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PRINCIPAL INVESTIGATOR: **Dr. Yin-Yuan Mo**

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

-University of Mississippi Medical Center, 2500 N. State St, Jackson MS 39216

Virginia Commonwealth University, 1101 E. Marshall S, Richmond, VA 23298

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U.S. Army Medical Research and Development Command
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**10. SPONSOR/MONITOR'S ACRONYM(S)**

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**13. SUPPLEMENTARY NOTES**

**14. ABSTRACT**
Diabetes mellitus is a devastating metabolic disease characterized by hyperglycemia that can occur through distinct mechanisms, such as β-cell destruction, β-cell failure, and insulin resistance in peripheral tissues. The increasing prevalence of diabetes also affects the military personnel. Several genes associated with diabetes have been identified in genome wide association studies, but their genetic validation as causative factors remains largely unexplored. In this grant proposal, we focused on one of these genes, *TGIF*, whose conditional overexpression in the pancreatic tissue led to severe hyperglycemia and diabetes. We proposed experiments to investigate how TGIF induces diabetes. The results showed that conditional overexpression of TGIF in pancreatic progenitor cells in mice resulted in diabetes by decreasing insulin production by islet β-cells. Mechanistic experiments demonstrate that TGIF suppresses insulin production by directly repressing the expression of Pdx1, the master transcription factor in β-cell, which drives expression of insulin. Loss-of-function genetic experiments demonstrated that TGIF plays an instrumental role in diabetes associated with obesity, thereby implicating TGIF as a possible target for attenuating diabetes in obese patients.

**15. SUBJECT TERMS**
**NONE LISTED**

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# 1. INTRODUCTION

Diabetes is a complex disease caused by the abnormal expression of multiple genes that govern critical aspects of pancreatic development and homeostasis. Several potential genes associated with diabetes have been identified by integrative genomic approaches, but their validation as causative factors remains to be established. In this proposal, we intended to focus our efforts on one of these candidate diabetes genes, *TGIF*, which encodes for a transcriptional repressor known to govern fundamental biological processes crucial for proper body development and maintenance of organ homeostasis throughout life. Our preliminary data showed that enforced expression of TGIF in the pancreatic epithelium in mice resulted in high hyperglycemia, reminiscent of diabetes. Based on this novel observation, we hypothesized that TGIF overexpression might affect insulin production by islets β-cells, thereby culminating in insulin insufficiency and attendant hyperglycemia and diabetes. To test this overreaching hypothesis, we proposed to conduct genetic experiments using mice harboring either conditional overexpression (*Tgif*+) or conditional knockout (*Tgif.KO*) of *Tgif* in the pancreatic tissue. We designed several approaches to conduct full analyses of the diabetic phenotype of *Tgif*+ mice, including pancreas histology, blood glucose levels, serum insulin levels, glucose tolerance, insulin tolerance and β-cell mass, proliferation, apoptosis and dedifferentiation. To corroborate the role of endogenous TGIF in driving β-cell dysfunction, we proposed to challenge *Tgif.KO* mice with high fat diet and carry out the same experiments as described earlier for *Tgif*+ mice. To delineate the molecular mechanisms by which TGIF affects β-cell homeostasis and insulin production, we designed molecular and biochemical studies aimed at elucidating whether TGIF functions to repress transcription of the *Pdx1* gene (pancreatic duodenal homeobox-1), which encodes for a master transcription factor that directly regulate synthesis and production of insulin by islet β-cells. To the best of our knowledge, this was the first study to show that TGIF plays a crucial role in β-cell function and glucose homeostasis.

