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

REPORT DATE: July 2022

TYPE OF REPORT: Annual

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

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
July 2022	Annual	01Jul2021-30Jun2022

4. TITLE AND SUBTITLE

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

5a. CONTRACT NUMBER
W81XWH-21-1-0523

5b. GRANT NUMBER
CA200914

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

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

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8. PERFORMING ORGANIZATION REPORT NUMBER

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

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

10. SPONSOR/MONITOR'S ACRONYM(S)

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

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

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 been productive by successfully relocating my lab to Baylor College of Medicine, publishing 3 papers, presenting a lecture at the international premier gastroenterology conference, Digestive Disease Week 2022, and continuing to share my work through talks at the Cincinnati Children's Hospital Medical Center Endoderm Club, BETRNet Annual Steering Committee Meeting, Michael E. DeBakey VAMC/CTRID Research Seminar Series and the Texas Medical Center Digestive diseases Center Annual Frontiers in Digestive Diseases Symposium.

15. SUBJECT TERMS: None listed.

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRDC
Unclassified	Unclassified	Unclassified	Unclassified	71	19b. TELEPHONE NUMBER *(include area code)*

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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?**

<u>Specific Aim 1: Characterize the effects of SOX2 loss on esophageal homeostasis and during injury.</u>
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
-66% complete
Subtask 3: Determine the effects of Sox2 loss upon DOC injury at 6, 9, and 12 months.
Target Date: 3/2024
-33% 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
-50% 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
-75% 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

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
-50% 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
-0% complete
Milestone Achieved: Characterization of effects of SOX2 overexpression in BE and EA; publication of 1 peer reviewed paper.
Target Date: 7/2023
-33% complete

- **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 1: IACUC regulatory protocol approval for animal studies.
Baylor College of Medicine IACUC approval was obtained (approval letter attached in appendices).

Subtask 2: Examine the effects of Sox2 loss at 6 weeks, 3 and 6 months.
Target Date: 10/2022

I have assessed the histological effects of Sox2 loss in the *Krt5$^{CreER/+}$; Sox2$^{flox/flox}$; ROSA26$^{LSLTdTomato/+}$* mice at 6 weeks and 3 months (**Figure 1**).

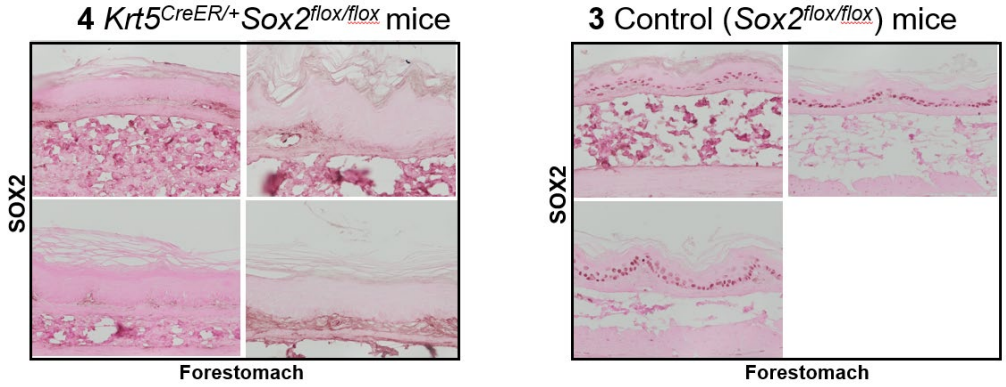


Figure 1. Sox2 loss induces histologic changes in the squamous epithelium at 3 months. Immunohistochemistry staining showing loss of SOX2 and thickening of the squamous epithelium in the forestomach of *Krt5$^{CreER/+}$; Sox2$^{flox/flox}$; ROSA26$^{LSLTdTomato/+}$* and control *Krt5$^{+/+}$; Sox2$^{flox/flox}$; ROSA26$^{LSLTdTomato/+}$* mice at 3 months.

I have also performed electron microscopy analysis of *Krt5$^{CreER/+}$; Sox2$^{flox/flox}$; ROSA26$^{LSLTdTomato/+}$* and control *Krt5$^{+/+}$; Sox2$^{flox/flox}$; ROSA26$^{LSLTdTomato/+}$* mice at 6 weeks (**Figure 2**).

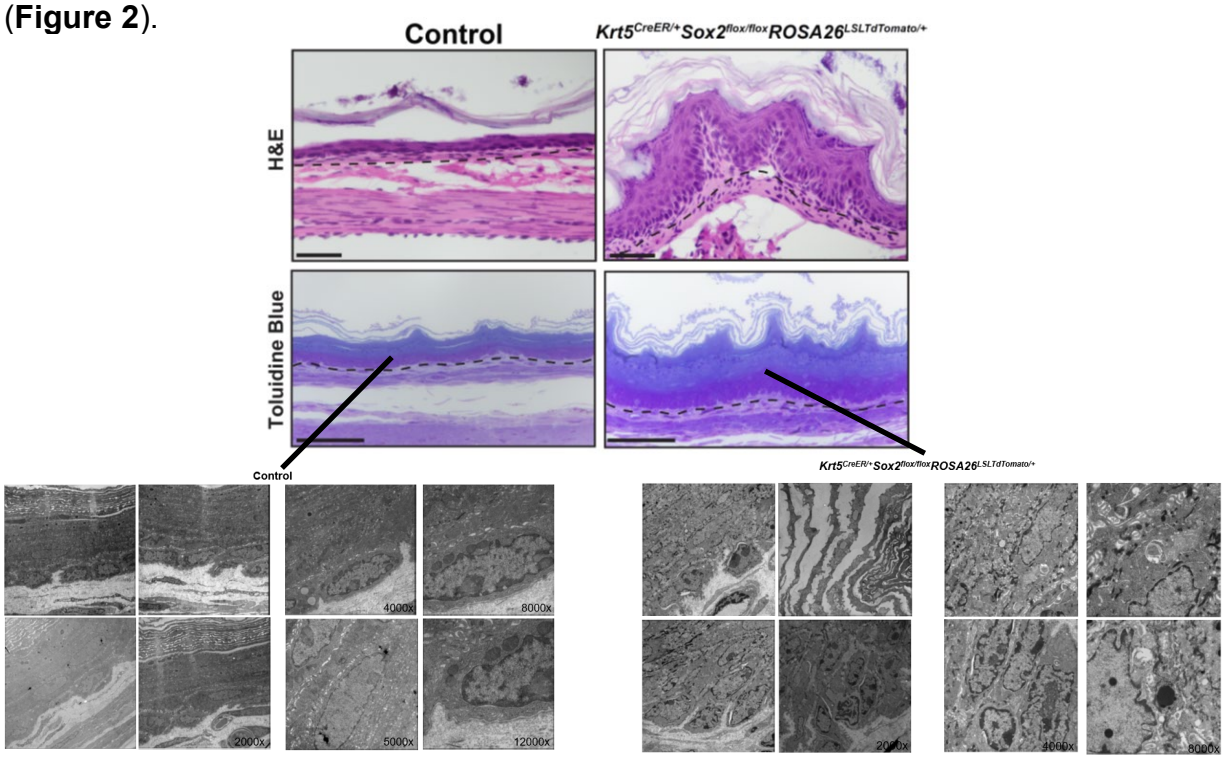


Figure 2. Electron microscopy analysis of *Krt5$^{CreER/+}$; Sox2$^{flox/flox}$; ROSA26$^{LSLTdTomato/+}$* and control *Krt5$^{+/+}$; Sox2$^{flox/flox}$; ROSA26$^{LSLTdTomato/+}$* mice at 6 weeks with H&E and Toluidine Blue staining. Insets showing control and *Krt5$^{CreER/+}$; Sox2$^{flox/flox}$; ROSA26$^{LSLTdTomato/+}$* mice electron micrographs at indicated magnifications.

Work on the longer term 6 month knock experiments are pending. In addition, I have determined the transcriptional changes due to *Sox2* loss in the murine squamous epithelium using Affymetrix GeneChip microarray analysis (**Figure 3**).

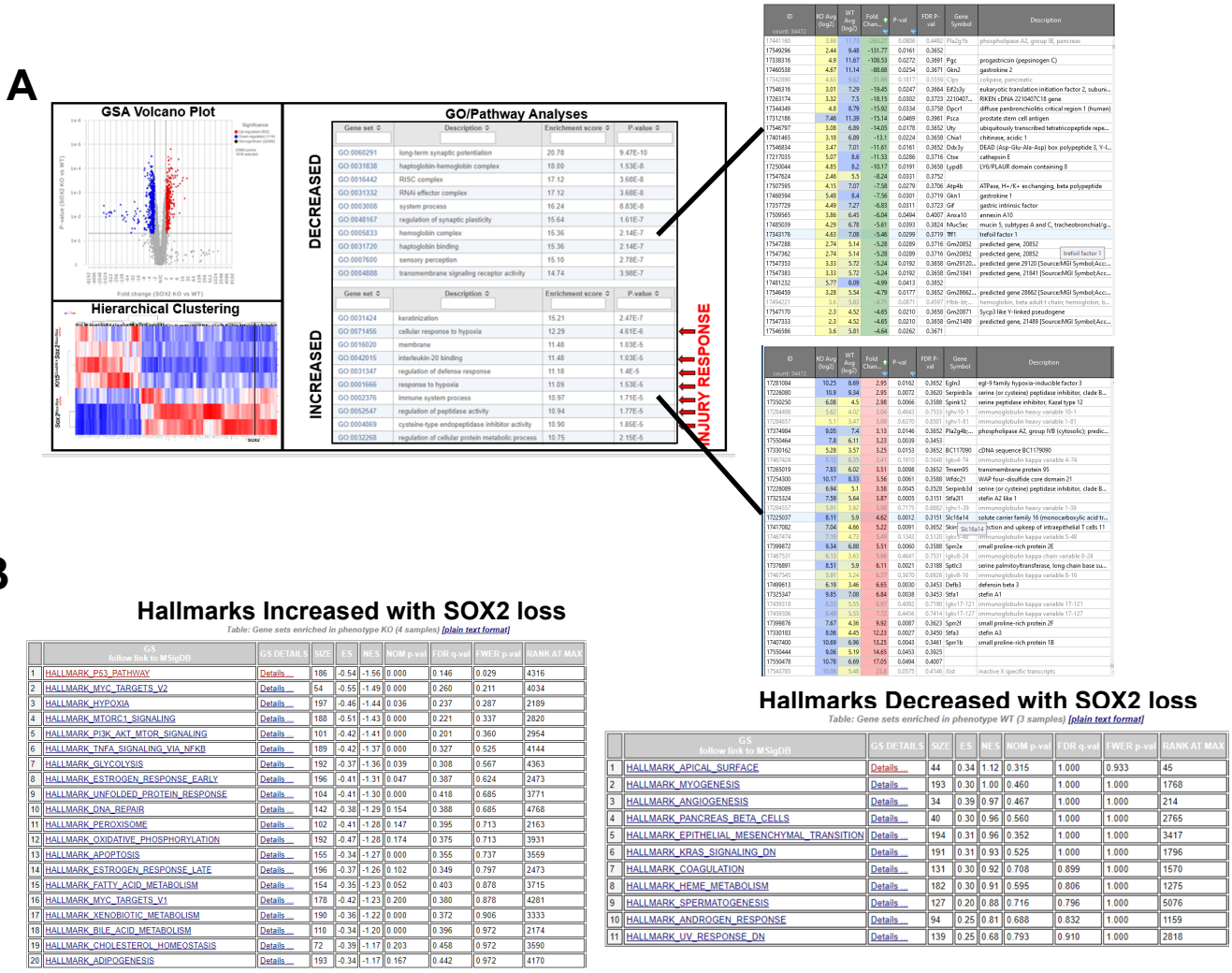


Figure 3. Transcriptional changes due to *Sox2* loss. A) GSA Volcano Plot and Hierarchical Clustering with blue showing genes decreased and red showing genes increased. GO/Pathway Analyses showing decreased and increased pathways and representative top gene lists. B) GSEA analysis with top Hallmark pathways increased and decreased with *Sox2* loss.

Additional CHiP-seq experiments determining the direct transcriptional targets of *Sox2* are pending. CHiP grade antibodies against SOX2 have been obtained (PMID: 31844668).

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

I have treated *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 death for many of the mice (**Figure 4**).

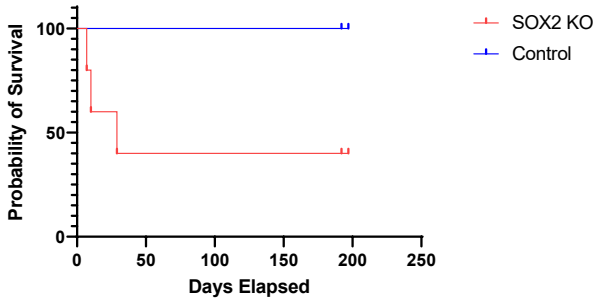


Figure 4. Kaplan-Meier survival analysis for *Krt5*$^{CreER/+}$*; Sox2*$^{flox/flox}$*; ROSA26*$^{LSLTdTomato/+}$ (5 mice) and control *Krt5*$^{+/+}$*; Sox2*$^{flox/flox}$*; ROSA26*$^{LSLTdTomato/+}$ (7 mice) mice with 0.3% DOC.

Histologic analysis from necropsies performed on *Krt5$^{CreER/+}$; Sox2$^{flox/flox}$; ROSA26$^{LSLTdTomato/+}$* mice showed marked disruption of the keratinized squamous epithelium (**Figure 5**). No changes were apparent for control mice. Of note, previous experiments confirmed that WT mice have minimal histologic changes for up to one year of DOC treatment.

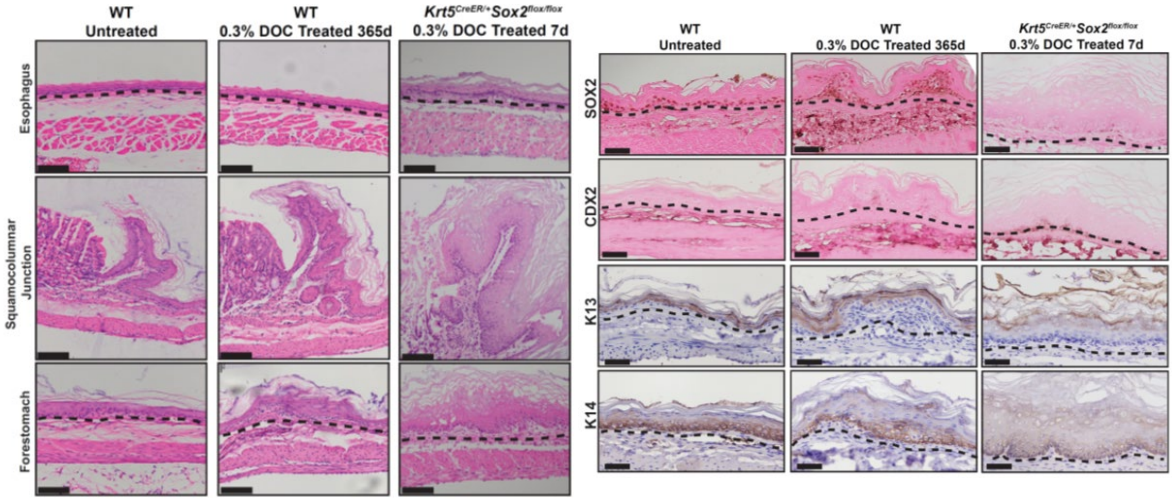


Figure 5. Histologic analysis of Control and *Krt5$^{CreER/+}$; Sox2$^{flox/flox}$; ROSA26$^{LSLTdTomato/+}$* mice treated with 0.3% DOC at 365 and 7 days (at time of necropsy), respectively. Staining for SOX2, CDX2, Keratin 13, and Keratin 14 are shown.

Krt5$^{CreER/+}$; Sox2$^{flox/flox}$; ROSA26$^{LSLTdTomato/+}$ mice that have survived to 6 months show distinctive histologic changes especially at the squamocolumnar junction (**Figure 6**).

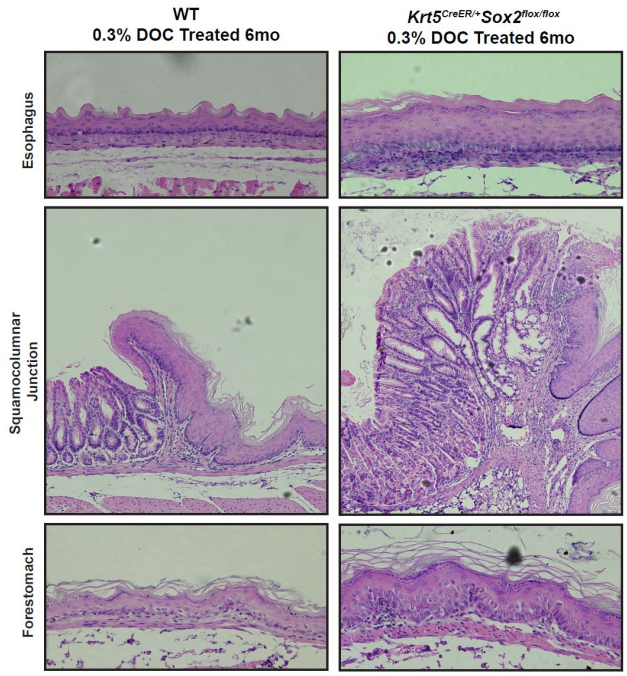


Figure 6. Histologic analysis of Control and *Krt5$^{CreER/+}$; Sox2$^{flox/flox}$; ROSA26$^{LSLTdTomato/+}$* mice treated with 0.3% DOC at 6 months.

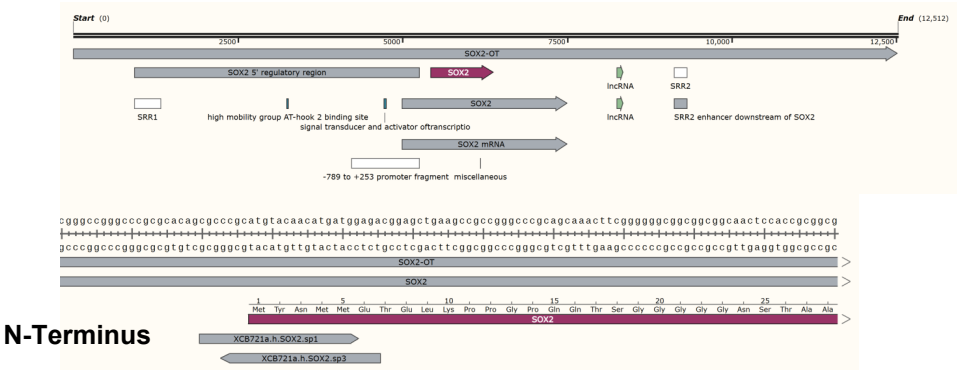
Additional studies characterizing Control and *Krt5$^{CreER/+}$; Sox2$^{flox/flox}$; ROSA26$^{LSLTdTomato/+}$* mice treated with 0.3% DOC at 6 months are pending. I am awaiting ongoing studies of Control and *Krt5$^{CreER/+}$; Sox2$^{flox/flox}$; ROSA26$^{LSLTdTomato/+}$* mice treated with 0.3% DOC for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

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.

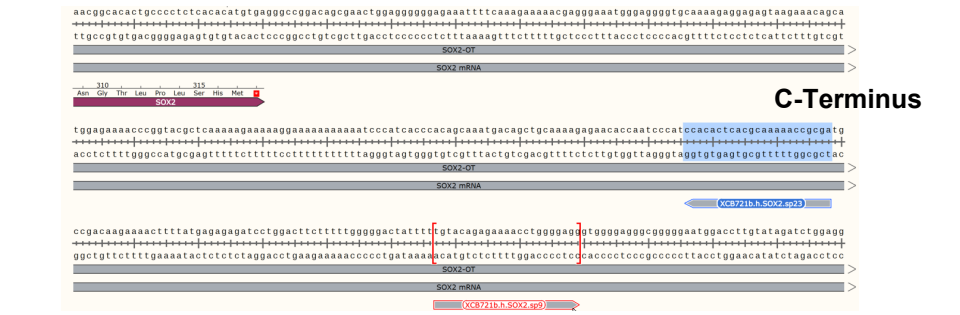
Target Date: 9/2021
Baylor College of Medicine IRB approval was obtained (approval letters attached in appendices). 2 IRB protocols were submitted and approved, H-50658 will allow use of human organoids brought from Washington University, and H-49631 will allow the generation of additional new human patient derived organoids at Baylor College of Medicine.

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

SOX2 CRISPR guide RNAs were successfully designed and RNPs have been prepared (**Figure 7**).



Name	Score	Doench16	gRNA	long_0	long_1	long_2	long_3	short_0	SNP	SNP_count
XCB721a.h.SOX2.sp1	99	0.568	CGCCCGCATGTACAACATGANGG	1	1	1	6	57	NA	0
XCB721a.h.SOX2.sp5	98	0.48	GTACATGCGGGCGCTGTGCGNGG	1	1	1	4	13	NA	0
XCB721a.h.SOX2.sp6	98	0.344	TACATGCGGGCGCTGTGCGCNGG	1	1	1	3	10	NA	0
XCB721a.h.SOX2.sp3	91	0.62	TCTCCATCATGTTGTACATGNGG	1	1	1	21	17	NA	0
XCB721a.h.SOX2.sp4	90	0.535	CTCCATCATGTTGTACATGCNGG	1	1	2	38	15	NA	0
XCB721a.h.SOX2.sp8	88	0.341	TGTGCGCGGGCCCGGCCCGCNGG	1	1	2	20	44	NA	0
XCB721a.h.SOX2.sp2	87	0.619	CATGTACAACATGATGGAGANGG	1	1	2	21	52	NA	0
XCB721a.h.SOX2.sp9	75	0.491	GCGCGGGCCCGGCCCGCCGGNGG	1	1	4	57	10	NA	0
XCB721a.h.SOX2.sp7	72	0.247	GCGGGCGCTGTGCGCGGGCCNGG	1	1	7	66	7	NA	0



Name	Score	Doench16	gRNA	long_0	long_1	long_2	long_3	short_0	SNP	SNP_count
XCB721b.h.SOX2.sp23	100	0.603	TCGCGGTTTTTGCGTGAGTGNGG	1	1	1	1	5	NA	0
XCB721b.h.SOX2.sp1	92	0.373	AACTTTTATGAGAGAGATCCNGG	1	1	1	13	28	NA	0
XCB721b.h.SOX2.sp9	86	0.604	TGTACAGAGAAAACCTGGGGNGG	1	1	1	20	39	NA	0
XCB721b.h.SOX2.sp20	92	0.526	AGTCCCCCAAAAAGAAGTCCNGG	1	1	2	16	121	NA	0
XCB721b.h.SOX2.sp3	89	0.236	GAGATCCTGGACTTCTTTTTNGG	1	1	2	17	33	NA	0
XCB721b.h.SOX2.sp10	81	0.611	GTACAGAGAAAACCTGGGGANGG	1	1	2	35	50	NA	0
XCB721b.h.SOX2.sp4	82	0.628	AGATCCTGGACTTCTTTTTGNGG	1	1	4	34	49	NA	0

Figure 7. gRNA design for human SOX2 at the N-terminus and C-terminus with off target analyses.

Subtask 3: Generate stable SOX2 knockout organoid cell lines from CRISPR-Cas9 RNPs developed in task 2.2.
Target Date: 3/2022

I have generated human esophageal organoids from EGD biopsy samples (WU011, WU012, WU013, and WU014) and from esophagectomy samples (BCM1, BCM2, BCM3) (**Figure 8**). However, I have been unable to maintain long term cultures of these organoids

with decrease in viability and growth of organoids through continued passages despite trying differing sources of starting material (EGD biopsies vs esophagectomy samples) and differing culture media conditions. This difficulty in maintaining human esophageal organoids in culture has been documented and detailed (PMID: 29552622). Discussions with multiple other researchers in the field have not yielded significantly improved results.

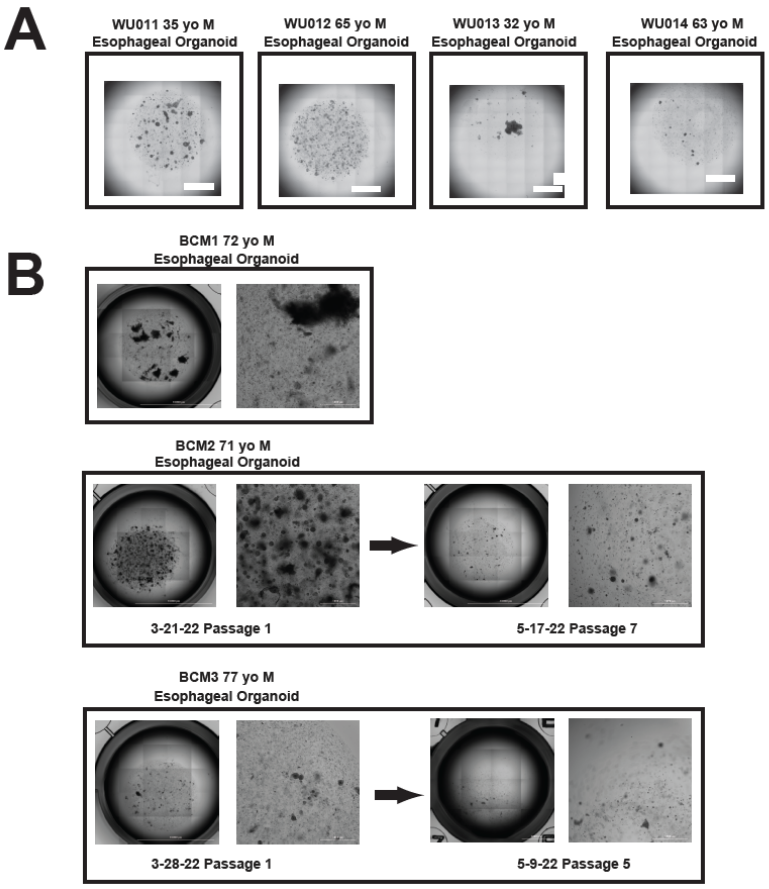


Figure 8. Human esophageal organoids. A) Generated from EGD biopsies. B) Generated from esophagectomy samples with passage time course.

As a result of the difficulty in maintaining human esophageal organoids, I have transitioned to using mouse esophageal organoids. Similarities between these have been well established (PMID: 29552622). Importantly, growth of these murine esophageal organoids is not limited by current organoid culturing techniques (**Figure 9**).

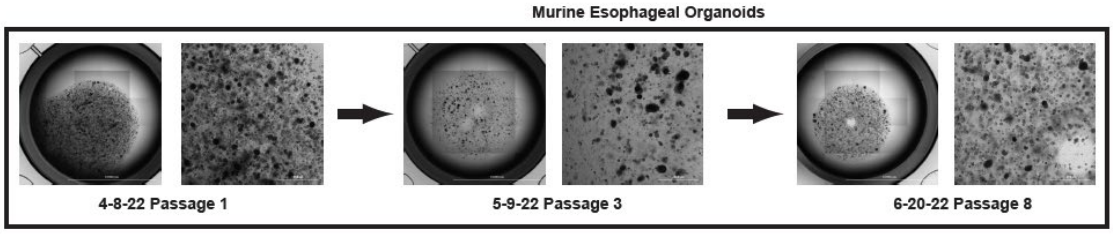


Figure 9. Murine esophageal organoids growth with passage time course.

