Determining the effects of TGF-β1 on *COL1A1* Expression in Hepatocellular Carcinoma

Grant Proposal

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Author Note

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Abstract

Hepatocellular Carcinoma (HCC), a major subset of liver cancer, stands as a global health concern, accounting for over 700,000 annual deaths annually. Current treatments offer limited prognostic efficacy, leading to persistent complications and tumor recurrence. A deeper comprehension of novel targets against HCC progression is imperative due to ineffective clinical treatments. Currently, many cell transduction pathways have been identified and correlated to excess Collagen I production in HCC, as Collagen I is an indicator of HCC progression; however, the involvement of Transforming growth factor-β1 (TGF-β1), which plays an important role in HCC development, and the gene encoding Collagen type I (COL1A1) in HCC remains unclear. This study aims to elucidate the previously unknown relationship of the TGFβ pathway and the COL1A1 gene in HCC by testing how TGF-β1 regulates the expression level of COL1A1 and its impact on the proliferation and migration of hepatocellular carcinoma cells. HepG2 cells were cultured and COL1A1 was knocked down via siRNA transfection to assess changes in proliferation and migration rates of HCC, in vitro. These cells were then treated with TGF-β1. Based on the findings, COL1A1 is overexpressed in HCC. Upregulation of COL1A1 facilitates the proliferation and migration of HCC cells through the TGFβ pathway. The correlation of TGF-β1 and COL1A1 upregulation introduces a novel therapeutic target against HCC, deterring HCC cell progression by reducing ambient levels of collagen I in the carcinoma tissue.

Keywords: COL1A1, hepatocellular carcinoma, Collagen I, TGF-β1, HepG2

Targeting COL1A1 Expression in TGF-β Pathways in Hepatocellular Carcinoma Through siRNA Silencing

Liver cancer is one of the most malignant types of cancer, accounting for over 700,000 annual deaths worldwide (Ferlay et al., 2015). The most common form of liver cancer is Hepatocellular Carcinoma (HCC), which has emerged as the third leading cause of cancer-associated mortality worldwide. Currently, strategies against HCC include transplantation and target drugs; however, these treatment options lack proper prognosis, causing complications and tumor recurrence to remain a common occurrence. The lack of effective clinical treatments requires a further understanding of novel targets against hepatocellular carcinoma progression.

Extracellular Matrix

The extracellular matrix (ECM), a three dimensional structure with distinct biochemical and biomechanical properties, is an important component of the tumor microenvironment. Changes in the composition of the ECM have been shown to promote tumor growth in HCC (Khalfallah et al., 2011). Collagens are essential in ECM and can regulate tumor cell behavior (Wang et al., 2020). Collagen Type I is a major component of the liver ECM and is significantly upregulated in 83.7% of human liver cancer specimens compared with their adjacent non tumor tissue (Liu et al., 2021). Excess collagen in the tumor microenvironment creates a cycle of upregulation, further elevating levels of collagen I in the HCC microenvironment. The type I collagen network helps the basement membranes interact with nearby cells, playing a crucial

role in cell migration and proliferation (Wang et al., 2020). The *COL1A1* gene, alongside another alpha1 and alpha2 chain, provides instructions for making one component of type I collagen, which is upregulated in HCC as HCC progresses towards late stage carcinoma (Alomowitch et al., 2009). The upregulation of *COL1A1* can generate a modified extracellular matrix environment that promotes the survival, proliferation, metastasis, and invasion of liver cancer cells (Wang et al., 2020). Therefore, the knockdown of the *COL1A1* gene is a promising target to reduce HCC cell progression and moderate disease progression.

TGF-β1

The process of HCC initiation, development, and progression involves various complex signaling pathways and networks, which are controlled by several oncogenes and tumor suppressor genes. Included in these pathways is the Transforming growth factor-β (TGF-β) signaling pathway, which plays important roles in several cellular processes, including proliferation and migration (Gonzalez-Sanchez et al., 2021). The TGF-β pathway's involvement in cancer progression presents as an excellent therapeutic target.

In the TGF-β pathway, Transforming growth factor-β1 (TGF-β1) binds to the TGF-β1 receptor, which induces the phosphorylation of Smad2/3 proteins. Phosphorylated Smad2/3 form a complex with Smad4, which translocates to the nucleus and regulates gene transcription through the interaction with various transcription factors (Ezzoukhry et al., 2016). TGF-β1 and Smad proteins are highly expressed in HCC cell lines and tissues, and play a significant role in the transcription of several genes, including *COL1A1* (Shi et al., 2020). It has been identified that TGF-β1 stimulates the expression of *COL1A1* and *COL1A2* genes at the transcriptional and protein level; however little is known about TGF-β1's relationship with *COL1A1* and collagen I

upregulation with respect to specific regulatory mechanisms relevant to HCC (Cutroneo et al., 2007). This study focuses on the role TGF-β1 plays in *COL1A1* upregulation in HCC via the SMAD pathway.

