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14. ABSTRACT

During the funding period, we have generated a number of novel mouse models and genome editing tools to identify the cellular origins of liver cancer. These new tools have helped identify genes involved in regulating liver tumorigenesis. Thus, we have made progress towards advancing basic knowledge on the origin of liver cancer.

15. SUBJECT TERMS

Liver cancer, hepatocyte progenitor cells, Tbx3, CRISPR-Cas9

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TABLE OF CONTENTS

Page

1. INTRODUCTION

The purpose of this project is to investigate whether liver cancer arises from Hepatocyte Progenitor Cells (HPC)s through the disruption of important genetic and cellular regulators of HPC function. During the funding period, we have generated a number of novel transgenic mouse models and genome editing tools to identify the cell of origin of liver cancer. These new tools and the studies conducted with them have lead to the exciting discovery that HPCs express a transcription factor, Tbx3, that acts as a guardian of cell cycle entry and epithelial to mesenchymal transition (EMT) in hepatocytes. When cell cycle entry and EMT is dysregulated, as a consequence of tumor suppressor loss and ectopic Tbx3 expression, tumorigenesis is initiated. Therefore, our studies have informed us of genes specifically involved in regulating both normal liver tissue homeostasis and liver tumorigenesis, and have advanced our understanding of the origin of liver cancer. We are currently writing our results into a manuscript, which we are planning to submit for publication.

Specific Aims:

Aim 1: Determine if liver cancer arises from hepatic progenitor cells.
Aim 2: Define the molecular function of TBX3 in hepatic progenitor cells and liver cancer cells.
Aim 3: Identify novel genetic regulators of liver proliferation and tumorigenesis.

2. KEYWORDS

Liver cancer, hepatocyte progenitor cells, Tbx3, CRISPR-Cas9.

3. ACCOMPLISHMENTS

Research

Specific Aim 1: Determine if liver cancer arises from hepatic progenitor cells.

To determine if HPCs are the cell of origin in adult derived liver cancer, we designed two genetic lineage tracing mouse lines. One included an Axin2-CreERT2; Rosa 26-mTmGflox line, that allows us to trace the fate of HPCs. We also generated an Albumin-CreERT2; Rosa 26-mTmGflox mouse line which traces all hepatocytes during development of liver cancer. Our original goal was to induce tumorigenesis in these mouse lines using a liver carcinogen, di-ethylnitrosamine (DEN), and then trace the fate of different liver cells using the genetic lineage tracing during cancer development.

We succeeded in obtaining ACURO approval (Milestone achieved on July, 25th, 2017), and generated a colony of Axin2-CreERT2; Rosa 26-mTmGflox and Albumin-CreERT2; Rosa 26-mTmGflox mice needed for Aim1's experiments. However, upon DEN treatment of these animals we failed to see tumor growth. This is possibly explained by work done by a previous study demonstrating that mice of a C57BL/6 background are resistant to the tumor inducing properties of DEN [1]. Our lineage tracing mice have a mixed background which is majority C57BL/6. We suspect that the background of our lineage tracing mice maybe a reason why DEN treatment did not induce tumors.

We predicted the background of our mice may reduce DEN induced tumorigenesis in our initial Projective Narrative, and proposed an alternative approach to switch to a different mouse model system. In these animals, the MET gene, a driver of hepatocellular carcinoma, is under the control of rtTA and doxycycline. Expression of MET in all hepatocytes leads to HCC development in about 60% of mice [2]. However, upon receiving these mice from a collaborator, we failed to detect the MET transgene in the colony. Fortunately, our work on Aim 3 has lead to the development of another approach to induce liver cancer.