I have designed SOX2 CRISPR guide RNAs and RNPs have been prepared to target murine Sox2. I will plan to proceed with generating stable SOX2 knockout organoid cell lines from CRISPR-Cas9 RNPs. In addition, I have generated murine esophageal/forestomach organoids from Control and *Krt5*$^{CreER/+}$*; Sox2*$^{flox/flox}$*; ROSA26*$^{LSLTdTomato/+}$ mice.

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

Target Date: 7/2022

From the 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 (**Figure 10**). Additional CHiP-seq experiments determining the direct transcriptional targets of *Sox2* are pending.

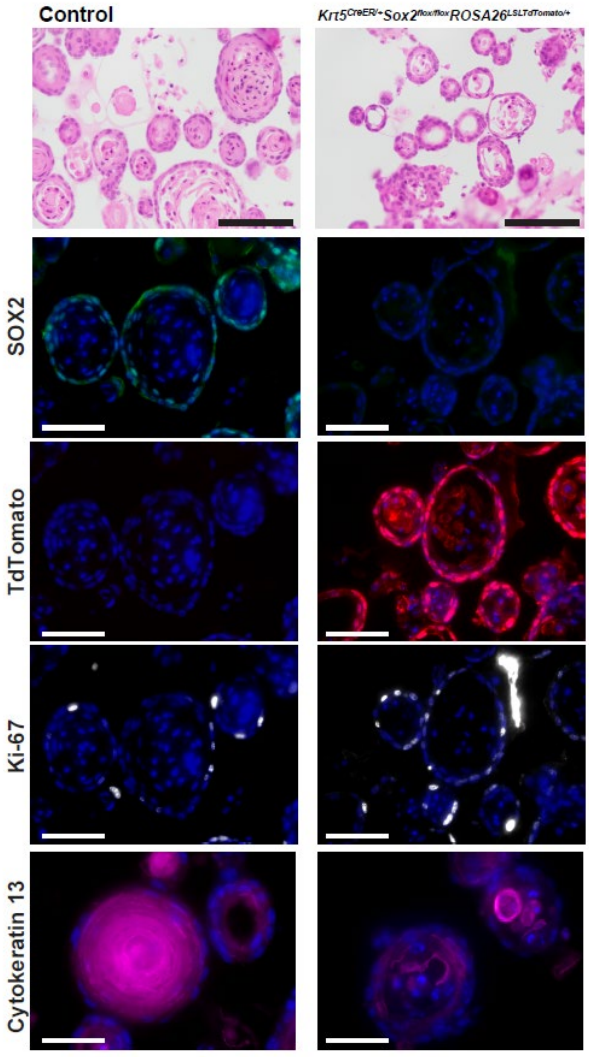


Figure 10. Histologic effects of SOX2 loss in esophageal organoids. Murine esophageal/forestomach organoids from Control and *Krt5*$^{CreER/+}$*; Sox2*$^{flox/flox}$*; ROSA26*$^{LSLTdTomato/+}$ mice with H&E staining and immunofluorescence staining for SOX2, TdTomato, Ki-67, and Cytokeratin 13.

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

In order to pursue this task, I first determined the baseline levels of SOX2 expression in our biobank of human BE organoids using both transcriptomic and confirmatory histologic analyses (**Figure 11**). Interestingly, I found that SOX2 levels varied among human BE organoids. Based on SOX2 levels I subgrouped the BE organoids in intestinal (lowest SOX2 expression), transitional (intermediate SOX2 expression), and foregut (highest SOX2 expression).

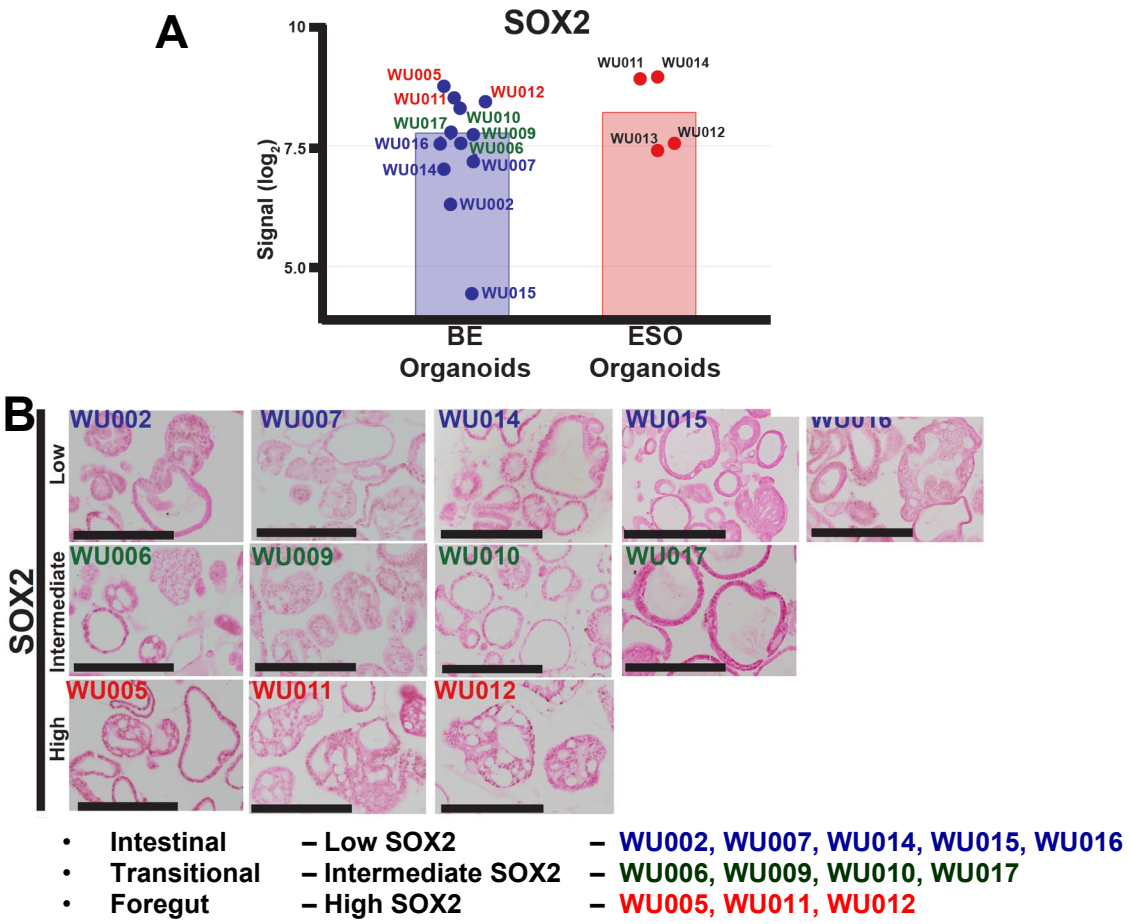


Figure 11. Variable SOX2 expression in human BE organoids. A) Transcriptomic analysis of BE organoids compared to normal esophageal organoids focused on SOX2 expression. B) Immunohistologic analysis of SOX2 expression for human BE organoids. Overall Intestinal, Transitional, and Foregut subgroups defined.

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, and WU012) and showed that SOX2 is expressed in a subset of cells (**Figure 12**).

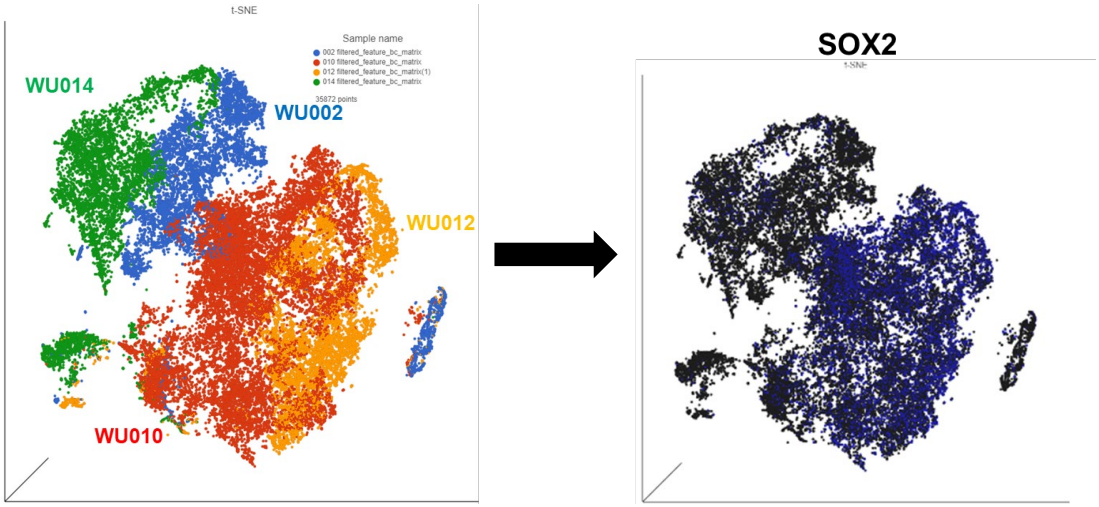


Figure 12. Single-cell RNA sequencing of human BE organoid lines WU014, WU002, WU010, and WU012 with SOX2 expressing cells shown in blue.

Based the SOX2 expression results, I have used human BE organoid line WU014 to overexpress SOX2. I have overexpressed a SOX2 GFP fusion plasmid in WU014 human BE organoids (**Figure 13**).

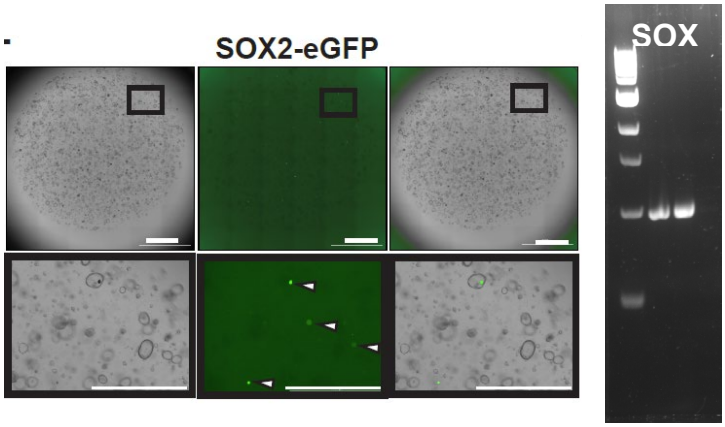


Figure 13. Establishment of SOX2-eGFP overexpression human BE organoids. Live florescent imaging showing abundant SOX2-eGFP expressing cells. Documentation of SOX2-eGFP plasmid by PCR from DNA extracted from SOX2-eGFP expressing human BE organoids.

Additional experiments to further characterize these SOX2-eGFP expressing human BE organoids are underway including transcriptomic analyses and CHiP-seq analyses.

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

Whole-Exome sequencing performed on the BE and EA organoids determined that line WU010 harbored a pathogenic *TP53* mutation (**Figure 14**). Excitedly, *TP53* mutations are highly correlated with the presence of dysplasia in BE (PMID: 29608884 and PMID: 34757142). Based on my further characterization and the pathology report on the patient biopsy sample, we have determined that WU010 is a dysplastic BE organoid.

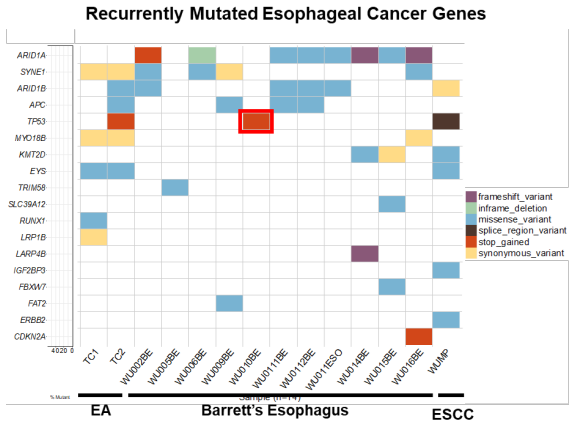


Figure 14. Whole-Exome sequencing of BE, EA and ESCC organoid samples with analysis of the top 30 genes recurrently mutated in gastroesophageal cancers shown.

I have subsequently overexpressed SOX2-eGFP in our dysplastic BE organoid line, WU010 (**Figure 15).**

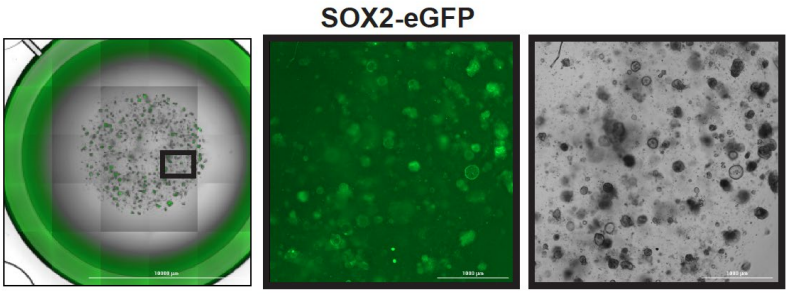


Figure 15. Establishment of SOX2-eGFP overexpression dysplastic human BE organoids. Live florescent imaging showing abundant SOX2-eGFP expressing cells.

Additional experiments to further characterize these SOX2-eGFP expressing dysplastic human BE organoids are underway including transcriptomic analyses and CHiP-seq analyses. Complementary experiments are also underway to use these techniques to overexpress SOX2 via SOX2-eGFP in esophageal adenocarcinoma organoids.

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

Using the dysplastic human BE organoid line, WU010, described above, 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 (**Figure 16**).

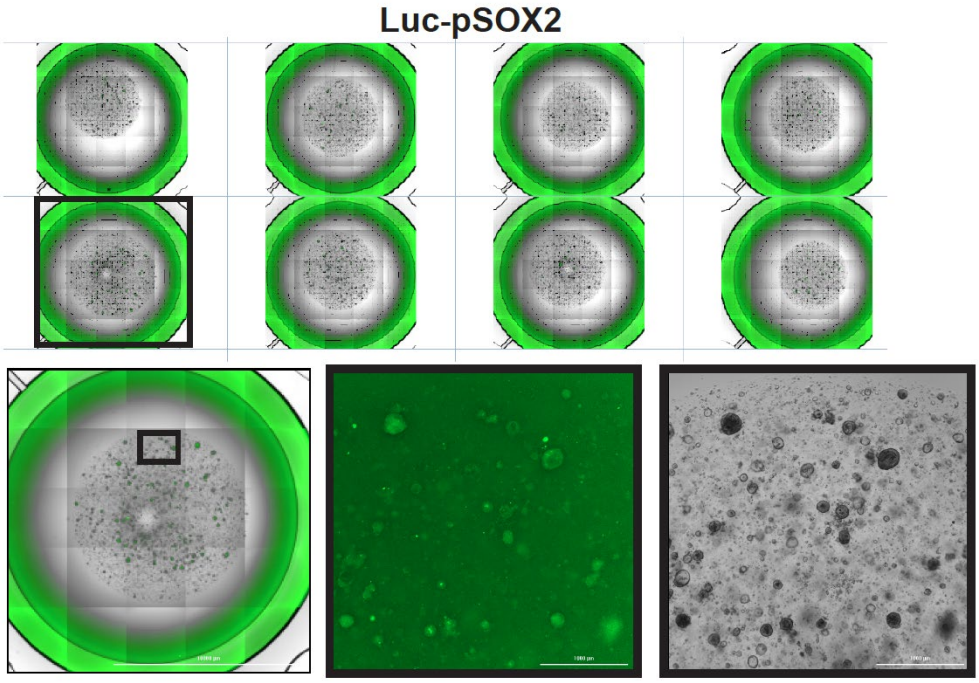


Figure 16. Establishment of Luc-pSOX2 human BE organoids. Live florescent imaging showing abundant Luc-pSOX2 cells.

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

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 to proceed with the proposed high throughput screen. Of note, complimentary HEK293 cells stably expressing Luc-pSOX2 have also been established. We will plan to use these cells to pilot any preliminary screening experiments.

- **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. In terms of professional development and skills, I have enrolled and completed the Career Advancement Series 2021-2022 at Baylor College of Medicine with goal to enhance leadership skills and assist with professional and personal goals for career advancement. Specifically, sessions involved mentorship & sponsorship, building your network and collaborations, pathways to promotion, conflict resolution, and thriving in a health sciences institution. 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 Digestive Disease Week 2022, the Cincinnati Children's Hospital Medical Center Endoderm Club, BETRNet 10th Annual Steering Committee Meeting, Michael E. DeBakey VA Medical Center/CTRID (Center for Translational Research on Inflammatory Diseases) Research Seminar Series, and the Texas Medical Center Digestive Diseases Center Annual Frontiers in Digestive Diseases Symposium. I have also participated in the AGA Abstract Review Committee for the Organoid Models of Gastrointestinal Disorders section for Digestive Disease Week 2022. Finally, I have served as a moderator for the Cellular and Molecular Biology of Gastric and Esophageal Disease Pathogenesis Session at Digestive Disease Week 2022.

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

  The results have been disseminated at multiple national/international meetings including an oral presentation at Digestive Disease Week 2022, a lecture at the Cincinnati Children's Hospital Medical Center Endoderm Club, a lecture at the BETRNet 10th Annual Steering Committee Meeting, a lecture at the Michael E. DeBakey VA Medical Center/CTRID (Center for Translational Research on Inflammatory Diseases) Research Seminar Series, and a poster presentation at the Texas Medical Center Digestive Diseases Center Annual Frontiers in Digestive Diseases Symposium.

  Shared developed organoids with Grady (Fred Hutchinson Cancer Center), Blum (Case Western Reserve University School of Medicine), Stachler labs (UCSF) and Wash U DDC, and organoid data or technical advice to Battle (Medical College of Wisconsin) and Souza (Baylor University Medical Center at Dallas) labs.

- **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 mice and using my murine organoids. Specifically, I will plan to assess for the direct transcriptional targets of SOX2 using CHiP-seq and integrate that data with my transcriptomic data already. Based on these results, I will corroborate results using immunohistochemistry. I also plan to further characterize overexpression of SOX2 in WU014 and WU010 (dysplastic BE organoid line) via transcriptomic and CHiP-seq analyses. I will also establish additional SOX2 overexpressing organoid lines including esophageal adenocarcinoma organoids. Finally, I will pursue my high throughput screen for drugs/compounds that induce *SOX2* expression using my now established Luc-pSOX2 human BE organoids. Collaborations have been made with the Texas A&M University Institute of Biosciences and Technology will allow potentially multiple different drug/compound libraries to be screened.

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 Task 2 we will continue to attempt to grow human esophageal organoids as discussed above. However, However, I have been unable to maintain long term cultures of these organoids with decrease in viability and growth of organoids through continued passages despite trying differing sources of starting material (EGD biopsies vs esophagectomy samples) and differing culture media conditions. This difficulty in maintaining human esophageal organoids in culture has been documented and detailed (PMID: 29552622). Discussions with multiple other researchers in the field have not yielded significantly improved results. As a result of the difficulty in maintaining human esophageal organoids, I have transitioned to using mouse esophageal organoids. Similarities between these have been well established (PMID: 29552622). Importantly, growth of these murine esophageal organoids is not limited by current organoid culturing techniques. Experiments and approach will remain unchanged.

- **Actual or anticipated problems or delays and actions or plans to resolve them**

Difficulties in culturing human esophageal organoids have been detailed above. I will plan to use mouse esophageal organoids that are readily maintained in current culture conditions.

- **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, **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. Published. Acknowledgement of federal support YES

Pang MJ, Burclaff JR**, Jin R**, Adkins-Threats M, Osaki LH, Han Y, Mills JC, Miao ZF, Wang ZN. Gastric Organoids: Progress and Remaining Challenges. Cell Mol Gastroenterol Hepatol. 2022;13(1):19-33. doi: 10.1016/j.jcmgh.2021.09.005. Epub 2021 Sep 20. Review. PubMed PMID: 34547535; PubMed Central PMCID: PMC8600088. Published. Acknowledgement of federal support YES

Stachler MD, **Jin RU**. Molecular Pathology of Gastroesophageal Cancer. Surg Pathol Clin. 2021 Sep;14(3):443-453. doi: 10.1016/j.path.2021.05.008. Epub 2021 Jul 8. Review. PubMed PMID: 34373095. 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, Wang JS, Li QK, Mills JC. The Role of UBE2C in Barrett's Esophagus Development and Progression to Esophageal Adenocarcinoma. Digestive Disease Week 2022, San Diego, CA (5/2022). Selected for Oral Presentation.

Jin RU, Wang JS, Li QK, Mills JC. The Role of UBE2C in Barrett's Esophagus Development and Progression to Esophageal Adenocarcinoma. Texas Medical Center Digestive Diseases Center Annual Frontiers in Digestive Diseases Symposium, Houston, TX (3/2022).

Jin RU, Wang JS, Mills JC. The Role of SOX2 and CDX2 in Barrett's Esophagus. Cincinnati Children's Hospital Medical Center Endoderm Club, Virtual (10/2021).

Jin RU, Wang JS, Li QK, Mills JC. The Role of UBE2C in Barrett's Esophagus and Progression to Esophageal Adenocarcinoma. BETRNet 10th Annual Steering Committee Meeting, Virtual (10/2021).

Jin RU, Wang JS, Mills JC. The Role of SOX2 and CDX2 in Barrett's Esophagus and Esophageal Adenocarcinoma. Michael E. DeBakey VA Medical Center/CTRID (Center for Translational Research on Inflammatory Diseases) Research Seminar Series, Virtual (8/2021).

- **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?**

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.
Funding Support:	Department of Defense Peer Reviewed Cancer Research Program Career Development Award: W81XWH-21-1-0523 ASCO 2021 Conquer Cancer Young Investigator Award: 2021YIA-8674301298 Barrett's Esophagus Translational Research Network (BETRNet) Developmental Research Program Award: U54 CA163060
Name:	Toni Nittolo
Project Role:	Research Technician
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	7 (hired 11/2021)
Contribution to Project:	Ms. Nittolo is responsible for routine lab procedures like cell culture, genotyping, immunohistochemistry, and mouse colony management.
Funding Support:	Additional support provided by Dr. Jason Mills (Career Mentor)

- **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**

  Nothing to report

- **What other organizations were involved as partners?**

  1. **Organization Name:**

     ASCO CCF

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

  3. **Partner's contribution to the projec**t *(identify one or more)*

     1. **Financial support**

     I have attached a letter in the appendices detailing the complementary yet separate experiments that have been proposed for both organizations.

The specific aims for the ASCO CCF award has also been included the appendices section.

8. **SPECIAL REPORTING REQUIREMENTS**
    - **COLLABORATIVE AWARDS:**
    - **QUAD CHARTS:**
9. **APPENDICES:**

April 12, 2021

Baylor College of Medicine
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RAMON JIN
BAYLOR COLLEGE OF MEDICINE
MEDICINE: HEMATOLOGY & ONCOLOGY

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,

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

March 4, 2022

RAMON JIN
BAYLOR COLLEGE OF MEDICINE
MEDICINE: HEMATOLOGY & ONCOLOGY

Baylor College of Medicine
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H-49631 - MOLECULAR DETERMINANTS OF GASTROINTESTINAL TRACT METAPLASIAS

APPROVAL VALID FROM 2/1/2022 TO 1/31/2027

Dear Dr. JIN

The Institutional Review Board for Human Subject Research for Baylor College of Medicine and Affiliated Hospitals (BCM IRB) is pleased to inform you that the research protocol and consent form(s) named above were reviewed and approved by Expedited procedures on 2/1/2022 by Board 1.

The study **does not require continuing review** but will require a 5 year renewal check in with the IRB Office. You will receive an email renewal reminder notice prior to study expiration; however, it is your responsibility to assure that this study is not conducted beyond the expiration date.

Please be aware that only IRB-approved informed consent forms may be used when written informed consent is required.

Any changes in study or informed consent procedure must be submitted to the IRB as an amendment for review and approval prior to implementation unless the change is necessary for the safety of subjects. In addition, you must inform the IRB of adverse events encountered during the study or of any new and significant information that may impact a research participants' safety or willingness to continue in your study.

Research that has been approved by the BCM IRB may be subject to further appropriate review and approval or disapproval by officials of the institution(s) where the research will be conducted. However, those institutional officials may not approve the research if it has not yet been approved by the IRB.

The BCM IRB is organized, operates, and is registered with the United States Office for Human Research Protections according to the regulations codified in the United States Code of Federal Regulations at 45 CFR 46 and 21 CFR 56. The BCM IRB operates under the BCM Federal Wide Assurance No. 00000286, as well as those of hospitals and institutions affiliated with the College.

Sincerely yours,

JULIE KATKIN, M.D., B.A.
Institutional Review Board for Baylor College of Medicine and Affiliated Hospitals

October 20, 2021

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RAMON JIN
BAYLOR COLLEGE OF MEDICINE
MEDICINE: HEMATOLOGY & ONCOLOGY

H-50658 - DE-IDENTIFIED HUMAN ORGANOIDS TO STUDY UPPER GI CANCERS

APPROVAL VALID FROM 10/5/2021 TO 10/4/2026

Dear Dr. JIN

The Institutional Review Board for Human Subject Research for Baylor College of Medicine and Affiliated Hospitals (BCM IRB) is pleased to inform you that the research protocol named above was reviewed and approved by Expedited procedures on 10/5/2021 by Board 1.

The study **does not require continuing review** but will require a 5 year renewal check in with the IRB Office. You will receive an email renewal reminder notice prior to study expiration; however, it is your responsibility to assure that this study is not conducted beyond the expiration date.

Please be aware that only IRB-approved informed consent forms may be used when written informed consent is required.

Any changes in study or informed consent procedure must be submitted to the IRB as an amendment for review and approval prior to implementation unless the change is necessary for the safety of subjects. In addition, you must inform the IRB of adverse events encountered during the study or of any new and significant information that may impact a research participants' safety or willingness to continue in your study.

Research that has been approved by the BCM IRB may be subject to further appropriate review and approval or disapproval by officials of the institution(s) where the research will be conducted. However, those institutional officials may not approve the research if it has not yet been approved by the IRB.

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Sincerely yours,

GABRIEL HABIB, M.D., M.S.
Institutional Review Board for Baylor College of Medicine and Affiliated Hospitals

Seminars in Cancer Biology xxx (xxxx) xxx

Contents lists available at ScienceDirect

Seminars in Cancer Biology

journal homepage: www.elsevier.com/locate/semcancer

Molecular pathogenesis, targeted therapies, and future perspectives for gastric cancer

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ARTICLE INFO

Keywords:
Cancer organoids
Paligenosis
Precision oncology
Cyclical hit model
Mouse models

ABSTRACT

Gastric cancer is a major source of global cancer mortality with limited treatment options and poor patient survival. As our molecular understanding of gastric cancer improves, we are now beginning to recognize that these cancers are a heterogeneous group of diseases with incredibly unique pathogeneses and active oncogenic pathways. It is this molecular diversity and oftentimes lack of common oncogenic driver mutations that bestow the poor treatment responses that oncologists often face when treating gastric cancer. In this review, we will examine the treatments for gastric cancer including up-to-date molecularly targeted therapies and immunotherapies. We will then review the molecular subtypes of gastric cancer to highlight the diversity seen in this disease. We will then shift our discussion to basic science and gastric cancer mouse models as tools to study gastric cancer molecular heterogeneity. Furthermore, we will elaborate on a molecular process termed paligenosis and the cyclical hit model as key events during gastric cancer initiation that impart nondividing mature differentiated cells the ability to re-enter the cell cycle and accumulate disparate genomic mutations during years of chronic inflammation and injury. As our basic science understanding of gastric cancer advances, so too must our translational and clinical efforts. We will end with a discussion regarding single-cell molecular analyses and cancer organoid technologies as future translational avenues to advance our understanding of gastric cancer heterogeneity and to design precision-based gastric cancer treatments. Elucidation of interpatient and intratumor heterogeneity is the only way to advance future cancer prevention, diagnoses and treatment.