## 2. KEYWORDS


- Diabetes mellitus
- Hyperglycemia
- Obesity
- Genome wide association studies
- Transcriptional co-repressor TGIF
- Pancreas-specific overexpression of *Tgif*
- Pancreas-specific deletion of *Tgif*
- Pancreatic progenitor cells
- Transcription factor Pdx1 (pancreatic duodenal homeobox-1)
- Insulin production
- Islet β-cells
- Histone deacetylase 9 (HDAC9)

# 3. ACCOMPLISHMENTS

### 3.A. What were the major goals of the project?

The observation that motivated our study on the role of TGIF in β-cell function and diabetes was serendipitous. Using transgenic mice bearing a conditionally activable *Tgif* allele, we discovered that overexpression of TGIF in the pancreatic tissue caused severe hyperglycemia. Extending our analysis to human diabetic patients using SNP-based GWAS data, we found that TGIF was significantly associated with diabetes ($p$= 0.015 and 0.008). Moreover, gene microarray analysis using public available data showed that *TGIF* is more expressed in obese diabetic patients than in obese healthy people ($p$= 0.015). In our efforts to unravel the molecular mechanisms by which TGIF affects glucose homeostasis, we found that overexpressing TGIF in the pancreatic tissue induced decreased expression of Pdx1 (pancreatic duodenal homeobox-1), a key mediator of β-cell development and insulin production. The *Pdx1* promoter possesses a conserved TGIF binding motif, raising the possibility that TGIF might function as a direct transcriptional repressor for the *Pdx1* gene. Since TGIF represses expression of many genes by recruiting HDACs to chromatin, we turned our attention on HDAC9, which we identified as a TGIF interacting partner in a yeast two-hybrid screening, and it is considered as a key regulator of β-cell function. Crucially, our SNP analysis using GWAS data clearly indicated that HDAC9 is associated with diabetes ($p$= 5.9E-6), an observation that was further supported by gene microarray datasets highlighting differential expression of HDAC9 in diabetic patients as compared to healthy people ($p$= 0.03). In light of these convergent observations, we hypothesized that TGIF may recruit HDAC9 to repress *Pdx1* expression and thereby compromises β-cell function, which in turn culminates in defective insulin production and attendant diabetes. We proposed to test this hypothesis in two specific aims:

***Specific Aim 1:*** **Achieve a comprehensive characterization of TGIF's ability to promote diabetes, with particular emphasis on blood glucose and serum insulin levels, glucose tolerance, and β-cell function and mass in mice bearing either conditional overexpression or conditional knockout of *Tgif* in the pancreatic tissue.**

***Specific Aim 2:*** **Explore the molecular mechanisms by which TGIF affects β-cell function, focusing on its ability to repress expression of the *Pdx1* gene, which encodes the master transcription factor in β-cells.**

To achieve this research proposal, we designed several experimental approaches as outlined in our statement of work:

**Major Task 1: Characterization of the diabetic phenotype of mice bearing conditional overexpression of *Tgif***

**MT1-Subtask 1:** Evaluation of hyperglycemia and serum insulin levels in mice with conditional overexpression of *Tgif* in the pancreatic tissue (*Tgif*+). Blood glucose levels will be determined using the ReliOn system and serum insulin levels will be determined using an ELISA kit.

**MT1-Subtask 2:** Glucose tolerance test using *Tgif*+ mice. Glucose (2g/kg body weight) will be injected intraperitoneally and the blood glucose levels will be determined at different times (0 to 120 min) by the ReliOn system.

**MT1-Subtask 3:** Insulin tolerance test using *Tgif*+ mice. Insulin (1U/kg body weight) will be injected intraperitoneally and the blood glucose levels will be determined at different times (0 to 120 min) using the ReliOn system.

**MT1-Subtask 4:** Evaluation of β-cell mass, proliferation, apoptosis and dedifferentiation in *Tgif*+ mice. Pancreatic tissues will be fixed in formalin, embedded in paraffin and sections will be subjected to immunofluorescence using appropriate antibodies.

**MT1-Milestone(s) Achieved:** The study will provide compelling evidence that enforced expression of TGIF can affect β-cell function, thereby leading to hyperglycemia and diabetes.

**Major Task 2: Investigate the role of endogenous TGIF in β-cell failure in *Tgif* conditional knockout mice undergoing high fat-induced diabetes.**

**MT2-Subtask 1:** Generation of experimental mice harboring pancreas-specific *Tgif* knockout (*Tgif.KO*) by crossbreeding the breeding pairs *Tgif.fl/fl;Pdx1.Cre* mice and *Tgif.fl/fl* mice.