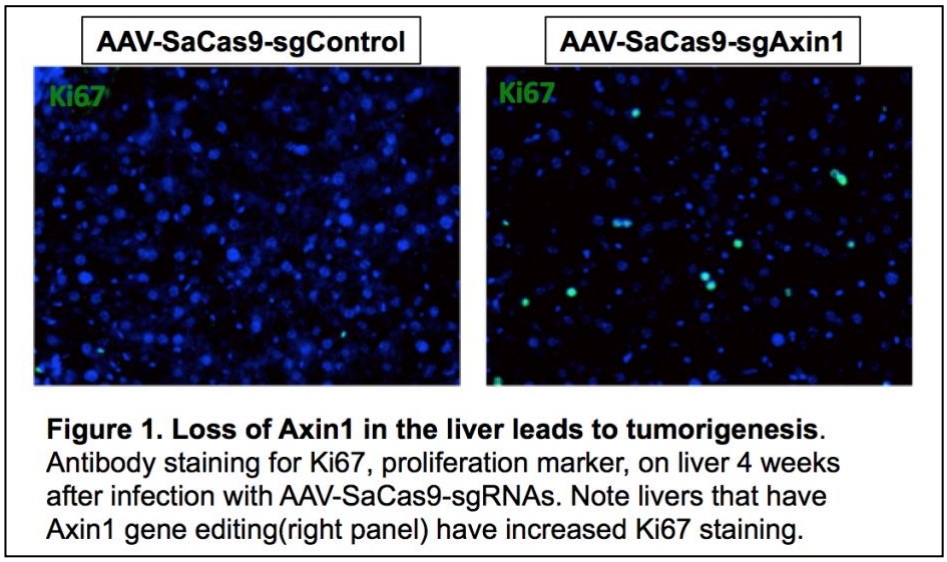

Figure 1. Loss of Axin1 in the liver leads to tumorigenesis. Antibody staining for Ki67, proliferation marker, on liver 4 weeks after infection with AAV-SaCas9-sgRNAs. Note livers that have Axin1 gene editing(right panel) have increased Ki67 staining.

Briefly, using the AAV-Cas9-sgRNA system in Aim 3, we have been able to design an sgRNA against Axin1, a potent tumor suppressor in the liver. Hepatocellular carcinoma arises in the liver 1 year after loss of Axin1 [3]. Injection of mice with the AAV-Cas9-sgAxin1 lead to upregulation of the proliferation marker Ki67, 4

weeks after infection (Figure 1). This increase in Ki67+ cells has also been observed in the livers of Axin1 knockout mice 3 months after knockout, and maybe a marker of initiation of tumorigenesis since cell proliferation is a very rare event in the normal liver even after AAV infection [4]. Our plan was to use the AAV-Cas9-sgAxin1 gene editing system to induce cancer in Axin2-CreERT2; Rosa 26-mTmGflox and Albumin-CreERT2; Rosa 26-mTmGflox lineage tracing mice to determine whether liver cancer arises from HPCs or other liver cells.

Since working with this system, the scientific community has given feedback that has helped us improve our system further. One piece of feedback included the concern that the Axin2-CreERT2 allele contains a genetic deletion of one of the *Axin2* alleles. Axin2 is a known tumor suppressor [4], and therefore haploinsuffciency of Axin2 may indirectly impact our studies on tumorigenesis. We, therefore, switched to another HPC lineage tracing system, an Axin2-rtTA (transgene); Teto-Cre; Rosa 26-mTmGflox line. This system contains a transgene for Axin2-rtTA, and therefore does not lead to loss of Axin2 protein. We are still working on characterizing tumor development in these models after gene editing of Axin1 by using AAV-Cas9-sgAxin1. Since it has taken some time to mate in all the mouse genetic alleles and it takes a year for tumorigenesis to occur with loss of Axin1[3], we were not able to reach final results or conclusions for this aim within the funding period given the numerous technical struggles we faced. However, working through these struggles led to the development of the Axin2rtTA lineage system. This system has been very useful for testing the molecular function of Tbx3 in the liver in Aim 2 (see Figure 4).

Specific Aim 2: Define the molecular function of Tbx3 in hepatic progenitor cells and liver cancer cells.

Tbx3 is a transcription factor that is expressed by both HPCs and liver cancer cells. Loss-of-function studies have suggested that Tbx3 controls cell identity and proliferation of both normal liver cells and liver cancer cells. However, it is not clear how Tbx3 controls these important functions. To further investigate the molecular function of Tbx3, our goal was to conduct chromatin immunoprecipitation (ChIP) sequencing on normal liver cells and liver cancer cells to determine which genes Tbx3 binds to. Furthermore, we proposed to confirm Tbx3 regulation of these candidate genes by genetic knockout strategies in normal liver and liver cancer cells. We completed these tasks within the funding period, and also further developed our findings with additional experiments to confirm function of target genes. Interestingly, our studies have demonstrated that Tbx3 inhibits cell cycle repressors, E2F7 and E2F8, thus permitting liver cells to progress through the cell cycle. The results, developments and conclusions of these studies are detailed below, and are currently being developed into a manuscript that we are planning to submit for publication in the near future.

Initial ChIP-sequencing in both normal liver cells and liver cancer cells (Hepg2 cells) revealed Tbx3 binding peaks at enhancers regions of key cell cycle regulators E2F7 and E2F8 (Figure 2A,B). We further confirmed binding of Tbx3 to these E2F7/8 enhancer regions by ChIP qPCR using qPCR primers targeting the enhancers target sites identified in the ChIP-sequencing (Figure 2 C) and by developing a luciferase reporter assay for the E2F7/8 enhancer regions (Figure 2D). Interestingly, when cells with the E2F7/8 enhancer luciferase reporters were transfected with a dominant negative version of Tbx3, E2F7/E2F8 luciferase activity was increase compared to control cells with normal Tbx3 expression (Figure 2 D), suggesting that Tbx3 binds to the enhancer regions to repress expression of E2F7/E2F8.