1. The clinical dilemma of gastric cancer

The advent of DNA sequencing technology and the "omics" revolution has heralded a new era of precision oncologic medicine [1,2]. The armamentarium for the oncologist is no longer limited to cytotoxic chemotherapies, and new more efficacious and safer molecularly targeted therapies are changing the way that we treat many solid malignancies [3–8]. While the same sequencing efforts have also characterized stomach cancer and revealed important molecular details [9,10], these new insights have not dramatically improved survival for gastric cancer patients as targeted therapies have shown only modest efficacy [11–13]. At the same time, the clinical burden of gastric cancer remains high throughout the world. In the United States for 2021, it is estimated that there will be more than 45,000 new gastric cancer cases to result in more than 26,000 deaths [14]. Worldwide, gastric cancer remains the fifth most common cancer by incidence causing the fourth-highest number of cancer-related deaths [15]. Survival for this disease also remains poor [16] as the five-year overall survival rates remain around 25% for all stages, and the five-year survival rates for gastric cancer patients diagnosed with advanced metastatic disease remain less than 5% [17]. It is clear that improved treatments for gastric cancer are needed.

We will present an overview of the current therapeutic paradigm to highlight the scarcity of molecular targeted therapies for gastric cancer [18,19]. Importantly, current gastric cancer treatments approach this disease as a *single entity*, and treatment strategies for gastric cancer can be divided based on either curative or palliative intent. For the former, applicable to locally advanced gastric cancers, the recommended treatment modality is based on a peri-operative multi-drug chemotherapy regimen [20–22]. Alternatively, the combination of chemotherapy and radiation [23–26] or chemotherapy alone [27–29] in the adjuvant setting after curative-intent resection has also been shown to

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https://doi.org/10.1016/j.semcancer.2021.12.004
Received 10 September 2021; Received in revised form 29 November 2021; Accepted 11 December 2021
Available online 18 December 2021
1044-579X/

be clinically beneficial depending on the adequacy of the surgery [30]. There is currently no evidence that targeted therapy or immunotherapy has any role in the treatment of gastric cancers for curative intent. Of note, the CheckMate 577 trial has recently shown that the addition of nivolumab, a humanized anti-programmed death 1 (PD-1) antibody improved survival among esophageal and gastroesophageal junctional cancer patients after neoadjuvant chemoradiotherapy and surgery with residual pathological disease [31]. For our discussion here we will be using the classic definition of gastric cancer along with Siewert type III gastroesophageal junctional tumors [32] given the clinical [33,34] and molecular similarities [9,35].

For advanced gastric cancers, traditional palliative intent treatment has been multi-agent cytotoxic chemotherapies [36,37]. And while clinical efficacy has been shown to increase with two and even three drug combinations, so does the side effect profile and treatment limiting toxicities [36,37]. Fortunately, given the need for efficacious and well-tolerated treatments for patients with advanced gastric cancer, targeted therapy and immunotherapy have emerged as adjuncts to traditional cytotoxic chemotherapy, Table 1. In terms of targeted therapy, there are currently only two clinically established molecular targets for advanced gastric cancer. The first is the human epidermal growth factor receptor 2 (HER2/neu or ERBB2), which is overexpressed or amplified in 10%–30% of gastric cancers [38]. To target this molecule, trastuzumab, a monoclonal antibody against HER2, was developed and has been shown in the ToGA trial to be efficacious as a frontline treatment for gastric cancer when combined with multiagent chemotherapy [39]. Multiple other HER2 targeting agents and strategies have not shown clinical efficacy [11]. It is only recently, through the DESTINY-Gastic01 trial, that an additional antibody-drug conjugate HER2 targeting drug, trastuzumab deruxtecan, has been approved as a single agent for advanced gastric cancer patients after progression on frontline treatment [40]. In addition to HER2/neu, the vascular endothelial growth factor (VEGF) signaling pathway has also emerged as a target for gastric cancer treatment. Specifically, the humanized antibody against VEGR receptor 2 (VEGFR2), ramucirumab, has been approved as a single agent [41] or in combination with paclitaxel chemotherapy [42] for patients with advanced gastric cancer after progression on frontline chemotherapy. In China, rivoceranib, an orally dosed VEGFR2 inhibitor is also approved for the treatment of advanced gastric cancer in the second-line setting [43]. Of note, targeting this VEGF signaling pathway has not been shown to be efficacious for treating gastric cancer in the frontline setting [11].

In addition to targeted therapies, immunotherapies that block PD-1 to restore cancer immunosurveillance [44] have also emerged as newly approved therapies for gastric cancer. Pembrolizumab, a monoclonal antibody against PD-1, has shown clinical efficacy as monotherapy in the third-line setting (this indication was recently voluntarily withdrawn in North America) for patients whose gastric tumor demonstrates positivity for programmed cell death ligand 1 (PD-L1) combined positive score (CPS) [45,46]. Currently, there is insufficient evidence showing single agent pembrolizumab is superior to chemotherapy in the frontline setting [47]. Nivolumab is another monoclonal antibody targeting PD-1. Similar to pembrolizumab, single agent nivolumab has been shown to be efficacious in patients with advanced gastric adenocarcinoma after progression on standard chemotherapy with approvals for use in this setting in Japan [48,49]. Recently, nivolumab has demonstrated efficacy in the frontline setting when combined with multi-agent chemotherapy based on the ATTRACTION-4 [50] and CheckMate 649 [51] trials. Finally, there are encouraging data regarding the possible combination of immunotherapy and targeted therapy as potential future treatment strategies including recent clinical data with the combination of pembrolizumab with trastuzumab [52,53].

In summary, we have detailed here the current treatment strategies including approved targeted therapies and immunotherapies for advanced gastric cancer. Despite our continued molecular characterization and understanding of gastric cancer, there are currently no targeted agents that have shown single agent frontline efficacy for gastric cancers. In fact, most of these novel treatments are still used in combination with traditional cytotoxic chemotherapies. While these agents described above have improved our treatment of gastric cancer patients, why has our basic science understanding of this disease not more fully translated to new efficacious treatments as in other solid tumor malignancies? The answer lies in the fact that current treatments for gastric cancers approach this disease as a homogeneous disease. Whereas, in reality, gastric cancers constitute a diverse number of diseases with separate molecular characteristics that we will explore in detail in the next section.

2. The molecular characterization of gastric cancers

Gastric cancers have traditionally been categorized based on tumor histology by Lauren classification (diffuse or intestinal types) or World Health Organization (WHO) classification (papillary, tubular, mucinous, or poorly cohesive types) [54,55]. Recently, the molecular profiling of gastric and gastroesophageal junctional adenocarcinomas has revealed distinct molecular and clinical characteristics. These advancements in sequencing have expanded our understanding of basic gastric cancer biology and the heterogeneity that is inherent to this disease. There have been several major efforts to this end for gastric cancer; work from the 'Singapore-Duke' study [56], the Asian Cancer Research Group (ACRG) study [57], and The Cancer Genome Atlas (TCGA) group [9], have provided the basis for the molecular classification of gastric cancer. Here we will discuss the results of these efforts and detail the unique molecular heterogeneity of gastric cancer, Table 2. Of note, besides the 'Singapore-Duke', the ACRG, and TCGA gastric cancer molecular classifications, there are numerous other gastric cancer molecular classification efforts that we will not review here [58,59].

The 'Singapore-Duke' study identified three major molecular subtypes of gastric cancer based on gene expression patterns from a selection of 248 Singapore stomach tumors and an additional 70 independent gastric cancer sample set [56]. Through this analysis, gastric adenocarcinomas were subclassified into 1) a proliferative subtype with a high number of *TP53* mutations, genomic instability, and DNA hypomethylation; 2) a metabolic subtype with "normal" gastric mucosa gene expression profile (including *MUC5AC, TFF2, MUC6, GIF, ATP4A, ATP4B,* and *CHGA*; Gene Ontology terms "digestion" and "secretion"*)*; and 3) an epithelial-mesenchymal transition (EMT) subtype (termed 'mesenchymal') with rare *TP53* mutations, decreased *CDH1* expression, and increased undifferentiated cell markers [56,58]. Interestingly, the proliferative subtype associates strongly with Lauren intestinal type histology, and the mesenchymal subtype displays mostly Lauren diffuse type histology [56]. When analyzed for patient survival metrics, the authors found no significant survival differences amongst the subgroups. However, the authors did show that response to treatment differed significantly among the subtypes. The proliferative subtype displayed the least sensitivity to 5-fluorouracil (5-FU) chemotherapy likely due to high frequency of *TP53* mutations [60–62], the metabolic subtype was more sensitive to treatment with 5-FU chemotherapy possibly due to low frequency of *TP53* mutations, and the mesenchymal subgroup responded particularly well to inhibition of the phosphatidylinositol-3-kinase (PI3K) pathway in part due to increased "cancer stem cell" signature mediated activation of this pathway [63].

Next, the Asian Cancer Research Group (ACRG) performed gene expression profiling, genome-wide copy number microarrays, and targeted gene sequencing on 300 gastric cancers [57]. Based on these studies, the ACRG classified the gastric tumors in their cohort into four subtypes: microsatellite instability (MSI), microsatellite stable (MSS)/EMT, MSS/TP53+, or MSS/TP53–. The ACRG first showed that these molecular subtypes associated with unique patient clinical characteristics; the MSI subtype had early-stage tumors located in the antrum with intestinal histology, MSS/EMT subtype consisted of significantly younger patients, and MSS/TP53+ tumors were more

Table 1
Targeted Therapies for Advanced Gastric Cancer. Molecular target, targeted agent, and pertinent clinical trial data including treatment strategy and clinical efficacy outcomes are presented for the major targeted therapies and immunotherapies used in advanced gastric cancer.

Molecular Target	Targeted Agent	Trial	Treatment	Clinical Efficacy Based on Primary Outcomes
HER2	Trastuzumab (Herceptin)	ToGA (Phase III) [39]	Advanced HER2 positive gastric or GEJ adenocarcinoma patients treated with firstline trastuzumab and chemotherapy (capecitabine plus cisplatin or fluorouracil plus cisplatin, n = 298) compared with chemotherapy alone (n = 296).	Median overall survival was 13.8 months in those assigned to trastuzumab plus chemotherapy compared with 11.1 months in those assigned to chemotherapy alone (hazard ratio 0.74; 95% CI 0.60–0.91; P = 0.0046).
	Trastuzumab deruxtecan (Enhertu)	DESTINY-Gastric01 (Phase II) [40]	Advanced gastric or GEJ adenocarcinoma patients treated with trastuzumab deruxtecan (n = 125) compared to chemotherapy (irinotecan or paclitaxel, n = 62) after progression on at least two previous therapies.	Objective response rate was 51% in patients treated with trastuzumab deruxtecan as compared with 14% of those in the physician's choice treatment group (P < 0.001).
VEGFR-2	Ramucirumab (Cyrmaza)	REGARD (Phase III) [41]	Advanced gastric or GEJ adenocarcinoma patients treated with ramucirumab (n = 238) compared to placebo (n = 117) after progression on first-line chemotherapy.	Median overall survival was 5.2 months in patients in the ramucirumab group and 3.8 months in those in the placebo group (hazard ratio 0.776; 95% CI 0.603–0.998; P = 0.047).
		RAINBOW (Phase III) [42]	Advanced gastric or GEJ adenocarcinoma patients treated with ramucirumab and palitaxel (n = 330) compared to palitaxel (n = 335) after progression on first-line chemotherapy.	Median overall survival was 9.9 months in patients treated with ramucirumab plus paclitaxel group compared to 7.4 months in the placebo plus paclitaxel group (hazard ratio 0.807; 95% CI 0.678–0.962; P = 0.017).
	Apatinib (Rivoceranib) *Approval in China only	Phase III Trial of Apatinib in Patients With Chemotherapy-Refractory Advanced or Metastatic Adenocarcinoma of the Stomach or Gastroesophageal Junction [43]	Advanced gastric or GEJ adenocarcinoma patients in China treated with apatinib (n = 176) compared to placebo (n = 91) after progression on at least two previous therapies.	Median overall survival was 6.5 months in the apatinib group compared with 4.7 months in the placebo group (hazard ratio 0.709; 95% CI 0.537 to 0.937; P = 0.0156).
PD-1	Pembrolizumab (Keytruda)	KEYNOTE-059 (Phase II) [46]	Advanced gastric or GEJ adenocarcinoma patients treated with pembrolizumab monotherapy (n = 259) after progression on at least two previous therapies.	Objective response rate was 11.6% in patients treated with pembrolizumab (30 of 259 patients; 95% CI 8.0%–16.1%).
		KEYNOTE-061 (Phase III) [45]	Advanced gastric or GEJ adenocarcinoma patients (combine positive score [CPS] of 1 or greater) treated with pembrolizumab (n = 196) compared to paclitaxel (n = 199) after progression on firstline chemotherapy.	Median overall survival was 9.1 months in patients treated with pembrolizumab and 8.3 months in patients treated with paclitaxel (hazard ratio 0.82; 95% CI 0.66–1.03; one-sided P = 0.0421).
		KEYNOTE-062 (Phase III) [47]	Advanced gastric or GEJ adenocarcinoma patients (combine positive score of 1 or greater) treated with firstline pembrolizumab (n = 256), pembrolizumab plus chemotherapy (cisplatin plus fluorouracil or capecitabine, n = 257), or chemotherapy (n = 250).	Pembrolizumab was noninferior to chemotherapy for median overall survival in patients with CPS ≥ 1 (10.6 vs 11.1 months; hazard ratio 0.91; 99.2% CI 0.69−1.18) and pembrolizumab prolonged median overall survival in patients with CPS ≥ 10 (17.4 vs 10.8 months; hazard ratio 0.69; 95% CI 0.49−0.97; *not statistically tested). Pembrolizumab plus chemotherapy was not superior to chemotherapy for median overall survival in patients with CPS ≥ 1 (12.5 vs 11.1 months; hazard ratio 0.85; 95% CI 0.70−1.03; P = 0.05) or CPS ≥ 10 (12.3 vs 10.8 months; hazard ratio 0.85; 95% CI 0.62−1.17; P = 0.16).
	Nivolumab (Optivo)	ATTRACTION-2 (Phase III) [49]	Advanced gastric or GEJ adenocarcinoma patients in asia treated with nivolumab monotherapy (n = 330) compared to placebo (n = 163) after progression on at least two previous therapies.	Median overall survival was 5.26 months in the nivolumab treated group and 4.14 months in the placebo treated group (hazard ratio 0.63, 95% CI 0.51−0.78; P < 0.0001).
		ATTRACTION-4 (Phase III) (Presented, unpublished) [50]	Advanced gastric or GEJ adenocarcinoma patients in asia treated with firstline nivolumab and chemotherapy (S-1 or capecitabine plus oxaliplatin, n = 362) compared to chemotherapy (n = 362).	Median progression free survival was 10.5 months in patients treated with nivolumab and chemotherapy compared to 8.3 months in patients treated with chemotherapy alone (hazard ratio 0.68; 98.51% CI 0.51−0.90; P = 0.0007). Median overall survival was 17.5 months in patients treated with nivolumab and chemotherapy compared to 17.2 months in patients treated with chemotherapy alone (hazard ratio 0.90; 95% CI 0.75−1.08; P = 0.257).
		CheckMate 032 (Phase I/II) [48]		

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Table 1 (*continued*)

Molecular Target	Targeted Agent	Trial	Treatment	Clinical Efficacy Based on Primary Outcomes
			Advanced gastric, esophageal, GEJ adenocarcinoma patients treated with nivolumab (n = 59) compared to nivolumab and ipilimumab (n = 49 with nivolumab 1 mg/kg plus ipilimumab 3 mg/kg, n = 52 with nivolumab 3 mg/kg plus ipilimumab 1 mg/kg) after progression on first-line chemotherapy.	Objective response rates were 12% (95% CI, 5%–23%) for the nivolumab treated group, 24% (95% CI, 13%–39%) for the nivolumab 1 mg/kg and ipilimumab 3 mg/kg treated group, and 8% (95% CI, 2%–19%) for the nivolumab 3 mg/kg and ipilimumab 1 mg/kg treated group.
		CheckMate 649 (Phase III) [51]	Advanced gastric, esophageal, GEJ adenocarcinoma patients treated with firstline nivolumab and chemotherapy (capecitabine or fluorouracil plus oxaliplatin, n = 789) compared to chemotherapy only (n = 792).	Nivolumab plus chemotherapy showed statistical significant improvements in overall survival (hazard ratio 0.71; 98.4% CI 0.59–0.86; P < 0·0001) and progression free survival PFS (hazard ratio 0.68; 98% CI 0.56–0·81; P < 0·0001) versus chemotherapy alone in patients with PD-L1 CPS ≥ 5.
Combination (HER2 and PD-1/PD-L1)	Trastuzumab and Pembrolizumab	First-line pembrolizumab and trastuzumab in HER2-positive oesophageal, gastric, or gastro-oesophageal junction cancer: an open-label, single-arm, phase II trial [52]	Advanced HER2 positive gastric, esophageal or GEJ adenocarcinoma patients treated with firstline trastuzumab, pembrolizumab, and chemotherapy (capecitabine or fluorouracil plus cisplatin or oxaliplatin, n = 37).	6 months progression free survival was 70% (26 out of 37 patients; 95% CI 54%–83%) for patients treated with trastuzumab, pembrolizumab, and chemotherapy.
		KEYNOTE-811 (Phase III) (Presented, unpublished) [53]	Advanced HER2 positive gastric or GEJ adenocarcinoma patients treated with firstline trastuzumab, pembrolizumab and chemotherapy (capecitabine plus oxaliplatin or fluorouracil plus cisplatin, n = 133) compared with trastuzumab and chemotherapy (n = 131).	Objective response rate was 74.4% for trastuzumab, pembrolizumab and chemotherapy treated patients compared to 51.9% for trastuzumab and chemotherapy treated patients (difference 22.7%; 95% CI, 11.2%–33.7%, P = 0.00006).

likely to be Epstein-Barr virus (EBV) associated. In contrast to the 'Singapore-Duke' study, patient survival differed among the ACRG subtypes. The MSI subtype had the best patient survival, followed by MSS/TP53+ and MSS/TP53− groups. The MSS/EMT gastric cancers showed the worst prognosis and survival metrics. Through genomic sequencing, the authors found that the MSI gastric cancers displayed hypermutation with frequent mutations in *KRAS*, *ALK*, *ARID1A,* and additional genes in the PI3K signaling pathway. The MSS/EMT group had a relatively low number of somatic alterations. The MSS/TP53- group was enriched in *TP53* mutations resulting in high genomic instability and genomic amplifications in *ERBB2*, *EGFR*, *CCNE1*, *CCND1*, *MDM2*, *ROBO2*, *GATA6*, and *MYC*. Finally, the MSS/TP53+ group displayed genomic stability but harbored a higher number of mutations in *APC*, *ARID1A*, *KRAS*, *PIK3CA*, and *SMAD4*. Interestingly, the authors noted that the MSS/TP53+ subgroup was associated with EBV infection, but found no molecular association with *H. pylori* infection. Together, the 'Singapore-Duke' and the ACRG studies not only have revealed unique molecular differences among gastric cancers, but also raise the possibility that molecular characterization may be used to prospectively predict patient treatment responses and survival.

In 2014, The Cancer Genome Atlas group performed a comprehensive molecular study of 295 primary gastric adenocarcinomas including detailed DNA, RNA, and protein analyses [9]. Through this work, they concluded that gastric cancer could be divided into four distinct entities: Epstein–Barr virus (EBV), Microsatellite instability (MSI), genomically stable (GS) and chromosomal instability (CIN) subtypes [9]. Of note, in 2018, Liu et al. analyzed 462 cases of upper GI adenocarcinomas that had molecular data available from the TCGA core platforms and categorized an additional rare small subset of hypermutated (non-MSI) tumors as a hypermutated single-nucleotide variants (HM-SNV) subtype [64]. Here, we will detail each major TCGA molecular subtype.

Epstein-Barr virus infection is clearly associated with the development of gastric cancers [65,66], and EBV-associated gastric carcinomas account for about 10% of gastric adenocarcinomas worldwide [67]. EBV-associated gastric cancers can be detected in a variety of ways including *in situ* hybridization via Epstein-Barr virus-encoded small RNA assays [68,69]. Accordingly, the TCGA found a distinct molecular subgroup of EBV-positive tumors that occur mostly in the fundus and body of the stomach with a male predominance [9]. The most significant molecular feature of EBV-positive tumors is its extreme CpG island methylator phenotype (CIMP) characterized by extensive hypermethylation (i.e., inactivation) of various cancer-related genes' promoter region CpG islands [70–72]. For example, *CDKN2A* promoter hypermethylation can be detected in 81.6% of EBV-positive tumors [73], and is a key molecular characteristic for these gastric cancers [74]. In addition, EBV-positive tumors also demonstrate frequent somatic mutations in *PIK3CA*, *ARID1A*, and *BCOR* [9,75,76], which may be leveraged for future drug development [10]. Importantly, 80% of EBV-positive gastric cancers have non-silent *PIK3CA* mutations that result in the activation of the PI3K-AKT signaling pathway [9,75,77–79]. Clinically, EBV-associated gastric cancers display the best prognosis among the TCGA subtypes [80–83]. The better prognosis of EBV-positive tumors may be due to the increased viral mediated immune response resulting in downregulation of cancer cell metabolic activity [81,84]. In addition, histologic subclassification and tumor-infiltrating lymphocytes (TILs) can be used to further predict recurrence-free survival (RFS) and disease-free survival (DFS) for patients with EBV-positive tumors [85]. CD8 + T lymphocytes are the major immune cells to target tumors during cancer progression [86], and accordingly, increased CD8 + T lymphocytes infiltration is often found in EBV-positive tumors [10,87]. Amplification of *CD274* or interferon-gamma (IFN-γ) mediated signaling via activation of IRF3 has been shown to cause PD-L1 overexpression [88]. Moreover, EBV-positive gastric cancers also demonstrate amplifications of *JAK2* and *PDCD1LG2 (PD-L2)* [9]. Thus, patients with EBV-positive tumors may preferentially benefit from anti-PD-1 therapy and JAK pathway inhibition [9,65]. Furthermore, preferential responses to immunotherapy may be a more general principle for all EBV-positive tumors [89–92].

MSI is a molecular phenotype described by the TCGA that is caused by impaired DNA mismatch repair (MMR) function [93,94], and readily identifiable clinically via immunohistochemistry or molecular analyses [68]. MSI gastric cancers have the unique molecular feature of hypermutation, and specifically there are several genes most commonly

Table 2
The Molecular Characterization of Gastric Cancer. Major molecular subgroup classifications are shown based on the the Singapore-Duke', the Asian Cancer Research Group (ACRG), and The Cancer Genome Atlas (TCGA) group studies with highlighted key molecular and clinical characteritics. Novel mouse models are displayed for several TCGA subgroups.

