Acute Respiratory Syndrome Coronavirus 2 for Barrett's Esophagus May Increase Susceptibility to Developing Coronavirus Disease 2019. *Gastroenterology.* 2021 May;160(6):2165-2168.e4. doi: 10.1053/j.gastro.2021.01.024. Epub 2021 Jan 20. PubMed PMID: 33484686; PubMed Central PMCID: PMC7816599.
3. **Jin RU**, Mills JC. Tumor Organoids to Study Gastroesophageal Cancer: A Primer. *J Mol Cell Biol.* 2020 Jul 11;. doi: 10.1093/jmcb/mjaa035. [Epub ahead of print] PubMed PMID: 32652008.
4. Park H, **Jin RU**, Wang-Gillam A, Suresh R, Rigden C, Amin M, Tan BR, Pedersen KS, Lim KH, Trikalinos NA, Acharya A, Copsey ML, Navo KA, Morton AE, Gao F, Lockhart AC. FOLFIRINOX for the Treatment of Advanced Gastroesophageal Cancers: A Phase 2 Nonrandomized Clinical Trial. *JAMA Oncol.* 2020 Aug 1;6(8):1231-1240. doi: 10.1001/jamaoncol.2020.2020. PubMed PMID: 32469386; PubMed Central PMCID: PMC7260693.

B. Positions, Scientific Appointments, and Honors

Positions and Scientific Appointments

2021 – present	Instructor in Medicine, Section of Hematology/Oncology, Baylor College of Medicine
2017 - 2021	Hematology-Oncology Fellow, Washington University School of Medicine, St. Louis, MO
2015 - 2017	Internal Medicine Intern/Resident, Barnes-Jewish Hospital, St. Louis, MO
2006 – 2015	Student in Medical Scientist Training Program, Washington University School of Medicine, St. Louis, MO

Honors

2021- present	Peer Reviewed Cancer Research Program (PRCRP) Career Development Award, US Department of Defense
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2022	Lecture Presentation, Digestive Disease Week 2022, AGA
2021	Conquer Cancer Young Investigator Award, Conquer Cancer, the ASCO Foundation
2021	Poster of Distinction, Digestive Disease Week 2021, AGA
2020	Keystone Symposia Scholarship Award, Keystone Symposia
2020	AGA Research Foundation COVID-19 Rapid Response Research Award, AGA
2020	Washington University DDRCC Pilot & Feasibility Program Award, Washington University School of Medicine
2020	ASCO Annual Meeting Conquer Cancer Merit Award, ASCO
2019-2021	BETRNet Developmental Research Program Award, BETRNet
2019	Knowlton Incentive for Excellence Award. Barnes-Jewish Hospital Foundation
2019	Selected for the AACR Molecular Biology in Clinical Oncology Workshop, AACR
2018	Selected for the R25 Cancer Genomics and Outcomes Research STRENGTH Program, Washington University School of Medicine
2018-2020	Chief Fellow, Divisions of Hematology and Oncology, Washington University School of Medicine
2012	Annual Meeting Travel Award, ASCI/AAP/APSA
2012	Selected to attend the AGA Attracting MD/PhD Students into Gastroenterology Workshop, AGA
2011	Viktor Hamburger Award, Department of Developmental Biology, Washington University School of Medicine
2009	Summer Research Conference: Gastrointestinal Tract XII: The Molecular and Integrative Basis for GI Development, Homeostasis and Disease Travel Award, FASEB
2006	Graduated <i>summa cum laude</i> , Case Western Reserve University
2005-2006	Case Alumni Association Junior/Senior Scholarship, Case Western Reserve University
2002-2006	Trustee's Scholarship, Case Western Reserve University

C. Contributions to Science

1. The Role of CFTR in Modulating the Timing of Puberty. My undergraduate research focused on the link between alterations in the gene that causes cystic fibrosis, *CFTR*, and the timing of puberty. Patients with cystic fibrosis are known to have delayed puberty independent of nutritional status. I was able to show using several genetic mouse models that altered functional CFTR protein directly affects the timing of puberty.
 - a. **Jin R**, Hodges CA, Drumm ML, Palmert MR. The cystic fibrosis transmembrane conductance regulator (*Cftr*) modulates the timing of puberty in mice. *J Med Genet.* 2006 Jun;43(6):e29. doi: 10.1136/jmg.2005.032839. PubMed PMID: 16740913; PubMed Central PMCID: PMC2564546.
2. Roles of MIST1 and RAB26 in Zymogenic (Chief) Cell Differentiation and Subcellular Organization. My graduate research involved investigation into the basic cell biological and developmental mechanisms that allow normal cells to rearrange their cellular architecture to change physiologic function. I found that a single transcription factor, MIST1, through its direct transcriptional targets, acts as a master regulator of cellular function. In its absence, normal exocrine secretory cells stop producing secretory granules, initiate degradation of existing acinar vesicles, and adopt additional cellular architectural changes to facilitate cell cycle re-entry. Interestingly, I found that these direct targets of MIST1, including the small protein GTPases RAB26 and RAB3D, form an integrative secretory maintenance network that not only promotes development of secretory vesicles, but also polarizes the apical cytoplasm and segregates the innate intracellular degradative machinery from the secretory compartment.
 - a. Tian X*, **Jin RU***, Bredemeyer AJ, Oates EJ, Błazewska KM, McKenna CE, Mills JC. RAB26 and RAB3D are direct transcriptional targets of MIST1 that regulate exocrine granule maturation. *Mol Cell Biol.* 2010 Mar;30(5):1269-84. doi: 10.1128/MCB.01328-09. Epub 2009 Dec 28. PubMed PMID: 20038531; PubMed Central PMCID: PMC2820885. *co-first authors
 - b. Capoccia BJ, **Jin RU**, Kong YY, Peek RM Jr, Fassan M, Rugge M, Mills JC. The ubiquitin ligase Mindbomb 1 coordinates gastrointestinal secretory cell maturation. *J Clin Invest.* 2013 Apr;123(4):1475-91. doi: 10.1172/JCI65703. Epub 2013 Mar 8. PubMed PMID: 23478405; PubMed Central PMCID: PMC3613919.
 - c. **Jin RU**, Mills JC. RAB26 coordinates lysosome traffic and mitochondrial localization. *J Cell Sci.* 2014 Mar 1;127(Pt 5):1018-32. doi: 10.1242/jcs.138776. Epub 2014 Jan 10. PubMed PMID: 24413166; PubMed Central PMCID: PMC3937772.

- d. Lo HG, **Jin RU**, Sibbel G, Liu D, Karki A, Joens MS, Madison BB, Zhang B, Blanc V, Fitzpatrick JA, Davidson NO, Konieczny SF, Mills JC. A single transcription factor is sufficient to induce and maintain secretory cell architecture. *Genes Dev.* 2017 Jan 15;31(2):154-171. doi: 10.1101/gad.285684.116. Epub 2017 Feb 7. PubMed PMID: 28174210; PubMed Central PMCID: PMC5322730.
3. Novel developmental and injury response pathways in the development of Barrett's esophagus and its progression to esophageal adenocarcinoma. Over the past several decades there has been an alarming increase in the incidence of both Barrett's esophagus and esophageal adenocarcinoma. Current treatment and surveillance strategies are based on patient clinical characteristics with unclear long term survival benefit. There is significant lack of knowledge about the pathogenesis of metaplasia in the esophagus and its neoplastic progression. My work involves elucidating the molecular steps involved in the development of Barrett's esophagus and its progression to esophageal adenocarcinoma. To this end, I am actively developing novel models of esophageal metaplasia and adenocarcinoma with SOX2 inducible knockout mice and human-derived organoid models of Barrett's esophagus and esophageal adenocarcinoma.
 - a. **Jin RU**, Mills JC. Are Gastric and Esophageal Metaplasia Relatives? The Case for Barrett's Stemming from SPEM. *Dig Dis Sci.* 2018 Aug;63(8):2028-2041. doi: 10.1007/s10620-018-5150-0. Review. PubMed PMID: 29948563.
 - b. **Jin RU**, Mills JC. The cyclical hit model: how paligenosis might establish the mutational landscape in Barrett's esophagus and esophageal adenocarcinoma. *Curr Opin Gastroenterol.* 2019 Jul;35(4):363-370. doi: 10.1097/MOG.0000000000000540. Review. PubMed PMID: 31021922.
 - c. **Jin RU**, Brown JW, Li QK, Bayguinov PO, Wang JS, Mills JC. Tropism of Severe Acute Respiratory Syndrome Coronavirus 2 for Barrett's Esophagus May Increase Susceptibility to Developing Coronavirus Disease 2019. *Gastroenterology.* 2021 May;160(6):2165-2168.e4. doi: 10.1053/j.gastro.2021.01.024. Epub 2021 Jan 20. PubMed PMID: 33484686; PubMed Central PMCID: PMC7816599.
 4. Patient-derived Xenografts and Organoids to guide treatment decisions in advanced gastroesophageal cancer patients. Stomach cancer is a leading cause of cancer death world-wide. One-size-fits-all chemotherapy remains the mainstay of treatment even as we begin to identify specific targetable gene mutations. One problem is that agents to target specific genes are chosen based on large clinical trials with so many diverse patients included that we lose the ability to learn what will work for individual patients. To improve outcomes and individualize cancer therapy, we need novel avenues to study and predict treatment effects at the level of the individual. To this end, we propose developing model systems where we can grow actual patient tumors in a Petri dish. We plan to establish the feasibility of these models through the current proposal and expand this novel approach to future clinical trials to truly tailor treatment for individual patients with gastroesophageal cancers.
 - a. Park H, **Jin RU**, Wang-Gillam A, Suresh R, Rigden C, Amin M, Tan BR, Pedersen KS, Lim KH, Trikalinos NA, Acharya A, Copsey ML, Navo KA, Morton AE, Gao F, Lockhart AC. FOLFIRINOX for the Treatment of Advanced Gastroesophageal Cancers: A Phase 2 Nonrandomized Clinical Trial. *JAMA Oncol.* 2020 Aug 1;6(8):1231-1240. doi: 10.1001/jamaoncol.2020.2020. PubMed PMID: 32469386; PubMed Central PMCID: PMC7260693.
 - b. **Jin RU**, Mills JC. Tumor organoids to study gastroesophageal cancer: a primer. *J Mol Cell Biol.* 2020 Aug 1;12(8):593-606. doi: 10.1093/jmcb/mjaa035. Review. PubMed PMID: 32652008; PubMed Central PMCID: PMC7683018.
 - c. Zeng Y, **Jin RU**. Molecular pathogenesis, targeted therapies, and future perspectives for gastric cancer. *Semin Cancer Biol.* 2021 Dec 18;. doi: 10.1016/j.semcancer.2021.12.004. [Epub ahead of print] PubMed PMID: 34933124.