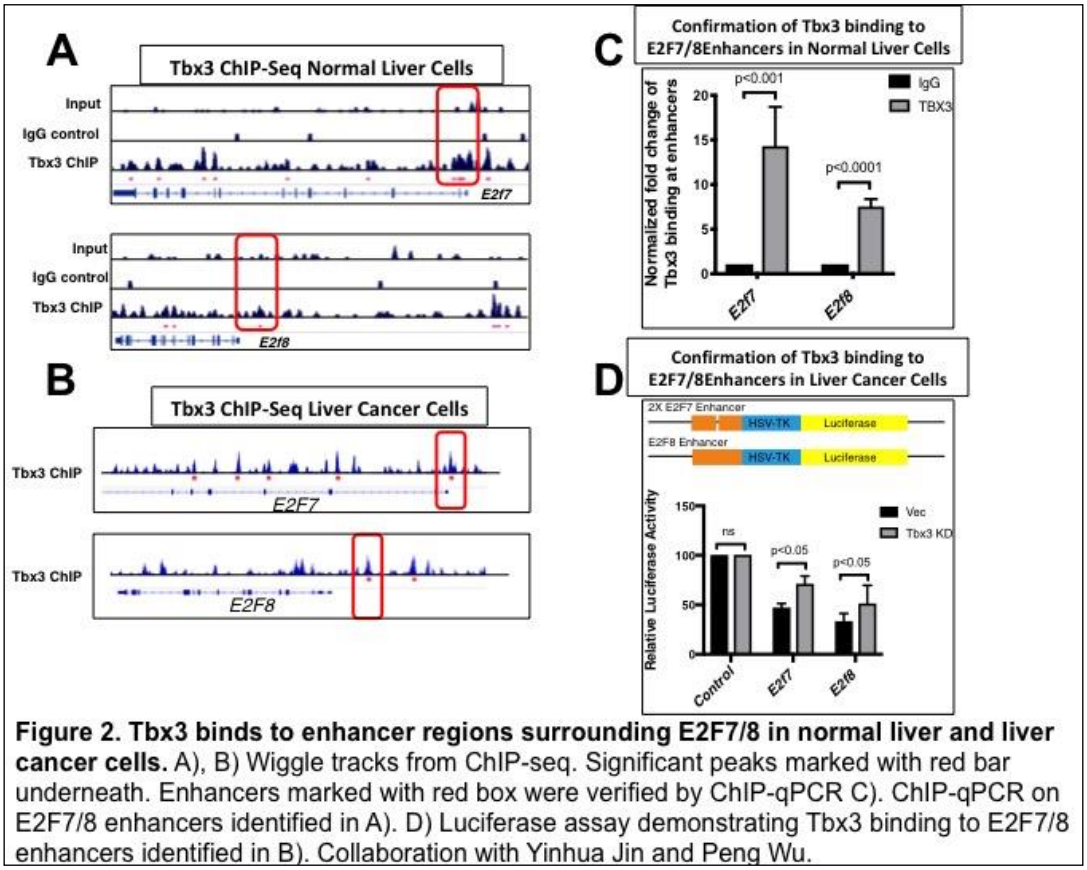

Figure 2. Tbx3 binds to enhancer regions surrounding E2F7/8 in normal liver and liver cancer cells. A), B) Wiggle tracks from ChIP-seq. Significant peaks marked with red bar underneath. Enhancers marked with red box were verified by ChIP-qPCR C). ChIP-qPCR on E2F7/8 enhancers identified in A). D) Luciferase assay demonstrating Tbx3 binding to E2F7/8 enhancers identified in B). Collaboration with Yinhua Jin and Peng Wu.

To further confirm that Tbx3 binds to the enhancers of E2F7/8 to repress expression of these genes, we conducted genetic knockout studies of Tbx3 in liver cells. Livers from Tbx3 control and mutant mice were isolated and tested for Tbx3 and E2F7/8 expression by qPCR (Figure 3). Tbx3 expression was near absent in livers isolated from Tbx3 mutant mice compared to Tbx3 control mice, which had

significant expression of Tbx3. In contrast, E2F7/8 expression was significantly increased in Tbx3 mutant livers compared to control livers, supporting the conclusion that Tbx3 represses E2F7/8 in the liver (Figure 3A). Furthermore, we developed a Tbx3 overexpression system by transfecting primary hepatocyte cultures with a sleeping beauty construct expressing Tbx3 (Figure 3B). In this overexpression system, Tbx3 expression was increased by 60 fold compared to control hepatocytes. However, E2F7/E2F8 expression was decreased in Tbx3 overexpressing hepatocytes (Figure 3C). Together, these results support the role of Tbx3 to repress expression of the cell cycle regulators E2F7/E2F8.