					Mesenchymal	**Proliferative**	**Metabolic**
"SINGAPORE –DUKE" Study Classification[53]	**Molecular Characteristics**				- Low frequency of *TP53* mutations; - Few copy number alterations; - KEGG pathways: Focal Adhesion and ECM Receptor Interaction; - GO pathways: Cell Adhesion, Cell Motility, and Angiogenesis - Activation of the Epithelial-Mesenchymal Transition and Cancer Stem Cell pathways; - Hypermethylation	- High frequency of *TP53* mutations; - High copy number alterations; - KEGG pathways: Cell Cycle and DNA Replication; - GO pathways: M phase and Mitotic Cell Cycle; - Activation of E2F, MYC and RAS pathways; - Genes amplified: *CCNE1, MYC, ERBB2,* and *KRAS*; - Hypomethylation	- Low frequency of *TP53* mutations; - KEGG pathways: Metabolic processes; - GO pathways: Digestion and Secretion ("normal gastric mucosa gene expression"); - Activation of Spasmolytic-Polypeptide-Expressing Metaplasia (SPEM) pathway
	Clinical Characteristics				- Mostly Lauren Diffuse histology type; - High histologic grade; - Potential Benefit from PI3K-PTEN-mTOR pathway inhibitors	- Mostly Lauren Intestinal histology type; - Low histologic grade; - Worse disease-free survival based on multivariate analysis	- No histologic correlation; - Beneficial effect of 5-FU treatment after surgery
		MSS/TP53+	**MSI**		**MSS/EMT**	**MSS/TP53-**	
ASIAN CANCER RESEARCH GROUP Classification[54]	**Molecular Characteristics**	- Intact TP53 activity signature; - High prevalence of mutations in *APC, ARID1A, KRAS, PIK3CA,* and *SMAD4;*	- Hypermutation in *KRAS, ALK, ARID1A*, and genes in the PI3K-PTEN-mTOR pathway; - Loss of *MLH1* by RNA expression; - Elevated DNA methylation signature; - Overexpression of PD-L1; - T cell infiltrate		- Lower number of mutation events; - Loss of *CDH1* by RNA expression; - Epithelial-to-Mesenchymal Transition gene expression signature	- Most common subtype; - Loss of TP53 activity signature; - Highest frequency of *TP53* mutations with low frequency of other mutations; - Highest genomic instability index with recurrent amplifications in *ERBB2, EGFR, CCNE1, CCND1, MDM2, ROBO2, GATA6* and *MYC*;	
	Clinical Characteristics	- Associated with EBV infection; - Intermediate patient survival	- Tumors predominantly located in the antrum; - Early-stage disease; - Mostly Lauren Intestinal histology type; - Best patient survival; - Low recurrence rate with preferential liver only metastases		- Significantly younger patient median age; - Late-stage disease; - Mostly Lauren Diffuse histology type; - Worst patient survival; - High recurrence rate with preferential peritoneal seeding	- Mostly Lauren Intestinal histology type; - Intermediate patient survival; - Preferential liver only metastases	
		EBV	**MSI**	**HM-SNV**[57]	**GS**	**CIN**	
The CANCER GENOME ATLAS Classification[9]	**Molecular Characteristics**	- EBV-CpG Island Methylator Phenotype; - DNA hypermethylation (*CDKN2A* silencing);	- Gastric-CpG Island Methylator Phenotype; - Hypermutation including mutations in *TP53, MSH6, EGFR, KRAS, BAX, CASPASE5, PLK1,*	- *POLE* mutations; - Hypermutation; - Heterogeneity in immune signature expression	- Frequent *CDH1, RHOA*, and *ARID1A* mutations, and *CLDN18– ARHGAP* fusions; - Cell Adhesion and Angiogenesis pathways	- Most common subtype; - Frequent mutations in *TP53*, cell-cycle mediator genes (*CCNE1, CCND1,* and *CDK6*), and β-catenin pathway genes (*APC* and *CTNNB1*);	

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Table 2 (*continued*)

		- Frequent mutations in *PI3KCA, ARID1A,* and *BCOR*; - PD-L1/2 Overexpression; - *JAK2, CD274* and *PDCD1LG2* amplifications; - CD8+ T cell infiltrate and IFN-γ immune signature	*BLM, HLA-B, B2M, E2F, RNF43, AGO2, PIK3CA,* and *MLK3*; - Hypermethylation of *MLH1* promoter (*MLH1* silencing); - Mitotic and DNA pathway enrichment; - CD8+ T cell infiltrate		enriched with increased Epithelial - Mesenchymal Transition signature; - B cell, CD4+ T cell and macrophage immune infiltrates	- Recurrent amplifications of various receptor tyrosine kinase signaling pathway genes (*ERBB2, EGFR, FGFR2, ERBB3, MET, KRAS, NRAS,* and *VEGFA*); - Loss of Heterozygosity (LOH); - T cell exclusion and high levels of CD68+ macrophages
	Clinical Characteristics	- Occurs predominantly in the fundus and body; - Male patient predominance; - Best prognosis in patients with resectable disease; - Potential benefit from anti-PD-1 antibodies and JAK2 inhibitors	- Occurs predominantly in the antrum; - Older age predominance (median age 72 years); - Lack of benefit from adjuvant chemotherapy; - Best benefit from anti-PD-1 antibodies	- May benefit from agents that enhance NK cell activity	- Occurs predominantly in the distal stomach; - Younger age predominance (median age 59 years); - Mostly Lauren Diffuse histology type; - Worst prognosis in patients with resectable disease; - Lack of benefit from chemotherapy; - May benefit from FAK inhibition, ROS1 inhibition, and immunotherapy	- Occurs predominantly in the GEJ/cardia; - Mostly Lauren Intestinal histology type; - Associated with *H. pylori* infection; - Greatest benefit from adjuvant chemotherapy; - Potential benefit from targeting receptor tyrosine kinase signaling pathways (e.g., *ERBB2* and *VEGFR*); - Potential lack of benefit from anti-PD-1 antibodies
	Novel Mouse Models[223]				GS-Wnt Model: *Anxa10-CreERT2;Cdh1*$^{fl/fl}$*;Kras*$^{G12D/+}$*;Apc*$^{fl/fl}$ GS-TGFB Model: *Anxa10-CreERT2;Cdh1*$^{fl/fl}$*;Kras*$^{G12D/+}$*;Smad4*$^{fl/fl}$	CIN Model: *Anxa10-CreERT2;Kras*$^{G12D/+}$*;Tp53*$^{R172H/+}$*;Smad4*$^{fl/fl}$

mutated including *TP53, MSH6, EGFR, KRAS, BAX, CASPASE5, PLK1, BLM, HLA-B, B2M, E2F, RNF43, AGO2, PIK3CA,* and *MLK3* [9,95–99]. These genes are involved in a variety of cellular processes such as signal transduction, transcriptional regulation, cell cycle progression/regulation, DNA integrity maintenance, chromatin remodeling, and apoptosis [100]. In detail, *PIK3CA* mutations demonstrate a strong association with MSI status [101], and TCGA molecular analyses reported *PIK3CA* gene mutations in 42% of the MSI gastric cancer tumors [9]. Lynch syndrome, a hereditary cancer predisposition syndrome caused by MMR-related germline gene mutations, is involved in about 15% of microsatellite instability-high (MSI-H) gastroesophageal cancers [10,102,103]. MSI gastric cancers demonstrate favorable survival outcomes compared to microsatellite stable tumors, again likely due to immunosurveillance with high infiltrating levels of CD8 + T cells [10, 100,104,105]. Furthermore, MSI gastric cancers also display frequent alterations in the major histocompatibility complex class I genes (e.g., *B2M* and *HLA-B*), resulting in the HLA class 1 complex expression loss and immune-surveillance escape [100]. MSI-H gastroesophageal cancers feature dense lymphocyte infiltration with a widespread increased expression of immune-checkpoint proteins including PD-L1 [104,106, 107]. MSI-H tumors demonstrated worse clinical response towards cytotoxic chemotherapy, but importantly, improved durable responses to immune checkpoint inhibitors (ICIs) [100,107,108], due to the increased CD8 + T cell infiltration and higher PD-L1 and IFN-γ protein expression levels [105,109–111]. This has been confirmed in clinical trials as MSI status and tumor mutational burden (a surrogate marker for hypermutation) are now routinely used clinically to determine the utility of immunotherapy for many cancers [112–115].

Gastric and gastroesophageal cancers lacking the molecular characteristics associated with any of the other subtypes are classified as genomically stable (GS) [9]. For example, other TCGA subtypes demonstrate mitotic network upregulation, such as increased mRNA levels of *AURKA, AURKB, E2F, FOXM1, PLK1*, and *MYC* activation targets, but the GS subtype uniquely does not [9]. These GS gastric tumors often arise in the distal stomach, are enriched for the diffuse histological variant, and frequently harbor *CDH1* and *RHOA* mutations and fusions involving RHO-family GTPase-activating proteins (e.g., *CLDN18-ARHGAP26* fusions) [9,116–119]. Consequently, these molecular aberrancies are associated with an increased epithelial-to-mesenchymal transition molecular signature [9,116–119]. *RHOA* regulates the formation of actin stress fibers during EMT [120,121], and *CDH1* encodes the E-cadherin (E-cad) protein, which is critical for normal epithelial cell-cell adhesion [120–122]. *CLDN18-ARHGAP26* fusions lead to *CLDN18* loss and *ARHGAP26* gain-of-function, which impairs gastric cellular epithelial barrier properties and results in EMT phenotypes [118]. In addition, the GS subtype gastric cancers display poor prognosis [10,81], and are often refractory to chemotherapy (especially in the adjuvant setting) due to their increased mesenchymal characteristics [10,81,123]. However, the GS subtype and its recurrent mutations may potentially benefit from targeted therapeutic strategies including FAK inhibition for those with tumors harboring *RHOA* mutations [124] and inhibition of ROS1 for patients with *CDH1* mutations [10,125]. Interestingly, GS tumors also demonstrate high levels of B cells, CD4 + T cells, and tumor-associated macrophages with the development of ectopic immune cell aggregations termed tertiary lymphoid structures (TLS) [126,127] possibly making these cancers promising candidates for immunotherapy [126].

The final TCGA subgroup of gastric cancers is the chromosomal instability (CIN) subtype. These CIN gastric cancers are the most common molecular subtype, found in at least 50% of gastric cancers, and they are associated with Lauren intestinal-type histology classification [128]. CIN gastric cancers are preferentially located at the gastroesophageal junction and proximal cardiac region of the stomach [9,10]. There exists heterogeneity in this subgroup of gastric cancers in of itself, and CIN gastric cancers may be a compilation of an even more molecularly heterogeneous group of tumors [68]. CIN gastric cancers display marked aneuploidy determined by large chromosome-level abnormalities amplifications [9,97] resulting in upregulation of many various growth factor signaling pathways [10]. CIN gastric cancers also display loss of heterozygosity (LOH) and genomic deletions leading to loss of tumor suppressor gene function [129,130]. In fact, high-levels of LOH are correlated with CIN associated intestinal or mixed-typed histology gastric cancers [131,132]. Determination of CIN gastric cancer subtype is difficult and requires oftentimes molecular analyses as there are no easily assayable biomarkers [68]. Many current additional diagnostic methods have been proposed to identify CIN gastric cancers including comparative genomic hybridization, single nucleotide polymorphism array, micronuclei counting, karyotyping, LOH analysis, and fluorescent *in situ* hybridization [133]. However, none of these methods are the optimal clinical diagnostic tools [68], and further work is needed to further define and diagnose CIN gastric cancers.

Despite their heterogeneity, CIN gastric cancers do display certain common molecular characteristics that include *TP53* mutation enrichment [9]; recurrent amplifications of various receptor tyrosine kinase signaling pathway genes (*ERBB2, EGFR, FGFR2, ERBB3, MET, KRAS*, *NRAS*, and *VEGFA);* mutations in cell-cycle mediator genes (*CCNE1, CCND1,* and *CDK6*); β-catenin pathway (*APC* and *CTNNB1*) loss-of-function mutations; and COSMIC signature 17 with common AA > AC nucleotide transversions [9,10,97,134,135]. Additional common molecular pathways induced by CIN tumors are being elucidated including the presence of double-stranded DNA in the cytosol and activation of the cGAS-STING anti-viral pathway [136], and changes in cellular physiology including autophagy and protein stress response pathways to tolerate aneuploidy [137]. Furthering the understanding of these pathways may allow the development of simplified IHC based, clinically applicable biomarkers. In terms of prognostic significance of the CIN subtype, studies have found that it has an intermediate prognosis (worse than the EBV subtype but better than the GS subtype), but with variability that is likely due to its greater intragroup heterogeneity [81]. Furthermore, the CIN subtype displayed the greatest benefit from adjuvant chemotherapy [81]. In addition, Derks et al. demonstrated that more than 50% of all CIN tumors exhibit T cell exclusion and high levels of CD68+ macrophages [126]. The associations of increased MYC activity and *CCNE1* amplification with immune-poor CIN gastric cancer may shed light on immune evasion mechanisms [126]. As discussed above, many of these shared CIN molecular pathways have been therapeutically targeted [9–11,134,138]. For example, trastuzumab and trastuzumab deruxtecan have activity in those CIN gastric cancers with overexpression of *ERBB2* [39,40], and the VEGFR2 targeting antibody ramucirumab has efficacy in those CIN gastric cancers with *VEGFA* gene amplifications [41,42].

This particular subtype of gastric cancer is also highly correlated with *Helicobacter pylori* infection, and the chromosomal instability may be a direct consequence of this pathogen [139]. Zhang et al. examined the mutational signature of 1703 gastric tumor tissue samples [140]. The *H. pylori*-positive group of gastric cancers displayed unique mutational signatures shared with the CIN molecular subtype [140]. Mechanistically, it is known that *H. pylori* infection affects the DNA damage response and impairs DNA repair in epithelial cells [141]. Specifically, *H. pylori* strains with the *cag* pathogenicity island (PAI) that produce the cytotoxin-associated gene A (CagA) are thought to induce more severe gastric epithelial damage and are more strongly associated with gastric cancer development [142–145]. *H. pylori* induces genomic damage through *cag* PAI mediated suppression of DNA damage response pathways [146,147], and other potential *cag* PAI independent mechanisms [148,149]. In particular, *H. pylori* infection is thought to dysregulate TP53 function through multiple mechanisms including increased frequency of *TP53* mutations [150,151], increased MDM2 mediated TP53 feedback loop [152], and alteration of *TP53* isoform expression [153]. Direct DNA damage and impaired DNA repair response due to *H. pylori* infection, ultimately result in genomic translocations and amplifications by breakage-fusion-bridge cycles (i.e., chromosomal instability)

[154–156]. Interestingly, certain telomere-proximal, actively transcribed regions demonstrate the most susceptibility to DNA damage induced by *H. pylori*, and these same genomic aberrations in susceptible genomic regions can be found in *H. pylori* associated gastric cancers as well [146].

Here we have detailed the molecular and clinical characteristics of the major molecular gastric cancer subgroups. There is still much work to be done to bridge the gaps between the basic and clinical understanding of these subgroups, and this will be critical given the future clinical translatability of gastric cancer molecular subtypes. Current molecular gene mutational profiling and gene expression based categorization are too cumbersome and time consuming to be clinically applicable. Immunohistochemical based tests that can serve as faithful biomarkers are needed and studies have started to develop algorithms based on histology and immunohistochemistry markers to approximate the molecular subtypes of gastric cancer [157–160], but these methodologies need to be validated by larger studies.

As a whole, it is clear that stomach cancer is comprised of different unique diseases each with its own pathogenesis. Gastric cancers oftentimes lack "early" oncogenic drivers such as *KRAS* mutations in pancreatic adenocarcinoma [161,162] and *APC* mutations in colorectal cancers [163]. Molecular subgroups have not been included in gastric cancer clinical decision making as gastric adenocarcinomas are still viewed as a common clinical entity. This monochromatic view of a complex disease is likely the reason why our translational efforts in gastric cancer have resulted in few new targeted agents with only modest clinical benefit (e.g., improvements in overall survival measured in weeks to months). To facilitate future clinical translational efforts, we need improved preclinical models of gastric cancer to study and elucidate the pathophysiological and developmental origins of gastric cancer molecular heterogeneity.

3. Imurine models of gastric cancer

Mus Musculus represents one of the best *in vivo* models to study carcinogenesis [164]. However, a challenge for gastric cancer mouse models is our theme of heterogeneity and the lack of unified oncogene driver mutations found in gastric cancers (especially for the most common CIN molecular subgroup). Unlike pancreatic adenocarcinoma mouse models in which two or three mutations can be targeted to specific pancreatic cell lineages to model human pancreas carcinogenesis [165,166], such simplicity is not available for gastric cancer mouse models [167–169]. A detailed review of these models is beyond the scope of this work and has been covered previously [167–169]. Our intention here is to highlight the progression of murine models of gastric cancer as this advancement has directly fueled our basic and translational understanding of gastric cancer. In this section we will highlight several mouse models available for gastric cancer research including chemical/infectious models, transgenic models, site-specific recombinase systems, and more recent human TCGA-based models.

To start, chemical agent and infectious-based mouse models were developed to recapitulate the pathogenesis of human stomach cancers. These models display differing tumor phenotypes with distinct tumorigenesis mechanisms. Specifically, we will elaborate on chemical carcinogenesis with N-methyl-N-nitrosourea (MNU) and pathogen-based carcinogenesis with *Helicobacter* infection models. The MNU model relies on a highly potent gastric carcinogen exposure for 30–50 weeks to induce adenocarcinoma formation in the glandular stomach [170–174]. The exact mechanism of MNU induced gastric tumorigenesis is still unclear [168], but this model has allowed researchers to elucidate many potential basic science pathways involved in gastric cancer formation [175–182]. After *H. pylori* was identified as a type I carcinogen and *H. pylori* infection was deemed to be the greatest risk for developing gastric cancer [139], researchers started to develop *Helicobacter* infection based gastric cancer mouse models. The *H. felis,* [183] and mouse-adapted *H. pylori strains* (e.g., Sydney strain, SS1) [184] infection models were developed as feasible means to study the progression from gastritis to adenocarcinoma as mice are resistant to human *H. pylori* colonization [185,186]. Chronic infection with *H. felis* results in inflammation, atrophy, and invasive adenocarcinoma [187]. Murine models utilizing *H. pylori* SS1 develop dysplasia after chronic infection [184], but only develop adenocarcinomas when the *cag* pathogenicity island is introduced, CagA-positive *H. pylori* SS1 (PMSS1) [188]. These animal models have been crucial in allowing researchers to understand the role of dietary exposure, inflammation, and infection on gastric carcinogenesis [167].

Transgenic mouse models were introduced to allow researchers in the gastric cancer field more precise control over the tumor initiating events. The INS-GAS transgenic mice were designed to excessively express the gastric hormone, gastrin, under control of the insulin promoter [189,190]. These mice develop gastric atrophy, dysplasia, and gastric cancer [167]. However, this process is exacerbated when combined with chronic *Helicobacter* infection as invasive gastric adenocarcinomas appear in these mice under 12 months of age [189,190]. Genetic knockout of trefoil factor 1, a protein with tumor suppressor role, in mice ($Tff1^{-/-}$) demonstrates a progression from hyperplasia to antral intramucosal carcinoma within five months in 30 percent of the mice [191] with increased mucosal inflammation through the activation of NF-κB [192] and COX2 signaling pathways [193]. The H/K-ATPase: IL-1β mice were designed to allow increased expression of the pro-inflammatory cytokine, IL-1β, in a stomach-specific manner under the control of the parietal cell specific H/K-ATPase promoter [194]. These mice develop gastric atrophy and metaplasia, and by 18 months of age, 30% of these mice progress to gastric cancer [194]. The inflammatory mechanisms of gastric carcinogenesis and the role of the microenvironment have been explored using these mice [195,196]. The next transgenic murine model we will touch upon is the $gp130^{F/F}$ mice, in which a mutation in the IL-6 receptor β-chain, gp130, results in upregulation of STAT signaling [197,198]. These mice develop gastric adenomas quickly by 3 months of age, but rarely do these tumors progress to invasive cancers [197,198]. A similar STAT signaling inflammation mediated model of gastric carcinogenesis has been shown for $Nfkb1^{-/-}$ mice with 95% developing gastric tumors by 18 months of age with the majority of these tumors displaying evidence of invasion [199,200]. Finally, oncogenic $Kras^{V12D}$ has been expressed under the keratin 19 (K19) promoter, which itself is expressed in the gastric isthmal neck zone [201]. Inflammation, metaplasia, dysplasia, and gastric adenocarcinomas form in one-third of the mice by 16 months of age [202]. These transgenic and genetic knockout mouse models have proven to be insightful tools, but they are often limited due to their additional tissue non-specific phenotypes [168]. For example, the INS-GAS and $Tff1^{-/-}$ mouse models have well documented intestinal phenotypes [191,203,204]. In addition, these transgenic and genetic knockout mouse models by themselves rarely demonstrated the aggressiveness and metastatic gastric adenocarcinoma phenotypes seen in the human disease [167].

To increase the specificity (i.e., targeting of genetic changes only in gastric cell lineages) and biological relevance of murine models of gastric cancer, researchers have turned to use site-specific recombinase, in particular, Cre recombinase technology to develop new gastric cancer mouse models [168,205]. Researchers have crossed *K19-*Cre^{ERT} knock-in mice with Lox-Stop-Lox-$Kras^{G12D}$ mice to allow inducible conditional expression of oncogenic K-ras in the K19 expressing cell lineages resulting in hyperplasia, metaplasias and adenomas in the stomach (but also in the oral cavity, colon, and lung) [206]. Other models make use of the intestinal and gastric antral *Villin-Cre* line and a more stomach specific *Foxa3-Cre* to delete the tumor suppressor, Krüppel-like factor 4 (*Klf4*) by crossing with $Klf4^{fl/fl}$ mice [182]. The authors showed that loss of *Klf4* results in metaplastic changes in the gastric antrum that develop into tumors in 29% of these mice by 80 weeks of age, a process that is accelerated with MNU treatment [182]. The applicability of these early "site-specific" mouse models of gastric cancer is also limited due to the

lack of unique stomach-specific promoters [167–169]. To this end, researchers have evaluated many gastrointestinal tissue stem/progenitor genetic markers such as *Tff1, Bmi1, Lrig1, Lgr5, Hopx, Sox2*, and *Sox9* [207–213] in an attempt to identify a better gene promoter to drive Cre-recombinase expression specifically in gastric cell lineages. However, the results for these markers have failed to identify the "perfect" gastric cell lineage marker as each marker exhibits potential extra-gastric expression. For example, *Tff1-CreERT2* mice were designed as a stomach specific (corpus and antral) inducible Cre recombinase that forms gastritis, metaplasia and gastric adenomas when crossed with $Kras^{LSL-G12D}$ (tumors in 30% of mice after 9 months) and $Braf^{LSLV600E}$ (tumors in 66% of mice after 8 months) mice in a STAT4 dependent manner [207]. However, the *Tff1-Cre* transgenic mouse (not *Tff1-CreERT2* mice) also showed recombination detectable in Brunner glands, cecum, and proximal colon in addition to the stomach suggesting the non-stomach specific nature of this gene promoter [214]. Interestingly, using this *Tff1-Cre* mouse, researchers found that activation of oncogenic K-ras or deletion of *Pten* resulted in the development of atrophy and spasmolytic polypeptide expressing metaplasia (SPEM) or pseudopyloric metaplasia without evidence of dysplasia even at 12 months of age [214]. Furthermore, the use of *Tff1-Cre* to delete *Cdh1* resulted in columnar epithelial loss and replacement with squamous epithelium [214].

More recently with the elucidation of the molecular subtypes of human gastric cancer, genetic mouse models have been designed to recapitulate the molecular characteristics of the human disease rather than previous candidate gene approaches. The Bass lab developed a genetic mouse model of genomically stable diffuse gastric cancer through overexpression of mutant $Rhoa^{Y42C}$ in combination with loss of *Cdh1* (common genomic events in GS gastric cancers as discussed above) using an inducible *Mist1-CreERT2* allele [124]. Of note, MIST1 is expressed in specific gastric cell lineages including gastric chief cells [215–217], but also in the pancreas [218], salivary glands [219], and plasma cells [220]. Histologically signet-ring tumors formed in most mice 14 months after Cre recombinase induction [124]. In addition, the authors developed an organoid based xenograft system to induce *in vitro* recombination and show that these genetically altered organoids recapitulated many key aspects of the human disease including peritoneal carcinomatosis and ascites formation when orthotopically transplanted into the gastric wall of nude recipient mice [124]. The same researchers also developed a model for the CIN gastric cancer subtype using *Mist1-CreERT2* and *Lgr5-CreERT2* to drive conditional deletion of *Tp53* and subsequent carcinogen (deoxycholate bile acid and MNU) exposure [221]. They showed that these mice formed dysplastic lesions *in vivo*, and organoids derived from these mice exhibited key features of CIN gastric cancer pathogenesis including genome doubling events and transcriptional upregulation of cell cycle and stem cell related pathways [221]. Again, it should be noted that neither of these genes are stomach specific with MIST1 expression discussed above, and LGR5 expression shown also in intestines [222]. This work has demonstrated the importance of gene-environment interactions in gastric carcinogenesis and is a novel murine model to study early molecular events of CIN gastric cancer formation.

The Stange lab has sought to further effectively recapitulate the human TCGA subgroups by developing a novel stomach and pan-gastric cell-type inducible Cre mouse line. Using bioinformatic data from murine gene expression databases, the authors found the *Annexin A10* (*Anxa10)* gene to have a stomach-specific expression pattern in all gastric cell lineages [223]. Interestingly, in humans, ANXA10 is also expressed in duodenal Brunner's glands, the urothelium, and certain pancreatobiliary cancers [224]. Utilizing a tamoxifen-inducible Cre recombinase within the *Anxa10* gene locus, Seidlitz et al. generated several stomach-specific cancer mouse models: a CIN mouse model (*Anxa10-CreERT2;* $Kras^{G12D/+}$*;* $Tp53^{R172H/+}$*;* $Smad4^{fl/fl}$) and two models for the genomically stable subtype (GS-TGFB, *Anxa10-CreERT2;* $Cdh1^{fl/fl}$*;* $Kras^{G12D/+}$*;* $Smad4^{fl/fl}$, GS-Wnt, *Anxa10-CreERT2;* $Cdh1^{fl/fl}$*;* $Kras^{G12D/+}$*;* $Apc^{fl/fl}$) [223]. For the CIN mouse model, the authors sought to genetically recapitulate the three most common altered molecular pathways exhibited by the human subtype [129,223]. After tamoxifen administration and Cre recombinase activation, in gastric cells expressing *Anxa10*, there would be concomitant activation of oncogenic $Kras^{G12D}$ mutant, expression of pathogenic mutant $Tp53^{R172H}$, and *Smad4* deletion. These mice develop gastric dysplasia within 2–3 weeks and early gastric cancer formation within 2–8 weeks that were histologically similar to human intestinal-type gastric cancer. Subsequently, subserosa invasive cancers form within 8–10 weeks, and these mice progress to advanced disease with lung and liver metastases after 10 weeks. Interestingly, the authors also show that this mouse model mirrors many aspects of the human pre-cancerous progression from SPEM to intestinal metaplasia, and that proliferating chief cells (i.e., cells that undergo paligenosis, see below) can be found in these early pre-cancerous lesions. For the GS-TGFB mouse model, the authors genetically combined loss of *Cdh1* with activation of oncogenic $Kras^{G12D}$ and *Smad4* deletion. After *Anxa10* Cre recombinase activation with tamoxifen, early signet ring cell containing cancerous lesions developed within one week, progressed to more locally advanced disease over the next 16 weeks, and finally resulted in metastatic disease in the lungs and peritoneum after 16 weeks. Similar to the CIN mouse model above, proliferating cells expressing chief cell markers were found in these tumors. Finally, for the GS-Wnt mouse model, the authors combined loss of *Cdh1* with activation of oncogenic $Kras^{G12D}$ and *Apc* loss. Early in these mice, dysplasia developed with proliferating cells expressing chief cell markers as well as persistent parietal cells (i.e., lack of oxyntic atrophy). The gastric tumors that later developed exhibited maximum invasion to the submucosa, and adenomatous serrated tooth-like structures histologically. Furthermore, *in vitro* organoids derived from the tumors of these three mouse models showed differential responses to treatment. For example, organoids derived from the CIN model were more refractory to epidermal growth factor receptor (EGFR) pathway inhibition with the MEK1/2 inhibitor, trametinib, and more responsive to docetaxel chemotherapy compared to the GS models.

The development and continued evolution of animal models of gastric cancer has allowed further understanding of the basic biology underlying this disease. While these different mouse models of human gastric cancer display important features of the human tumors there are key differences. For example, human gastric cancer tumorigenesis (CIN molecular subtype) is a process that involves years of injury/inflammation with induction of early *TP53* mutations resulting in later chromosomal amplifications and activation of receptor tyrosine kinase pathways. These mouse models [223] do not recapitulate the same molecular sequence of events, but rather rely on concomitant activation of genetic oncogenic "drivers". In addition, questions remain about the ultimate faithfulness and clinical translatability of these models. How are the immune/stromal components of these mouse models? How well do these mouse models respond to standard of care chemotherapy treatment *in vivo*? Do these tumors express the same predictive biomarkers and response to targeted treatment? Further collaborations between basic scientists and clinical physicians working on gastric cancer will fuel future improvements in our ability to model gastric cancers using animal models. In addition, further "omics" based comprehensive analyses, lineage tracing cell-of-origin studies, and microenvironment investigations will continue to uncover the underlying mechanisms of human gastric cancer development and progression. Specifically, improvements in animal modeling have uniquely enabled novel insights to be made into the early molecular events during gastric cancer formation. Next, we will describe one newly emerging molecular process to view and understand the development of gastric cancers.

4. Paligenosis: a novel lens to view gastric cancer

On a tissue level, the stomach exhibits marked cellular plasticity

[225–227]. Spasmolytic polypeptide expressing metaplasia or pseudopyloric metaplasia is generally thought to be a reversible, regenerative response to injury of gastric glands to chronic inflammation and damage usually induced by *H. pylori* infection [215,228–230]. This process is often termed "atrophy", as acid-secreting parietal cells die, and mature chief cells in the base of the gland become metaplastic [215,228–234]. In patients infected with chronic *H. pylori*, the stomach undergoes continued SPEM that may be accompanied by less-reversible intestinal-type metaplasia [228–230]. It is thought that continued infection and inflammation leads to a progression of SPEM to intestinal metaplasia, and to dysplasia and cancer; a process termed the "Correa pathway" [235–237]. Here, we will discuss the emerging evidence that the molecular basis for the tissue plasticity of the stomach may contribute to inflammation and injury induced cancer risk.

