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14. ABSTRACT

Stomach cancer arises within a field of precancerous metaplastic lineages. The present proposal focuses on understanding the earliest stages of gastric carcinogenesis to define therapies that can prevent or reverse pre-cancerous lesions. Two types of metaplasia are observed in the atrophic human stomach: intestinal metaplasia and Spasmolytic Polypeptide (TFF2) Expressing Metaplasia (SPEM). Both of these metaplasias are associated with development of intestinal type cancers in the stomach. We have recently developed a novel mouse model for gastric pre-neoplasia, the Mist1-Kras mouse. In this mouse, tamoxifen treatment induces expression of activated Kras(G12D) in mature chief cells in stomach, which incites a series of metaplastic transitions over the following 4 months including first the evolution of SPEM, followed by the emergence of intestinal metaplasia. We now seek to utilize the Mist1-Kras mouse to provide insights into the factors that lead to the evolution of metaplasia into neoplasia. We hypothesize that discrete populations of pre-neoplastic stem cells exist within metaplastic lesions and represent cancer-initiating cells. We will pursue two specific aims: 1) we will utilize gastric metaplasia organoids derived from Mist1-Kras mice to determine the carcinogenic properties of metaplastic lineages and 2) we will identify putative gastric cancer stem/progenitor cell populations.

15. SUBJECT TERMS

None listed.

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1. Introduction

Stomach cancer arises within a field of precancerous metaplastic lineages. The present proposal focuses on understanding the earliest stages of gastric carcinogenesis to define therapies that can prevent or reverse pre-cancerous lesions. Two types of metaplasia are observed in the atrophic human stomach: intestinal metaplasia and Spasmolytic Polypeptide (TFF2) Expressing Metaplasia (SPEM). Both of these metaplasias are associated with development of intestinal type cancers in the stomach. We have recently developed a novel mouse model for gastric pre-neoplasia, the Mist1-Kras mouse. In this mouse, tamoxifen treatment induces expression of activated Kras(G12D) in mature chief cells in stomach, which incites a series of metaplastic transitions over the following 4 months including first the evolution of SPEM, followed by the emergence of intestinal metaplasia. Thus, this model recapitulates all of the pre-cancerous metaplastic changes observed in humans. We have sought to utilize the Mist1-Kras mouse to provide insights into the factors that lead to the evolution of metaplasia into neoplasia. We hypothesize that discrete populations of pre-neoplastic stem cells exist within metaplastic lesions and represent cancer-initiating cells.

2. Keywords

Metaplasia, gastric cancer, pre-cancer, cancer stem cell, stem cell, organoid, gastroid

3. Accomplishments

Our aims remain unchanged

Specific Aim 1: We will utilize gastric metaplastic organoids derived from Mist1-Kras mice to determine the carcinogenic properties of metaplastic lineages. Currently, there is no gastric cancer cell line, which reflects the full spectrum of cancerous metaplastic conversions to gastric adenocarcinoma. We will establish cultured metaplastic organoids marked with membrane-associated EGFP isolated from Mist1-Kras-mTmG mouse stomachs 4 months after induction of active Kras. These lines will be used to evaluate *in vitro* whether the metaplastic cells evolve to dysplastic or cancerous cells. Mist1-Kras organoids will also be re-implanted into the gastric submucosa of mice and we will evaluate *in vivo* their progression to invasive metaplasia, invasive cancer and metastatic cancer.

Specific Aim 2: We will identify putative gastric cancer stem/progenitor cells. For these studies, we will isolate putative cancer stem cells from Mist1-Kras;Sox9-EGFP mice 4 months after induction of Ras activation in chief cells. Flow sorting for GFP along with the metaplasia-associated CD44 variant 9 (CD44v9) will allow isolation of putative cancer stem cells. We will also be able to isolate normal stem/progenitor cells by isolation of GFP-positive, CD44v9 negative cells. These putative stem cells will be characterized by single-cell RNA sequencing and we will evaluate their stem cell characteristics in clonal 3-dimensional culture and by re-implantation into the mouse gastric mucosa.

Final report: We have organized this final report to describe progress and accomplishments for each of the Aims as outlined in the Statement of Work.

Specific Aim 1:

Subaim 1A: Major Task 1:
This task was completed in Year 2 and published in *Nature Commmunications*.