Project Approach

In this study, HepG2 cells will be cultured and administered with COL1A1 siRNA in vitro. The upregulation of COL1A1 and its relation to carcinoma progression will be assessed through a migration and proliferation assay. Proliferation of the HepG2 cells will be measured through a MTT cell viability assay, which assesses the number of healthy HCC cells over a period of 48-72 hours. A cell counter will also be employed for the proliferation assay. For the migration assay, a scratch assay will be employed to compare COL1A1 knock-down against a control group. mRNA levels of COL1A1 will be measured using a quantitative real-time polymerase chain reaction (qRT-PCR). The results from both assays hope to show that the knockdown of COL1A1 reduces the proliferation and migration of HCC cells.

The involvement of TGF-β1 with COL1A1 upregulation in HCC will be assessed by treating HepG2 cells with TGF-β1. Expression of COL1A1 mRNA after 48h, 72h, and 96h of TGF-β1 treatment will be assessed using qRT-PCR. The confirmation that COL1A1 is upregulated after TGF-β1 treatment validates novel HCC targets in the TGF-β1 -COL1A1 pathways.

Section II: Specific Aims

This proposal's objective is to uncover the underlying molecular mechanisms of HCC by analyzing the association of TGF-β1 and COL1A1 in HCC progression. This study seeks to elucidate the precise involvement of COL1A1 genes within the TGF-β and Smad signaling pathway in HCC proliferation and migration. To unravel the relationship between COL1A1

expression and HCC proliferation and migration, understanding the functional implications of the COL1A1 gene is crucial. Because COL1A1 is responsible for encoding the α1 chain of type I collagen–a major extracellular matrix component of the liver–it plays a large role in the progression of liver cancer. The upregulation of COL1A1 promotes the proliferation and metastasis of HCC cells through cell transduction pathways (Hayashi et al., 2014).

While some transduction pathways, such as the FAK-Src pathway, have been well-studied in HCC, the relationship between COL1A1 and TGF-β1 remains unclear in HCC (Wang et al., 2020). The long-term goal of this study is to draw a correlation between TGF-β downstream pathways and the COL1A1 gene to communicate the potential of COL1A1, TGF-β1, and Smad 4 as a novel therapeutic target. By effectively knocking out TGF-β1/Smad factors responsible for the upregulation of the COL1A1 gene, it can be hypothesized that the proliferation and migration rates of HCC will be reduced.

Specific Aim 1: Identify the extent to which directly knocking down COL1A1 plays a role in the migration and proliferation of HepG2 cells. The effects of COL1A1 knockdown will be identified through depleting COL1A1 mRNA using short interfering RNAs (siRNAs).

Specific Aim 2: Assess the role of TGF-β1 in COL1A1 upregulation in HCC by treating HepG2 cells with TGF-β1.

Specific Aim 3: Reduce HCC cell migration through siRNA-induced knockdown of Smad pathways to silence COL1A1 expression. The silencing of the COL1A1 gene will decrease levels of collagen I within the HCC cell microenvironment.

The expected outcome of this work is to be able to identify the extent to which inhibiting TGF-β1 and Smad pathways with siRNA can reduce mRNA levels of COL1A1 to remove excessive levels of collagen I from the ECM of HCC.

Section III: Project Goals and Methodology

Relevance/Significance

HCC is one of the most prevalent and aggressive forms of liver cancer worldwide. Understanding the molecular mechanisms driving its progression is crucial for developing effective therapeutic strategies. Due to the global commonality of liver cancer and the lack of effective treatments, the overall survival rate for HCC is 5 years (Wang et al., 2020). The large population this disease severely affects makes it necessary to understand the molecular mechanisms of the transduction pathways in hepatocellular carcinoma to identify novel therapeutic targets.

Because collagen is the most abundant protein in the human body and can regulate the microenvironment and behavior of cells, it plays an important role in the progression of HCC. In a study conducted by Zheng et al. (2017), collagen I expression was upregulated in 83.7% of HCC cell samples, suggesting that the production of collagen plays an important role in HCC progression. A transduction pathway involved with HCC-related collagen I is TGF-β. TGF-β1 promotes type I collagen secretion in many organs and cellular models, including the liver (Ezzoukhry et al., 2016). The downstream pathways of TGF-β are also involved with Collagen I activity. Important downstream factors of the TGF-β pathway are the Smad transcription factors. Members of the Smad family, including Smad2 and Smad 4, play a large role in activating the promoter region of the COL1A1 gene (Sysa et al., 2009). Due to the important role

collagen I and TGF-β pathways play in HCC promotion, investigating the interaction between TGF-β and COL1A1 expression in HCC provides valuable insights into specific molecular events contributing to tumor development and progression.

The relationship between TGF-β1 and COL1A1 upregulation also poses the potential for a novel therapeutic target. The detailed mechanisms of the complex relationship between the important transduction pathway and collagen I encoding gene serve as a gateway to elucidate previously unresearched targets against the tragic complexities of HCC.

Innovation

While transduction pathways relating to collagen I upregulation in HCC have been explored, the relation of TGF-β pathways and the COL1A1 gene remains unknown in HCC. The relationship of upregulated COL1A1 and TGF-β1—with attribution to potential downstream targets—will enable the discovery of potential therapeutic targets for HCC, in vitro.