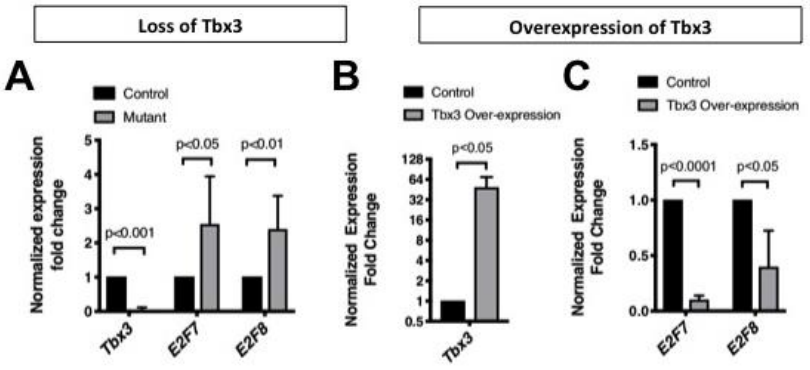

Figure 3. Tbx3 represses E2F7/8 in liver cells. A)qPCR for Tbx3, E2F7 and E2F8 on Tbx3 control and mutant livers. B) Generation of Tbx3 overexpression system in primary hepatocyte culture. Over-expression system induces 60 fold increase in Tbx3. C) qPCR for E2F7 and E2F8 in control and Tbx3 over-expression hepatocytes described in (B). Collaboration with Yinhua Jin.

Since E2F7/8 acts to repress cell cycle progression [5], we next tested whether Tbx3 functions to regulated liver cell proliferation. We first mated in our Tbx3 knockout mouse line to our Axin2rtTA lineage tracing system, developed in Aim 1, to simultaneously induce Tbx3 loss and trace HPC proliferation (Figure 4 A). We treated these mice for 1 week with Doxycycine (Dox) water to simultaneously knockout Tbx3 and lineage trace the fate of HPCs. Right after Dox treatment, T0, HPC expansion, represented in this system by the expansion of GFP+ cells, was limited to a single layer of cells surrounding the central vein. In contrast, 2 weeks after Dox treatment, wild type HPCs (CT) expanded outward from the central vein across the liver lobule. Strikingly, loss of Tbx3 (Tbx3 mutant) repressed expansion of GFP+ cells. We also confirmed Tbx3's role in regulated liver cancer cell proliferation by introducing our dominant negative Tbx3 mutant into Hepg2 cells. Cells transfected with the dominant negative Tbx3 failed to form colonies compared to Hepg2 cells with normal Tbx3 (Figure 4D).