Recent work has shown that many differentiated cells such as chief cells in the stomach and pancreatic acinar cells use an evolutionarily conserved program termed paligenosis to reprogram their metabolism and readopt a progenitor state as a reparative response to injury [238–241]. This process of inherent tissue plasticity is likely to be important for an even broader range of differentiated cells including those in the lung [242,243], kidney [244], intestine [245–247], liver [248,249], and skin [250,251]. The potential universality of a paligenotic injury response [241,252,253] has also been demonstrated during neurite regeneration after axotomy in the murine dorsal root ganglion, regeneration after limb amputation in the amphibian axolotl, and gut stem cell recruiting oxidative stress injury for *Drosophila*

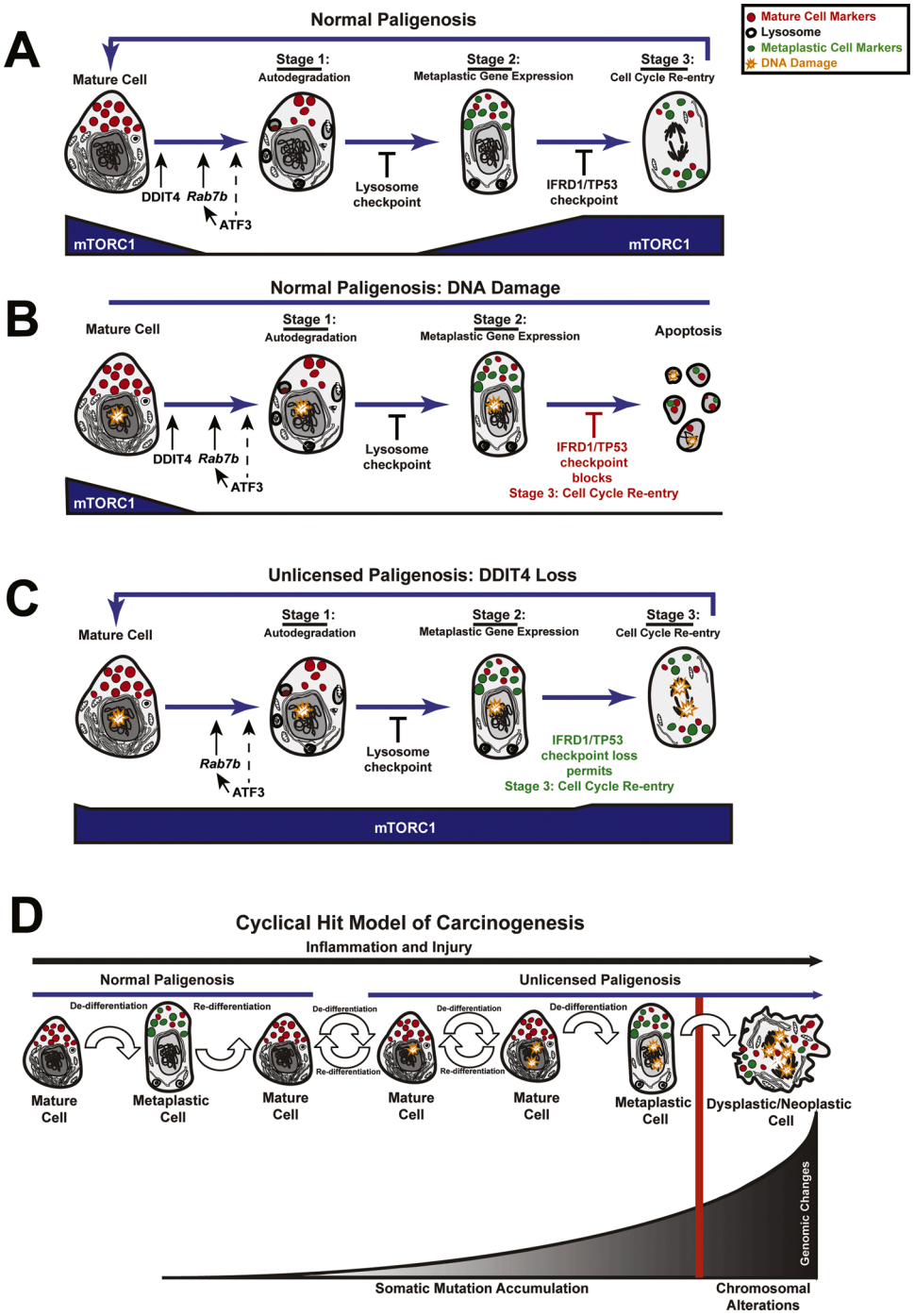

Fig. 1. Paligenosis and The Clinical Hit Model for Gastric Carcinogenesis. A) Normal paligenosis is a conserved molecular process that involves a stepwise progression through three regulated stages allowing mature differentiated cells to reprogram their metabolism and readopt a progenitor state as a reparative response to injury. Key genetic determinants, molecular processes, and checkpoints are shown including bi-phasic mTORC1 activation. B) Normal paligenosis enables "sensing" of DNA damage through an IFRD1/TP53 mediated checkpoint resulting in blockade of cell cycle re-entry (i.e., Stage 3) as a safety mechanism to maintain genomic integtrity and minimize cancer development risk. C) Unlicensed paligenosis through loss of key regulators such as DDIT4 permits inappropriate cell cycle entry of cells harboring DNA mutations through failure to suppress mTORC1 activity. D) Continued unlicensed paligenosis permits mature cells to undergo cycles of de-differentiation and re-differentiation with each entry into the cell cycle increasing the risk of cancer-predisposing mutations. Cancer cells-of-origin accumulate mutations over time with little effect until the cells acquire certain key changes and become trapped in a proliferative and more embryonic-like (i.e., oncogenic) state in a process that leads to carcinogenesis.

melanogaster [238]. At its core, paligenosis is a fundamental cellular and molecular process that explains how cells previously thought to be nondividing can become proliferative again. The molecular regulation of this conserved cellular regeneration program has recently been elucidated. As with any conserved cellular process, paligenosis is comprised of distinct stages with carefully regulated intervening checkpoints: Stage 1 involves autodegradation of differentiated cellular architecture; Stage 2 necessitates upregulating of progenitor-associated or metaplasia-associated gene expression; and Stage 3 results in cell cycle re-entry [238,239,254], Fig. 1**A**. After these differentiated cells divide through paligenosis, it is thought that they may "redifferentiate" though the molecular details of these steps are still yet unclear. As a whole, the process of paligenosis allows tissue healing following injury as differentiated cells are able to divide and initiate repair [239]. Specifically, upon inflammation and injury in the stomach, zymogenic chief cells that normally have abundant mTORC1 activity to drive zymogen production, decrease mTORC1 activity and massively upregulate lysosomes/autophagosomes (i.e., autophagy) to completely repurpose cellular architecture and metabolism [216]. In addition, at this initial Stage 1 phase, *activating transcription factor 3 (Atf3)* is induced to transcriptionally activate lysosomal trafficking genes such as *Rab7b* and facilitate autodegradation [255]. Loss of *Atf3*, results in a failure to increase RAB7-positive autophagic and lysosomal vesicles and eventual cell death due to "failed" paligenosis progression [255]. Subsequently, after Stage I, damage and progenitor associated metaplastic genes such as *Sox9* and *Cd44* are upregulated and mTORC1 is reactivated in preparation for cell cycle re-entry [239]. As mentioned before, this process has multiple exquisitely regulated checkpoints centered around this biphasic mTORC1 signaling. For example, blocking the reemergence of mTORC1 signaling during Stage II to Stage III transition still allows induction of autophagy and metaplastic genes (Stage I and Stage II), but prevents cell cycle re-entry at S-phase (Stage III) [239], Fig. 1**B**. In addition, two highly conserved genes, *DNA damage-induced transcript 4 (DDIT4)* and *interferon-related developmental regulator 1* (*IFRD1*), have been found to increase during paligenosis as key regulators of this process [238]. Increasing DDIT4 initially suppresses mTORC1 during Stage I to allow autodegradation. As cells progress through Stage I, DDIT4 decreases and TP53 becomes activated to continue mTORC1 suppression to maintain cell quiescence. Later in paligenosis, increased IFRD1 suppresses TP53 activity to allow mTORC1 reactivation and cell cycle entry. Of note, *Ddit4*$^{-/-}$ cells never suppress mTORC1 and bypass the later IFRD1-TP53 proliferation checkpoint resulting in less cell death and increased proliferation, whereas *Ifrd1*$^{-/-}$ cells do not complete paligenosis due to persistent TP53 mediated prevention of mTORC1 reactivation resulting in more cell death and less proliferation [238]. Together, DDIT4 and IFRD1 cooperate to allow only "healthy" cells to re-enter the cell cycle. They are the safety mechanisms built into this regenerative process to minimize the risk of cancer development while still permitting normal tissue healing.

On a cellular level, unregulated (i.e., "unlicensed") paligenosis increases cancer risk over time, Fig. 1**C**. In the stomach, this may allow mature chief cells to aberrantly re-enter the cell cycle during chronic inflammation induced injury [225]. This unlicensed plasticity permits chief cells to undergo cycles of de-differentiation and re-differentiation with each entry into the cell cycle increasing the risk of cancer-predisposing mutational accumulation [225]. On the other hand, during transient periods of redifferentiation, these increasing mutational signatures may be "hidden" within these seemingly normal differentiated chief cells evading cancer immunosurveillance [256]. These cancer cells-of-origin may acquire mutations over the years or decades of the cells' lifespan accumulating with little effect until the cells acquire certain key changes and become trapped in a proliferative and more embryonic-like state [225,228,239,257,258]. Once redifferentiation is blocked, dysplastic oncogenic clones emerge may in a process that leads to carcinogenesis. This we term "the cyclical hit model" [225,259], Fig. 1**D**. The initial events that may lead to the emergence of these pathogenic clones may involve disfunction of key regulators of paligenosis, such as *DDIT4*, *IFDR1* or *TP53*. The oncogenic consequences of unlicensed paligenosis have been clearly demonstrated [260]. As discussed, *Ddit4*$^{-/-}$ chief cells never suppress initial mTORC1 activity. This results in inappropriate cell cycle entry of chief cells harboring potential DNA mutations. After multiple cycles of unregulated paligenosis with concomitant carcinogen exposure, these *Ddit4*$^{-/-}$ mice had increased rates of spontaneous gastric tumorigenesis [260]. Clearly, unlicensed paligenosis affords differentiated cells the potential to divide in an unregulated manner, and it becomes immediately clear why under these circumstances, dysplasia and cancer may develop from chronic inflammation-induced areas of metaplasia [225,259,260]. The emergence of these unlicensed pathogenic clones harboring heterogeneous somatic mutational burdens can give rise to cancer sporadically and at potentially different chronologic times. Thus, the gastric cancers that arise may be genomically and phenotypically heterogeneous. The cyclical hit model explains, incorporates, and unifies key molecular features of gastric cancer (in particular the CIN subtype) discussed earlier including the occurrence of early *TP53* mutations, molecular and clonal heterogeneity, and subsequent activation of multiple oncogenic pathways. Many studies using animal models and high-resolution genomic and transcriptomic analyses of metaplastic and cancerous tissues have demonstrated a critical role for SPEM lineages as precursor lesions for gastric cancer [216,231,261–263]. However, there are studies that support a gastric cancer cell-of-origin higher in the gastric gland [264–266]. The chief cell centric model is certainly not the only model for the origin of gastric metaplasia or cancer, nor is there reason to believe there is only one route to gastric tumorigenesis.

Paligenosis and the cyclical hit model lie at the nexus of basic science and clinical medicine. Continued elucidation of the pathways involved in the stages of paligenosis will undoubtedly further our fundamental understanding of the stomach and the cellular origins of gastric cancer. More importantly, understanding the origins of gastric cancer through the perspective of paligenosis and the cyclical hit model allows new avenues for diagnosis and treatment. Developing assays to determine and identify cells that have undergone cycles of paligenosis or have started to display unlicensed paligenosis may clue into cells that are at risk for neoplastic progression. In addition, elucidation of genes specific to paligenosis will allow the development of specific "biomarkers" that indicate when this process is occurring akin to the markers we have now for other fundamental cellular processes like apoptosis. Leveraging our understanding of paligenosis may become a route for chemoprevention to prevent the formation of gastric cancer. We may be able to "re-differentiate" cells at risk for cancer development in an effort to block further unlicensed cycles of paligenosis and prevent them from re-entering the cell cycle. In addition, tumors that arise via aberrant paligenosis may continue to exhibit an abnormal paligenotic response to stress. Eventual treatment in the form of chemotherapy or radiation may be interpreted by these tumor cells to be a paligenosis-inducing injury or stress. In response, the cancer cells may exploit paligenosis as a survival pathway to proliferate and survive these treatments. Targeting paligenosis might be a promising adjunct strategy for cancer treatment especially as paligenosis does not occur during normal organ homeostasis, and it is not a process used by the normal constitutively active organ stem cells. Undoubtedly, these are exciting avenues for future lines of translational investigation as we learn more about the emerging concept of paligenosis.

5. Future perspectus

Here we have detailed the molecular pathogenesis of gastric cancer in an attempt to explain why targeting specific pathways has been such a difficult translational task for this disease. The key to overcoming these hurdles will be continued elucidation of the interpatient and intrapatient heterogeneity that exists in gastric cancer. In other words, we must appreciate that each tumor may contain unique mixed populations

of cancer cells that may respond differently to treatment. To start, we must increase the resolution by which we are able to molecularly characterize cancer. Continued genomic analyses have now integrated over 2,600 sequenced genomes from 38 differing tumor types [267], and established a pan-cancer DNA mutational "roadmap". Specifically, for gastric cancer (especially for the most common CIN molecular subtype), the findings corroborate previous findings of *TP53* mutations frequently occurring as an early event with chromosomal alterations occurring later as subclonal events as discussed above. However, our understanding of cancer must advance towards single cell resolution. The advent of single-cell "omics" now allows important insights towards this goal of fully understanding cancer heterogeneity [268]. The use of whole-genome and exome sequencing of single-cell nuclei has enabled investigators to detail the mutational clonal diversity of single cells within a breast cancer tumor mass [269] and also compare the genomic makeup of the primary colorectal cancer tumor cells compared to metastatic tumor cells and adjacent normal cells [270].

In addition to genomic profiling cancers at single cell resolution, detailed single-cell transcriptome analyses have also been performed in an attempt to further elucidate the heterogeneity of cancers. Several studies have laid the foundation for a single-cell transcriptomic map of the normal upper gastrointestinal tract [271]. Building upon this, recent work has used single-cell RNA sequencing to study gastric cancer. In a study by Zhang et al., the authors assessed the single cell transcriptomic profile from 9 primary gastric adenocarcinomas and were able to identify unique expression profile subgroups [272]. Importantly, they also identified the molecular characteristics of these intratumor subgroups including assignment of "differentiation degrees" within the tumors of these gastric cancers as a means to explain interpatient and intrapatient responses to treatment and prognosis. The power of single-cell transcriptomics has also been applied to the stroma to elucidate the tumor microenvironment of gastric cancer [273]. Analyzing tumor cells from seven gastric cancer patients and one patient with gastric intestinal metaplasia, Sathe et al. used single-cell RNA sequencing to detail the tumor microenvironment including unique molecular characteristics of tumor-associated stromal and immune cells, T cell exhaustion mechanisms among cytotoxic T cells, and distinct intercellular signaling pathways within the tumor microenvironment. More recently, several studies have used this technology to study the molecular changes that occur between primary gastric cancer and metastases including those to the lymph nodes [274] and to the peritoneum [275]. These above-detailed studies rely on the input of single cell suspensions, which in of itself results in certain transcriptional changes and loss of spatial resolution. However, new spatial transcriptomic platforms have been able to closely match the resolution power of single-cell RNA sequencing technology, while still maintaining tissue spatial relationships as a means to study gastric cancer tumor heterogeneity [276], and specifically the importance of recognizing this intratumor diversity as these diverse regions differ in the targetable genomic and transcriptomic molecular profiles.

The functional determinants of gastric cancer development and pathogenesis are not limited to the genome or transcriptome. Epigenomic changes are now recognized as important mechanisms for gastric cancer carcinogenesis including changes in DNA methylation, histone modification (methylation and acetylation markers) and noncoding RNAs [277] with analyses to describe these processes being developed at the single cell resolution [278] as well as single-cell chromatin accessibility assays [279]. In addition, advancements in mass spectrometry have also enabled the possibility of single cell metabolomics and proteomics of human tissues [280]. With these seemingly unlimited expansive layers of data describing the cellular composition of tumors, there also needs equal advancements in our ability to integrate and interpret these complex data sets [280–283]. Continued integration of these data sets into meaningful translation and clinically applicable information will be an additional important future target. The goal of these studies is to parse through the tumor to ultimately find targetable pathways and be able to predict resistance mechanisms in an effort to improve treatment for individual cancer patients. How can we use the data gathered from these high resolution "omics" methods to prospectively predict patient response to treatment?

Organoids are an established model system to study cancer biology [284–288], and human-derived organoid "biobanks" have been generated for many GI cancers that faithfully reproduce key aspects of and reflect the heterogeneity of the original tumor enabling characterization of molecular pathways of carcinogenesis, genetic modification for disease modeling, and drug screening to predict treatment efficacy and resistance [289,290]. Specifically, for gastric cancer, Seidlitz et al. established a biobank of twenty gastric cancer organoids with appropriate genomic, transcriptomic, and histologic characterization [291]. The authors show that these established gastric cancer organoids have differing responses to five standard chemotherapies as well as targeted therapies including trastuzumab for *ERBB2* mutations and palbociclib for *CDKN2A* mutations. In a similar set of experiments, Steele et al. characterized seven human gastric cancer organoid lines and demonstrated a correlation between organoid treatment response and *in vivo* patient response to standard chemotherapies [292]. Cancer organoids have also been used to demonstrate important genotype-phenotype correlations (e.g., mutations in *CHD1* and *TP53* correlate with Wnt pathway independence) that may uncover new targetable pathways for gastric cancer treatment [293]. In addition, Yan et al. established a more expanded gastric cancer organoid biobank comprised of 63 organoids derived from 34 patients [294]. They performed genomic, transcriptomic, and histologic characterization of their organoids to detail similarities with the actual *in vivo* tumors [294]. Their gastric cancer organoid system was shown as an important tool to recapitulate intra-tumoral heterogeneity and model tumor clonal evolution. Importantly, Yan et al. were able to demonstrate concordance of organoid and *in vivo* treatment response to chemotherapy and targeted therapy, and perform a 37 anticancer drug screen to assay for large-scale cancer treatment sensitivity. Finally, several groups have demonstrated the utility of patient-derived cancer organoids to study the tumor microenvironment and response to immunotherapy in gastric cancers using advanced air-liquid interface or immune cell co-culture techniques [295–297].

Recently, studies have demonstrated the translatable application of cancer organoids as a tool to potentially prospectively predict cancer patient treatment response. Ganesh et al. established a biobank of 65 rectal cancer organoids and showed that the organoids *ex vivo* and when transplanted into transplanted in mouse rectums faithfully recapitulates *in vivo* patient responses to both chemotherapy and radiation treatments [298]. Yao et al. similarly derived rectal cancer organoids from 80 patients and directly compared organoid responses to the actual patient responses to radiation, 5-FU, and irinotecan [299]. The authors found that these cancer organoids were able to predict patient clinical outcomes with 78% sensitivity, 92% specificity, and 84% accuracy. More recent studies have begun to integrate cancer organoids prospectively into clinical oncology. The Tumor Organoids: feasibility to predict sensitivity to treatment in cancer patients (TUMOROID) study was a prospective observational trial to study the ability of colon cancer organoids to predict patient chemotherapy response [300]. The study was able to predict patient responses to single agent irinotecan and combination irinotecan chemotherapy with 80% and 83%, respectively with timely turnaround times of 2–3 weeks. The culmination of this work has been the Selecting Cancer Patients for Treatment Using Tumor Organoids (SENSOR) trial in which organoids were used to predict and guide patients with investigational agents [301]. This innovative trial generated 31 organoid lines from 54 enrolled advanced colorectal cancer patients prior to their last standard of care treatment. The authors were able to perform drug screening on 25 of the organoid lines against an 8 targeted therapy panel: vistusertib to block mTOR signaling, capivasertib to block AKT signaling, selumetinib to block MEK signaling, gefitinib to block EGFR signaling, palbociclib to inhibit CDK4/6

signaling, axitinib to block VEGFR signaling, gedatolisib to block PI3K/mTOR signaling, and glasdegib to block SMO signaling. Based on their organoids responses, three patients were treated with vistusertib and three additional patients were treated with capivasertib. However, none of these selected patients had predicted clinical responses. This trial demonstrates the future challenges in cancer organoid integration into clinical oncology, mainly, the ability to generate these organoids in a timely and efficient manner while the patient maintains good treatment tolerability and, most importantly, the ability to objectively decide and select pathways to test and target.

6. Conclusions

Clearly, the advancement of single cell genomics, transcriptomics, and protein analyses will enable unprecedented insight into the intra- and interpatient heterogeneity of gastric cancers. Human-derived organoid culture techniques will also enable unique experimental potential to explore and exploit this heterogeneity. However, new challenges remain. How do we interpret and integrate in a timely manner the big data that results from these advanced single cell studies? How do we also improve the prospective potential for organoid treatment screening? The answer lies in combining the resolution and precision of single cell “omics” with the functional potential of organoids. In other words, using single cell “omics” to determine the pathways and molecular vulnerabilities of the cancer, while using functional organoid drug screens to determine the best combination of drugs and compounds to block those signaling pathways. Clearly, when appropriately selected there is clinical efficacy to a molecularly targeted approach for gastric cancer [302]. Overall, we must progress beyond thinking about gastric cancers as a singular entity and disease. We must recognize that each gastric cancer is uniquely different, and every line of treatment should be tailored based on the individual features of that patient’s cancer. Only with patient-oriented precision oncology treatments can we truly improve survival for gastric cancers.

Declaration of Competing Interest

The authors report no declarations of interest.

Acknowledgements

RUJ: Department of DefenseW81XWH-20-PRCRP-CDA, American Society of Clinical Oncology (ASCO)2021YIA-8674301298, and NIH NCIU54 CA163060.

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CELLULAR AND MOLECULAR GASTROENTEROLOGY AND HEPATOLOGY

Gastric Organoids: Progress and Remaining Challenges

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SUMMARY

We introduce the differing methods for culturing healthy gastric tissue from adult tissues, pluripotent stem cells or gastric cancer tissue. We also discuss the promise these systems have for preclinical drug screens and highlight the applications of organoids for precision medicine.

The stomach is a complex and physiologically necessary organ, yet large differences in physiology between mouse and human stomachs have impeded translation of physiological discoveries and drug screens performed using murine gastric tissues. Gastric cancer (GC) is a global health threat, with a high mortality rate and limited treatment options. The heterogeneous nature of GC makes it poorly suited for current "one size fits all" standard treatments. In this review, we discuss the rapidly evolving field of gastric organoids, with a focus on studies expanding cultures from primary human tissues and describing the benefits of mouse organoid models. We introduce the differing methods for culturing healthy gastric tissue from adult tissues or pluripotent stem cells, discuss the promise these systems have for preclinical drug screens, and highlight applications of organoids for precision medicine. Finally, we discuss the limitations of these models and look to the future to present potential ways gastric organoids will advance treatment options for patients with GC. *(Cell Mol Gastroenterol Hepatol 2022;13:19–33; https://doi.org/10.1016/j.jcmgh.2021.09.005)*

Keywords: Stomach; Organoids; Gastric Cancer; Tumor Microenvironment; Preclinical Trial.

The mammalian stomach is lined by an ordered epithelium consisting of invaginated gastric units housing varied cell types (mucous pit cells, acid-secreting parietal cells, zymogenic chief cells, and proliferative and intermediate populations).[1] Research into gastric epithelial biology has long been hindered by lack of accurate models, as gastric tissue is historically difficult to culture and there are notable physiological differences between mouse and human stomachs.[2] Recent advances in our ability to culture 3-dimensional (3D) self-renewing organoids from mouse and human stomachs have opened many possibilities for studying gastric cells.[3–5] In the past decade, researchers have used gastric organoids to better probe basic stomach biology, identify cell plasticity, analyze interactions between the gastric epithelium and immune cells or pathogens such as *Helicobacter pylori*, and gain valuable insights into the progression and treatment of gastric cancer (GC).[6–9]

GC is a major public health issue, ranking as the fifth most common malignancy and the fourth-leading cause of cancer-related deaths worldwide. One study attributed about 770,000 global deaths to GC in 2020 alone.[10] Therefore, it is necessary to improve GC treatments both in terms of efficacy as well as safety. GC prognosis is poor, with surgery currently the only curative option.[11] However, surgery alone is oftentimes not enough and multimodal treatments including incorporation of per-operative chemotherapy is now routine to increase survival rates.[12] For metastatic or advanced GC, systemic chemotherapy, targeted therapy, and immunotherapy are the only treatments. Current 2- to 3-drug chemotherapy regimens only modestly benefit overall survival, with median overall survival under 12 months.[13,14] Another novel treatment is molecular targeted therapy, but there are only 2 targeted therapies currently approved and although many new therapeutics are tested every year, very few are validated clinically.[15]

One major hurdle for developing effective treatments for human cancers is the lack of accurate experimental platforms to identify new therapies and to test efficacy of

Abbreviations used in this paper: 2D, 2-dimensional; 3D, 3-dimensional; ALI, air-liquid interface; CagA, cytotoxin-associated gene A; DC, dendritic cell; EGF, epidermal growth factor; ESC, embryonic stem cell; FGF10, fibroblast growth factor 10; GC, gastric cancer; ORISC, organ-restricted adult stem cell; PD-L1, programmed death ligand 1; PDO, patient-derived organoid; PDX, patient-derived tumor xenograft; PSC, pluripotent stem cell.

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2352-345X
https://doi.org/10.1016/j.jcmgh.2021.09.005

therapies on individual patients to personalize their treatment. Animals are useful research models for some aspects of developing anticancer therapy, such as testing safety and efficacy of new regimens, yet many drugs that clear preclinical animal trials fail during clinical trials due to differences in animal and human physiology.[16,17] As an alternative, organoids have recently become a popular in vitro culture model for developmental biology, drug screening, and disease modeling.[18] Organoids can be developed from human and animal cells and tissues, recapitulate more of the cellular complexity of actual tissues, and are a common preclinical model.[16,17] As well as their use in the field of cancer, gastric organoids have shed light on stomach development and progressed our knowledge of pathogenic infection and immune response in the stomach. Here, we describe the application of gastric organoids to the study of basic stomach biology and disease states and elaborate on their potential for implementation in clinical practice as a guide for precision medicine.