Complete List of Published Work:

https://www.ncbi.nlm.nih.gov/myncbi/18iewzt9_54/bibliography/public/

PHS OTHER SUPPORT
For All Application Types – DO NOT SUBMIT UNLESS REQUESTED

There is no "form page" for reporting Other Support. Information on Other Support should be provided in the format shown below.

*Name of Individual: Jin, Ramon.
Commons ID: RAMON_JIN

Other Support – Project/Proposal

*Title: The Role of SOX2 in Barrett's Esophagus Development and Progression to Esophageal Adenocarcinoma.

*Major Goals: Determine if SOX2 functions to maintain foregut squamous epithelial identity and to inhibit columnar and intestinal specification of the esophageal mucosa, and if its loss is a critical step during BE development and EA progression.

*Status of Support: Active.

Project Number: W81XWH-20-PRCRP-CDA.

Name of PD/PI: Jin, Ramon.

*Source of Support: Department of Defense Peer Reviewed Cancer Research Program Career Development Award.

*Primary Place of Performance: Baylor College of Medicine.

Project/Proposal Start and End Date: (MM/YYYY) (if available): 07/2021 – 06/2024.

*Total Award Amount (including Indirect Costs):.

*Person Months (Calendar/Academic/Summer) per budget period.

Year (YYYY)	Person Months (##.##)
1. 2021	7.20
2. 2022	7.20
3. 2023	7.20

***Overlap** (summarized for each individual):

In terms of current support, the PI will have the majority of his effort devoted to the W81XWH-20-PRCRP-CDA Department of Defense Peer Reviewed Cancer Research Program Career Development Award. For the current Texas Medical Center Digestive Diseases Center Pilot/Feasibility Award proposal (Aim 1) I will confirm by immunohistochemistry and quantitative PCR the transcriptional changes found through the mice generated and the experiments completed in Aim 1 of the DoD proposal. In addition, I will propose (Aim 2) to use a novel Cleavage Under Targets and Tagmentation (CUT&Tag) assay to elucidate the direct transcriptional targets of SOX2 using WT and Sox2 deleted squamous organoids generated from experiments completed in Aim 1 of DoD proposal. The experiments proposed in this Texas Medical Center Digestive Diseases Center Pilot/Feasibility Award are complementary and non-overlapping with the experiments proposed in the Department of Defense proposal. **Funds from the Texas Medical Center Digestive Diseases Center Pilot/Feasibility Award will allow further lines of**

Name of Individual: Jin, Ramon
Commons ID: RAMON_JIN

investigation initiated through the DoD proposal to confirm transcriptional changes upon Sox2 loss and to elucidate the direct transcriptional targets of SOX2 in the mature squamous epithelium. These Texas Medical Center Digestive Diseases Center Pilot/Feasibility Award proposed experiments will provide the key data to allow the PI to apply for a K08 Clinical Investigator Award through the NIH NIDDK.

I, Dr. Ramon Jin, certify that the statements herein are true, complete and accurate to the best of my knowledge, and accept the obligation to comply with Public Health Services terms and conditions if a grant is awarded as a result of this application. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties.

*Signature:  _____
Date: 09/23/2022 _____

Research Plan

1. Specific Aims

Barrett's esophagus (BE) is a precancerous condition defined as replacement of the normal esophageal squamous epithelium with metaplastic columnar intestinal epithelium caused by chronic gastroesophageal reflux damage. It has been well established that the presence of Barrett's esophagus confers the greatest risk for developing esophageal adenocarcinoma (EA). Alarming, both BE and EA diagnoses have increased in the US over the past several decades. Current screening and surveillance guidelines are based largely on patient symptomatology and risk factors with unclear effectiveness at decreasing risk of cancer related deaths. These data reflect a deficiency of knowledge regarding the pathogenesis of Barrett's metaplasia development and progression to esophageal adenocarcinoma.

To this end, a thorough elucidation of the development context in which BE emerges will uncover novel avenues to treat BE and halt the progression to EA. Spatial specification of the developing digestive tract occurs through a series of regulated transcriptional cascades. At embryonic day 9, the dorsal foregut is marked by expression of SOX2, a transcription factor of the Sry-like HMG box family, which is required for esophageal development. SOX2 continues to be expressed in the esophagus throughout adulthood as a key homeostatic maintenance factor. Importantly, SOX2 expression is decreased or lost completely in BE. Surprisingly, little is known about the molecular function, direct transcriptional targets, or functional consequences of SOX2 loss during BE development.

I hypothesize that SOX2 functions to maintain foregut squamous epithelial identity, and its loss is a critical step during Barrett's esophagus development and the progression to esophageal adenocarcinoma. To assay the molecular effects of SOX2 loss in the foregut, I have generated a novel inducible Sox2 knockout mouse model, *Krt5^{CreER/+}; Sox2^{flox/flox}; ROSA26^{LSLTdTomato/+}*, and have assessed the histologic and transcriptional effects of SOX2 loss on squamous epithelial homeostasis. Interestingly, the top increased genes are "injury/squamous" related and the top decreased genes are "digestion/gastric" related. I propose the following specific aims:

Aim 1: Validate the transcriptional changes due to SOX2 loss in the murine foregut squamous epithelium. Using qPCR and immunohistochemical analyses, I will confirm the top increased and decreased genes upon SOX2 loss in the *Krt5^{CreER/+}; Sox2^{flox/flox}; ROSA26^{LSLTdTomato/+}* mice.

Aim 2: Assay for direct transcriptional targets of SOX2 in the murine foregut squamous epithelium. Using a novel Cleavage Under Targets and Tagmentation (CUT&Tag) assay and foregut squamous tissues/organoids generated from *Krt5^{CreER/+}; Sox2^{flox/flox}; ROSA26^{LSLTdTomato/+}* mice, I will determine the direct transcriptional targets of SOX2 important in the adult murine squamous epithelium.

This proposal will address for the first time the role of SOX2 in adult foregut squamous epithelium using a novel genetic mouse model. Together, these experiments will elucidate the molecular pathways involved in BE development and reveal new therapeutic avenues to treat BE and prevent esophageal cancer.

2. Significance and Innovation:

Barrett's esophagus is a precancerous condition defined as replacement of the normal esophageal squamous epithelium with metaplastic columnar intestinal epithelium caused by chronic gastroesophageal reflux damage that confers the greatest risk for developing esophageal adenocarcinoma^{1, 2}. In the United States, there has been an increasing incidence of both BE^{3, 4} and EA⁵⁻⁷ over the past several decades. EA remains a deadly disease with limited treatment advancements⁸⁻¹⁰ resulting in 5 year overall survival rates under 20%¹¹. Given that almost all cases of EA likely arise from BE¹², an improved understanding of BE will inevitably lead to better

EA treatment and prevention strategies as current BE surveillance guidelines have unclear effectiveness at reducing cancer-related deaths¹³⁻¹⁵. In addition, there are no biomarkers in routine clinical practice to predict BE cancer risk¹⁶, effective chemopreventative strategies to prevent progression to EA^{17, 18}, or representative experimental models that fully recapitulate the complexity of these conditions¹⁹⁻²¹. These data demonstrate a lack of knowledge regarding the pathogenesis of Barrett's esophagus.

To understand the aberrant intestinal programming of the normal esophagus that defines Barrett's metaplasia, we need to elucidate the developmental mechanisms underlying BE formation. An overlapping set of transcription factors determines and maintains GI tract differentiation. SOX2 is a key transcription factor in the normal esophagus as high levels are expressed during embryonic development²²⁻²⁴ and maintained throughout adult esophagus homeostasis²⁵⁻²⁷. Normal SOX2 expression becomes decreased in BE, and is also lost during neoplastic progression^{28, 29}, **Figure 1**. Early embryonic deletion of *Sox2* results in esophageal atresia, a columnar (rather than normal squamous) esophageal epithelium, and ectopic expression of intestinal genes²³. **No studies have detailed loss of SOX2 in the adult esophagus**, but deletion of *Sox2* in the glandular stomach de-represses expression of intestinal and metaplasia associated genes, and potentiates Wnt³⁰/NOTCH³¹ signaling mediated tumorigenesis; whereas ectopic overexpression of *Sox2* in the mouse intestine causes an "anterior" foregut phenotype³². Interestingly, loss of SOX2 expression also correlates with worse prognosis and survival for gastroesophageal adenocarcinoma patients³³⁻³⁵. Thus, maintained expression of SOX2 is crucial for normal esophageal squamous epithelial homeostasis to prevent metaplasia and neoplasia.