Subaim 1A: Major Task 2:
We have evaluated the effects of putative pro-proliferative cytokines or autocrine factors secreted from dysplastic cells on the metaplasia progression. We have performed co-culture studies using Meta1 organoids (SPEM

organoids) with cytokines such as IL-13 and IL-4. These studies have unexpectedly identified a very high affinity receptor for IL-13 that also shows a lower effect by IL-4 on the activation of phospho-Stat6 (Figure 1). Since the IL-13 receptor is a heterodimer with the IL-4 receptor, usually the IL-13 effects are seen as equal to or less than IL-4. The findings indicate that metaplastic gastric cells harbor an IL-13 receptor with unusual binding properties, perhaps suggestive f an unrecognized co-receptor. This possibility will be the focus of future studies.

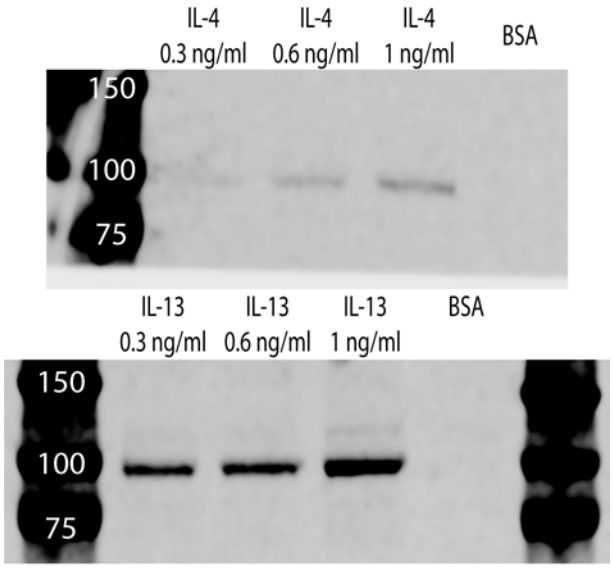

Figure 1: Meta1 metaplastic gastroids have a high affinity receptor for IL-13. Meta 1 organoids were cultured with either IL-4 or IL-13 (0.3-1 ng/ml) and the induction of phospho-STAT6 was assessed by western blot. Molecular weight markers (kDa) are shown at left. The lower panel shows a strong increase in pSTAT6 with IL-13 with far less pSTAT6 stimulated with IL-4.

We have previously assessed that conditioned media obtained from Meta4 organoid culture contains a number of growth factors including amphiregulin. We have also examined the effect of an autocrine factor, amphiregulin secreted from Meta4 organoids (dysplastic organoids) on the metaplasia progression using the Meta1 organoids. To examine changes in growth or behaviors of Meta1 organoids by amphiregulin, we performed an antibody-mediated neutralization assay of amphiregulin to inhibit the functional activity of the amphiregulin present in Meta4-conditioned media. Meta1 organoids were exposed to Meta4-conditioned media containing either non-specific IgG control ab or anti-amphiregulin ab for 1 week. The Meta1 organoids cultured in Meta4-conditioned media with non-specific IgG showed increased budding structures compared to Meta1 organoid with control media. However, no significant changes were observed in the Meta1 organoids cultured in Meta4-conditioned media with either non-specific IgG or anti-amphiregulin ab. These results indicate that amphiregulin is not the autocrine factor released from the dysplastic Meta4 cells, which promotes metaplasia progression.

This task is now completed.

Subaim 1B: Major Task 1:
This task was completed in Year 2 and published in *Nature Communications*.

Specific Aim 2:

Major Task 1: Animal approval and breeding
This task was completed in Year 1.

Major Task 2: Subaim 2A
We have generated single cell RNA-sequencing data from control, and Mist1-Kras;Sox9-EGFP mice, 1 and 4 months after induction of Ras activation in chief cells. The data have been applied to alignment, filtering, and data visualization pipelines to compare SPEM (1 month Kras induction) and dysplasia (4 month Kras induction). The data demonstrate increases in biomarkers associated with SPEM at 1 month indicating Kras induction during this period produces a transcriptomic signature consistent with SPEM. Interestingly, new populations of SPEM-associated changes in endocrine cells and associated inflammatory cytokines may be a contributing factor to SPEM induction and progression to dysplasia. Interestingly, there were no overt differences in the SPEM (1 month) cell clustering and the dysplasia (4 month) cell clustering, despite clear dysplasia in the histology of 4-month Kras induced mice. We attribute this to two potential issues, 1) is that the mouse used for single cell RNAseq had less induction of Kras compared to the littermates used for histological analysis, or 2) the number of cells that were able to be obtained from the 4-month induced mice were less abundant and thus smaller but significant changes were not able to be observed. We have induced additional mice for the 4-month dysplastic condition. More biological replicates and cells will be applied to scRNAseq analysis in 3-weeks at the end of this long induction.