**MT2-Subtask 2:** Exposure of *Tgif.KO* mice to high fat diet and assessment of hyperglycemia and serum insulin abundance. Blood glucose levels will be determined using the ReliOn system and serum insulin levels will be determined using an ELISA kit.

**MT2-Subtask 3:** Glucose tolerance test using *Tgif.KO* mice. Glucose (2 g/kg body weight) will be injected intraperitoneally and the blood glucose levels will be determined at different times (0 to 120 min) by the ReliOn system.

**MT2-Subtask 4:** Insulin tolerance test using *Tgif.KO* mice. Insulin (1U/kg body weight) will be injected intraperitoneally and the blood glucose levels will be determined at different times (0 to 120 min) by the ReliOn system.

**MT2-Subtask 5:** Evaluation of β-cell mass, proliferation, apoptosis and dedifferentiation in *Tgif1.KO* mice subjected to high fat diet. Pancreatic tissues will be fixed in formalin, embedded in paraffin and sections subjected to immunofluorescence using appropriate antibodies.

**MT2-Milestone(s) Achieved:** Completion of these experiments will provide strong evidence that endogenous TGIF plays crucial roles in β-cell failure under diabetic conditions resulting from obesity.

**Major Task 3: Investigate the molecular mechanisms by which TGIF represses *Pdx1* expression**

**MT3-Subtask 1:** Quantification of *Pdx1* mRNA and protein by qRT-PCR and Western blotting, respectively. We will use pancreatic tissues from *Tgif*+ or *Tgif.KO* mice and their appropriate control. We will also perform gene reporter assays using Min6 cells transfected with *Pdx1-Luc* in the absence or presence of TGIF or siRNAs targeting TGIF.

**MT3-Subtask 2:** Assessment of TGIF binding to the Pdx1 promoter by chromatin immunoprecipitation (ChIP) and pull-down assays using chromatin or protein lysates from Min6 cells or *Tgif*+ and *Tgif.KO* and their appropriate control.

**MT3-Subtask 3:** We will investigate the interaction between TGIF and HDAC9 by coimmunoprecipitation and ChIP. We will use chromatin extracts or protein lysates from Min6 cells or *Tgif*+ and *Tgif.KO* and their appropriate control.

**MT3-Milestone(s) Achieved:** Achievement of this study will provide compelling evidence that TGIF affects β-cell function through its ability to repress expression of Pdx1, the master transcription factor in β-cells. In addition, our findings will indicate whether TGIF represses *Pdx1* expression by recruiting HDAC9, a general transcriptional corepressor that plays an important role in the pathogenesis of diabetes.

**3.B. What was accomplished under these goals?**

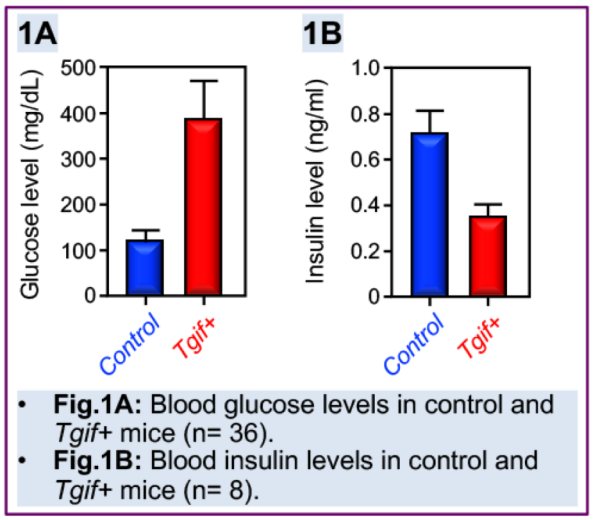
Our specific aims remained unchanged during the entire funding period. Overall, we performed the majority of experiments described in our original grant application, and the data clearly showed that TGIF expression in the pancreatic tissue plays a key role in diabetes development. Beyond this grant

proposal, our new data also allowed us to progress significantly in another ongoing project related to the mechanisms by which TGIF promotes pancreatic cancer, which is highly associated with diabetes and obesity.