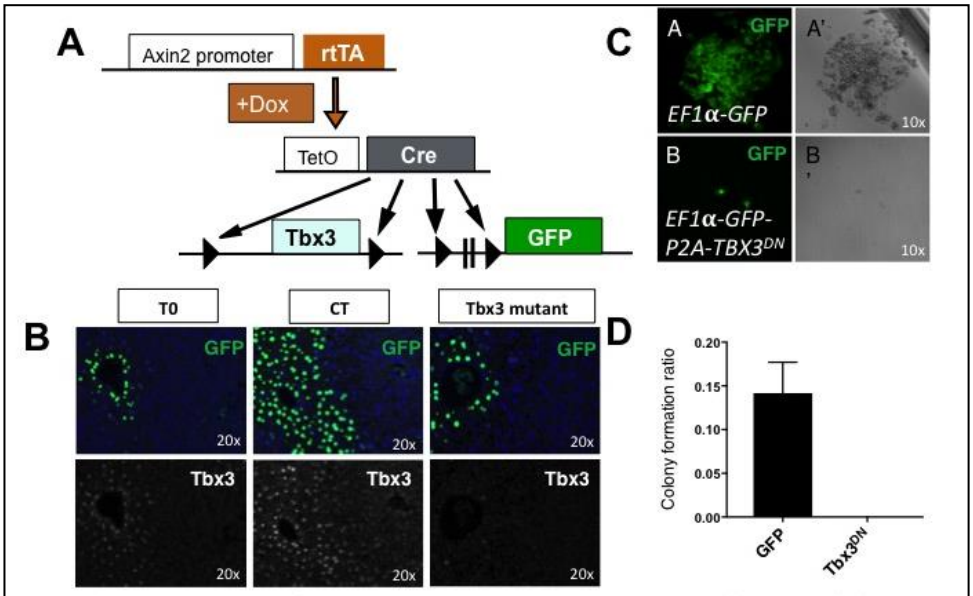

Figure 4. Tbx3 is required for normal liver and liver cancer cell proliferation. A) Genetic system to both mutate Tbx3 and trace cell proliferation in mouse liver. B) Lineage tracing of control and Tbx3 mutant cells in system describe in A). Tbx3 mutant cells fail to proliferate. C) System to inactivate Tbx3, through dominant negative mutation, in liver cancer cells. D) Colony formation quantification between liver cancer cells with normal Tbx3 or dominant negative Tbx3. Collaboration with Eric Rulifson.

In summary, our results support the role of Tbx3 in promoting liver cell proliferation by repressing cell cycle repressors E2F7/8.

Specific Aim 3: Identify novel genetic regulators of liver proliferation and tumorigenesis.

Our previous RNA sequencing analyses of HPCs allowed us to identify a list of genes that may play a role in regulating HPC growth or identity, but whose function in the liver is currently unknown. Our goal in this aim was to create a crispr-cas9 genome editing *in vivo* system to directly test the function of these candidate genes and determine if they played critical roles in liver proliferation and tumorigenesis. Figure 5 demonstrates our methodology for creating this crispr-cas9 genome editing *in vivo* system for the gene *Apc*, a gene that is a potent suppressor of liver tumorigenesis and is a positive control to make sure our gene editing system worked. First, we introduced a vector containing Cas9 and our candidate sgRNAs against our target gene into NIH 3T3 cells to induce gene editing of the target gene. Following, we extracted DNA from edited cells, and performed an indel assay to verify gene editing (Fig. 5A). Finally, we introduced selected gRNAs against our target genes into an AAV8-SaCas9-sgRNA virus, and injected mice with high titers of this virus. We re-confirmed gene editing with indel assays and antibody staining. Apc deletion is known to induce ectopic expression of glutamine synthetase(GS) in the liver, and staining for GS allowed us to identify individual cells that had lost Apc through SaCas9 induced gene editing (Figure 5B-D).

Additional analyses of livers with AAV8-SaCas9-sgApc gene editing have lead to several important findings that have expanded our understanding of the role of Tbx3 in the liver (see Aim 2). One discovery was the observation that loss of Apc leads to the ectopic expression of Tbx3 in the liver (Figure 6A). Apc is a key negative regulator of the Wnt signaling pathway, one of the most commonly mutated pathways in liver cancer [6]. Therefore, our observation that Tbx3 is ectopically expressed with loss of Apc supports the conclusion that Tbx3 expression is regulated by Wnt signaling in the liver. Furthermore, ectopic expression of Tbx3, by loss of regulated Wnt signaling, may be one of the earliest signs of tumorigenesis since it allows hepatocytes that are not dividing to enter the cell cycle by repressing E2F7/8 (see results in Aim 2) thereby promoting abnormal growth. Another observation we made in the Apc mutant livers was that hepatocytes that ectopically expressed Tbx3 had lost their E-cadherin junctions (Figure 6B). Loss of E-cadherin junctions is a required process of epithelial to mesenchymal transition (EMT), an important step in the progression of liver cancer[7]. Therefore, development of the AAV8-SaCas9-