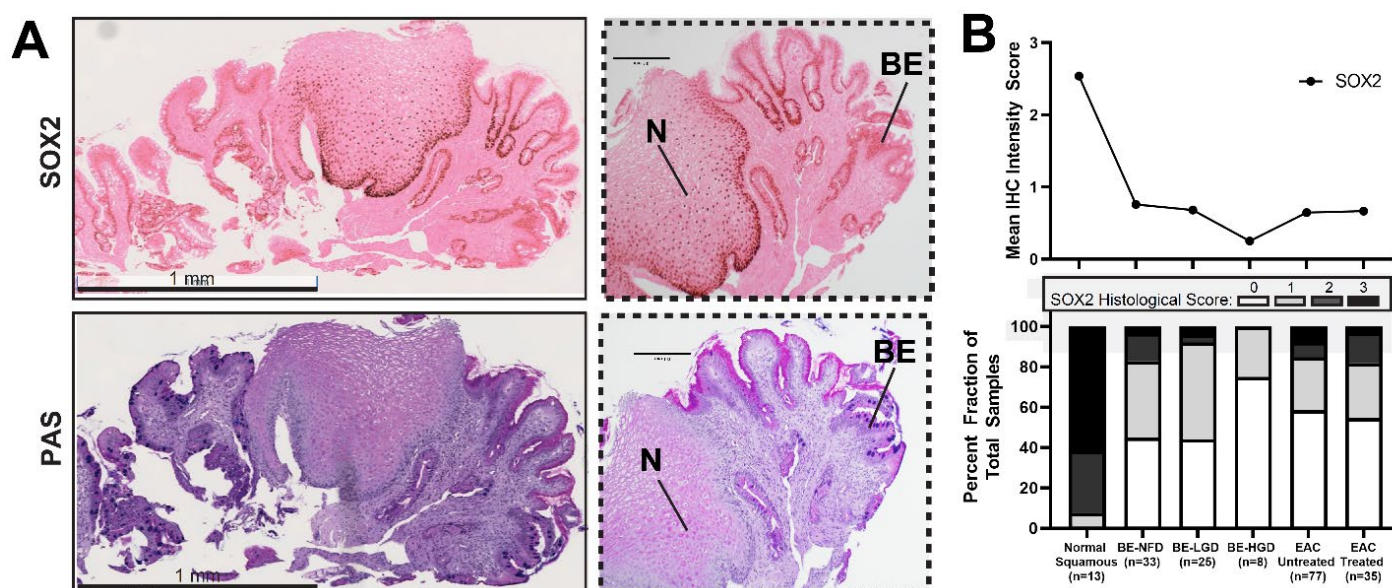


Figure 1. SOX2 is lost during BE neoplastic progression. A) Human tissue immunohistochemical analysis of SOX2 expression and PAS staining showing area of normal esophagus ('N') and adjacent Barrett's esophagus ('BE'). B) Human tissue microarray with normal squamous, non-dysplastic BE ('BE-NFD'), BE with low grade dysplasia ('BE-LGD'), BE with high grade dysplasia ('BE-HGD'), and neoadjuvant treated and treatment naïve esophageal adenocarcinoma samples stained for SOX2 intensity (from score 0 meaning undetectable to 3 most intense). *Top* – average histological score for each phenotype. *Bottom* – relative fraction of tissue cores with each score plotted.

3. Preliminary Studies:

I have generated reporter mice with esophageal- and forestomach-specific inducible Cre-recombinase to conditionally delete *Sox2* (*Krt5^{CreER/+}*; *Sox2^{flox/flox}*; *ROSA26^{LSL}TdTomato/+*). Histologic analyses show abnormal thickened esophagus and forestomach squamous epithelium, increased basal cell proliferation, and decreased esophageal differentiation markers (Cytokeratin 13),

Figure 2A,B. Bulk transcriptomic studies show many “digestion-related/gastric” genes increased and many “injury-related/squamous” genes decreased in expression, **Figure 2C,D.**

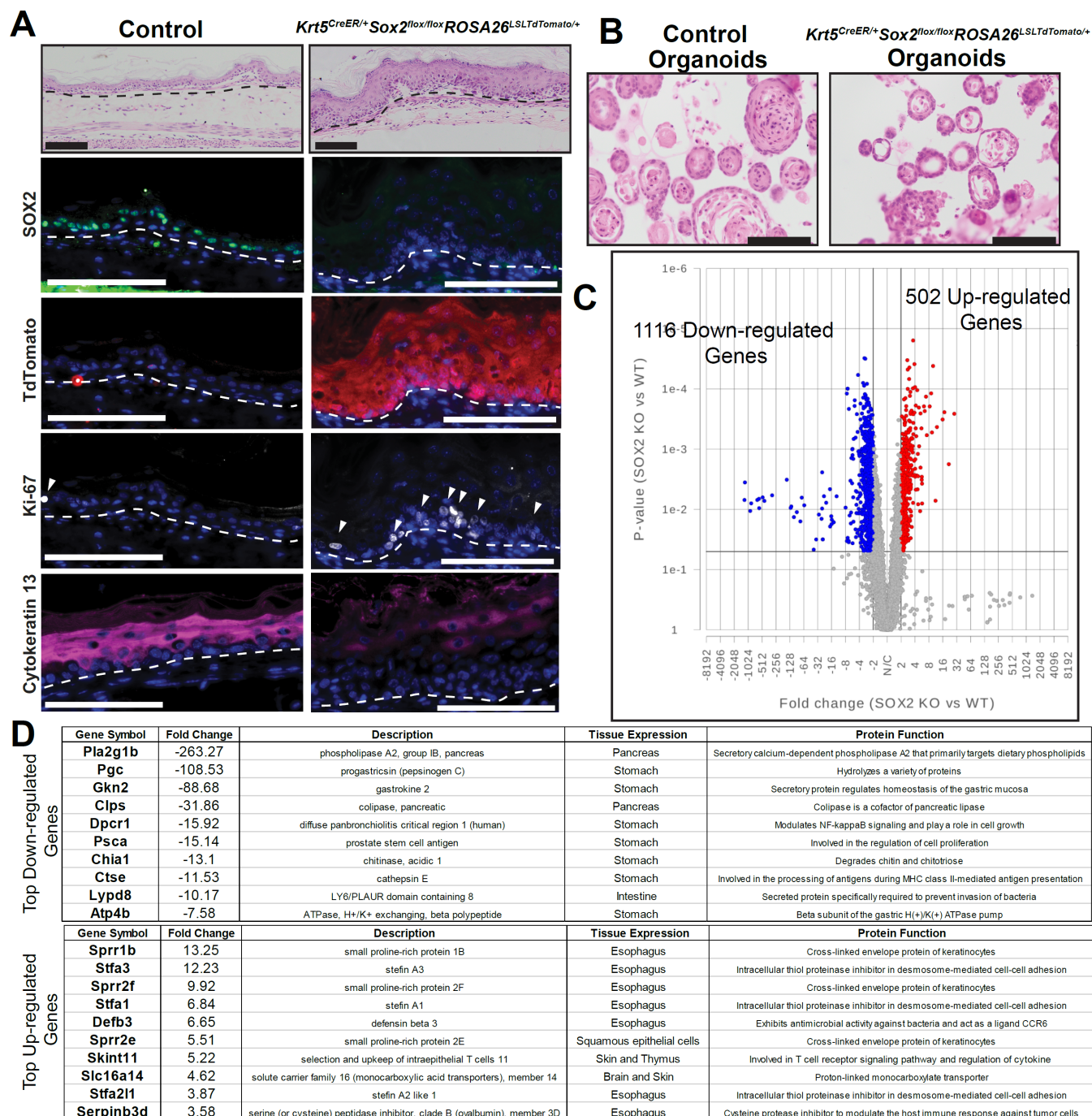


Figure 2. Loss of SOX2 in the adult squamous epithelium. A) *Krt5^{CreER/+}; Sox2^{flox/flox}; ROSA26^{LSLTdTomato/+}* mice with thickened squamous epithelium compared to control. Immunofluorescence showing loss of SOX2 (green), expression of TdTomato (red), increased Ki-67 (white), and decreased Cytokeratin 13 (purple) staining. B) Organoids generated from squamous epithelium of Control and *Krt5^{CreER/+}; Sox2^{flox/flox}; ROSA26^{LSLTdTomato/+}* mice. Scale bars 100µm. C) Bulk microarray transcriptomics volcano plot of Control (n=3) and *Krt5^{CreER/+}; Sox2^{flox/flox}; ROSA26^{LSLTdTomato/+}* (n=4) mice showing 1116 down-regulated (blue) and 502 up-regulated genes (red) upon SOX2 loss. D) Top down-regulated and up-regulated genes with Fold Change, Description, Tissue Expression and Protein Function (data adapted from <https://www.proteinatlas.org/>).

4. Research Approach:

Aim 1: Validate the transcriptional changes due to SOX2 loss in the murine foregut squamous epithelium.

Rationale: Barrett's esophagus involves an abnormal intestinal "reprogramming" of the normal esophagus. SOX2 is a key transcription factor in the normal esophagus during embryonic development²²⁻²⁴ and adult homeostasis²⁵⁻²⁷. Normal SOX2 expression becomes decreased in BE development and neoplastic progression^{28, 29}, **Figure 1**. While no studies have detailed loss of SOX2 in the adult foregut squamous epithelium or its direct transcriptional targets, conditional deletion of *Sox2* in the gastric antrum de-represses expression of intestinal and metaplasia associated genes, alters chromatin accessibility at forestomach lineage loci³¹, and exacerbates Wnt³⁰/NOTCH³¹ signaling mediated tumorigenesis. I have generated reporter mice with foregut squamous epithelium specific inducible Cre-recombinase to conditionally delete *Sox2* (*Krt5*^{CreER/+}; *Sox2*^{flox/flox}; *ROSA26*^{LSLTdTomato/+}), **Figure 2**. Of note, the stomach of rodents differs from humans; it is divided into a non-glandular, squamous epithelial-lined anterior forestomach that shares similarity to the esophagus, and a distal glandular portion³⁶⁻³⁸. In *Krt5*^{CreER/+}; *Sox2*^{flox/flox}; *ROSA26*^{LSLTdTomato/+} mice euthanized 6 weeks after tamoxifen treatment to induce Cre-recombinase and *Sox2* deletion, preliminary data show abnormal thickened esophagus and forestomach, increased basal cell proliferation, and decreased esophageal differentiation markers (e.g. Cytokeratin 13), **Figure 2A, B**. Microarray transcriptomic analyses of squamous epithelia from *Krt5*^{CreER/+}; *Sox2*^{flox/flox}; *ROSA26*^{LSLTdTomato/+} (n=4) compared to wildtype (Control, n=3) mice show unique down-regulated (1116) and up-regulated (502) genes, **Figure 2C**. Consistent microarray transcriptomic changes were found in squamous organoids³⁹ generated from *Krt5*^{CreER/+}; *Sox2*^{flox/flox}; *ROSA26*^{LSLTdTomato/+} compared to wildtype (Control) mice, **Figure 2B**. Interestingly, the top down-regulated genes include "digestion-related/gastric" genes, and the top up-regulated genes include "injury-related/squamous" genes, **Figure 2D**. Further molecular characterization and confirmation of these transcriptional changes will be performed in Aim 1.