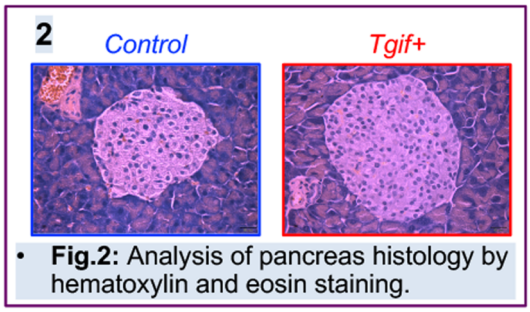
## ***Results and Key findings***

### *Pancreas-specific overexpression of Tgif suppresses insulin production*

To investigate the role of TGIF in pancreas development, we conducted genetic studies using mice with pancreas-specific overexpression of *Tgif* (*Tgif*+). Accordingly, we crossed mice bearing a Cre-activable *Tgif* transgene, *CAG-Loxp-STOP-Loxp-2HA-Tgif* (*LSL-Tgif*), with *Pdx1.Cre* mice, which express Cre recombinase in all pancreatic progenitor cells. *Tgif1*+ mice were born at the expected Mendelian ratio, showed no evidence of any gross anatomic or physiological abnormalities, and had normal weight at birth, indicating that TGIF overexpression in the pancreas glandular did not affect pancreas development. Intriguingly, measuring blood glucose of 4-week-old *Tgif*+ animals revealed severe hyperglycemia, which was associated with low circulating levels of insulin (Figures 1A and 1B). At necropsy, pancreas of *Tgif*+ mice did not show any defects in islets, as gauged by hematoxylin and Eosin (H&E) staining (Figure 2). In efforts to investigate the mechanisms behind this severe diabetic phenotype, we performed immunofluorescence experiments using anti-insulin antibody to analyze β-cell function. We detected a marked decrease in insulin staining in *Tgif*+ mice relative to wild-type littermates (Figure 3A). Noteworthy, we did not observe any difference in β-cell numbers between wild-type and *Tgif*+




- **Fig.1A:** Blood glucose levels in control and *Tgif*+ mice (n= 36).
- **Fig.1B:** Blood insulin levels in control and *Tgif*+ mice (n= 8).




- **Fig.2:** Analysis of pancreas histology by hematoxylin and eosin staining.