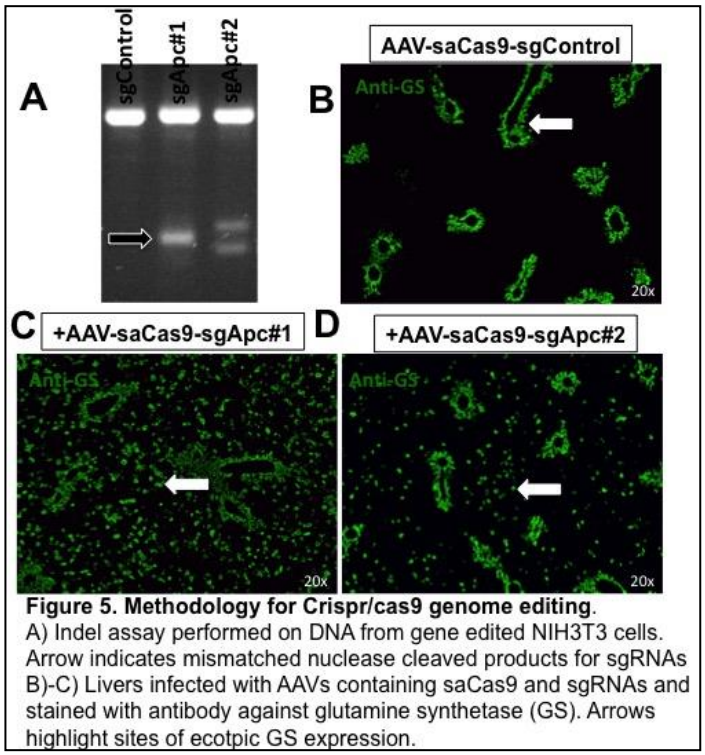

Figure 5. Methodology for Crispr/cas9 genome editing.
A) Indel assay performed on DNA from gene edited NIH3T3 cells. Arrow indicates mismatched nuclease cleaved products for sgRNAs
B)-C) Livers infected with AAVs containing saCas9 and sgRNAs and stained with antibody against glutamine synthetase (GS). Arrows highlight sites of ecotpic GS expression.

From our last report, one particularly intriguing candidate that appeared in our gene editing work was Peg3. Peg3 is highly expressed in HPCs in the normal liver, and has been associated with different metabolic roles including controlling leptin levels, body fat and obesity [8]. We predicted that loss of Peg3 in the liver may lead to fat accumulation and fatty liver disease, a condition that can increase cancer risk through impairment of hepatocyte function. We have identified an efficient sgRNA against Peg3 and are currently testing possible phenotypes of eliminating the function of the gene.

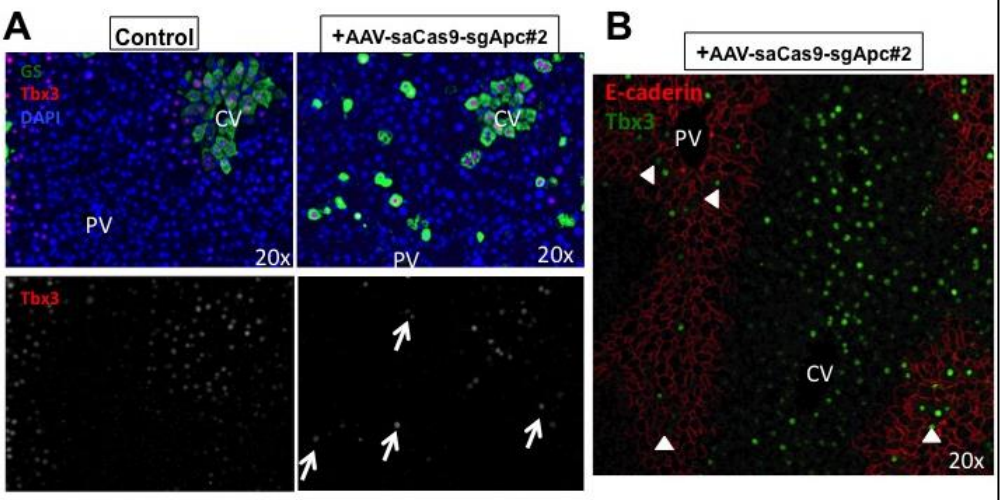

Figure 6. Loss of Apc induces ectopic expression of Tbx3 and EMT.
A) Tbx3 expression in control and Apc mutant livers (described in Fig. 5).
B) Arrows highlight Apc mutant, GS+, cells with ectopic Tbx3 expression. Apc mutant liver. Arrows highlight ectopic tbx3 cells where e-cadherin is lost.

In summary, although we were not able to complete the entire work proposed in Aim3, our work for this aim using the AAV8-SaCas9-sgApc gene editing system has strengthened our understanding of how dysregulation of Wnt signaling promotes tumorigenesis by inducing cell proliferation, through ectopic expression of theTbx3, and by inducing EMT, through repression of E-cadherin. Figure 7 summarizes these findings.