Major Task 3: Subaim 2B

Mist1-Kras;Sox9-EGFP mice that were Kras-induced for 1- or 4-months demonstrate increased numbers of Sox9EGFP cells by histology. Unfortunately, there was an unexpected pre-mature death in the 4-month Kras-induced mice which substantially reduced the number of mice that reached the full 4-month time point. The mice that did reach the endpoint were used for the single cell RNAseq analysis, precluding FACS analysis for CD44v9:Sox9EGFP studies proposed. Alternative breeding strategies were employed to increase the numbers of control and Mist1-Kras;Sox9-EGFP mice available for the long 4-month induction. Currently we have sufficient numbers of mice that will reach the endpoint of the 4-month Kras induction in 3-weeks. While this task was partially derailed, we have been able to make progress on the methodological aspects that are required once the mice become available for FACS analysis of CD44v9:Sox9EGFP cells. Progress was made on downstream analytics to evaluate transcriptomic and functional changes in cells isolated from metaplastic/dysplastic state. Automated Cell Raft Array (CRA) analyses and collection has been optimized to increase the number of single cells that can be evaluated for clonal organoid growth and collection. We have developed a highly sensitive RNAseq method to analyze individual clonal dysplastic organoids derived from single cells isolated from the various subpopulations of cells isolated from dysplastic mice. Using a dysplastic organoid line derived from Mist1-Kras mice, we have been able to demonstrate substantial differences in transcriptomic signatures of single dysplastic organoids that derive from a single cell isolated from parental dysplastic organoids. These data suggest heterogeneity in cells that contribute to dysplasia and the trajectory of proliferation and differentiation. These analyses will be applied to the CD44v9:Sox9EGFP from Mist1-Kras mice in 3 weeks when the 4-month timepoint ends. We anticipate the increased throughput of the automated method will allow enough dysplastic organoids to be used for transcriptomic analyses and functional analyses by transplantation.

Professional development:

Drs. Goldenring, Choi and Magness have received the following new grant:

DOD Peer Reviewed Cancer Research Program Impact Award
CA190172
Co-Principal Investigators
07/01/2020 – 06/30/2023

Dr. Choi has received the following new grant:
NIH NCI Merit Award
R37 CA244970
Principal Investigator
07/10/2020 – 6/30/2023

Training development:

Dr. Jimin Min has received the DOD Peer Reviewed Cancer Research Program Horizon Award
CA191242
Principal Investigator
07/01/2020 – 06/30/2022

Funding from this proposal has been used to train pre-doctoral student, Jarrett Blitton. He has developed an F31 proposal based on the topics related to this proposal. Planned submission: 12/8/2020

4. Impact

Our results have demonstrated that we can produce gastric organoids from metaplasia in mice that can be passaged continuously and still maintain their original characteristics. This makes these organoid cultures a unique resource for understanding the properties of precancerous lesions in the stomach. In addition, our investigations have shown that we can isolate putative cancer stem cell population from metaplastic mouse organoids and perform clonal analyses. In the future in studies beyond the scope of the present investigations we hope that our experience with culturing mouse metaplastic organoids will allow us to use similar techniques to isolate, culture and characterize metaplastic organoids from humans as powerful models of pre-cancer in the human stomach.

5. Changes/Problems

None

6. Products

PUBLICATIONS:

1. GOLDENRING, J. R. (2018) Pyloric metaplasia, pseudopyloric metaplasia, ulcer-associated cell lineage and spasmolytic polypeptide-expressing metaplasia: reparative lineages in the gastrointestinal mucosa. J. Pathology. 245(2):132-137.

2. Pinzon-Guzman, C., Meyer, A.R., Wise, R., Choi, E., Muthupalani, S., Wang, T.C., Fox, J.G., Goldenring, J.R. (2018) Evaluation of Lineage Changes in the Gastric Mucosa Following Infection With Helicobacter pylori and Specified Intestinal Flora in INS-GAS Mice. J Histochem Cytochem. 2019 Jan;67(1):53-63

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5. Min, J., Vega, P.N., Engevik, A.C., Williams, J.A., Yang, Q., Patterson, L.M., Simmons, A.J., Lau, K.S., Magness, S.T., GOLDENRING, J.R., and Choi, E. (2019) Heterogeneity and dynamics of active Kras-induced pre-neoplastic lineages from mouse stomach. Nature Commun. 2019 Dec 5;10(1):5549.