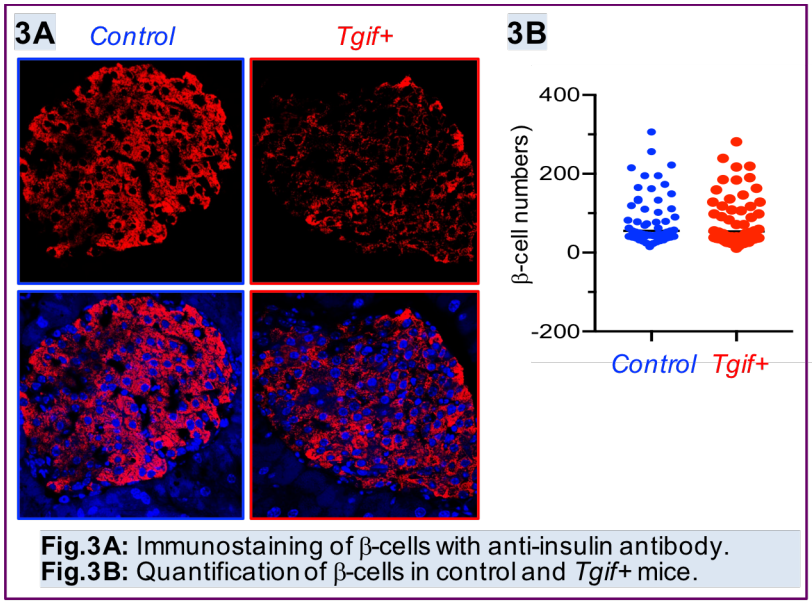
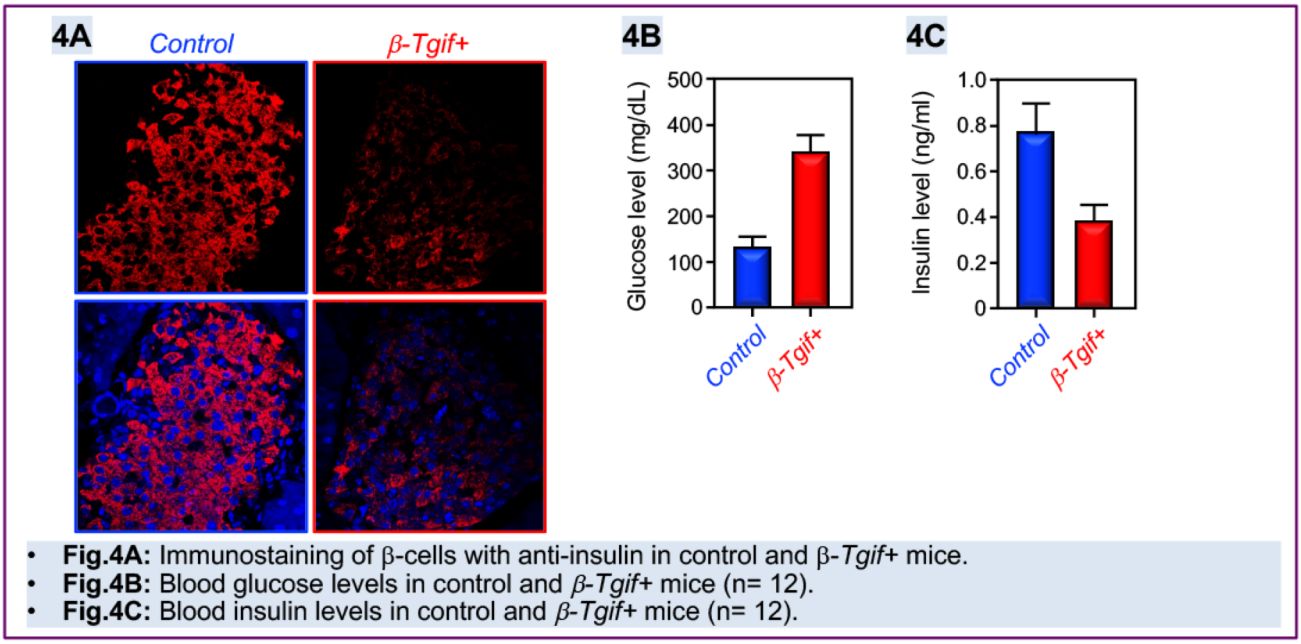



**Fig.3A:** Immunostaining of β-cells with anti-insulin antibody.
**Fig.3B:** Quantification of β-cells in control and *Tgif*+ mice.