Gastric Organoid Culture

Organoids are stem cell-originated, self-organized 3D clusters of organ-specific cells capable of maintaining aspects of the functionality and molecular and cellular heterogeneity of the originating organ. Organoids have been cultured from many tissues including the brain, retina, kidney, liver, and intestine.[19–23] However, even with the inherent self-organization capacity of stem cells, elaborate physiologically relevant tissues cannot be formed in all conditions. Rather, the experimental environment is paramount in steering cellular development in a highly context-dependent manner.[24]

Organoids can be derived from 2 sources of stem cells: organ-restricted adult stem cells (ORISCs) and pluripotent stem cells (PSCs),[24,25] both in the form of induced PSCs and embryonic stem cells embryonic stem cells (ESCs) (Figure 1, Table 1). PSC-derived organoids are obtained by mimicking the sequential signaling interactions operating during in vivo development, whereas organoids derived from ORISCs are obtained by replicating signaling cues native to the respective adult tissues.[24] Compared with ORISCs, PSCs possess a broader potency that allows for directed differentiation into organoids resembling many adult tissues, and their increased potency also allows for coordinated generation of cells from multiple germ layers. Both fundic and antral gastric organoids have been developed from PSCs by the Wells group.[4] These PSC-derived organoids do not require a biopsy and comprise diverse populations of gastric epithelial cells (like functional parietal and chief cells) and a surrounding layer of undifferentiated mesenchymal cells.[4] On the other hand, ORISCs are epithelial derived, and do not contain components of the tissue microenvironment, highlighting their restricted potential compared with PSCs. Nonetheless, ORISC-based organoids faithfully recapitulate homeostatic conditions and regenerative processes of the adult tissue.[26]

Ultimately, a major application of *in vitro* organoid cultures is for the study of the in vivo stem cell niche. Simulation of these niche signaling cues in an in vitro culture system allows stem cells to proliferate and differentiate into tissue-specific cell types. Human gastric organoids are commonly cultured by seeding gastric glands from human gastric resection tissue in a basement matrix and culturing in a medium containing epidermal growth factor (EGF), R-spondin-1, Noggin, Wnt-3a, fibroblast growth factor 10 (FGF10), gastrin, an inhibitor of ALK5/4/7 (aka TGFBR1, ACVR1B, or ACVR1C), the small molecule inhibitor A83-01, and SB202190.[27] Using these growth conditions, gastric glands can grow into gastric organoids.[28,29] Alternate methods exist which rely on co-culturing organoids with stromal elements rather than extrinsically added growth factors such as the air-liquid interface (ALI) technique (Figure 2).[30] In the following section, we will describe technical aspects of gastric organoid modeling and their derivation from cells that have stem-like characteristics.

Organoids From Organ-Restricted Adult Stem Cells

ORISC-derived gastric organoids are developed from primary human stomach tissues. Multiple strategies have been used to enable long-term growth of stomach tissue into organoids structures. One protocol uses a collagen type I gel (Trevigen, Gaithersburg, MD) with an ALI to support the growth of organoid epithelial structures.[31] Organoids can be grown with fetal calf serum alone, but growth is improved by supplementation with R-spondin1, similar to ALI intestinal organoids.[30]

A second protocol relies on distinct growth factors and extracellular support provided by laminin-rich Matrigel to support epithelial growth.[32] Notably, Bartfeld et al[29] used this method, based on a protocol developed earlier for culture of mouse antrum,[33] to generate gastric organoids from human antral/pyloric stomach tissue. They isolated gastric glands from human gastric corpus tissue then seeded them in Matrigel with media supplemented with various growth factors (Wnt-3a, R-Spondin-01, Noggin, N2, B27, FGF10, EGF, gastrin, nicotinamide, etc.). Organoids can be generated from the gastric cardia and expanded similarly under the same culture conditions. Matrigel is used to provide a suitable environment for the embedded gastric glands and to provide extracellular support. After seeding, human gastric glands seal and form small cysts that subsequently expand.

Organoids From PSCs

Gastric organoids derived from ORISCs have limitations. Establishing gastric organoids from ORISCs requires access to human tissue samples, which is not commonly available to many laboratories.[4] Even when available, the quality of these tissue samples is widely variable and heavily dependent on timely processing.[4] Moreover, ORISC-derived organoids are also further limited for use in cancer studies due to their lack of microenvironment. An alternate approach is to generate gastric organoids from human PSCs, which include both human ESCs and induced PSCs. The unique ability of PSCs to both self-renew and differentiate into cell

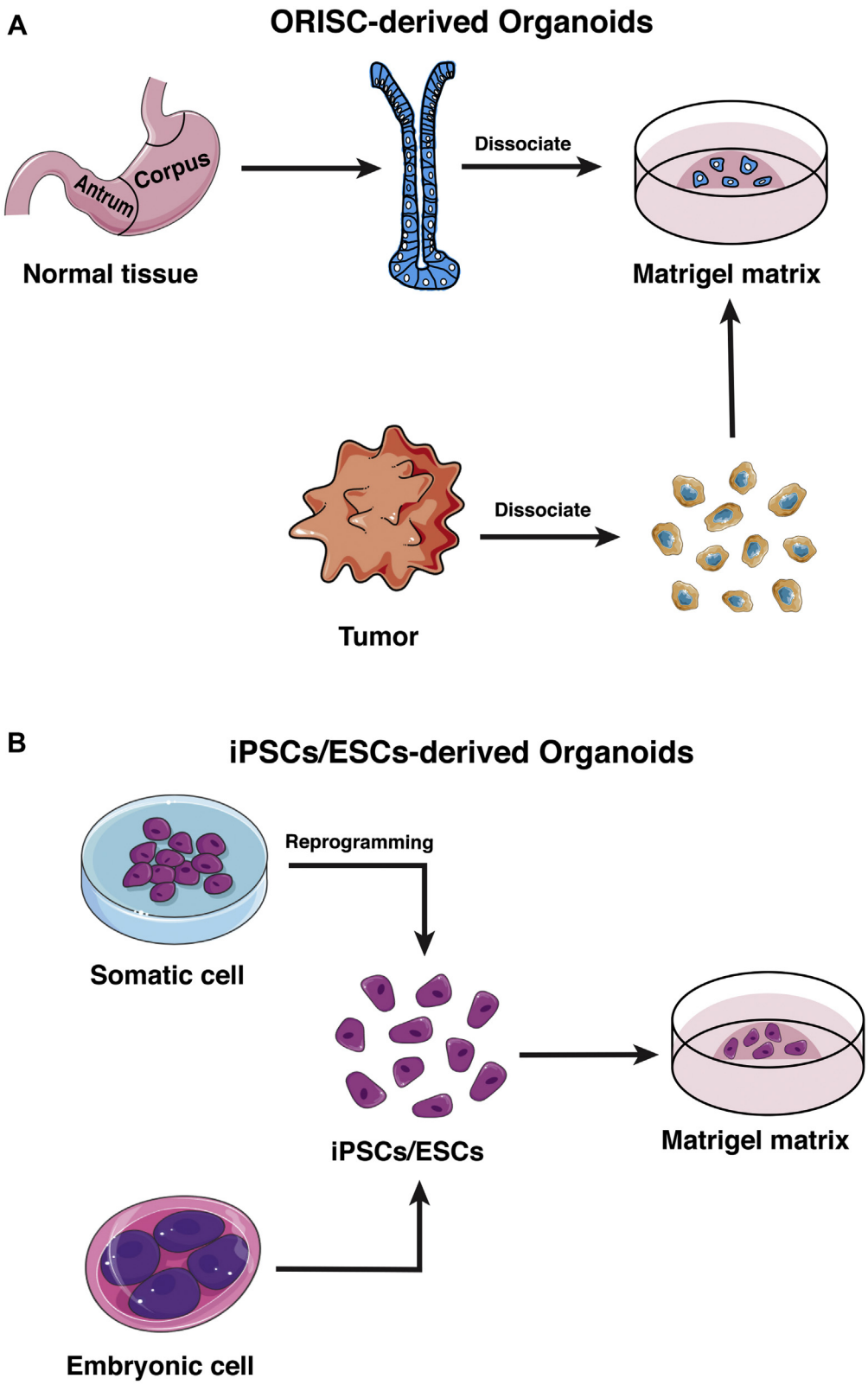

Figure 1. An overview of current approaches to develop gastric organoids in vitro. Gastric organoids can be generated from 2 sources of stem cells: ORISCs and PSCs. ORISCs are mainly derived from human gastric tissues samples such as endoscopic biopsy specimens. Gastric gland cells or cancerous cells were collected and plated into the Matrigel matrix. PSCs include both induced pluripotent stem cells (iPSCs) and ESCs; iPSCs can be derived from reprogramed adult somatic cells or blastocysts. Typically, isolated ORISCs or PSCs were embedded into Matrigel matrix domes and cultured with media supplemented with necessary growth factors.

Table1. Characteristics of 2 Different Sources of Organoids

Feature	PSC-Derived Organoids	ORISC-Derived Organoids
Pluripotency	Yes	Limited
Time needed	6–8 wk	1 wk
High-throughput screening	Yes	Yes
Modeling human disease	Yes	Yes
Modeling organogenesis	Yes	Limited
Precision medicine	Limited	Yes

ORISC, organ-restricted adult stem cell; PSC, pluripotent stem cell.

types from multiple lineages permits modeling the whole organogenesis process in cell culture.[24]

Recently, Noguchi et al[5] used a method to differentiate mouse ESCs into organoids consisting of gastrointestinal endoderm surrounded by mesoderm. However, this approach used mouse ESCs aggregation and spontaneous differentiation, resulting in heterogeneous organoids.[7] Moreover, stomach morphology and regionalization differ greatly across species,[34,35] making animal models like the mouse stomach suboptimal for simulating the major structure and physiology of the human stomach.[7,29,36,37] For example, the largest volume of the postprandial mouse stomach is composed of the forestomach, a nonglandular squamous epithelium similar to the esophagus. The forestomach is not present in humans.[29] Thus, to effectively study human gastric development, physiology, and disease, it is imperative to use a human model system.

McCracken et al[38] identified a step-wise differentiation approach to generate human gastric organoids, whereby PSCs were sequentially differentiated into definitive endoderm, patterned to posterior foregut, then specified into a pure antral epithelium with normal antral cell types. They began by differentiating PSCs into definitive endoderm by adding activin A, a transforming growth factor β family member that stimulates the highly conserved Nodal signaling pathway required for endoderm formation across vertebrate species.[39] Endoderm was then patterned into anterior and foregut endoderm by inhibiting BMP signaling with Noggin. Foregut spheroids were directed into posterior foregut by activation of the retinoic acid signaling pathway. Wnt activation promoted the development of human fundic gastric organoids, whereas simultaneous MEK inhibition and activation of the BMP signaling pathway promoted the differentiation of acid-secreting parietal cells that could be stimulated by histamine.[4,7] These gastric organoids represent the first human antrum fully derived in vitro that recapitulates many of the most important aspects of stomach physiology.[38] So far, PSC-derived gastric organoids have been used as an in vitro system to identify signaling mechanisms that regulate human stomach development and physiology, and to model the pathophysiological response of the gastric epithelium to *Helicobacter pylori* infection.[7,38,40,41]

Organoids From Gastric Tumors

To distinguish organoids derived from tumor tissue from those derived from normal organoids, tumor organoids are often called tumoroids or patient-derived organoids (PDOs). A common culture protocol for culturing PDOs follows steps similar to those used for ORISC-derived organoids.[29] In short, tumor tissue is mechanically disrupted and enzymatically digested, then seeded in Matrigel and supplied with a certain mixture of growth factors, finally leading to PDO formation.[29] The ALI method has also been used to propagate PDOs by embedding the minced tissues inside a collagen gel with an ALI to support the growth of organoids epithelial structures.[31] Different from normal organoids, the ALI method allows combined culture of epithelial and mesenchymal/stromal components like native immune cells (T, B, natural killer, macrophages) to develop together with the PDOs,[31,42] using a technology already applied to intestinal organoids.[43,44] Moreover, unlike normal PDOs, ALI-cultured PDOs can grow without A83-01, FGF10, and Wnt3a.[11]

One obstacle to obtaining pure PDO cultures is that patient-derived nonmalignant organoids commonly develop from healthy cells within the tumor samples.[19,45–48] Despite increased cell division in tumor-derived organoids, the nontumor organoids can eventually overgrow the tumor-derived organoids.[45] The reason for this seemingly paradoxical competitive advantage is not clear, but it may be due to a higher rate of mitotic failure and subsequent cell death in tumor cells.[19] Another factor involved may be that seemingly normal cells near a tumor may also harbor many cryptic mutations that confer faster growth than truly normal tissue from a patient without precancerous or cancerous lesions.[49,50] To circumvent these issues, multiple strategies have been developed to eliminate contaminating normal organoids. The first approach targets mutational patterns dictating growth factor dependency of the organoid culture (Figure 3).[45] Many GCs exhibit p53 pathway mutations,[51] and this feature can be selected for using the small molecule Nutlin-3, an MDM2 inhibitor, which stabilizes TP53 by disrupting the binding of TP53 to its negative regulator E3 ubiquitin ligase, MDM2.[45] Notably, ROCK inhibition plays a significant role in the recovery of individualized normal organoid cells. Second, an additional strategy used has been to culture PDOs in a ROCK inhibitor (Y-27632)-free medium to enrich for RHO-dysregulated GC organoids.[52] Third, organoids isolated from tumors harboring EGF receptor signaling pathway mutations can be selected for by EGF withdrawal or EGF receptor inhibition, which leads to loss of the patient-derived nonmalignant organoids.[45] Furthermore, if a culture displays a clear mix of populations, the normal organoids can be simply removed by phenotype-based manual selection.[45] Finally, clonal PDOs can be established from single cells collected via flow-cytometry based cell sorting.[45] However, all of these approaches may lessen cellular heterogeneity compared with

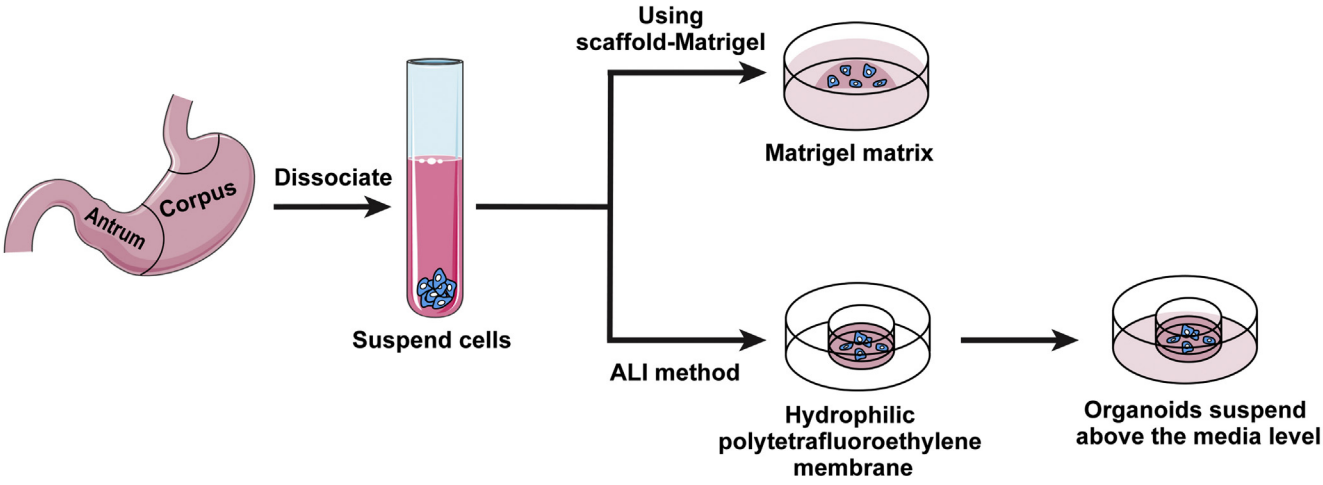

Figure 2. Differences between scaffold-Matrigel and ALI methods to generate gastric organoids. Gastric organoids can be generated from both scaffold-Matrigel and ALI methods. The scaffold-Matrigel culture system is a common and effective method to establish gastric organoids. First, isolated cells are mixed with Matrigel, the Matrigel is then allowed to polymerize, then culture medium supplied with various grow factors is added. For the ALI method, cells are placed onto a hydrophilic polytetrafluoroethylene membrane at the bottom with collagen solution. Different from the scaffold-Matrigel method, the top layer of the cells is directly exposed to the air instead of submerged in culture media, with only the basal surface keeping contact with the liquid culture media.

the initial cultures, so it is recommended that early passages of established cultures be frozen to serve as references for initial cell content.[45]

Another consideration for PDO cultures is source tissue quantity. While surgical resection supplies an abundance of tissue for initiating organoid cultures, surgery is invasive to patients and many metastatic or advanced GC patients never undergo resection. Therefore, esophagogastroduodenoscopy is ideal for obtaining tissues for organoid creation, especially for advanced patients lacking good treatment options and for whom testing potential treatments on PDOs is most beneficial. To address this, Gao et al[53] innovated a new method to develop gastric tumoroids from endoscopic biopsies of patients with gastric adenocarcinoma. They found that a single endoscopic biopsy of GC can generate organoids that are reflective of the overall primary tumor and may be used for patient-related testing.[53] As collection of GC tissues via endoscopy poses minimal risks to patients, it may be a plausible way to culture tissue from metastatic or advanced GC patients who would never undergo resection.

Modeling Disease in Human/Mouse Organoids

Chronic *H. pylori* infection is one of the single most critical factors increasing risk for GC worldwide. Long-term *H. pylori* infection in the stomach in many individuals causes a multistep histopathological cascade known as the Correa pathway which ultimately leads to GC.[54] The use of gastric organoids has enabled many important discoveries regarding *H. pylori* pathogenesis. For example, only a small percentage of people infected with *H. pylori* will develop GC,[55] likely owing to genetic factors and/or specific interactions between host, microbial, and environmental determinants.[56] Strains of *H. pylori* that produce the virulence factor cytotoxin-associated gene A (CagA) substantially increase stomach cancer risk compared with strains lacking CagA. Binding of CagA to the ASPP2 (apoptosis-stimulating protein of p53-2) causes mislocalization of PAR members, predisposing the infected cells to lose their cell polarity and gain an EMT-like phenotype promoted by the interaction of CagA with Par1b.[57] Human gastric organoids were used to show that a Cherry-tagged CagA-binding ASPP2 peptide could act as a sponge to reduce the CagA-induced phenotypes, abrogating the loss of cell polarity and reducing *H. pylori* colonization.[57]

Dendritic cells (DCs) in the human gastric mucosa are thought to be the major antigen-presenting cells that induce protective immune responses to *H. pylori* infection. Recently, Sebrell et al. developed an in vitro co-culture model by adding human monocyte-derived DCs isolated from peripheral blood mononuclear cells to organoid cultures.[58] They found that bidirectional crosstalk between gastric mucosal DCs and epithelial cells that were infected with *H. pylori* by microinjection contributed to the maintenance of gastric homeostasis and found that DC recruitment to the gastric epithelium is driven mostly by CXCL1, CCL20, and possibly CXCL8 following *H. pylori* infection.[58]

Another unique finding from in vitro human studies is that pathogenic *H. pylori* infection induces gastric epithelial cells to express programmed death ligand 1 (PD-L1), an immune checkpoint ligand known to suppress the immune system by shutting down T cell effector function.[59] Holokai et al[59] found that gastric organoids or monolayers derived from PSCs or adult tissues could survive chronic inflammation by expressing the immunosuppressive ligand PD-L1 throughout the infection and progression to cancer. This signifies that once a patient progresses to a metaplastic

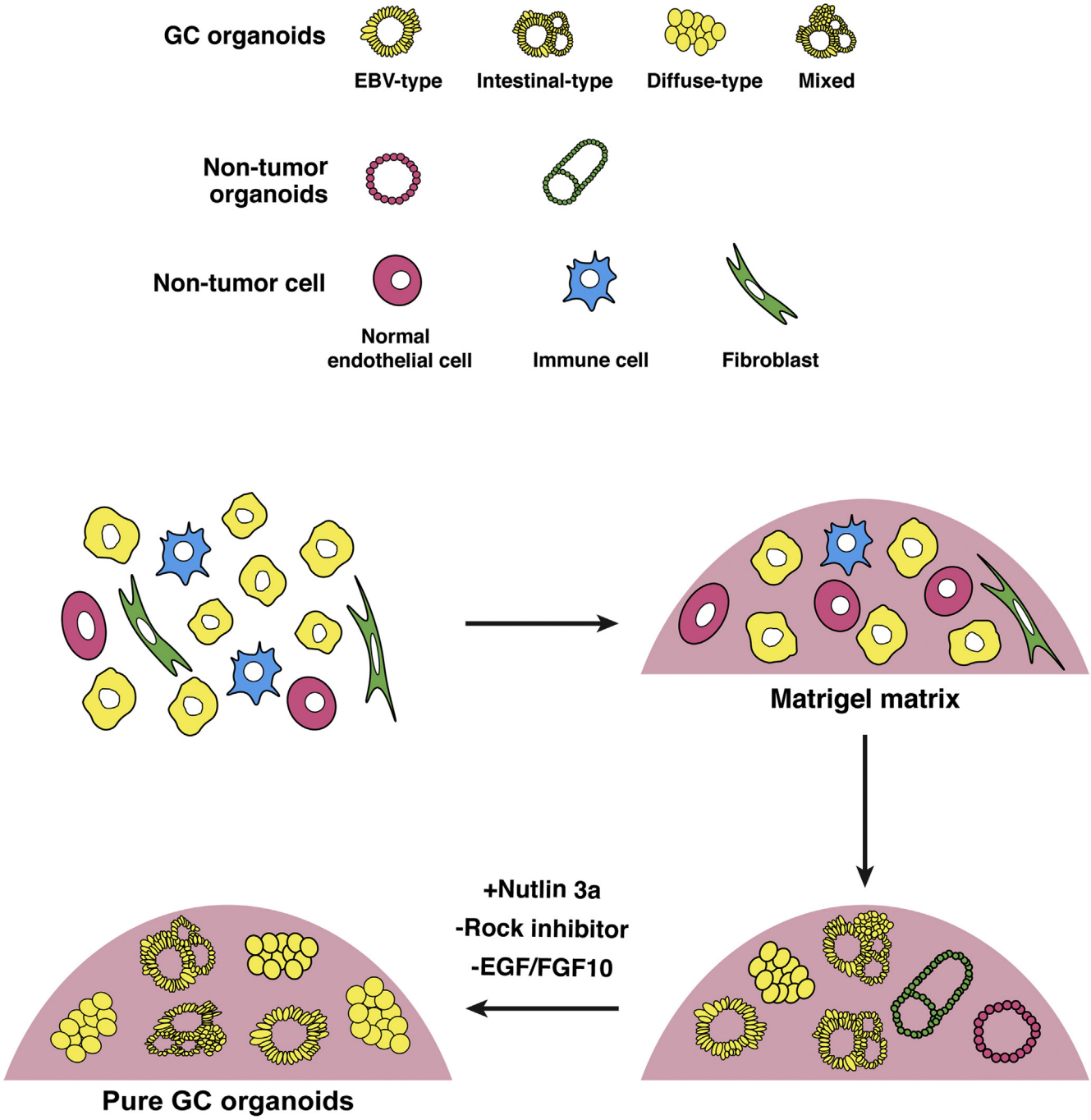

Figure 3. Establishing pure GC organoids. As primary tumor tissues often contain nonmalignant cells, nontumor organoids are commonly seen in patient-derived GC organoid cultures and eventually outgrow the tumor organoids. Therefore, it is essential to establish pure GC organoids for specific experiments like drug screening. Removing or adding certain growth factors in the culture medium allows for establishing pure GC organoids derived from various kinds of lesions such as primary tumors, metastases, and carcinomatous ascites with noncancerous tissues eventually lost.

state, eradication of *H. pylori* may not decrease the risk of developing GC.[60] Furthermore, a meta-analysis showed that PD-L1 expression lasts through GC development, and up to 69% of all GC expresses PD-L1.[61]

The use of murine gastric organoids has gained popularity in recent years as well. While these murine models do not fully replicate human stomach physiology or genetics, mouse models and organoids derived from mouse stomach mucosa have become a vital tool for studying mechanisms of tumorigenesis. Mouse gastric organoids are generally cultured in a Matrigel matrix with growth factors similar to those described for human culture.[33] Dedicated progenitor cells within adult tissues have been considered the main candidate cells of origin for cancer,[8] yet recent work

suggests that mature differentiated cells may contribute as much or more to cancer initiation in the stomach and other organs, especially in organs without dedicated progenitor stem cells like the pancreas and liver.[62,63] In fact, the idea that cancers could arise via recruitment of differentiated cells had been one of the prevailing theories prior to the dominance of the stem cell theory in the latter half of the 20th century.[64] Lineage tracing reporter mice, as well as tracing of cells that incorporate DNA analogs, have shown that multiple cells are capable of exhibiting progenitor activity in the stomach in both homeostatic and metaplastic (ie, precancerous) states. Gastric corpus organoids have been shown to be able to originate from normal epithelial progenitors or from proliferating chief cells.[65,66] For example, lineage tracing using a chief cell-specific marker, Troy, validated the existence of multipotent progenitor cells at the gland base in the gastric corpus. Sorted Troy^{+} chief cells were able to form long-lived organoids that can differentiate toward the mucus-producing cell lineages of the neck and pit region.[65] The cellular process by which mature chief cells re-enter the cell cycle in response to tissue damage was found to be regulated by mTORC1 and conserved among other cell types from a variety of differing organs.[67] This process has been termed paligenosis.[68] Loss of key genes regulating paligenosis such as *Ifrd1/Ddit4* can result in growth changes in corpus organoids, indicating that paligenotic chief cells contribute to organoid outgrowth.[69]

The ability to genetically manipulate mouse models allows for more specific studies on disease initiation and progression. Dysplasia is a key transition state between precancer and cancer in gastric tumorigenesis.[70] Min et al[71] derived gastric organoids from a Mist1-Kras(G12D) mouse model and examined functional roles for Kras activation in the progression of dysplasia by inhibiting MEK, a downstream mediator of Kras signaling. Kras activation was found to control cellular dynamics and progression to dysplasia, and dysplastic stem cells appeared to contribute to cellular heterogeneity in dysplastic cell lineages.[71] Similarly, another group found that Kras^{G12D} expression or p53 loss cause gastric organoids to develop dysplasia and easily generate adenocarcinoma upon *in vivo* transplantation.[72] Furthermore, TFF1-knockout) mouse–derived gastric organoids readily led to a proinflammatory phenotype with a cascade of gastric lesions that include low-grade dysplasia, high-grade dysplasia, and adenocarcinomas, indicating that gastric tumorigenesis may be suppressed by TFF1 impeding the IL6-STAT3 proinflammatory oncogenic signaling axis.[73]

Mouse organoids have also been well used to better understand mechanisms of disease development, especially with regards to *H. pylori* pathogenesis.[74] Morey et al[41] used organoids from both mouse and humans to demonstrate that *H. pylori* expression of cgt (the first in a series of *H. pylori* enzymes) reduced cholesterol levels in infected gastric epithelial cells, thereby blocking interferon gamma signaling to allow the bacteria to escape the host inflammatory response. The Zavros group has also made extensive use of mouse organoids as a model to analyze how gastric tissue responds to *H. pylori* infections, notably implicating the gene CD44 as promoting the increased proliferation following infection.[75] Through these varied uses, mouse gastric organoids have greatly increased our knowledge of the gastric epithelial response to injury and disease.

Personalized Medicine for GC Patients Using Pdos

With the advent of next-generation sequencing, single-cell RNA sequencing, and novel preclinical modeling strategies, GC research is undergoing a radical shift toward precision medicine.[76] PDOs comprise effective tools for genetic evolution studies, biomarker identification, drug screening, and preclinical evaluation of personalized medicine strategies for GC patients. Subsequently, we discuss how PDOs have contributed to current GC research and discuss their future possibilities.