Experimental Design: To test the effects of *Sox2* loss in the murine foregut, I have generated *Krt5*^{CreER/+}; *Sox2*^{flox/flox}; *ROSA26*^{LSLTdTomato/+} mice to induce deletion of *Sox2* and activation of the lineage tracing allele, *ROSA26*^{LSLTdTomato}, using *Krt5*^{CreER} with 7 consecutive days of tamoxifen IP injections (1mg/20g mouse body). Adult mice (aged at least 8 weeks) will be euthanized at 6 weeks following tamoxifen induced recombination. Control mice will be co-housed, sex-matched *Krt5*^{+/+}; *Sox2*^{flox/flox}; *ROSA26*^{LSLTdTomato/+} littermates that will be injected with tamoxifen and euthanized in an identical fashion. Upon sacrifice, esophagus and forestomach tissues will be processed for histologic (formalin fixation, paraffin embedding, and sectioning through the Tissue Analysis and Molecular Imaging Core), RNA and protein analyses. Immunohistochemistry will be performed to analyze a panel of markers delineating the top down-regulated (PLA2G1B, PGC, GKN2, CLPS, DPCR1, PSCA, CHIA1, CTSE, LYPD8, ATP4B) and the top up-regulated (SPRR1B, STFA3, SPRR2F, STFA1, DEFB3, SPRR2E, SKINT11, SLC16A14, STFA2L1, SPERINB3D) genes determined from the microarray experiments, **Figure 2D**. RNA extraction will be performed using Qiagen isolation kits, and assayed for the same markers (top down-regulated and top up-regulated genes) using qRT-PCR.

Expected Results, Alternative Approaches, and Future Directions: Given the results of my microarray transcriptomic analyses of *Krt5*^{CreER/+}; *Sox2*^{flox/flox}; *ROSA26*^{LSLTdTomato/+} (n=4) compared to wildtype (Control, n=3) mice, I expect to confirm these top down-regulated and up-regulated transcriptomic changes by IHC and by qRT-PCR. Interestingly, many of the top down-regulated genes are normally expressed in the glandular stomach (i.e. corpus). It is unlikely that these are from glandular stomach "contamination" as consistent microarray transcriptomic changes were found in squamous organoids generated from *Krt5*^{CreER/+}; *Sox2*^{flox/flox}; *ROSA26*^{LSLTdTomato/+} compared to wildtype (Control) mice. Given the role of SOX2 in the glandular stomach, histologic analyses of the corpus from these mice can also be performed. For all *in vivo* mouse histology and qRT-PCR experiments, generation of foregut organoids will be performed to replicate and

validate findings through the Gastrointestinal Experimental Model Systems Core. Western blotting will be used to confirm above histologic findings. In the future, I plan to combine *Sox2* loss with other genetic modifiers including ectopic *Cdx2* gain-of-function (*ROSA26^{LSL-Cdx2-mCherry}*)⁴⁰, Ras signaling activation (*Kras^{LSLG12D}*), and p53 pathway inactivation (*p53^{fllox}*)⁴¹ to further model BE development and progression to EA. In addition, alternative injury mechanisms including combination of acidic bile salts⁴² or a high-fat diet²⁰ will be explored.

Aim 2: Assay for direct transcriptional targets of SOX2 in the murine foregut squamous epithelium.

Rationale: SOX2 is a key transcription factor in the normal foregut squamous epithelial development²²⁻²⁴ and maintenance²⁵⁻²⁷. No studies have detailed loss of SOX2 in the adult esophagus, and specifically there are no studies that have elucidated the direct transcriptional targets of SOX2. Interesting work has shown that SOX2 is expressed in gastric progenitor cells, and importantly, through chromatin immunoprecipitation sequencing (ChIP-seq), that the targets of *Sox2* are tissue specific, related to development/Wnt signaling, and involved in carcinogenesis³⁰. Specifically, *Sox2* loss directly increased Wnt signaling through Tcf/Lef activity and enhanced intestinal metaplasia-associated gene expression to augment oncogenesis³⁰. In related work, embryonic deletion of *Sox2* resulted in abnormal forestomach squamous epithelium development³¹, and consequentially *Sox2* was found to induce forestomach specific genes through direct transcriptional regulation and maintenance of chromatin accessibility³¹. Cleavage Under Targets and Tagmentation (CUT&Tag) assays has improved signal-to-noise data compared to ChIP-seq from decreased cellular inputs⁴³. Given the importance of SOX2 to normal foregut homeostasis, its decrease seen in BE, and no studies elucidating the molecular function or significance of *Sox2* loss in the adult foregut squamous epithelium, a detailed study of the direct transcriptional targets of SOX2 in the adult foregut squamous epithelium is warranted and will be performed using CUT&Tag in Aim 2.

Experimental Design: *Krt5^{CreER/+}*, *Sox2^{fllox/fllox}*, *ROSA26^{LSLTdTomato/+}* and littermate control mice will be used to delete *Sox2* in the foregut squamous epithelium as described in Aim 1. Primary foregut squamous tissues and generated squamous organoids³⁹ will be used for CUT&Tag assays that will be performed according to established protocols⁴³. Briefly, 100,000 cells will be collected, washed, and incubated with Concanavalin A-coated magnetic beads. Primary antibody (ABIN2855074, anti-SOX2 validated for CUT&Tag^{44, 45}) and secondary antibody will be incubated with cells followed by addition of hyperactive pA-Tn5 transposase, tagmentation, and DNA extraction. PCR amplification using indexed i5 and i7 primers⁴⁶, library preparation, paired-end sequencing, and downstream for peak visualization/sequence alignment will be performed through the Functional Genomics and Microbiome Core with collaborator, Dr. Michele Battle^{47, 48}.

Expected Results, Alternative Approaches, and Future Directions: I have performed microarray transcriptomic analyses of *Krt5^{CreER/+}*, *Sox2^{fllox/fllox}*, *ROSA26^{LSLTdTomato/+}* (n=4) compared to wildtype (Control, n=3) mice resulting in unique down-regulated (1116) and up-regulated (502) genes. Through these proposed CUT&Tag experiments, I expect to confirm many of these differentially expressed genes as direct SOX2 transcriptional targets including the genes to be explored in Aim 1. The use of primary tissue and squamous organoids generated from these mice will allow me alternative approaches for the CUT&Tag experiments. ChIP-seq using previously validated approaches^{30, 31} can be pursued as an additional method to complement CUT&Tag experiments. The results of these experiments can be compared to previous *Sox2* ChIP-seq analyses performed in the adult glandular stomach³⁰ or embryonic foregut³¹. Once the direct transcriptional network for SOX2 is determined through these proposed experiments, further mechanistic investigations into the molecular mechanisms involved in SOX2 loss in BE may be performed. I plan to combine *Sox2* loss with ectopic *Cdx2* gain-of-function (*ROSA26^{LSL-Cdx2-mCherry}*)⁴⁰ in the murine foregut. Future experiments can elucidate using CUT&Tag the direct transcriptional targets of CDX2 in this setting, and the interplay between these important transcription factors.

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CAREER DEVELOPMENT PLAN:

My mentor and I have developed the following career development program that will build upon my strengths and fortify my weaknesses. The plan specifically incorporates new technical research skills that will be complemented by further expanding grantsmanship, leadership skills, translational research skills, and integration into the greater research community to ensure a successful independent research career studying esophageal cancer. This individualized plan will be reviewed frequently with my mentor to identify additional deficiencies and areas for improvement. I will have sufficient effort to ensure the successful completion of this proposal as greater than 75% of my time is protected for research, and the aims detailed in this proposal represent the focus of my research. My mentor, Dr. Jason Mills, is a recognized leader in the field of GI development, stem cell biology, and cancer biology studying cellular plasticity in metaplasias and tumorigenesis. He is actively involved with the BCM research community as Chief of Research for the section of Gastroenterology and Hepatology and Co-Director of the TMC DDC. Dr. Mills has developed many local, national, and international collaborations in the GI cancer field. Through these contacts, Dr. Mills has a rich collaborative network of informal co-mentors and future colleagues for my career development. Dr. Mills has an excellent track record for successful mentorship. Recent members of the Mills lab have had success in launching independent research careers. Specifically, Dr. Mills has already graduated 7 PhD students and placed 6 postdoctoral fellows in either tenure-track faculty or hybrid academic-biotech positions. We meet weekly for formal lab meetings and at least weekly for one-on-one research/career discussions.

CAREER GOALS – DURING THE PERIOD OF THIS AWARD

Technical Skills: I will continue to gain experience with molecular characterization of the upper GI tract from murine and human tissues including biomolecular imaging in collaboration with the DDC Tissue Analysis and Molecular Imaging Core, bioinformatics through collaboration with the DDC Functional Genomics and Microbiome Core, and continue to develop novel GI 3D organoid culture and primary co-culture models with assistance from the DDC Gastrointestinal Experimental Model Systems Core, the BCM Gene Vector Core, and the Cell Based Assay Screening Service Core.

Grantsmanship: I will attend the BCM Section of Gastroenterology T32 Lecture Series, which includes regular presentations on topics like writing grants and manuscripts, developing a career development plan, and establishing research budget. Furthermore, I will utilize the expertise of the Mills' lab senior editor, Robert Lawrence, to discuss, edit, and prepare future grant applications.

Leadership Skills: I have completed ad hoc reviews for scientific journals and research grants. I have also participated in the AGA Abstract Review Committee served as a moderator for the Cellular and Molecular Biology of Gastric and Esophageal Disease Pathogenesis Session at DDW 2022. In the upcoming conference season, I plan to continue to sit on selected committees and volunteer to review submitted abstracts.

Translational Research Skills: I will continue to engage through the BCM section of GI Oncology in translation research and clinical trial development by proposing new trial concepts and completing current ongoing trials.