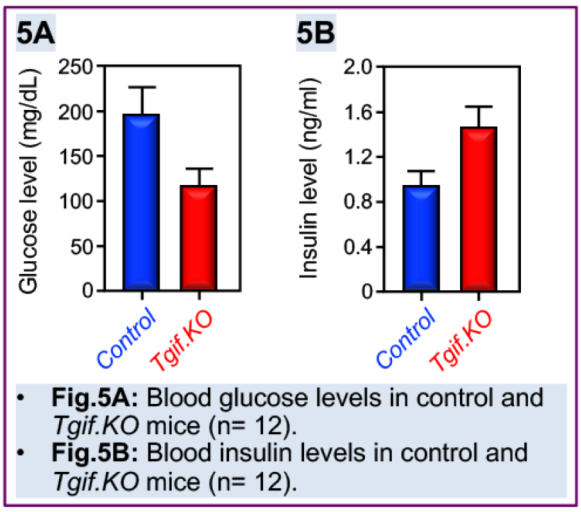
mice (Figure 3B), indicating that TGIF overexpression does not affect β-cell proliferation or survival. As such, these findings raise the interesting possibility that TGIF overexpression might block insulin synthesis. To corroborate our finding, we generated mice overexpressing TGIF in β-cells (*β-Tgif*+) by crossbreeding *LSL-Tgif* mice with *Ins2.Cre* mice. Here again, there was no change in islet morphology or size. Likewise, we were not able to see any difference in β-cell number between control and *β-Tgif*+ mice. However, we detected a marked decrease in insulin staining in *β-Tgif*+ mice when compared to wild-type littermates (Figure 4A). In support of this findings, *β-Tgif*+ mice displayed high hyperglycemia (Figure 4B), which was associated with decreased circulating insulin levels (Figure 4C). Collectively, these findings strongly suggest that TGIF overexpression drives diabetes by suppressing insulin production.




- **Fig.4A:** Immunostaining of β-cells with anti-insulin in control and β-*Tgif*+ mice.
- **Fig.4B:** Blood glucose levels in control and *β-Tgif*+ mice (n= 12).
- **Fig.4C:** Blood insulin levels in control and *β-Tgif*+ mice (n= 12).

***Pancreas-specific deletion of TGIF suppresses diabetes associated with fatty pancreas formation***

To investigate the role of endogenous TGIF in pancreatic function, we generated mice with pancreas-specific deletion of *Tgif* (*Tgif.KO*) as described in MT2-Subtask 1. As for *Tgif*+ mice, *TGIF.KO* mice were born at the expected Mendelian ratio, showed no evidence of any gross anatomic or physiological abnormalities, and had normal weight throughout postnatal life, indicating that TGIF is dispensable for pancreas development. To investigate the role of endogenous TGIF in diabetes, we challenged *Tgif.KO* mice with high-fat diet for 24 weeks, which is known to induce obesity and diabetes. Interestingly, although *Tgif.KO* mice gained more weight than wild-type mice, they showed a significant decrease in fasting blood glucose (Figure 5A). *Tgif.KO* mice also showed an increase in blood insulin levels when compared to control mice fed high-fat diet had (Figure 5B), providing further evidence that TGIF suppresses insulin production. Collectively, these experiments demonstrate that TGIF plays an instrumental role in the pathogenesis of diabetes under obesity conditions.