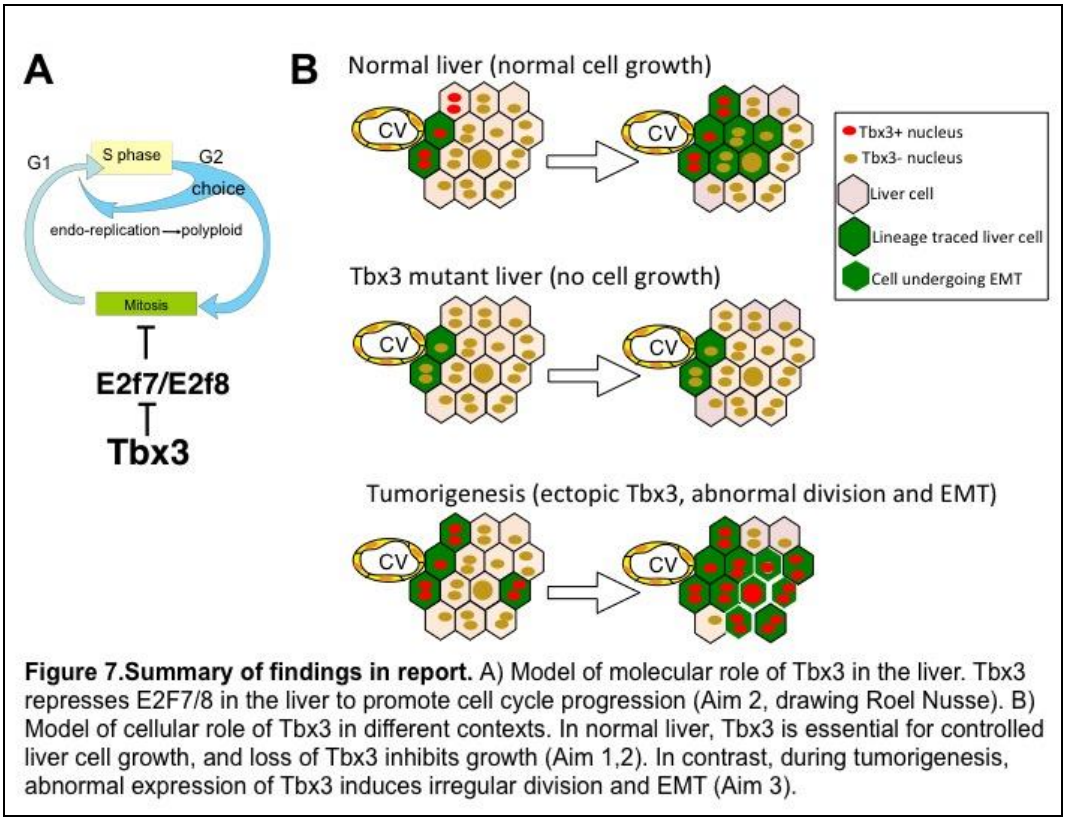

Figure 7.Summary of findings in report. A) Model of molecular role of Tbx3 in the liver. Tbx3 represses E2F7/8 in the liver to promote cell cycle progression (Aim 2, drawing Roel Nusse). B) Model of cellular role of Tbx3 in different contexts. In normal liver, Tbx3 is essential for controlled liver cell growth, and loss of Tbx3 inhibits growth (Aim 1,2). In contrast, during tumorigenesis, abnormal expression of Tbx3 induces irregular division and EMT (Aim 3).

Training

July 2017-July 2018

Since starting my training, I have attended several workshops and have given several department presentations to both improve my educational development in liver cancer research and gain feedback on my research. Specifically, I attended a Hepatobiliary Cancer Research Symposium at USCF on April 28th, 2017. I also attended, the Annual Center for Definitive and Curative Medicine Symposium at Stanford on February 3rd, 2018, where several speakers talked about the use of gene editing to treat liver disease. I gave a talk on my research at the Stanford Developmental Biology Retreat on September 13th, 2017 and have given several departmental talks for the Department of Developmental Biology and the Stem Cell Institute at Stanford through out the academic year (August 2017-June 2018).

July 2018-October 2019

Since the last reporting period, I have continued improving my training through attendance at national meetings and by giving talks on the work included in this report. Specifically, I attended the American Association for the Study of Liver Disease Meeting from November 9th-13th, 2018. I attended this meeting, instead of the American Association for Cancer Research, because it had a very large section on liver cancer with talks by several respected researchers in the field. These talks helped informed me of key questions remaining in the liver cancer field. I also attended and presented the work in this report at the UCSF Liver Center Symposium on May 30th, 2019 and at the Stanford Department of Developmental Biology Retreat on September 10th, 2019, where I gave a talk on the work. Presentations at both these events lead to valuable feedback from other researchers in the field, which helped improve the work in this report.

Plans for next reporting period

Nothing to report- Final report

4. IMPACT

The completion of these studies has broaden our understanding of the cellular, molecular and genetic basis of the development of liver cancer**.** Specifically, we have identified the role of the transcription factor Tbx3 as a guardian of liver cell division and cell identity, two key processes that are dysregulated during the development of liver cancer.

Furthermore, our studies have lead to the design of CRISPR based gene editing technology in the liver that could be utilized in the future for gene editing in diseased liver, such as the ablation of genes that enable cancer growth.