Integration into the Research Community / Foster Local and National Collaboration: I will continue to participate in conferences at BCM including Hematology Oncology Grand Rounds, Ben Taub Multidiscipline GI Tumor Board, TMC DDC GI Research Forum, Dan L. Duncan Comprehensive Cancer Center Molecular Tumor Board, and GI Oncology Clinical Research meetings. In addition, I have been able to present my research at multiple national/international meetings including The FASEB GI Tract XX Conference, Keystone Symposium, DDW 2021/2022, 2020 ASCO Annual Meeting, the Cincinnati Children's Hospital Medical Center Endoderm Club, BETRNet Annual Steering Committee Meeting, Michael E. DeBakey VAMC/CTRID Research Seminar Series, and the DDC Annual Frontiers in Digestive Diseases Symposium.

Build an Independent Research Program: I have recently hired a research technician and am also mentoring a senior oncology fellow, Dr. Huili Zhu, in translational GI research.

Publications: During the upcoming year, I plan to publish two high impact publications. In addition, I will maintain current and develop new collaborations with other members of the Mills lab, the BCM scientific community, and the larger Barrett's esophagus and GI oncology scientific communities.

Grant Applications: During the upcoming year, I will begin preparing my K08 application with plans to submit in 2023. This should give me ample time to resubmit if necessary.

MEDIUM & LONG TERM (2-5 YEAR) CAREER GOALS

I plan to develop a national leadership presence, continue to publish high impact papers that advance the upper GI cancer field, build an innovative research program that combines basic science with translational oncology, apply for R series grants, and train and mentor future young scientists.

Jason C. Mills, M.D., Ph.D., AGAF

Brown Endowed Professor, Departments of Medicine,
Pathology & Immunology, Molecular Cell Biology
Chief of Research, Section of Gastroenterology & Hepatology
Vice Chief, Section of Gastroenterology & Hepatology
Co-Director, Texas Medical Center Digestive Disease Center

September 22, 2022

Mentor Letter of Support for Dr. Ramon Jin

Applicant Background and Nature of Relationship with Mentor: I write to enthusiastically commit my support for Dr. Ramon Jin's application for the 2022/2023 DDC Pilot Feasibility Program. Ramon is an instructor in oncology in the section of Hematology/Oncology at Baylor College of Medicine after having graduated from the highly selective Wash U Physician Scientist Training Program (PSTP) and Wash U Medical Scientist Training Program (MSTP) after receiving his MD/PhD in Molecular Cell Biology. I knew that Ramon was well-trained in molecular, cellular, and developmental biology when he re-joined my lab during his oncology fellowship training with a number of prior publications demonstrating his skill in cell culture, molecular characterization of transcription factors, cell microscopy, and mouse genetics. His previous work has had high impact. For example, Ramon was a pioneer in establishing how a single transcription factor, *MIST1*, directly activates effector genes including the small protein GTPases, *RAB26* and *RAB3D* specifically in GI secretory acinar cells (e.g., gastric zymogenic chief cells and pancreatic acinar cells) to change cell shape and function. After this pioneering work at the intersection of cell and developmental biology, Ramon realized he wanted to use his rich basic science background to do studies he could translate to benefit oncology patients. It was with that in mind that he rejoined my lab to develop his future independent career as an oncology physician scientist. Specifically, he wanted to apply his background in cellular molecular biology to study upper GI cancers and their pre-cancerous metaplastic lesions through novel human-derived organoid modeling.

Applicant's Research in Mentor Lab: Ramon chose my lab based on a new perspective we have taken on the origins of cancers that also has implications for cancer therapy. In 2018, my lab published a paper showing that differentiated cells have access to an evolutionarily conserved program that allows them to re-enter the cell cycle after injury. Lesions like metaplasia that increase cancer risk often arise from differentiated cells returning to the cell cycle by this program (*paligenosis*). Tumors form from metaplasias after paligenosis takes old cells with many somatic mutations accumulated over decades and sends them back into the cell cycle, where oncogenic mutations cause outgrowth of clones that can no longer redifferentiate or die.

Ramon's novel, exciting idea about paligenosis is: If most tumors form by this abortive differentiation change then such tumors may "think" they are constantly responding to perceived injury and proliferating to repair that injury, i.e., they may be in constant cycling paligenosis. The implications would be that certain tumor cells may be resistant to anti-mitotic therapeutic approaches because they act like "differentiated", non-dividing cells. Damage to their mitotic neighbors may stimulate those cells to use paligenosis to reenter the cell cycle and "repair" damage to the tumor. Ramon wants to use this perspective to synchronize tumors in either differentiated or dedifferentiated stages and combine those approaches with anti-mitotic and pro-apoptotic agents.

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Given the lack of good *in vitro* models of metaplasia and cancer in the upper GI tract, Ramon started his expansion of scientific focus in my lab by developing a novel methodology to generate human organoids from Barrett's metaplasia, gastric intestinal metaplasia, and gastroesophageal cancer lesions. His pursuits have been highly successful, and in short time, he has established and thoroughly characterized organoid 'biobanks' with accompanying patient primary pathology and clinical characteristics for Barrett's esophagus and gastroesophageal cancer patients. His work has resulted in him being awarded an ASCO YIA award, a Department of Defense Peer Reviewed Cancer Research Program Career Development Award, and a Barrett's Esophagus Translational Research Network (BETRNet) Developmental Research Program award. In addition, these novel tools have been made available to the greater scientific community. In fact, the utility of his new tools has been applied to studying COVID-19 in the upper GI tract resulting in an AGA-Takeda COVID-19 Rapid Response Research Award with me and another post-doctoral GI instructor in the lab, Dr. Jeffrey Brown (now an Assistant Professor at Wash U), and publication of a manuscript entitled "Tropism of Severe Acute Respiratory Syndrome Coronavirus 2 for Barrett's Esophagus May Increase Susceptibility to Developing Coronavirus Disease 2019" (Jin et al., *Gastroenterology* 2021). Ramon's work has directly resulted in publication of other manuscripts (Jin and Mills, *Dig Dis Sci.*, 2018; *Curr Opin Gastroenterol.*, 2019; *J Mol Cell Biol.*, 2020; Spatz, Jin, and Mills, *Development*, 2021; Stachler and Jin, *Surg Path Clin.*, 2021; Zeng and Jin, *Semin Cancer Biol.*, 2021;), and oral presentations at The FASEB GI Tract XX: Making and Breaking the Gut Conference and Digestive Disease Week 2022.

Ramon has also continued his dedication to translating his basic science findings to directly benefit his patients in the clinic. He has worked alongside Dr. Haeseong Park, an upper GI oncologist at Wash U, to use these cancer organoid systems, integrating them into current and future clinical trials, as a method to make prospective predictions about cancer therapy response and resistance. His work has resulted in a publication reporting the results of a Phase II clinical trial using FOLFIRINOX chemotherapy in advanced gastroesophageal cancer (Park, Jin et al., *JAMA Oncol*, 2020) and a Conquer Cancer Merit Award poster presentation at the 2020 ASCO Annual Meeting.

Finally, with respect to the current proposal, Ramon is interested in the developmental origins of Barrett's esophagus and esophageal adenocarcinoma. He has found that cells stop expressing the normal esophageal developmental transcription factor, SOX2, during oncogenic progression from Barrett's esophagus to esophageal adenocarcinoma. He has developed the tools to explore the functional role of SOX2 in Barrett's esophagus and esophageal adenocarcinoma with a new genetically engineered mouse model of SOX2 loss in the foregut. Using this model in which he can inducibly delete *Sox2* in the murine esophagus and forestomach, Ramon has found that the adult squamous epithelium upon SOX2 loss exhibits altered epithelium homeostasis with increased proliferation and decreased mature esophageal makers, e.g., mature cytokeratins. Ramon's work focuses on the role of transcription factors in cell identity in terms of changing a mature differentiated squamous cell of the esophagus to become a differentiated columnar intestinal cell upon development of Barrett's esophagus. It is congruent with his idea about paligenosis and the application of this idea of moving between differentiated and dedifferentiated stages to develop new chemo preventative and cancer targeted treatments.

Jason C. Mills, M.D., Ph.D., AGAF

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Co-Director, Texas Medical Center Digestive Disease Center

Commitment of Institution and Mentor to Applicant: Ramon is currently being supported by a Department of Defense Peer Reviewed Cancer Research Program Career Development Award until 2024, and in this application, he seeks support to complement this support. Martha Mims, Chief of the Hematology and Oncology Section, along with me (Chief of Research and Vice Chief Gastroenterology & Hepatology), represent the strong support of the institution towards Ramon's career. The Section of Hematology and Oncology has made Ramon an Instructor, and I have provided him with his own bench space and private office to begin building his own independent program. In return, the Section of Hematology and Oncology has asked only for minimal clinical responsibilities that include a half day clinic every week. Thus, Ramon has sufficient protected time for research. I also have the resources, should it come to it, to support him from my external and internal funds. Ramon is welcome to take every aspect of the proposed research with him as he advances towards an independent research career. In the time that we have worked together, we have already coauthored three first author reviews (see above) and a manuscript detailing application of his Barrett's esophagus organoid biobank as a tool to understand potential for the SARS-CoV-2, the virus causing COVID19 to infect patients with Barrett's via an orogastric or aspiration route. I fully expect that this productivity will continue with the aforementioned projects. In addition to his own research, Ramon has acted as ad hoc reviewer for *Physiology Reports*, *The Journal of Physiology*, *Oncogene*, *JoVE*, *Stem Cells*, *Helicobacter*, *Gastric Cancer*, *Nature Communications*, *Experimental Physiology*, *European Journal of Pharmacology*, *Clinical Medicine Insights: Oncology*, *The American Journal of Physiology-Gastrointestinal and Liver Physiology*, and *EMBO Molecular Medicine*. He has reviewed research grants for UW Madison CALS Hatch Proposal, National Science center (Poland), The Cancer Society of New Zealand, and University of Sharjah. Ramon has also participated in the AGA Abstract Review Committee for the Organoid Models of Gastrointestinal Disorders section and served as a moderator for the Cellular and Molecular Biology of Gastric and Esophageal Disease Pathogenesis Session at Digestive Disease Week 2022. He will be well positioned to submit for a K08 in 2023 considering his productivity (including manuscripts currently under review he will have 17 publications, 7 of which are first author).