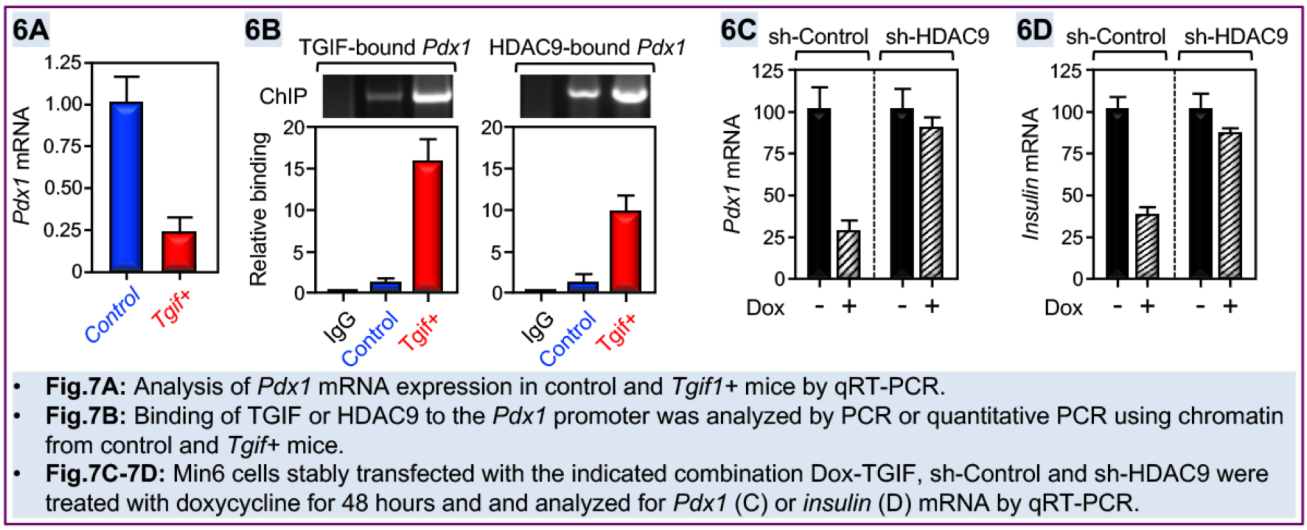



- **Fig.5A:** Blood glucose levels in control and *Tgif.KO* mice (n= 12).
- **Fig.5B:** Blood insulin levels in control and *Tgif.KO* mice (n= 12).

***TGIF functions as a direct transcriptional repressor for Pdx1***

Our preliminary data showed that pancreas-specific overexpression of TGIF led to a marked decrease in the expression of the Pdx1 protein, raising the intriguing possibility that TGIF might inhibit insulin synthesis and secretion by islet β-cells. In this study, we undertook a variety of experimental approaches to investigate whether TGIF could directly repress the expression of the *Pdx1* gene, and, if so, whether HDAC9 is involved in this process. First, we performed qRT-PCR using pancreatic tissues from *Tgif*+ and control mice, and detected a marked decrease in the expression of *Pdx1* mRNA in *Tgif*+ mice relative to control mice (Figure 6A). Second, an in-silico search identified a TGIF consensus binding site within the *Pdx1* promoter. To directly investigate whether TGIF could bind directly to the *Pdx1* promoter, we performed ChIP assays using pancreatic chromatin from *Tgif*+ and control mice. The results showed a marked increase in the binding of TGIF to the *Pdx1* promoter in *Tgif*+ mice when compared to control mice (Figure 6B). A similar increase was observed when the binding of HDAC9 to the *Pdx1* promoter was examined (Figure 6B), suggesting that TGIF might function to recruit or stabilize the binding of HDAC9 to the *Pdx1* promoter. Third, we assessed the ability of TGIF to induce expression of

endogenous Pdx1 in vitro using Min6 cells stably expressing a Doxycycline-inducible TGIF transgene (Min6-Dox-TGIF). We found that treating Min6-Dox-TGIF with doxycycline for 48 hours decreased the expression of the *Pdx1* mRNA (Figure 6C). As Pdx1 directly regulates the expression of insulin, we also examined *insulin* mRNA expression, and detected a significant decrease in Min6-Dox-TGIF cells exposed to doxycycline for 48 hours (Figure 6D). Finally, we also generated Min6-Dox-TGIF stably expression a shRNA targeting HDAC9, and found that depletion of HDAC was to block the ability of TGIF to repress the expression of both *Pdx1* and *insulin* genes (Figures 6C and 6D). Collectively, these findings provide strong evidence that TGIF functions as a direct transcription repressor for *Pdx1*, thereby supporting a model in which TGIF functions in islet β-cells to compromise insulin synthesis.




- **Fig.7A:** Analysis of *Pdx1* mRNA expression in control and *Tgif1*+ mice by qRT-PCR.
- **Fig.7B:** Binding of TGIF or HDAC9 to the *Pdx1* promoter was analyzed by PCR or quantitative PCR using chromatin from control and *Tgif*+ mice.
- **Fig.7C-7D:** Min6 cells stably transfected with the indicated combination Dox-TGIF, sh-Control and sh-HDAC9 were treated with doxycycline for 48 hours and and analyzed for *Pdx1* (C) or *insulin* (D) mRNA by qRT-PCR.

### 3.C. What opportunities for training and professional development has the project provided?

A PhD student (Creighton Friend) in my lab is currently working on the role of TGIF in diabetes and its link to other pancreatic diseases.