Drug Screening

Standard 2-dimensional (2D)–cultured human cell lines (in other words, standard tissue culture cell lines predominately isolated from malignant cells and cultured on plastic) have been particularly important in drug studies, and have been the primary source for studying drug responsiveness to identify novel drug targets for decades. Indeed, results from cell culture studies are a significant factor in deciding whether or not a drug should advance beyond preclinical trials in humans.[77,78] However, the high failure rate of new drugs that show efficacy and activity in these traditional preclinical studies has substantially increased the associated costs of drug development, demonstrating the need for more representative models of human organ systems for drug screening during the preclinical phase.[78] Moreover, 2D cell lines are prone to genotypic drift and cross-contamination, may fail to establish permanent cell lines after long-term culture, and can exhibit loss of tumor heterogeneity.[79] Therefore, organoids (especially from tumors) that lack tumor stroma and vasculature fall in between purely 2D cancer cell lines and patient-derived tumor xenografts (PDXs) (Table 2).[79] Although significant medical advances have been made using standard 2D cell culture models, these static models cannot effectively recapitulate the physiology of living tissues.[9] In physiological conditions, cells reside in a complex environment constantly interacting with other cells and the extracellular matrix; and these interactions are critical for proper tissue differentiation and function. Organoid culturing techniques promote cell-to-cell interactions and can oftentimes more precisely mimic physiological and pathological conditions.[80] For example, a recent study using organoids derived from human breast cancer showed higher levels of reactive oxygen species production and increased resistance to cisplatin compared with standard 2D cell cultures.[81] Importantly, organoids have been shown to exhibit different drug metabolism and secretion properties due to their different environmental cues, making them well suited for the study of drug therapies.[80] Because organoids more accurately represent human disease, they have tremendous potential for predicting in vivo drug sensitivity and responses.[82]

Table2. Comparison of Organoids With 2D Cell Lines and PDX Models

Feature	2D Cell Lines	Organoids	PDX
Heterogeneity	-	+	++
Genome editing	+++	++	-
High-throughput screening	+++	++	-
Modeling organ development/ disease	+	+++	++
Modeling tumor micro-environment	-	++	+

For the representation of each respective feature, +++ indicates the best, ++ indicates suitable, + indicates possible, and - indicates unsuitable.
2D, 2-dimensional; PDX, patient-derived tumor xenograft.

As PDOs retain the heterogeneity and histological characteristics of the primary tumor, they represent an ideal model for drug screening. Hence, establishing large PDO libraries function as living biobanks and combining with drug screening might be a powerful tool to delineate novel therapeutic strategies in GC generally.[83] Recently, Yan et al[84] established a primary GC organoids biobank encompassing normal, dysplastic, and cancerous stomach tissue as well as lymph node metastases from 34 patients. This unique GC organoid biobank encompassed nearly all known GC molecular subtypes and different stages of disease. The authors used this biobank to demonstrate that large-scale drug sensitivity screening is feasible.[84] Their organoid-based drug screen of 37 anticancer drugs was timely (taking <2 weeks), and, most importantly, was able to correlate with actual patient in vivo responses for several new targeted anticancer drugs including VE822, an ATR inhibitor.[84] These data suggest that organoid based preclinical testing may help guide future cancer therapeutic choices.

In addition to standard 2D cell cultures, the PDX model, in which patient cancer cells or tissue are implanted into immunodeficient mice to recapitulate the patient's tumor biology, is also an important preclinical model.[85] However, these PDX models have many inherent disadvantages including significant time and resource constraints in comparison to PDO models (Table 2). The organoid drug screening platform will likely be a more practical route to informing patient treatment and as a screening tool in clinical trials to accelerate anti-cancer drug development.[84] Moreover, GC organoid biobanks may serve as a useful tool for drug screening by bridging the gap between ex vivo and in vivo models by more accurately portraying the genetic profile of these cancers while decreasing time and resource costs.[83,86] However, despite the numerous advantages of organoid culture, the various techniques used to propagate the cultures are still relatively new and need to be further improved to enhance drug response and testing.[78] Standardizing the procedures for initiating and propagating gastric tumor organoids would be beneficial in maintaining biological relevance and predictability across studies.[87] In the next section, we will discuss the use of PDOs to optimize personalized cancer treatments.

Precision Medicine

GC is a heterogeneous disease featuring many different histological and molecular subtypes. Specifically, GC can be categorized into 4 major genomic subtypes: microsatellite instability, Epstein-Barr virus, intestinal (chromosome instability), and diffuse (genomically stable).[51] Each group is characterized by a distinct molecular profile of genes that are dysregulated, implicating unique therapeutic targets within that subgroup of GCs to be further evaluated in clinical trials. However, despite the pronounced interpatient and intratumor heterogeneity of GC and our further understanding of the molecular subtypes that make up this disease, cancer therapy approaches for GC have remained more or less homogeneous, with uniform treatment strategies used for virtually all patients.

With the advent of novel preclinical modeling strategies such as in vitro organoid cultures, GC treatment studies are increasingly focusing on precision medicine.[76] The goal of personalized or precision medicine is to tailor therapy to specific patients expressing a certain molecular abnormality to maximize efficacy and minimize side effects. If PDOs are going to help us realize the promise of personalize medicine, it will be critical to ensure that the organoids will mimic the intratumor and interpatient genetic heterogeneity.[88] Updated molecular genetic profiling of GC has yielded promising new therapeutic targets such as receptor tyrosine kinases, RAS, and PI3K signaling proteins.[76] Thus, the integration of GC genotype, phenotype, and drug sensitivity testing using PDOs models promises to accelerate the use of personalized anticancer therapy, to improve treatment outcomes, and to assist in future clinical trial design and personalized medicine strategies.[26,89]

Outside classical chemotherapy, only 2 targeted treatments have been approved by the U.S. Food and Drug Administration to take advantage of genetic alterations as molecular targets for novel treatment options: the anti-HER2 agent trastuzumab and the antiangiogenesis agent ramucirumab.[11,88,90–92] Thus, organoid lines may constitute innovative molecular subtype–specific model systems to test individualized treatment regiments.[90] GC organoids have already been used to help predict patient response to targeted therapies such as HER2 inhibition.[88] Steele et al[88] optimized culturing organoids in a 96-well-plate format for use in drug testing within 3 days of the patient's surgery,

making it is feasible to study molecular subtype, perform a drug screen and provide guidance on individualized adjuvant therapy for each patient within 5–6 days after surgery. Similarly, Vlachogiannis et al[93] cultured cancer-derived organoids from patients with gastrointestinal metastatic cancers and treated them with commonly used therapeutics to predict treatment response. They found 100% sensitivity, 93% specificity, 88% positive predictive value, and 100% negative predictive value for organoids forecasting the patient response to targeted agents or chemotherapy.[93] Importantly, they were able to mimic interpatient tumor differences using patient-derived organoids and also distinguish intra-patient tumor heterogeneity in response to chemotherapeutic drugs.[93] A third study showed that chemoradiation responses in patients were highly matched to rectal cancer organoid responses, with 84.43% accuracy, 78.01% sensitivity, and 91.97% specificity.[94] These studies indicate the strength of organoids to predict tumor-specific responses and potentially represent a first step toward personalized treatment regimens using PDOs.[95]

As mentioned previously, Gao et al[53] published a novel technique for establishing GC organoids from endoscopic biopsies, which may yield clinically relevant results for patients who are ineligible for surgical intervention. Their technique yielded abundant esophagogastroduodenoscopy-derived organoids that could be tested with multiple standards of care drug regimens and combination therapies within 2 weeks,[53] providing a valuable model for predicting therapies for individual patients with advanced metastatic GC. PDOs cultured from diagnostic biopsy procedures may also allow for simultaneous testing of multiple drug regimens to guide therapy in a clinically relevant time interval, an important step towards personalized medicine for GC patients.[96] As nearly all potential GC patients undergo endoscopic or diagnostic biopsies during their treatment course, biopsy-initiated organoids may also allow for widespread biobanking of not only GC organoids from many different patients, but also GC organoids from the same patient during meaningful time intervals throughout their disease course.[96]

The promise of in vitro organoid systems for personalized medicine also extends to analyzing or even modulating the patients' immune response to disease. Given the advent of checkpoint inhibitors and immunotherapy in frontline GC treatment,[97–99] better models are needed to study and optimize these treatments. PDO models are unable to mimic the in vivo tumor microenvironment completely, as they normally lack blood vessels for studying cancer angiogenesis and metastasis, and also lack the immune component found within the native tumor environment. However, recent studies have demonstrated that co-cultures of organoids and immune cells can partially overcome this limitation. The ALI culture method can preserve the complex histological tumor microenvironment architecture with tumor parenchyma and stroma, including functional tumor-infiltrating lymphocytes.[31] Moreover, this PDO system allows for in vitro modeling of tumor microenvironment–intrinsic immune cell responses as opposed to those driven by peripheral immune populations.[31] Dijkstra et al[100] demonstrated that co-culturing tumor organoids with autologous peripheral blood lymphocytes can enrich for tumor-reactive T cells from peripheral blood of patients with mismatch repair–deficient colorectal cancer and non-small cell lung cancer. These activated T cells can then kill tumor cells from the same patient. This provided proof of concept to generate a novel class of tumor-specific T cell products derived from the peripheral blood, and established a means to assess the sensitivity of tumor cells to T cell-mediated attack in individual patients.[100] Moreover, Zavros' group co-cultured transgenic mouse–derived gastric tumoroids with autologous immune cells specifically for the study of PD-L1/PD-1 interactions within the tumor microenvironment.[101] Presently, the exploration of organoids co-cultured with immune cells is still in a relatively early stage, but these findings are significant and demonstrate that PDOs recapitulate many key aspects of cancer immunobiology such as upregulation of checkpoint proteins like PD-L1 that promote immune evasion and microbial persistence.[61,102] These co-culture models may help optimize the response of effector T cells specifically against a patient's tumor or provide a means to generate large numbers of effector T cells targeted to tumor cells for potential adoptive cell transplantation.[103] While still early in development, these GC patient-specific model systems hold great promise for implementing personalized medicine and targeted therapy. It is clear that future advances in PDO GC models will allow for combining traditional genome- and phenotype-based strategies to rapidly advance precision medicine applications.[76]

Future Perspectives and Limitations

Since the Clevers group discovered the combination of culture factors needed to maintain the division and differentiation of intestinal stem cells in a 3D environment a decade ago,[3] the worldwide application of organoid technology has resulted in unprecedented advances for many organs and diseases, including the study of GC.

Owing to the heterogeneous nature of tumors, especially in GC, no drug can be effective for all patients. Thus, personalized medicine is needed to advance cancer therapy. Tumor-derived organoids are rapidly becoming an important tool to realize this goal. Drug screening using human primary cancer organoids can aid in developing personalized treatment strategies, and PDOs from early-stage human cancers may even help identify early molecular aberrations to be used as biomarkers and prevention targets.[103] Moreover, organoids can be generated from rounds of biopsies over time from cancer patients to continuously assess their treatment response, to detect any developing drug resistance, and to prospectively predict their response to future therapy options.[104] In 2019, Clevers' group identified that tumor organoids can be used to establish individualized ex vivo model systems to support T cell–based therapies and to study the interactions between T cells and tumor cells.[100,105] They were able to test for tumor cell sensitivity and resistance to immunotherapy, potentially allowing for future unbiased generation of patient-specific T cell products.

Further incorporation and integration of microenvironment components will enable gastric organoids and tumoroids to more faithfully represent in vivo physiology. Currently, there are many disparate and evolving techniques using organoids to study epithelial and niche interactions. A thorough discussion of this topic is beyond the scope of this review and is covered in detail elsewhere.[101] Specifically, microenvironment components may be added individually to gastrointestinal epithelial organoids such as immune components.[95,101,104] mesenchymal cells,[106,107] and even neurons.[108] Developmentally based co-culturing techniques have also been developed using PSCs that enables differentiation of the mesenchyme together with gastric epithelial organoids.[36] Finally, the ALI technique developed by the Kuo Lab is an additional 3D gastrointestinal organoid culturing technique that incorporates multiple components of the microenvironment including mesenchymal and immune components.[29,30,109] Much work is needed to optimize these co-culturing techniques and to fully elucidate the limits of this in vitro system.

Techniques for how to best recapitulate the stomach's physiology are also rapidly evolving. Human "organs-on-a-chip," a multichannel 3D microfluidic cell culture chip that simulates the activities, mechanical properties, and physiological responses of the organ and organ system, is emerging as a new direction for constructing organoids models with higher physiological relevance. While organoids rely heavily on spontaneous self-assembly to generate their organized tissue structure, organoids-on-a-chip provide an artificial bioengineered system to arrange cells to simulate tissue or organ physiology.[110] In the near future, additional bioengineering approaches such as live imaging, genome editing, and single-cell genomics may also be incorporated into these systems to better study human organogenesis, diseases and personalized medicine,[110] possibly opening new avenues to advance this "next generation" of ex vivo organoid models.

Organoids are robust tools for studying human development and disease. However, it is important to note their limitations. Drawbacks to gastric organoids in general are difficult access to the lumen (require microinjection)[28] and the fact that organoids that are passaged over time in vitro lose the ability to differentiate into functional chief and parietal cells. Meanwhile, gastric organoid models help overcome the technical and biological restrictions of in vivo studies, but they lack nonepithelial cellular components including mesenchymal tissue, neural cells, and immune cells, as discussed previously. Another practical limitation for organoid cultures is the necessity of Matrigel or other animal-derived matrices in most organoid models to enable cells to aggregate into organoids.[104] The composition of these matrices is poorly defined, and their efficient removal is pivotal for subsequent DNA or RNA isolation, CRISPR/Cas9 editing, or cryopreservation.[38,104] In addition, their use may preclude PDOs from truly being able to be integrated into CLIA-certified clinical applications. When studying tumors ex vivo, obtaining pure cancer organoids represents another crucial hurdle, as tumor organoids can be overgrown and contaminated by normal organoids derived from healthy gastric epithelial cells intermixed in the starting tissue material.[19,45–47,52] Investigators have begun to develop sophisticated means to molecularly select for tumor cells.[52,84] While these limitations need to be noted and accounted for in experimental design, few are insurmountable. In addition, these limitations are also outbalanced by the many advantages of tumor organoid models such as their ease of use, benefits of their 3D physiology, and the ability to test tumor tissue from a patient in a time frame that allows for clinical decision making. It is clear that advancing organoid culturing techniques will improve our understanding of stomach physiology and pathology.

Conclusions

In summary, even though current organoids systems show limitations and require additional optimization for use in disease modeling and personalized medicine, they have opened up important new avenues for regenerative medicine and, in combination with additional bioengineering approaches, they will continue to be invaluable tools in preclinical and clinical research.

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Received January 20, 2021. Accepted September 13, 2021.

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Conflicts of interest
The authors disclose no conflicts.

Funding
Zhi-Feng Miao is supported by the National Natural Science Foundation of China (82072724) and the Department of Education of Liaoning Province (QNZR2020004). Joseph R. Burclaff is supported by Grant NO. F32-DK124929 (National Institutes of Health, National Institute of Diabetes and Digestive and Kidney Diseases). Mahliyah Adkins-Threats is supported by Grant Nos. T32-DK077653 (National Institutes of Health, National Institute of Diabetes and Digestive and Kidney Diseases) and R25-GM103757 (National Institute of General Medical Sciences, National Institutes of Health). Zhen-Ning Wang is supported by the National Key R&D Program of China (MOST-2017YFC0908300), the National Natural Science Foundation of China (81961128026, U1908207), the Major Scientific and Technological Special Project of Liaoning Province of China (2019020176-JH1/103), and the Overseas training project of Liaoning general higher education (2019GJWYB022). Jason C. Mills is supported by Grant Nos. R01DK094989, R01DK105129, R01DK110406, and R01CA239645; the Alvin J. Siteman Cancer Center–Barnes Jewish Hospital Foundation Cancer Frontier Fund; the National Institutes of Health National Cancer Institute Grant Nos. P30 CA091842 and R01 CA246208; and the Barnard Trust.

Molecular Pathology of Gastroesophageal Cancer

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KEYWORDS

• Esophageal squamous cell carcinoma • Esophageal adenocarcinoma • Gastric adenocarcinoma
• Molecular pathology

Key points

- Esophageal squamous cell carcinoma and esophageal adenocarcinoma are separate entities with differing molecular pathology.
- Gastric adenocarcinomas can be classified into 4 distinct molecular subtypes that may suggest treatments unique to the subtypes.
- Esophageal adenocarcinoma and chromosomal unstable–type gastric adenocarcinoma are very similar to each other and likely constitute a spectrum of the same disease.

ABSTRACT

Upper gastroesophageal carcinomas consist of cancers arising from the esophagus and stomach. Squamous cell carcinomas and adenocarcinomas are seen in the esophagus and despite arising from the same organ have different biology. Gastric adenocarcinomas are categorized into 4 molecular subtypes: high Epstein-Barr virus load, microsatellite unstable cancers, chromosomal unstable (CIN) cancers, and genomically stable cancers. Genomically stable gastric cancers correlate highly with histologically defined diffuse-type cancers. Esophageal carcinomas and CIN gastric cancers often are driven by high-level amplifications of oncogenes and contain a high degree of intratumoral heterogeneity. Targeted therapeutics is an active area of research for gastroesophageal cancers.

OVERVIEW

Upper gastrointestinal cancers comprise malignancies of the esophagus and stomach. Although most gastrointestinal cancers are adenocarcinomas, esophageal cancers come in both adenocarcinoma and squamous cell carcinoma. Despite being derived from the same organ, esophageal adenocarcinoma (EAC) and esophageal squamous cell carcinoma (ESCC) are quite different at both cellular and molecular levels and should be treated as separate entities.[1] Traditionally, adenocarcinomas of the esophagus and stomach were considered two separate types of cancer and treated as such. Recent evidence has suggested, however, that EAC is very similar to intestinal-type gastric adenocarcinomas of the proximal stomach.[1,2] Although they are discussed separately, they should be considered as a spectrum of the same disease.[3] In the United States, gastroesophageal cancers represent a significant source of cancer morbidity and mortality with more than 45,000 new cases resulting in more than 26,000 deaths estimated for 2021.[4] The lack of early endoscopic surveillance guidelines and the often subtle clinical symptoms have resulted in many patients presenting at time of diagnosis with advanced metastatic disease and 5-year survival rates under 20%.[5] As understanding of these

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Surgical Pathology 14 (2021) 443–453
https://doi.org/10.1016/j.path.2021.05.008

complex cancers continues to improve, new more efficacious and better tolerated targeted therapies are being developed.

ESOPHAGEAL SQUAMOUS CELL CARCINOMA

ESCC arises in the upper and middle esophagus and has a widely varying regional incidence, with highest rates in China, South Africa, and South America.[6] Risk factors also vary according to region, but common ones include tobacco, diet, and alcohol.[6] The molecular alterations present in ESCC have been well studied. As in other squamous cell carcinomas, ESCCs typically have a moderately high mutation burden and frequent copy number alterations. A recent The Cancer Genome Atlas (TCGA) article,[1] as well as others,[7,8] describe frequent activation of the RAS and PI(3)K pathways, loss of cell-cycle regulation, chromatin remodeling dysregulation, and alterations in transcription factors/cell differentiation pathways. RAS and PI(3)K pathway alterations include frequent amplifications of *EGFR* and *FGFR1* with *ERBB2*, *KRAS*, and *MET* less commonly amplified and common activating mutations in *PIK2CA*. *PTEN*, a negative regulator of *PIK3CA*, is inactivated through deletion or loss of function mutations in approximately 10% of cases. Commonly altered genes involved in cell-cycle regulation include very frequent deletions of *CDKN2A* (approximately 75% of ESCCs), deletions or mutations in *RB1*, and amplifications of *CCND1* and/or *CDK6*. Genes involved in chromatin remodeling are altered in approximately a third of cases with mutations or deletions of *SMARCA4*, *KDM6A*, and *KMT2D* the most common. Transcription factors or other genes involved in cell differentiation also commonly are altered. Amplifications involving genomic regions that contain *TP63*/*SOX2* are seen in approximately half of ESCCs with mutations in *NOTCH1* and *ZNF750* also somewhat common. Finally, a few other genes also commonly are altered. These include *TP53* mutations in more than 80% of cases, *MYC* amplifications, and less commonly *SMAD4* mutations or deletions.

ESCC arises from dysplastic (premalignant) lesions similar to other squamous cancers. Studies comparing ESCC and dysplasia adjacent to ESCC found remarkably similar aggregate mutational and copy number profiles, with areas of dysplasia having a similar frequency of events in genes commonly altered in ESCC.[9,10] Despite a similar frequency of alterations, when paired ESCC and dysplasia samples from the same patient were compared with each other, there still was a high degree of genomic heterogeneity as well as private, nonshared events. This suggests that fields of dysplasia may consist of an oligoclonal population, where 1 of these clones eventually develops an invasive phenotype to become ESCC. When dysplasia adjacent to ESCC was compared with dysplasia from patients without ESCC, 2 important differences were identified.[10] First, although *TP53* mutations still were identified in patients with only dysplastic tissue, a second event affecting the alternative allele was very rare. This is in contrast to ESCC and dysplasia adjacent to ESCC, where finding 2 alterations of *TP53* was extremely common. Second, the number of mutations and CNVs in patients with only dysplastic tissue was lower than both low-grade dysplasia and high-grade dysplasia taken adjacent to ESCC. These results raise the possibility of using molecular alterations to better stratify patients with esophageal squamous dysplasia into high and low risk.

ESOPHAGEAL ADENOCARCINOMA

EAC arises in the lower esophagus out of a field of columnar metaplasia that develops a varying degree of intestinal differentiation (called Barrett's esophagus [BE]). Although traditionally EAC was rare, with ESCC the predominate cancer type of the esophagus, there has been a dramatic rise in incidence of EAC within European and North American countries.[11–14] Combined with the low 5-year survival rate, this increase in incidence has driven an increased interest in understanding the molecular alterations that are present in this cancer. Several large studies have characterized the landscape of alterations present, including both by the TCGA[1] and the International Cancer Gene Consortium.[15] Like ESCC and many other cancers, pathways that commonly are altered in EAC include receptor tyrosine kinases (RTK) and their downstream signaling partners (Ras signaling), cell-cycle control, transcription factors/cell differentiation, chromatin remodeling, and transforming growth factor (TGF)-β signaling. Oncogenic activation through the RTK pathway typically occurs through amplification of *ERBB2*, *EGFR*, or *KRAS* which are present in approximately 25%, 15%, and 10% to 15% of cancers, respectively. Less commonly, amplifications can be seen in *IGFR1*, *FGFR1*, *FGFR2*, and *MET*. Additionally, amplifications in *VEGFA* are seen in 10% to 20% of EACs. Loss of cell-cycle regulation occurs through inactivation of *CDKN2A* in 75% of cases and amplifications of *CCNE1*, *CCND1*, and *CDK6*, all of which occur in 10% to 30% of EACs, with *CCND1* reported to be the most commonly amplified.[16] The majority of *CDKN2A*

inactivation in EAC occurs through promotor methylation and less commonly through deletions or mutations. The transcription factors *GATA4* and *GATA6*, which both have a role in cellular differentiation and development, are amplified in approximately 20% of EACs each and usually (but not always) are mutually exclusive. Although not as common as in ESCC, loss of function alterations in genes involved in chromatin remodeling can be seen in EAC. The most commonly altered genes include *SMARCA4* and *ARID1A*, both of which are altered in approximately 10% of cases. Deletions and loss of function mutations in *SMAD4* and *SMAD2*, which are mediators of TGF-β signaling, are seen in approximately 25% of EACs. *MYC* amplifications can be seen in 20% to 30% of these cancers. Loss of normal *TP53* function has been proposed to play a vital role in EAC progression and can be seen in approximately 75% of EACs with *MDM2* amplifications seen in some of the *TP53* wild-type cancers.[17]

EACs typically emerge from premalignant lesions within the lower esophagus, termed BE. BE, which is the replacement of the normally squamous lined esophagus with columnar epithelial cells that develop intestinal differentiation, is thought to form in response to injury induced by chronic bile and acid reflux and the resultant inflammation. The prevalence of BE is thought to be much higher than EAC and has been estimated to exist in 1% to 10% of adults in the United States.[18] The vast majority of those with BE never progresses to cancer, complicating the understanding of BE progression to EAC. In order to understand this process, several groups have either studied paired genomic profiles of EAC and adjacent BE or BE samples with known long-term follow-up to characterize the evolution of cancer from precursor lesions. These studies have identified that *TP53* inactivation is a common early event that can occur in nondysplastic BE. This is followed by the development of aneuploidy, often including development of genome doubling.[17,19–25] Transformation of dysplastic lesions to EAC is thought to occur via acquisition of high-level focal amplifications of oncogenes (as described previously), often in the context of complex genomic disruptions.[17,26,27]

GASTRIC ADENOCARCINOMA

Gastric cancer is one of the world's leading causes of cancer mortality, with an estimated 783,000 deaths in 2018.[28,29] Similar to esophageal cancer, the incidence is highly variable according to geographic region. Most cases of gastric cancer are associated with *Helicobacter pylori* or Epstein-Barr virus (EBV) infection and a small subset are associated with germline mutations in *CDH1* (E-cadherin) or mismatch repair genes (Lynch syndrome).[30,31] Gastric adenocarcinomas traditionally are classified by histology. The Lauren classification divides gastric cancer into diffuse and intestinal types whereas the World Health Organization uses papillary, tubular, mucinous, and poorly cohesive.[32,33] Recent comprehensive molecular characterization has suggested, however, a classification system based on genomic and methylation differences. TCGA Research Network gastric cancer study, suggests gastric cancers should be categorized in 4 molecular subtypes (**Table 1**).[2] Although more work needs to be done to better correlate the molecular findings with clinical parameters, these molecular subtypes provide more insight into the biology of the tumor and give some suggestions for targeted therapies. The first molecular subtype includes gastric cancers that are EBV positive. These tumors tend to have extensive DNA methylation of gene promotors and low overall mutation and copy number alteration rates and often are found in the gastric body or fundus. EBV-positive gastric adenocarcinomas almost always have *CDKN2A* promotor methylation and have high rates of *PIK3CA* and *ARID1A* mutations and low rates of *TP53* mutations. Amplifications involving *CD274* (programmed death ligand [PD-L] 1 protein), *JAK2*, and *ERBB2* can be seen in approximately 15%, 12%, and 12% of EBV-positive gastric cancers, respectively. The second molecular subtype of gastric cancers are the microsatellite instability (MSI) gastric cancers. These cancers are characterized by hypermethylation with methylation of (and thus inactivation of) the *MLH1* gene promotor. This leads to defective mismatch repair and highly elevated mutation rates. Prominent alterations in MSI gastric cancers include mutations in *PIK3CA*, *ERBB3*, *KRAS*, *NRAS*, *PTEN*, and *RASA1*. High-level amplifications are rare in MSI gastric cancers but occasionally are found involving *PIK3CA*. The third molecular subtype of gastric cancer is the genomically stable subgroup. These gastric cancers are EBV-negative and microsatellite stable with a low level of copy number alterations. This subgroup is enriched for the diffuse-type gastric cancers in the Lauren classification. As such, frequent alterations in *CDH1* can be found. Other commonly altered genes include *ARID1A* and *RHOA*. Although copy number alterations are rare, activating amplifications or mutations in *FGFR2*, *ERBB2*, *KRAS*, *NRAS*, and *PIK3CA* can be seen in 5% to 10% of cancers for each gene. The fourth molecular subtype is the chromosomal instability (CIN) subtype that is characterized by a

Table 1
Molecular classification of gastric adenocarcinomas

Subgroup	Defining Characteristic	Methylation Status	Mutation Rates	Copy Number Variant Rates	Associations
EBV positive	High EBV burden	Extensive DNA promotor methylation (CIMP)	Low to moderate	Low to moderate	Enriched in gastric fundus and body
MSI	Microsatellite unstable	Hypermethylation with methylation of *MLH1* promotor	High	Low to moderate	Loss of mismatch repair through mutation (Lynch syndrome) or MLH1 promotor methylation
Genomically stable	Low degree of genomic complexity	Variable (moderate)	Low	Low	Enriched for diffuse-type cancers
CIN	High degree of genomic complexity	Variable (moderate)	Moderate	High	Enriched in proximal stomach

Abbreviations: CIMP, CpG island methylator phenotype.

high degree of copy number changes. This subtype is found more commonly in the proximal stomach and is very similar to EACs. Like EAC, the CIN gastric cancers have frequent *TP53* mutations, amplifications in the RTK/RAS pathway (*ERBB2*, *EGFR*, *FGFR2*, *ERBB3*, *MET*, *KRAS*, and *NRAS*) and in cell-cycle mediators (*CCNE1*, *CCND1*, and *CDK6*). Loss-of-function mutations in the β-catenin pathway (*APC* and *CTNNB1*) also can be seen.