Resources Provided for Candidate's Career Development: Ramon presents every 8 weeks in our group lab meetings, which comprise a 2–3-hour research update with extensive questioning and guidance from myself and lab members. This format affords Ramon the opportunity to present data and defend its interpretation in a friendly, informal, interactive environment that encourages scientific rigor. Ramon Attends and presents in the Hematology Oncology Grant Rounds, Ben Taub Hospital Multidiscipline GI Tumor Board, Texas Medical Center Digestive Disease Center GI Research Forum, Dan L Duncan Comprehensive Cancer Center Molecular Tumor Board, and GI Oncology Clinical Research meetings. Nationally and internationally, I believe in my trainees' routinely attending and presenting unpublished work at meetings. Ramon has and will continue to attend the yearly AACR, ASCO, and DDW meetings, as well as the summer luminal GI FASEB meeting (every other year) and other meetings as appropriate. Ramon is an outstanding writer and will continue to draft manuscripts and grant applications that I will chiefly edit and guide.

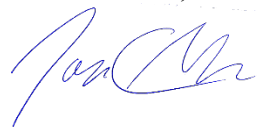
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Mentor's Resources and Statement: The lab currently houses 2 other Instructors and 2 post-doctoral fellows with either PhD or MD/PhD backgrounds as well as 4 graduate students and two supporting scientists. Recent members of the Mills lab have had success in launching independent research careers. Specifically, Dr. José Sáenz, MD PhD, garnered an AGA Scholar in Gastric Cancer, a Burroughs-Wellcome award, and has received a K08 (he is now a tenure-track Assistant Professor at Wash U); Dr. Spencer Willet was a Gastric Cancer AACR fellow and has been awarded a K01 (he is now an Instructor at Wash U); and Dr. Jeffrey Brown MD PhD, is a recipient of the DoD PRCRP-CDA for gastric cancer and recently received a K08 (he was just appointed as a tenure-track Assistant Professor at Wash U). I have already graduated 7 PhD students and placed 6 postdoctoral fellows in tenure-track faculty positions and 2 in hybrid academic-biotech positions. Ramon and I meet weekly for formal lab meetings and at least weekly for one-on-one research/career discussions. I have graduated 7 PhD students and placed 6 postdoctoral fellows in either tenure-track faculty positions or hybrid academic-biotech positions. Ramon is a future colleague, a physician-scientist who will be an outstanding physician and a top-notch basic scientist. I am convinced Ramon will be starting his own lab in about two years, following continued high productivity as a fellow and junior faculty member, and that I will be addressing him as a colleague physician-scientist. Please do not hesitate to ask me to provide additional information for this star!

Sincerely,

Jason C. Mills, M.D., Ph.D.



Jason C. Mills, M.D., Ph.D.
Brown Endowed Professor of Medicine-Gastroenterology
Baylor College of Medicine

BIOGRAPHICAL SKETCH

Provide the following information for the Senior/key personnel and other significant contributors.
Follow this format for each person. **DO NOT EXCEED FIVE PAGES.**

NAME: Mills, Jason C.

eRA COMMONS USER NAME (credential, e.g., agency login): JCMILLS

POSITION TITLE: Brown Endowed Professor

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, include postdoctoral training and residency training if applicable. Add/delete rows as necessary.)*

INSTITUTION AND LOCATION	DEGREE (if applicable)	Completion Date MM/YYYY	FIELD OF STUDY
Washington University, St. Louis, MO	A.B.	05/1989	Biology & Russian
University of Pennsylvania School of Medicine	M.D., Ph.D.	05/1997	Cell Biology
Barnes-Jewish Hospital Residency Training, St. Louis		06/2000	Pathology
Washington University, St. Louis, MO		12/2003	Postdoctoral Training

A. Personal Statement

I am a human pathologist and cell and developmental biologist. My laboratory now works on metaplasia and regulation of stem cell homeostasis in the GI tract. In particular, we are interested in how gastric epithelial cells in adults form from the stem cell and how this process goes askew during metaplasia. We are also interested in how mature, differentiated cells can be recruited back into the cell cycle during tissue regeneration and in tumorigenesis. We propose that metaplasia arises in large part from that kind of dedifferentiation or transdifferentiation process, which appears to be highly conserved across tissues and species. We have proposed, in fact, that there is a specific cellular-molecular program that mature cells can use to become regenerative, which we call Paligenosis. Paligenosis, especially when iterative throughout life, allows for accumulation of mutations in cells that cycle in between long-lived, differentiated states and bursts of proliferation; we call this warehousing-unmasking of mutations the Cyclical Hit theory of tumorigenesis.

In general, the lab uses approaches that range from structural studies at the protein level to clinical trials with oncologists to test agents like tamoxifen that may affect metaplasia and progression to cancer in humans. I have extensive bioinformatics expertise and my lab has published our own in-house expression analysis software/algorithms. Our work has been most focused on stomach, but we also study intestines, pancreas and esophagus, the latter especially in re: Barrett's metaplasia in mice and humans.

Ongoing and recently completed projects that I would like to highlight include:

R01DK094989

Mills (PI)

09/11/2012-04/30/2023

Regulation of atrophy-induced progenitor cells in the gastric corpus

R01DK105129

Mills (PI)

09/30/2015-08/31/2024

Mechanisms of chief cell dedifferentiation

R01 CA246208

Mills (PI)

5/01/2020-4/30/2025

Mechanisms and biomarkers in aberrant paligenosis-induced stomach tumorigenesis

R01 CA239645

Mills (PI)

06/10/2020 – 05/31/2025

Mechanisms Governing Expansion of Embryonic Progenitor Cells (EPCs) in Metaplasia

B. Positions, Scientific Appointments, and Honors

Positions and Appointments

2021-	Professor, Section of Gastroenterology and Hepatology, Department of Medicine, Department of Pathology & Immunology, Department of Molecular and Cellular Biology, Baylor College of Medicine, Houston, TX
2021-	Chief of Research, Section of Gastroenterology and Hepatology, Baylor College of Medicine
2021-	Vice Chief, Section of Gastroenterology and Hepatology, Baylor College of Medicine
2021-	Co-Director, Digestive Disease Center, Texas Medical Center
2016-2021	Professor, with tenure, Departments of Medicine, Pathology & Immunology, and Developmental Biology, Washington University School of Medicine, St. Louis, MO
2016-2021	Director of Research, Division of Gastroenterology
2011-2016	Associate Professor, with tenure, Departments of Medicine, Pathology & Immunology, and Developmental Biology, Washington University School of Medicine, St. Louis, MO
2004-2010	Assistant Professor, Department of Pathology & Immunology, Department of Developmental Biology, Washington University School of Medicine, St. Louis, MO
2003-2004	Instructor, Department of Molecular Biology and Pharmacology, Washington University Medical School, St. Louis, MO

Honors

2022	Inducted into American Association of Physicians
2021	American Gastroenterological Association (AGA) Cellular & Molecular Gastroenterology CMG Section Mentor of the year
2021	Named Herman Brown Endowed Professor of Medicine
2018	Named Visiting Professor, China Medical University, Shenyang, Liaoning Province, China (this position comes only with honorary diploma and is not a faculty appointment)
2017	Elected Vice-Chair with Chairmanship to follow, AGA CMG Section (Chair and Vice-Chair program all symposia at the annual GI, Digestive Disease Week (DDW) conference
2016	Promoted to full professor in three departments
2016	Named Director of Research, Gastroenterology Division
2015	Organizer, XVI annual summer FASEB meeting on the luminal GI tract
2015	Appointed standing member to NIH CIMG Study Section
2014	Named Outstanding Graduate Student Faculty Mentor, Graduate Student Senate
2013	Appointed standing member to American Cancer Society DDC Study Section
2013	Inducted as a Fellow of the American Gastroenterological Association (AGAF)
2013	Appointed Councilor, AGA, Regulatory Peptides, Cell Signaling & Molecular Biology (now CMG) Section
2012	Inducted into American Society for Clinical Investigation
2011	Promoted to Associate Professor with tenure in three departments
2010	Named an Associate Editor for the American Physiological Society journal <i>Physiological Genomics</i>
2010	Graduate Student Senate and Graduate School of Arts and Sciences Award for excellence in graduate student mentoring
2010	Named the 2010 AGA Foundation Funderburg Research Scholar in Gastric Biology Related to Cancer

C. Contribution to Science

1. Molecular characterization of the **normal epithelial stem cell**: Of all the stem cells constitutively active in adult mammals, the one fueling the quotidian regeneration of the epithelium of the stomach has been arguably the least characterized. We have applied, over the years, early laser-capture microdissection techniques and early Affymetrix microarrays to develop gene expression profiles of these cells. We have shown that these

cells require a ©-ERK→CD44→STAT3 signaling cascade to respond to certain types of injury (e.g., those caused by infection with the bacterium *Helicobacter pylori*). We have also investigated how cytokines and drugs regulate proliferation of the stem cell *in vivo* and in organoid models (“gastroids”). Most recently, we have shown how energetic regulation by the energy sensing kinase AMPK modulates cell fate/differentiation decisions and maturation of stem cells. Understanding how the stomach responds to *H. pylori*, which greatly increases risk for gastric cancer, should allow us to modulate the risk of infected patients to developing gastric cancer.