### 3.D. How were the results disseminated to communities of interest?

"Nothing to Report"

### 3.E. What do you plan to do during the next reporting period to accomplish the goals?

"Not Applicable"

# 4. IMPACT

**4.A. What was the impact on the development of the principal discipline(s) of the project?**

"Nothing to Report"

**4.B. What was the impact on other disciplines?**

Given that diabetes is associated with other human diseases, such as chronic pancreatitis and pancreas cancer, we explored whether TGIF could contribute to these phenotypes, which could help achieve a major research project of the lab dedicated to unravel mechanistic paradigms of these two devastating diseases. For instance, we found that *TGIF* plays key roles in pancreatitis and pancreatic ductal adenocarcinoma formation. We believe that our data shed tantalizing insights into mechanism paradigms of several pancreas-related diseases, including diabetes, pancreatitis and pancreatic ductal adenocarcinoma.

**4.C. What was the impact on technology transfer?**

"Nothing to Report"

**4.D. What was the impact on society beyond science and technology?**

"Nothing to Report"

# 5. CHANGES / PROBLEMS

**5.A. Changes in approach and reasons for change**

"Nothing to Report"

**5.B. Actual or anticipated problems or delays and actions or plans to resolve them**

"Nothing to Report"

**5.C. Changes that had a significant impact on expenditures**

"Nothing to Report"

**5.D. Significant changes in use or care of human subjects**

"Nothing to Report"

**5.E. Significant changes in use or care of vertebrate animals**

"Nothing to Report"

**5.F. Significant changes in use of biohazards, and/or select agents**

"Nothing to Report"

# 6. PRODUCTS

## 6.A. Publications, conference papers, and presentations

Parajuli P, Singh P, Wang Z, Li L, Eraganreddi S, Ozkan S, Ferrigno O, Prunier C, Razzaque, M, Xu K and Atfi A. TGIF1 functions as a tumor suppressor in pancreatic ductal adenocarcinoma, EMBO Journal 38: e101067, 2019.

Parajuli P, Thien Ly N, Prunier C, Razzaque MS, Xu K and Atfi A. Pancreatic Ductal Adenocarcinoma Triggers Selective Depletion of beta-Cells, Life Science Alliance (under revision).

## 6.B. Website(s) or other Internet site(s)

"Nothing to Report"

## 6.C. Technologies or techniques

"Nothing to Report"

## 6.D. Inventions, patent applications, and/or licenses

"Nothing to Report"

## 6.E. Other Products

"Nothing to Report"

# 7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

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

- Azeddine Atfi: no change
- Hao Mei: no change
- Parash Parajuli: no change

| | |
|---|---|
| Name: | Dr. Yin-Yuan Mo |
| Project Role: | PI (Dr. Yin-Yuan Mo became the PI of this award since I left from the University to Mississippi Medical Center. Dr. did not contribute to any experiment, conceptually or experimentally. |
| Researcher Identifier: | https://orcid.org/0000-0002-4980-0186 |

Nearest person month worked: 0 months

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

**R01CA210911 (NIH/NCI)**
4/1/2017 - 6/31/2022
Title: Targeting the TGIF/Twist1 network in osteosarcoma
Role: Azeddine Atfi, Principal Investigator
There is no overlap with DOD **PR152164**

**R01CA240484 (NIH/NCI)**
06/01/2019 - 06/30/2024
Title: Exploiting the Twist1 in network in cancer Cachexia.
Role: Azeddine Atfi, Principal Investigator
There is no overlap with DOD **PR152164**

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

Virginia Commonwealth University. Dr. Azeddine Atfi was the previous PI of this grant before he moved to Virginia Commonwealth University. A subcontract was established between the University of Mississippi Medical Center and Virginia Commonwealth University. The Department of Defense approved this subcontract.

Virginia Commonwealth University provided all required for completion of this research proposal (administration, facilities, computers, etc.).

# 8. SPECIAL REPORTING REQUIREMENTS

**8.A. Collaborative Awards:**

"Nothing to Report"

**8.B. QUAD CHARTS:**

"Nothing to Report"

# 9. APPENDICES

“Nothing to Report”