6. Meyer, A.R., Engevik, A.C, Willet, S.G., Williams, J.A., Zou, Y., Massion, P.P, Mills, J.C., Choi, E., and Goldenring, J.R. (2019) Cystine/Glutamate Antiporter (xCT) is required for chief cell plasticity after gastric injury. Cell Mol Gastroenterol Hepatol. 2019;8(3):379-405.

7. Riera, K.M., Jang, B., Min, J., Roland, J.T., Yang, Q., Fesmire, W.T., Camilleri-Broet, S., Ferri, L., Kim, W., Choi, E., Goldenring, J.R. (2020) Trop2 is upregulated in the transition to dysplasia in the metaplastic gastric mucosa. J Pathol. 2020 Jul;251(3):336-347.

MEETING ABSTRACTS:

1. FASEB 2017. Understanding tumorigenic potential of metaplasia using a novel metaplasia organoid system. Eunyoung Choi, Amy C. Engevik, James R. Goldenring.

2. DDW 2018. Selumetinib, a MEK inhibitor, suppresses active kras inducedmetaplasia progression toward gastric neoplasia. Eunyoung Choi, Amy C. Engevik, James R. Goldenring.

3. AACR 2018. Progression and regression of gastric preneoplasia through MEK inhibition. Eunyoung Choi, Amy C. Engevik, James R. Goldenring.

4. DDW 2018. Evaluation of mucosal lineage changes in the mouse gastric mucosa following infection with helicobacter pylori and intestinal flora. Carolina Pinzon-Guzman, Rachel Wise, Eunyoung Choi, Sureshkumar Muthupalani, Timothy C. Wang, James G. Fox, James R. Goldenring.

5. DDW 2018. Amelioration of metaplasia and re-emergence of normal gastric lineages after treatment of H. pylori-infected gerbils with a MEK inhibitor. Sachiyo Nomura, Qing Yang, Tomohiko Yasuda, Takeshi Toyoda, Eunyoung Choi, Eiji Uchida, Yasuyuki Seto, James R. Goldenring.

6. Keystone 2019. Heterogeneity of active Kras-induced gastric pre-neoplasia in mouse stomach. Eunyoung Choi, Paige Vega, Jimin Min, Qing Yang, Alan J. Simmons, Ken Lau, Scott T. Magness, James R. Goldenring.

7. DDW 2019. Trop2 upregulation in mouse pre-neoplastic stomach lineages. Katherine Riera, Eunyoung Choi, James R. Goldenring.

8. DDW 2019. Identification of putative cancer stem cells in gastric precancerous lesions. Jimin Min, Scott Magness, James R. Goldenring, Eunyoung Choi.

9. DDW 2019. Cystine/Glutamate Antiporter (XCT) is required for chief cell plasticity after gastric injury. Anne Meyer, Amy C. Engevik, Eunyoung Choi, James R. Goldenring.

11. Keystone 2019: A high throughput cell culture platform for phenotypic analysis of metaplastic gastroids at single cell resolution. R. Jarrett Bliton, Eunyoung Choi, James R. Goldenring, Scott Magness

12. ISSCR 2020. Evaluation of clonal organoid heterogeneity at single gastroid resolution. R. Jarrett Bliton, Eunyoung Choi, James R. Goldenring, Scott Magness

7. Participants & Other Collaborating Organizations

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Funding Support:	NIDDKD, Department of Veterans Affairs

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Nearest person month worked:	1.8
Contribution to Project:	Co Investigator, oversees metaplastic gastric organoid preparation and implantation into mice
Funding Support:	NIDDKD, Department of Veterans Affairs, AACR, DOD

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Contribution to Project:	Postdoctoral Felllow, development and characterization of metaplastic gastric organoids
Funding Support:	DOD

Name:	Scott Magness, PhD
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Funding Support:	NIDDKD, NCI, DOD

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Funding Support:	DOD

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Contributio to Project:	Technical Support, Development of mouse models, Cell Raft Arrays, and clonal gastroid analyses
Funding Support:	DOD

8. Special Reporting Requirements

None

9. Appendices

None