- a. Khurana SS, Riehl TE, Moore BD, Fassan M, Rugge M, Romero-Gallo J, Noto J, Peek RM, Stenson WF, **Mills JC**. The hyaluronic acid receptor CD44 coordinates normal and metaplastic gastric epithelial progenitor cell proliferation. *J Biol Chem*; 2013;288:16085-16097; PMC3668764 (Chosen for Cover Illustration)
- b. Radyk MD, Burclaff J, Willet SG, **Mills JC**. Metaplastic Cells in the Stomach Arise, Independently of Stem Cells, via Dedifferentiation or Transdifferentiation of Chief Cells. *Gastroenterology* 2018; 154:839-843 PMC5847468 (Faculty of 1000 Prime selection)
- c. Sáenz JB, Vargas N, **Mills JC**. Tropism for Spasmolytic Polypeptide-Expressing Metaplasia Allows *Helicobacter pylori* to Expand Its Intragastic Niche. *Gastroenterology* 2019; 156:160-174 PMC6309511
- d. Burclaff J, Willet SG, Sáenz JB, **Mills JC**. Proliferation and Differentiation of Gastric Mucous Neck and Chief Cells During Homeostasis and Injury-induced Metaplasia. *Gastroenterology* 2020; 158: 598-609. PMC7010566

2. Defining the “scaling factor”, a developmentally regulated transcription factor devoted to scaling up or down subcellular architecture. We have shown that there are evolutionarily conserved transcription factor modules that invoke specific subcellular architecture programs. We call such transcription factors “Scaling Factors” and have worked to understand how they enhance efficiency of specific cellular functions. The prototypic Scaling Factor is the bHLH MIST1 (BHLHA15), whose ortholog in flies is DIMM. MIST1 and DIMM are necessary and sufficient to scale up the entire secretory granule apparatus during differentiation of long-lived, professional secretory cells. Loss of MIST1/DIMM does not affect what cells secrete (i.e., their lineage fate choices), just how effective they are at secreting what they have been specified to secrete. *Mist1* expression depends on X-box binding protein 1 (XBP1), which directly upregulates *Mist1* while also vastly expanding rough endoplasmic reticulum (rER) to produce the proteins that the MIST1-governed secretory granule apparatus then stores and secretes. Protein-secreting cells throughout the body use the XBP1→MIST1 cassette to develop and maintain high-level of secretion: gastric chief cells, antibody-secreting plasma cells, and pancreatic acinar cells. Recently, we have shown that the transcription factor HNF4α directly upregulates XBP1.

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- c. Moore BD, Jin RU, Lo H, Jung M, Wang H, Battle MA, Wollheim CB, Urano F, **Mills JC**. Transcriptional Regulation of X-Box-binding Protein One (XBP1) by Hepatocyte Nuclear Factor 4α (HNF4A) Is Vital to Beta-cell Function. *J Biol Chem*; 2016; 291:6146-6157. PMC4813565
- d. Lo HG, Jin RU, Sibbel G, Liu D, Karki A, Joens MS, **Madison BB, Zhang B, Blanc V, Fitzpatrick JA, Davidson NO, Konieczny SF, Mills JC**. A single transcription factor is sufficient to induce and maintain secretory cell architecture. *Genes Dev*. 2017; 31:154-171 PMC5322730

3. Paligenosis: Differentiation had largely been thought irreversible until Yamanaka and colleagues dramatically showed that cells could reprogram back to the progenitor/stem cell state. In recent years, it has become clear that this reversal of differentiated fate is actually an evolutionarily conserved response to tissue injury, wherein mature cells are recruited to other fates and/or called back into the cell cycle to serve as reserve stem cells. We have been showing that gastric zymogenic chief cells have this capacity to reverse their fate (“dedifferentiate”) and help maintain tissue integrity during *Helicobacter*-like injury that results in loss (atrophy) of the acid-secreting parietal cells in the stomach. The change in chief cell differentiation is what underlies a substantial portion of the metaplasia that is seen in humans infected with *Helicobacter*. We have introduced the drug Tamoxifen to study how chief cells dedifferentiate, proliferate, and redifferentiate, because

> 3mg/20 g mouse weight induces metaplasia in the stomach. Recently, we have shown that the process or reprogramming from a mature to a regenerative state may be similar across cells and species by comparing the stages of reprogramming in stomach and pancreatic metaplasia. Because cells go through a sequence of inhibitable, cellular-molecular steps that are genetically controlled, we have proposed that this sort of reprogramming is a general feature of cells, like apoptosis or mitosis. We term it “paligenosis”.

- a. Huh WJ, Khurana SS, Geahlen JH, Kohli K, Waller RA, **Mills JC**. Tamoxifen induces rapid, reversible atrophy and metaplasia in mouse stomach. *Gastroenterology* 2012;142:21-27. PMC3708546
- b. Burclaff J, Osaki LH, Liu D, Goldenring JR, Mills JC. Targeted apoptosis of parietal cells is insufficient to induce metaplasia in stomach. *Gastroenterology* 2017; 152:762-766. PMC5391042
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- d. Miao ZF, Lewis MA, Cho CJ, Adkins-Threats M, Park DK, Brown JW, Sun JX, Burclaff JR, Kennedy S, Lu JY, Mahar M, Vietor I, Huber LA, Davidson NO, Cavalli V, Rubin DC, Wang ZN, **Mills JC**. A dedicated evolutionarily conserved molecular network licenses differentiated cells to return to the cell cycle. *Dev Cell* 2020; 55: 178-194. PMC7606764 (**Featured on Cover**; Commentaries in: PMC7606764, PMID33108751)

4. Identify myosin II as the driving force for membrane blebbing during apoptosis. During my graduate study, apoptosis was first being identified as a genetically programmed, basic cellular process. My contribution was to show that the dramatic morphological changes that apoptotic cells undergo during their final stages of dying were energy-dependent and included orchestrated cytoskeletal changes. Specifically, I showed microtubule phosphorylation/dephosphorylation was required, and, for the first time, that the hallmark apoptotic membrane blebbing depended on Rho and Myosin-light-chain kinase regulation of non-muscle myosin II.

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- b. **Mills JC**, Lee VM, Pittman RN. Activation of a PP2A-like phosphatase and dephosphorylation of tau protein characterize onset of the execution phase of apoptosis. *J Cell Sci* 1998;111:625-636. PMID: 9454736
- c. **Mills JC**, Stone NL, Erhardt J, Pittman RN. Apoptotic membrane blebbing is regulated by myosin light chain phosphorylation. *J Cell Biol* 1998;140:627-636. PMC2140178
- d. **Mills JC**, Stone NL, Pittman RN. Extranuclear apoptosis: The role of the cytoplasm in the execution phase. *J Cell Biol* 1999;146:703-708. PMC2156138

5. Bioinformatic tools and expertise in mouse and human organoid culture and generation. During my postdoctoral fellowship, microarrays and large, comprehensive gene expression profiling were new tools. I pioneered some of the first studies of individual cell lineage-specific gene expression patterns (specific stem cells of the stomach and intestine). As a fellow and later as an independent investigator, I developed early bioinformatic tools to analyze large, gene expression profiles. This work led to a basic understanding of how adult stem cells of various tissues share core, gene expression profiles that make them more like each other than the differentiated cells to which they give rise. My lab has also been an early adopter of organoid technology, generating and molecularly manipulating both mouse and human gastric organoids. This expertise has led to both data manuscripts and high-impact commentaries. Our most recent work, described in the stem cell section above, used organoids to help show how metformin and its target AMPK regulate stem cell differentiation

- a. Sáenz JB, **Mills JC**. Biological techniques: Stomach growth in a dish. *Nature* 2017; 541:160-161.
- b. Osaki LH, Bockerstett KA, Wong CF, Ford EL, Madison BB, DiPaolo RJ, **Mills JC**. Interferon-γ directly induces gastric epithelial cell death and is required for progression to metaplasia. *J Pathol* 2019; 247:513-523. PMC6402979
- c. Miao ZF, Adkins-Threats M, Burclaff JR, Osaki LH, Sun JX, Kefalov Y, He Z, Wang ZN, **Mills JC**. A Metformin-Responsive Metabolic Pathway Governs Differentiation and Maturation from Epithelial Progenitor Cells. *Cell Stem Cell* 2020; 26:910-925. PMC7275895
- d. Sáenz JB, Vargas N, Cho CJ, **Mills JC**. Regulation of the double-stranded RNA response through ADAR1 licenses reprogramming in gastric epithelium. *JCI Insight* 2022; 7:e153511. PMID: 35132959; PMC8855806

Complete List of Published Work in MyBibliography:

<https://www.ncbi.nlm.nih.gov/myncbi/jason.mills.1/bibliography/public/>

Jason C. Mills, M.D., Ph.D., AGAF

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September 22, 2022

Letter Explaining Dr. Ramon Jin's Pathway to Independence

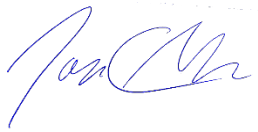
I write to detail Dr. Ramon Jin's pathway to independence over the upcoming years. I have known Ramon well through both my time at Washington University and currently at Baylor College of Medicine. Ramon is currently an instructor in the section of Hematology/Oncology at Baylor College of Medicine after having graduated from the Wash U Physician Scientist Training Program (PSTP) upon completion of his hematology oncology fellowship training. Ramon joined my lab during his PSTP hematology oncology fellowship to use his rich basic science background to benefit oncology patients. Specifically, he wanted to apply his background in cellular molecular biology to study the molecular mechanisms involved in upper GI cancers and their pre-cancerous metaplastic lesions.

His pursuits in my lab have been highly successful and productive, and in short time, his work has resulted in him being awarded an ASCO YIA award, a Department of Defense Peer Reviewed Cancer Research Program Career Development Award, a Barrett's Esophagus Translational Research Network (BETRNet) Developmental Research Program award, and an AGA-Takeda COVID-19 Rapid Response Research Award with me and another post-doctoral GI instructor in the lab, Dr. Jeffrey Brown. Ramon's work has directly resulted in publication of multiple high impact manuscripts (17 publications, 7 of which are first-author including recent work in *Gastroenterology* and *Development*), oral presentations at The FASEB GI Tract XX: Making and Breaking the Gut Conference and Digestive Disease Week 2022, and a previous Wash U DDRCC Pilot & Feasibility Program Grant Award. In addition to his own research, Ramon has also started to independently establish himself in the scientific community by acting as ad hoc reviewer for *Physiology Reports*, *The Journal of Physiology*, *Oncogene*, *JoVE*, *Stem Cells*, *Helicobacter*, *Gastric Cancer*, *Nature Communications*, *Experimental Physiology*, *European Journal of Pharmacology*, *Clinical Medicine Insights: Oncology*, *The American Journal of Physiology-Gastrointestinal and Liver Physiology*, and *EMBO Molecular Medicine*. He has reviewed research grants for UW Madison CALS Hatch Proposal, National Science center (Poland), The Cancer Society of New Zealand, and University of Sharjah. Ramon has also participated in the AGA Abstract Review Committee for the Organoid Models of Gastrointestinal Disorders section, and served as a moderator for the Cellular and Molecular Biology of Gastric and Esophageal Disease Pathogenesis Session at Digestive Disease Week 2022.