Nothing to report yet on technology transfer, society beyond science and technology

5. CHANGES/PROBLEMS

As previously stated in the ACCOMPLISHMENTS section, we underwent numerous technical challenges including inability to induce carcinogenesis in mice with DEN because of genetic background, lack of MET expression in our MET mouse transgenic line, and haploinsuffiency of Axin2 in our Axin2CreERT2 mouse line. As a consequence, we were not able to complete Aim 1.Our work for Aim 1 helped us develop a better lineage tracing model for HPCs, which helped us demonstrate that Tbx3 regulates HPC proliferation in the liver in Aim 2.

6. PRODUCTS

Nothing to Report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

Name: Dr. Yinhua Jin
Project Role: Collaborator, Roeland Nusse Lab Member
Nearest Person month worked: 5
Contributions to Project: Collaboration to help perform/analyze results from Tbx3 ChIP-sequencing and Tbx3 overexpression assay (see Figure 2 and 3 in Accomplishments Section).
Funding Support: Howard Hughes Medical Institute

Name: Dr. Peng Wu
Project Role: Collaborator, Roeland Nusse Lab Member
Nearest Person month worked: 1
Contributions to Project: Collaboration to design Tbx3 enhancer luciferase assay (Figure 2 in Accomplishments Section).
Funding Support: Howard Hughes Medical Institute

Name: Dr. Eric Rulifson
Project Role: Collaborator, Roeland Nusse Lab Member
Nearest Person month worked: 1
Contributions to Project: Collaboration to design Tbx3 dominant negative system (Figure 4 in Accomplishments Section).
Funding Support: Howard Hughes Medical Institute

Nothing to report for changes on active other support or other organizations.

8. SPECIAL REPORTING REQUIREMENTS

None to Report

9. APPENDICES

References

1. Diwan BA., Rice JM., Ohshima M., Ward JM. Interstrain differences in susceptibility to liver carcinogenesis initiated by N-nitrosodiethylamine and its promotion by phenobarbital in C57BL/6NCr, C3H/HeNCrMTV- and DBA/2NCr mice.*Carcinogenesis* 1986: 7: 215-220.
2. Wang, R., et al., Activation of the Met receptor by cell attachment induces and sustains hepatocellular carcinomas in transgenic mice. *J Cell Biol.* 2001*:* 153(5), 1023-34.
3. Feng, G.J., Cotta, W., Wei, X.Q., Poetz, O., Evans, R., Jardé, T., Reed, K., Meniel, V., Williams, G.T., Clarke, A.R., et al. Conditional disruption of Axin1 leads to development of liver tumors in mice. *Gastroenterology* 2012: 143, 1650–1659.

4. Liu W., Dong X., Mai M., Seelan R.S., Taniguchi K., Krishnadath K.K., Halling K.C., Cunningham J.M., Boardman L.A., Qian C. et al., Mutations in AXIN2 cause colorectal cancer with defective mismatch repair by activating beta-catenin/TCF signalling. *Nat. Genet.* 2000: 26: 146-147.
5. Chen HZ, Ouseph MM, Li J, et al. Canonical and atypical E2Fs regulate the mammalian endocycle. *Nat Cell Biol*. 2012;14(11):1192–1202.
6. Perugorria, M.J., Olaizola, P., Labiano, I. *et al.* Wnt–β-catenin signalling in liver development, health and disease. *Nat Rev Gastroenterol Hepatol* 2019: 16, 121–136.
7.Brabletz, T., Kalluri, R., Nieto, M. *et al.* EMT in cancer. *Nat Rev Cancer* 2018: 18, 128–134.
8.Curley JP, Pinnock SB, Dickson SL, Thresher R, Miyoshi N, Surani MA & Keverne EB. Increased body fat in mice with a targeted mutation of the paternally expressed imprinted gene Peg3. *FASEB J.* 2005: 19, 1302–4

List of Abbreviations, Acronyms, and Symbols

ACURO – Animal Care and Use Review Office
HPC - Hepatocyte Progenitor Cell
DEN - Dethylnitrosamine
AAV- adeno-associated virus
sgRNA - single guide RNA
SaCas9 - *Staphylococcus aureus* Crispr associated endonuclease
Cas9 - CRISPR Associated Protein 9
Crispr - Clustered Regularly Interspaced Short Palindromic Repeats
NIH - National Institute of Health
RNA - ribonucleic acid
Tbx3 - T box transcription factor 3
ChIP – Chromatin Immunoprecipitation
qPCR - quantitative polymerase chain reaction
Apc - adenomatous polyposis coli
EMT – epithelial to mesenchymal transition
Peg3 - paternally expressed gene 3