Two different forms of metaplasia have been described in the stomach. The first, gastric intestinal metaplasia, is histologically similar to BE. In one study, genomic and methylation–based profiling of gastric intestinal metaplasia showed that it harbored several recurrent genomic alterations and methylation patterns different than normal gastric epithelium.[34] This study, which looked at a mix of metaplasia from patients with regressive/stable disease and a lower number of patients in which the metaplasia progressed to high-grade dysplasia or cancer, found an overall lower mutational and copy number burden compared with gastric adenocarcinomas. Despite this, recurrent hot spot mutations in *FBXW7* and rarer mutations in *TP53* and *ARID1A* still were identified. In addition, copy number gains of 8q involving the oncogene *MYC* were seen. When metaplasia from patients who progressed were compared with those who did not progress, a trend for increased numbers of mutations, copy number alterations, and shorter telomeres was seen in the intestinal metaplasia from progressors.

The second type of metaplasia is termed, spasmolytic polypeptide–expressing metaplasia (SPEM) or pseudopyloric metaplasia. The exact relationship of gastric intestinal metaplasia and SPEM to each other and to gastric cancers is controversial and an area of ongoing research. Few studies have looked at the genomic landscape of SPEM; however, Srivastava and colleagues performed paired targeted sequencing on a small number of gastric cancer patients who had concurrent intestinal metaplasia and SPEM.[35] In this study, they found SPEM to have a much lower number of mutations compared with the paired intestinal-type gastric adenocarcinomas whereas the regions of intestinal metaplasia had similar numbers of mutations as the cancers. Further studies are needed to better delineate the genomic progression of gastric precancerous lesions to the different subtypes of gastric cancer.

INTRATUMORAL GENOMIC HETEROGENEITY IN ESOPHAGEAL AND GASTRIC ADENOCARCINOMA

As described previously, both esophageal and CIN-type gastric adenocarcinoma develop from preneoplastic lesions where early *TP53* mutations are common. This is followed by the development of aneuploidy and significant disruption of normal chromosomes. It is through this process that most of these cancers get their source of oncogenic signaling, namely development of high-level

amplifications of oncogenes late in the progression process. This is in contrast to gastrointestinal adenocarcinomas of other sites where activating mutations in important oncogenes occur relatively early in the progression process. For example, *KRAS* mutations in colon or pancreatic adenocarcinoma. This highly unstable state seen in esophageal and CIN-type gastric adenocarcinoma can lead to significant heterogeneity within the late preneoplastic lesion and the invasive cancer. Several recent studies have looked at multiregion primary and metastatic tumor sequencing and found a high degree of heterogeneity.[24,36] This heterogeneity potentially includes targetable oncogenic drivers. Pectasides and colleagues[24] found that between paired primary and metastatic samples nearly half of patients had discrepant pathogenic alterations. When they looked at samples with activating alterations in RTKs, a major focus of targeted therapy, more than half of patients had discrepant results between samples depending on the cohort utilized. This heterogeneity in important driver genes may be a major source for failure of precision medicine/targeted therapy in these diseases and points toward the need of careful sample selection for clinical testing. There is some suggestion that sequencing of plasma circulating tumor DNA may be a better predictor of response to targeted therapy.[24,37]

PRECISION MEDICINE IN UPPER GASTROINTESTINAL CANCERS

As understanding of the molecular mechanisms underpinning upper gastrointestinal cancers has improved, new more efficacious and better tolerated targeted therapies, including immunotherapeutics have advanced the landscape of treatment beyond cytotoxic chemotherapy, summarized in **Table 2**. To date, however, many of these therapies have shown only modest success. Therefore, improved understanding of the genomic heterogeneity and other mechanisms of resistance will be vitally important to further improve treatment strategies. These novel treatments and how they are tailored based on patient histology, anatomic location, and pathologic biomarkers are discussed.

The emergence of genomics and its clinical accessibility has changed the way cancer treatment is approached. Molecular characteristics of the cancer now are just as important in clinical oncology decision making as cancer anatomic location and histology. Specifically, for gastroesophageal cancers, detailed sequencing studies have revealed shared subtypes with common molecular pathogenesis.[1,2] Growth factor signaling pathway activation is a shared trait for the most prevalent CIN subtype of gastroesophageal cancer. Thus, targeting these signaling cascades has translated well clinically. The human epidermal growth factor receptor 2 (HER2/*ERBB2*) is overexpressed or amplified in 10% to 30% of gastroesophageal cancers.[47] The landmark ToGA trial examined the efficacy of targeting this pathway using trastuzumab, a monoclonal antibody against HER2, for HER2-positive (ie, 3+ staining on immunohistochemistry [IHC] or [fluorescence in situ hybridization positive]) gastroesophageal junction and stomach adenocarcinomas.[38] Although no esophageal cancer patients were included in this study, these results are applied to advanced esophageal cancer patients due to molecular similarities between gastric adenocarcinoma and EAC, and similar rates of HER2 positivity.[48] Addition of trastuzumab to chemotherapy in the first-line treatment setting significantly improved survival metrics and has now become standard-of-care treatment of HER2-positive patients.

In the second-line treatment setting, targeting the vascular endothelial growth factor (VEGF) signaling pathway has proved clinically efficacious. In particular, ramucirumab, a monoclonal antibody blocking human VEGF receptor 2 (*VEGFR2*) has been shown superior to single-agent chemotherapy in two large phase III clinical trials.[40,41] The first trial, REGARD, showed that monotherapy with ramucirumab was superior to placebo in the second-line setting for gastric or gastroesophageal junction adenocarcinomas.[40] The RAINBOW trial also showed clinical improvements with the addition of ramucirumab to single-agent paclitaxel chemotherapy in the second-line setting for gastric or gastroesophageal junction adenocarcinomas.[41] Again, as discussed previously, these results have been extrapolated to EACs given their similarities to gastric and gastroesophageal junction adenocarcinomas. Unlike trastuzumab, ramucirumab is approved to be used in gastroesophageal adenocarcinoma patient without an *a priori* biomarker test.

Currently, these 2 agents are the only targeted agents approved for advanced gastroesophageal cancers. Multiple other pathways have been examined but have not proved clinically efficacious.[49] Much work remains to not only develop better pathway targeting agents but also elucidate new ways to predict and select patients that most likely would benefit from these treatments. One new agent that recently has gained Food and Drug Administration (FDA) breakthrough therapy

Table 2
Approved targeted therapies for gastroesophageal cancer

Targeted Agent	Mechanism of Action	Biomarker	Clinical Trial	Histology	Line of Therapy	Anatomic Location	Efficacy
Trastuzumab (Herceptin)	Monoclonal antibody against human epidermal growth factor receptor 2 (HER2/ERBB2)	HER2-positive tumors (3+ staining on IHC or FISH positive)	ToGA[38]	Adenocarcinoma	First	Gastroesophageal junction and stomach	Improved survival
Fam-trastuzumab Deruxtecan (Enhertu)	Antibody drug conjugate targeting human epidermal growth factor receptor 2 (HER2/ERBB2)	HER2-positive tumors (3+ staining on IHC or 2+ staining on IHC and FISH positive)	DESTINY-Gastric01[39]	Adenocarcinoma	Third	Gastroesophageal junction and stomach	Improved survival
Ramucirumab (Cyramza)	Monoclonal antibody against human VEGFR2	None	REGARD[40]	Adenocarcinoma	Second	Gastroesophageal junction and stomach	Improved survival
			RAINBOW[41]	Adenocarcinoma	Second	Gastroesophageal junction and stomach	Improved survival

Pembrolizumab (Keytruda)	Monoclonal antibody against PD-1 receptor	PD-L1 positive tumors (CPS 1 or higher)	KEYNOTE-061[42]	Adenocarcinoma (79%), tubular adenocarcinoma (10%), signet ring cell carcinoma (4%)	Third	Gastroesophageal junction and stomach	Did not improve survival in the second-line setting but better adverse event profile compared with paclitaxel monotherapy
		PD-L1 positive tumors (CPS 10 or higher)	KEYNOTE-181[43]	Squamous cell carcinoma and adenocarcinoma	Second (FDA approved only for squamous cell carcinoma histology in the second-line setting)	Esophagus and Siewert type 1 gastroesophageal junction	Improved survival
		MSI-HIGH tumors	KEYNOTE-061[42]	Adenocarcinoma (79%), tubular adenocarcinoma (10%), signet ring cell carcinoma (4%)	Second	Gastroesophageal junction and stomach	Improved survival
			KEYNOTE-158[44]	Any solid tumor	Second	Any solid tumor	Improved survival
		Tumor mutational burden (at least 10 mutations per megabase)	KEYNOTE-158[45]	Any solid tumor	Second	Any solid tumor	Improved survival
Nivolumab (Optivo)	Monoclonal antibody against PD-1 receptor	None	ATTRACTION-3[46]	Squamous cell carcinoma	Second	Esophagus	Improved survival

designation is Fam-trastuzumab deruxtecan, a HER2 antibody-drug conjugate that was shown to have clinical activity in a cohort of heavily pretreated HER2-positive gastric/gastroesophageal junction adenocarcinoma patients.[39] This promising new agent demonstrates the potential of targeted agents to not only improve survival but also incur fewer treatment related toxicities compared with cytotoxic chemotherapies.

Given the chronic injurious nature that spurs formation of gastroesophageal cancers[50,51] (ie, smoking for ESCCs, acid reflux for EACs, and *Helicobacter pylori* infection for gastric adenocarcinomas), it is not surprising that these entities have been found to accumulate somatic mutations.[52] These genomic changes likely result in neoantigens, which ultimately are targeted by the immune system through cancer immunosurveillence.[53] Thus, immunotherapy and specifically targeting programmed death-1 (PD-1) receptor to block immunosuppressing ligands (PD-L1 and PDL-2) have resulted in new approved therapies for gastroesophageal cancer patients. The first agent, pembrolizumab, is approved in the United States to be used in concert with a combined positive score (CPS)[54] designed to preferentially select patients with higher PD-L1 levels and a higher probability of response. Specifically, for gastroesophageal adenocarcinomas, pembrolizumab is approved to be used for CPS score of 1 or higher in the third-line treatment setting based on results from KEYNOTE-061 study[42] showing no significant clinical efficacy for these patients as second-line therapy. Pembrolizumab also is approved to be used after progression on one or more prior treatments (ie, second-line treatment) for ESCCs that expresses high PD-L1 levels (CPS $\geq$10) based on the KEYNOTE-181 study.[43] In addition, pembrolizumab is approved to be used for tumor histology agnostic treatment of any solid tumor with defective mismatch repair (MSI-high) or high tumor mutation burden ($\geq$10 mut/Mb).[42,44,45] A second immunotherapy with a similar mechanism of action, nivolumab, is approved in the United States in the second line to treat ESCCs regardless of PD-L1 levels based on results of the ATTRACTION-3 trial.[46] These immunotherapy treatments have not only provided new safer avenues to treat gastroesophageal cancer patients but also have changed the basic approaches to cancer treatment. Multiple trials have recently completed or are ongoing to investigate the efficacy of these agents as part of combination systemic therapy. The promise of immunotherapy is evidenced by multiple recent FDA approvals. In the metastatic setting, immunotherapy is now approved for use in combination with frontline chemotherapy based on results of the CheckMate 649 [PMID: 34102137], ATTRACTION-4 [PMID: 30566590], and KEYNOTE-590 [PMID: 30735435] trials. In fact, the use of immunotherapy is now also favored in HER-2 positive patients [PMID: 33167735]. Furthermore, in the adjuvant setting after curative intent tri-modality therapy, immunotherapy has been approved based on the CheckMate 577 data [PMID: 33789008].

This article details examples of how understanding the molecular pathology of gastroesophageal cancers can have a direct impact on patient care. The complexity and heterogeneity of all cancers, including gastroesophageal cancers, mandate personalization of oncologic treatment. One-size-fits-all chemotherapy no longer is the ideal treatment of many of these patients. Elucidating the underlying pathogenesis of these diseases has resulted in and will continue to lead to important advancements in cancer diagnosis, prognosis, and individualized treatments.

CLINICS CARE POINTS

- ESCC and EAC are separate entities with differing molecular pathology.
- Gastric adenocarcinomas can be classified into 4 distinct molecular subtypes that may suggest treatments unique to the subtypes.
- EAC and CIN-type gastric adenocarcinoma are driven by a high degree of CIN and high-level amplifications of oncogenes, which leads to significant intratumor heterogeneity. This heterogeneity can lead to the wrong treatment being assigned if not testing the lesion that is wanted to treat.
- Targeted therapy in upper gastroesophageal cancers is an active area of research and is evolving rapidly.

DISCLOSURE

Dr. Stachler: NIH National Institute of Diabetes and Digestive and Kidney Diseases (K08 K08DK109209) and Doris Duke Charitable Foundation. Dr. Jin: NIH National Heart, Lung, and Blood Institute (T32HL007088), and the Barrett's Esophagus Translational Research Network (NCI U54CA163060).

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April 8, 2021

Re: 2021 Conquer Cancer Young Investigator Award (2021YIA-8674301298)

Dear Conquer Cancer, the ASCO Foundation,

I am writing this letter in response to the email inquiry sent by Dr. Andrew Smith copied below:

> "Thank you for providing your letter discussing the overlap of your DoD and YIA awards. We would like to get a better understanding of the distinctions between the work that would be funded by each grant, since the aims remain the same. To facilitate our review, can you please provide us with:
>
> - A brief list of the experiments that will be done for each aim in the DoD grant, and each aim for the YIA grant, as well as their anticipated outcomes. We do not need full experimental methods, but a bulleted list or table of the planned experiments and outcomes for each aim will be helpful as we review.
> - The budget for the DoD award, at least as it relates to the 3 aims. If it is possible to break down the budget by aim that would be helpful, but we can review the full budget as well."

First, I would like to describe briefly the two aims of my DoD award proposal. The first aim is to characterize the effects of SOX2 loss on esophageal homeostasis and during injury. Specifically, this aim will generate an inducible mouse model to delete *Sox2* in the adult murine foregut and characterize the resulting foregut phenotype at homeostasis and with chronic exposure to bile acid injury. This aim has no overlap with my YIA proposal as this work will be done in mouse tissue or organoids generated from mouse tissue.

The second aim of my DoD proposal is to determine the effects of forced expression of SOX2 on the progression of BE and EA. **My YIA proposal has complementary and non-overlapping experiments.** Additional details are described below. Briefly, the DoD proposal budget allowed limited funds to be used for human organoid experiments given the need for salary support and mouse experiments. As such, I proposed economical transient transfection experiments to assess for short term transcriptional changes upon SOX2 overexpression. The compound screen proposed in the DoD is also limited, and will only be performed at one concentration level. Given the additional funds available through the YIA, I have proposed more innovative and differing human Barrett's esophagus and gastroesophageal cancer organoid experiments. I will be generating stable expressing lines using a new PiggyBac transposon vector system that will allow better elucidation of long term morphologic and phenotypic changes. I will be performing these experiments on a more extensive biobank of available human Barrett's esophagus and gastroesophageal cancer organoids. I will also be utilizing a new CUT-and-TAG (T5 transposase based) direct transcription factor binding assay with improved specificity and sensitivity. Finally, I will be using a more clinically applicable kinase inhibitors screen at multiple different concentrations for drugs that can induce SOX2 expression.

I will elaborate on these details and describe the exact separate experiments for each award and the anticipated results below:

- For my DoD proposal **Aim 2A**, I propose to transiently transfect Barrett's organoid lines (WU002-WU014) with pcDNA3.3_SOX2 plasmid from Addgene (#26817) and assess the transcriptional changes

after 7 days including direct transcriptional targets of SOX2 (using RNA-seq and ChIP-seq) as well as assess for any general short term morphologic changes.
    - I would not expect great morphologic changes to occur with transient overexpression of SOX2 for 7 days as this is a developmental transcription factor that canonically functions through transcription factor cascades. I would expect to see transcriptional changes including binding to known direct transcriptional targets. **These SOX2 transcriptional changes are the key data to be generated here.**
- For my YIA **Aim 1**, I propose to generate stably SOX2 overexpressing human patient derived Barrett's esophagus organoid lines using additional and differing organoid lines (WU002-WU017). I will be using a different SOX2-T2A-GFP plasmid in combination with a novel PiggyBac transposon vector system to generate these stably SOX2 overexpressing human Barrett's esophagus lines. I plan to assess morphologic changes including markers for esophageal, gastric, intestinal, and metaplastic characteristics after at least 4 weeks post-transfection (including 3 weeks to allow line expansion and one week for Puromycin selection). I will also plan to assess transcriptional changes using microarrays and direct transcriptional targets of SOX2 using a novel CUT-and-TAG assay.
    - I would expect to see in these long term stable SOX2 overexpressing experiments more pronounced morphologic changes including a more esophageal phenotype with decreased expression of intestinal and metaplasia markers. We will also expect to see more relevant long term transcriptional changes. The novel CUT-and-TAG transcription factor binding assay will also increase the sensitivity and specificity of our approach to identify direct transcriptional targets. **These experiments will more fully address both the morphologic and phenotypic changes that arise from long term SOX2 overexpression.**

- For my DoD proposal **Aim 2B**, I plan to transiently transfect human gastroesophageal cancer organoid lines (TT, MP, SD, WT, CB, MH, CP, TC, JC, AF, RS, PB, JS, SB, RS2, GA) with pcDNA3.3_SOX2 plasmid from Addgene (#26817) and assess the transcriptional changes after 7 days including direct transcriptional targets of SOX2 (using RNA-seq and ChIP-seq) as well as assess for morphologic changes.
    - As above, I would not expect to see great morphologic changes to occur with transient overexpression of SOX2. In fact, I might even expect many of the SOX2 overexpressing gastroesophageal cancer lines to die. However, I would expect to see transcriptional changes including binding to known direct transcriptional targets. **These transcriptional changes are again the key data to be ascertained from these experiments.**
- For my YIA **Aim 2**, I propose to employ the SOX2-T2A-GFP plasmid and the PiggyBac transposon vector system to generate stably SOX2 overexpressing human gastroesophageal cancer organoid lines using additional and differing gastroesophageal cancer organoid lines (TT, MP, TW, CB, MH, EP, TC, JC, AF, RS, PB, JS, SB, RS2, GA, RK, MB, LS, WR, RW, DS, BI). I plan to assess morphologic changes including assays for organoid growth (including markers for cell division and growth factor pathway activation), invasion, and colony formation after at least 4 weeks post-transfection (to include 3 weeks for line expansion and one week to allow for Puromycin selection). I will also plan to assess transcriptional changes using microarrays and direct transcriptional targets of SOX2 using CUT-and-TAG.
    - In these long term stably SOX2 overexpressing gastroesophageal organoid lines, I would expect to see overt differences in organoid morphology as manifested in growth, invasion, and colony formation assays. We will also expect to see more relevant transcriptional changes, and the novel CUT-and-TAG transcription factor binding assay will increase the sensitivity and specificity of our approach. **These experiments will more fully address both morphologic and phenotypic changes that arise from SOX2 overexpression, and determine the transcriptional changes that arise.**

- For my DoD proposal **Aim 2C**, I seek to use human Barrett's esophagus organoids that stably overexpress a luciferase *SOX2* promoter reporter plasmid using nucleofection to induce genomic integration to screen the ICCB Known Bioactives library (Enzo) for compounds that induce SOX2 expression.

    - I expect to find compounds that induce *SOX2* expression using Barrett's esophagus organoids. Previous reported compounds including 4-hydroxytamoxifen, Nabumetone, and E-616452 will serve as positive controls.
- For my YIA **Aim 3**, I seek to generate human Barrett's esophagus organoids that stably overexpress a luciferase *SOX2* promoter reporter plasmid. However, I will be using a completely different approach. I have generated a new luciferase SOX2 promoter reporter plasmid that is now compatible with the PiggyBac transposon vector system. I propose to use this along with the newly derived human Barrett's esophagus organoids to generate the organoids that will be used for the throughput assay to identify agents that induce SOX2 expression. In addition, I will screen for different drugs/compounds. Instead of the ICCB Known Bioactives library (Enzo), I will use the 436 drug Kinase Inhibitors library (SelleckChem). This drug library will be more applicable to eventual clinical trial applications as these kinase inhibitors class of drugs have already been FDA approved and will be easier to directly translate to human patients.
    - I expect to find drugs and kinase inhibitors that induce SOX2 expression in human Barrett's esophagus organoids**. This approach will be more applicable to clinical translation and will allow easier integration into potential future clinical trials, and will also more readily identify specific signaling pathways that are crucial to induce SOX2 expression.**

I have included the full yearly budget and budget justification documents for my DoD award. This award is a 3 year award with direct costs. A significant portion of this award is going towards my salary and a technician. In addition, as described above, the first aim is mouse based. per year will be going towards mouse housing costs. Half of the budgeted for Materials, supplies and consumables will also be used for mouse based experiments. This will leave remaining to be used for the human Barrett's esophagus and gastroesophageal cancer organoid project (Aim 2). Specifically, this will be used towards transient SOX2 transfections of the Barrett's esophagus and gastroesophageal cancer organoids, and subsequent limited characterization experiments. There is also budgeted for the ICCB Known Bioactives library (Enzo) compound screen, As you know, these primary culture organoids are expensive to maintain as they need specific growth media and growth matrix support. The limited DoD budget was a large reason that I was unable to propose more elaborate and novel long term stable line generation experiments.

The proposed funds for the YIA will be applied directly to these additional crucial and expensive human organoid experiments. I have developed several new human Barrett's esophagus and gastroesophageal cancer lines, and will be used to culture and maintain these new expanded human Barrett's esophagus and gastroesophageal cancer organoid lines. Given that we will be assessing more organoid lines for longer time periods with more detailed phenotypic characterization and novel direct transcription factor binding assays, these additional funds will be needed. In addition will be for development of these new SOX2-T2A-GFP and luciferase *SOX2* promoter reporter plasmids to be used with the PiggyBac transposon vector system. Again, these are unique expenses to the YIA proposal. Finally, will be used for the Kinase Inhibitors library. We plan to perform unique triplicate experiments utilizing at least two different drug library concentrations. These are unique experiments that will only be funded by the YIA proposal.

I am appreciative of the decision made by the Conquer Cancer Young Investigator Award Grant Selection Committee to fund my proposal. Thank you for your time and consideration.

Sincerely,

Ramon Jin, MD, PhD

SPECIFIC AIMS:

Esophageal cancer is a leading cause of cancer mortality and morbidity world-wide. Alarmingly and for unclear reasons, the rates of esophageal adenocarcinoma (EA) have been increasing in the US. Surgery, radiation, and one-size-fits-all toxic chemotherapy remain the mainstay of our current poor treatment of this disease, as only one out of five patients will live longer than 5 years. Quite simply, we do not know why this disease occurs, how to prevent it from forming, or how to best treat it. What we do know is that there is a precursor condition termed Barrett's esophagus (BE) that occurs prior to the development of esophageal adenocarcinoma. This precancerous condition is defined by replacement of the normal esophagus lining with an intestinal-like lining in response to long term gastroesophageal reflux. If we understand how and why Barrett's esophagus develops, then we can possibly reverse this condition to prevent it from ever progressing to esophageal adenocarcinoma.

Key to initiation of Barrett's esophagus and progression to esophageal adenocarcinoma is a developmental reprogramming of the normal esophageal epithelium. The upper gastrointestinal tract transcription factor, SOX2 is necessary for epithelial fate specification during foregut development, and its expression is maintained in the proliferative basal cell layer of the adult esophageal epithelium. Upon chronic acid and bile induced injury, and the development of BE, SOX2 expression is decreased and an aberrant intestinal gene signature becomes activated. Moreover, SOX2 expression is further repressed and almost completely lost during progression to esophageal adenocarcinoma. Surprisingly little is known about the molecular function, direct transcriptional targets, or functional significance of decreased expression of SOX2 during this pathogenic process. I hypothesize that SOX2 functions to maintain esophageal squamous epithelial identity and to inhibit aberrant intestinal reprogramming, and its loss is a critical step during Barrett's esophagus development and the progression to esophageal adenocarcinoma. To test this hypothesis, I propose the following aims:

Aim 1: Determine the effects of SOX2 re-expression in Barrett's esophagus. I propose to use a SOX2-T2A-GFP plasmid in combination with a novel PiggyBac transposase vector system to generate stably SOX2 overexpressing human Barrett's esophagus lines. I plan to assess longterm morphologic changes including markers for esophageal, gastric, intestinal, and metaplastic characteristics, and assess transcriptional changes using microarrays and direct transcriptional targets of SOX2 using a novel CUT-and-TAG transposase-based assay.

Aim 2: Determine the effects of SOX2 re-expression in esophageal adenocarcinoma. I will employ the SOX2-T2A-GFP plasmid and the PiggyBac transposase vector system to generate stably SOX2 overexpressing human gastroesophageal cancer organoid lines. I plan to assess longterm morphologic changes including assays for organoid growth, invasion, and colony formation. I will also plan to assess transcriptional changes using microarrays and determine direct transcriptional targets of SOX2 using CUT-and-TAG.

Aim 3: Screen for drugs that induce SOX2 expression. I seek to generate human Barrett's esophagus organoids that stably overexpress a new luciferase SOX2 promoter reporter plasmid using the PiggyBac transposase vector system, and will develop a high-throughput screen using the SelleckChem 436 drug library for compounds that increase SOX2 expression.

This proposal will explore the mechanistic role of SOX2 in BE and EA. As there are limited experimental models for BE or EA, these organoids are a critical, unrivaled tool to perform the proposed experiments. In summary, I seek to study how a key esophageal transcription factor, SOX2, is lost during Barrett's esophagus development and progression to esophageal adenocarcinoma, and how prevention of this loss can be leveraged therapeutically.