Ramon is welcome to take every aspect of the proposed research with him as he advances towards an independent research career. Ramon is currently being supported by a Department of Defense

Peer Reviewed Cancer Research Program Career Development Award until 2024. Martha Mims, Chief of the Hematology and Oncology Section, along with me (Chief of Research and Vice Chief of Gastroenterology & Hepatology), represent the strong support of the institution towards Ramon's career. The Section of Hematology and Oncology has made Ramon an Instructor, and I have already provided him with his own bench space and office to begin building his own scientific program. He has independently hired a research technician that has been able to augment his productivity. He will be well positioned to submit for a K08 in 2023 considering his above detailed productivity and career trajectory. **The work detailed in this proposal will provide Ramon crucial preliminary data to aid in his K08 proposal.** With this funding, Ramon will be able to further expand his lab for his own scientific pursuits. In addition, during this important time of transition, Ramon will be allowed the opportunity to be a corresponding author on papers that are a collaborative effort between our labs. He will continue to develop technical skills, grantsmanship, leadership skills, translational research skills, and integration into the local and national research community. I am convinced Ramon will be starting his own lab in about two years with continued high productivity as an independent faculty member.

Sincerely,

A handwritten signature in blue ink, appearing to read "Jason C. Mills".

Jason C. Mills, M.D., Ph.D.
Brown Endowed Professor of Medicine-Gastroenterology
Baylor College of Medicine

Theme of the DDC: GI Infection and Injury

Barrett's esophagus (BE) is a precancerous condition defined as replacement of the normal esophageal squamous epithelium with metaplastic columnar intestinal epithelium caused by chronic gastroesophageal reflux damage (GERD) that confers the greatest risk for developing esophageal adenocarcinoma. Little is known about the molecular mechanisms that drive BE formation or cancer progression. Barrett's metaplasia can be viewed as an abnormal intestinal programming of the normal esophagus. To begin to understand this process and improve diagnostic/treatment modalities, we need to elucidate the developmental mechanisms underlying BE formation. Spatial specification of the developing digestive tract occurs through a series of regulated transcriptional cascades. At embryonic day 9, the dorsal foregut is marked by expression of SOX2, a transcription factor of the Sry-like HMG box family, which is required for esophageal development. SOX2 continues to be expressed in the esophagus throughout adulthood as an important homeostatic maintenance factor. Importantly, SOX2 expression is decreased or lost completely in BE. Surprisingly, little is known about the molecular function, direct transcriptional targets, or functional consequences of SOX2 loss during BE development. To assay the molecular effects of SOX2 loss in the foregut, I have generated a novel inducible Sox2 knockout mouse model, *Krt5^{CreER/+}; Sox2^{flox/flox}; ROSA26^{LSLTdTomato/+}*. I hypothesize that SOX2 functions to maintain foregut squamous epithelial identity, and its loss is a critical step during Barrett's esophagus development and the progression to esophageal adenocarcinoma. In Aim 1, I will validate the transcriptional changes due to SOX2 loss in the murine foregut squamous epithelium. Using qPCR and immunohistochemical analyses, I will confirm the top increased and decreased genes upon SOX2 loss in *Krt5^{CreER/+}; Sox2^{flox/flox}; ROSA26^{LSLTdTomato/+}* mice. In Aim 2, I will assay for direct transcriptional targets of SOX2 in the murine foregut squamous epithelium. Using a novel Cleavage Under Targets and Tagmentation (CUT&Tag) assay and tissues/organoids generated from *Krt5^{CreER/+}; Sox2^{flox/flox}; ROSA26^{LSLTdTomato/+}* mice, I will determine the direct transcription factor targets of SOX important in the adult murine squamous epithelium. This proposal will address for the first time the role of SOX2 in adult foregut squamous epithelium using a novel genetic mouse model. Together, these experiments will elucidate the molecular pathways involved in BE development and response to chronic gastroesophageal reflux damage, and reveal new therapeutic avenues to treat BE and prevent esophageal cancer progression.

Project Timeline:**Research-Specific Tasks:**

	Months	Performed at BCM
Specific Aim 1: Validate the transcriptional changes due to SOX2 loss in the murine foregut squamous epithelium.		
Major Task 1: Validate the top transcriptional changes due to SOX2 loss using immunohistochemistry.		
Subtask 1: Assess the expression of the top 10 transcriptionally increased and decreased genes upon SOX2 loss. Animals used: <i>Krt5</i> ^{CreER/+} , <i>Sox2</i> ^{flox/flox} , <i>ROSA26</i> ^{LSLTdTomato/+} and <i>Krt5</i> ^{+/+} , <i>Sox2</i> ^{flox/flox} , <i>ROSA26</i> ^{LSLTdTomato/+} (control) mice. 4 mutant and 4 control mice to be used (8 mice total).	1-3	Dr. Ramon U. Jin
<i>Milestone Achieved: Identify and confirm histologic protein effects of SOX2 loss in the adult murine squamous epithelium.</i>	4	Dr. Ramon U. Jin
Major Task 2: Validate the top transcriptional changes due to SOX2 loss using quantitative RT-PCR.		
Subtask 1: Pick and validate primers for the top 10 transcriptionally increased and decreased genes upon SOX2 loss found via microarray analysis.	1-2	Dr. Ramon U. Jin
Subtask 2: Assess the expression of the top 10 transcriptionally increased and decreased genes upon SOX2 loss. Animals used: <i>Krt5</i> ^{CreER/+} , <i>Sox2</i> ^{flox/flox} , <i>ROSA26</i> ^{LSLTdTomato/+} and <i>Krt5</i> ^{+/+} , <i>Sox2</i> ^{flox/flox} , <i>ROSA26</i> ^{LSLTdTomato/+} (control) mice. 4 mutant and 4 control mice to be used (8 mice total).	3-8	Dr. Ramon U. Jin
<i>Milestone Achieved: Identify and confirm the transcriptomic effects of SOX2 loss in the adult murine squamous epithelium.</i>	9	Dr. Ramon U. Jin
Specific Aim 2: Assay for direct transcriptional targets of SOX2 in the murine foregut squamous epithelium.		
Major Task 3: Characterize the direct transcriptional targets of SOX2 in the murine foregut using CUT&Tag.		
Subtask 1: Perform CUT&Tag on squamous organoids generated from <i>Krt5</i> ^{CreER/+} , <i>Sox2</i> ^{flox/flox} , <i>ROSA26</i> ^{LSLTdTomato/+} and <i>Krt5</i> ^{+/+} , <i>Sox2</i> ^{flox/flox} , <i>ROSA26</i> ^{LSLTdTomato/+} (control) mice.	1-6	Dr. Ramon U. Jin

Cell lines used: Murine squamous organoids generated from <i>Krt5</i> ^{CreER/+} ; <i>Sox2</i> ^{flox/flox} ; <i>ROSA26</i> ^{LSLTdTomato/+} and <i>Krt5</i> ^{+/+} ; <i>Sox2</i> ^{flox/flox} ; <i>ROSA26</i> ^{LSLTdTomato/+} (control) mice.		
Subtask 2: Perform CUT&Tag on primary squamous foregut tissues from <i>Krt5</i>^{CreER/+}; <i>Sox2</i>^{flox/flox}; <i>ROSA26</i>^{LSLTdTomato/+} and <i>Krt5</i>^{+/+}; <i>Sox2</i>^{flox/flox}; <i>ROSA26</i>^{LSLTdTomato/+} (control) mice. Animals used: <i>Krt5</i>^{CreER/+}; <i>Sox2</i>^{flox/flox}; <i>ROSA26</i>^{LSLTdTomato/+} and <i>Krt5</i>^{+/+}; <i>Sox2</i>^{flox/flox}; <i>ROSA26</i>^{LSLTdTomato/+} (control) mice. 4 mutant and 4 control mice to be used (8 mice total).	1-10	Dr. Ramon U. Jin
<i>Milestone Achieved: Identify direct transcriptional targets of SOX2 in the murine foregut squamous epithelium.</i>	11	Dr. Ramon U. Jin

Proposed Use of DDC Cores:

My proposal will utilize the services offered through TMC Digestive Disease Center Cores. Specifically, to accomplish Aim 1 in validating the transcriptional changes due to SOX2 loss in the murine foregut squamous epithelium, I will rely on the **Tissue Analysis and Molecular Imaging Core** for tissue processing, paraffin embedding, and slide mounting. For the organoid experiments proposed in Aim 2, I will use the **Gastrointestinal Experimental Model Systems Core** to purchase conditioned media and for additional advice and assistance with murine squamous organoid experiments. To accomplish the proposed CUT&Tag experiments in Aim 2, I will utilize the **Functional Genomics and Microbiome Core** for assistance in sequencing and bioinformatic results analyses.

I will also engage in the additional career enrichment activities offered through the DDC. The Section of Gastroenterology runs a T32 Lecture Series as part of its enrichment activities, which includes regular presentations on topics like writing grants and manuscripts, developing a career development plan, and establishing research budget. I plan to attend and present at these lectures. In addition, I will continue to participate and present my research including the results of these proposed studies in the weekly DDC GI Research Forum and the DDC Annual Frontiers in Digestive Diseases Symposium.

April 12, 2021



RAMON JIN
BAYLOR COLLEGE OF MEDICINE
MEDICINE: HEMATOLOGY & ONCOLOGY

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AN-8552: MOUSE MODELS OF GASTROINTESTINAL TRACT METAPLASIAS

APPROVAL VALID FROM April 12, 2021 thru April 11, 2024

Dear Dr. JIN

The animal care and use of your research protocol indicated above has been approved by the Institutional Animal Care and Use Committee of Baylor College of Medicine.

Baylor College of Medicine has an Animal Welfare Assurance approved by the Office of Laboratory Animal Welfare (OLAW), meeting the requirements of the Public Health Service Policy on Humane Care and Use of Laboratory Animals. The Assurance number is D16-00475.

As principal investigators of this research, you are required to provide a copy of the approved protocol and all subsequently approved amendments to each individual working on this project for their reference.

You will be notified should any U.S.D.A., Baylor College of Medicine, VAMC (if applicable) or other policies come into effect that would require you to amend your project.

Respectfully,

A handwritten signature in black ink that reads "Fred Pereira".

FREDERICK PEREIRA, PH.D., B.SC.
Institutional Animal Care and Use Committee