Methodology

In Silico Analysis of Cancer Expression

COL1A1 and TGF-β1 gene expression profiling and correlative studies will be performed using the Gene Expression Omnibus (GEO) human hepatocellular carcinoma microarray dataset and The Cancer Genome Atlas (TCGA) liver cancer hepatocellular carcinoma (LIHC) cohort. Data from TCGA will be analyzed using the TCGA portal using the University of California Santa Cruz (UCSC) Xena functional genomics explorer (Ma et al., 2019).

Cell Culture

The HepG2 cells will be cultured at 37°C, in a 5% humidified carbon dioxide incubator. Cells were cultured in media composed of DMEM, 10% FBS, and 1% penicillin-streptomycin, and will be grown for 48 hours in a 10 cm culture dish. Media will be changed daily, and cells will be split weekly in accordance with assays.

Testing *COL1A1* siRNA Knockdown Efficiency

Prior to conducting the proliferation and migration assays, the knockdown efficiency of *COL1A1* siRNA will be confirmed. Reverse siRNA transfection will be utilized by plating *COL1A1* siRNA and Non-targeting control (NTC) siRNA in 12-well plates. NTC and *COL1A1* siRNA will be mixed with 6 µL of Dharmafect-1 and 1188 µL of optimem. 478.8 µL of siRNA/transfection solution composed of 50 µM NTC5 siRNA, DPBS, and Optimem will be added to each well of a 12-well dish. HepG2 cells will be transferred and resuspended in 8640 µL of 16% FBS. 720 µL of HepG2 cells will be added to each well, and cells will be incubated in siRNA/transfection solution for 72 hours at 37°C before harvesting. After treatment, cells will be harvested, and total RNA was extracted using TRIzol reagent. After following harvesting protocols, mRNA levels of *COL1A1* will be measured using a quantitative real-time polymerase chain reaction (qRT-PCR).

Cell Viability Assay

The MTT solution will be prepared by mixing MTT with culture media. HepG2 cells will be seeded in a medium containing 10% FBS 24 hours before performance of assay. 50 µL of serum-free media and 50 µL of MTT solution will be added into each well. The plate will be incubated at 37°C for 3 hours, and after incubation 150 µL of MTT solvent will be added into each well. Absorption will be read at OD=540 within 1 hour of added solvent. Cell proliferation analysis will be conducted by averaging the duplicate reading for each sample and subtracting

the culture medium background from the assay reading to get the correct absorbance proportional to cell number. siRNA knockdown efficacy will be quantified using qtPCR.

Wound Healing Migration Assay

Cells will be seeded in 6-well plates with medium containing 10% FBS, then cultured to 95–100% confluence. A scratch along the center of the confluent adherent cells layer will be made with a sterile p10 pipette tip. Cell migration images will be captured at 0, 12, 24 and 48 hours after the scratch was made under a microscope and analyzed using microscopy imaging analysis.

Western Blotting Analysis

Cellular protein lysates will be isolated using a Protein Extraction Kit. An equal amount of protein lysate sample will be loaded in each lane and subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) along with a molecular weight marker following manufacturers instructions. Relative band intensity will be analyzed using a Bio-Rad image software.

Statistical Tests

All assays will be performed in triplicate. Comparisons between control and target groups with normal distribution between the groups was tested using a t-test or one-way ANOVA test. Comparison of means without a normal distribution between the groups will be done using Mann-Whitney U-test or Kruskal-Wallis nonparametric tests. A p-value of <0.05 will be considered statistically significant.

Specific Aim #1: Identify the extent to which directly knocking down *COL1A1* plays a role in the migration and proliferation of HepG2 cells. The effects of *COL1A1* knockdown will be identified through depleting *COL1A1* mRNA using short interfering RNAs (siRNAs).

Justification and Feasibility. By first measuring proliferation and migration levels of HCC in HepG2 cells through the inhibition of *COL1A1* by siRNA depletion, we can establish a relationship between *COL1A1* expression levels and carcinoma progression in HepG2 cells.

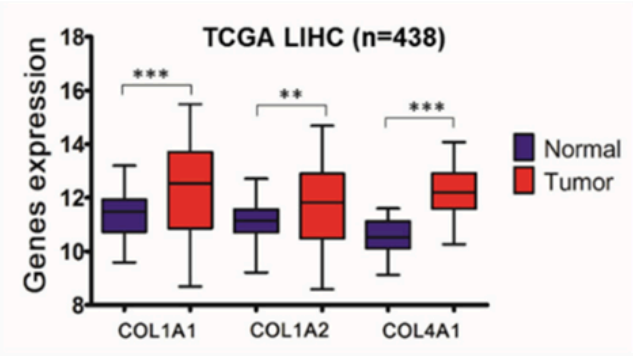

Figure 1: Graphical representation of the differential expression of COL1A1, COL1A2 and COL4A1 in normal liver or HCC samples from The Cancer Genome Atlas (TCGA) liver cancer hepatocellular carcinoma.

COL1A1 expression is elevated in HCC tumor samples, making it an important target against Collagen I upregulation (Figure 1). By depleting *COL1A1* at the mRNA level, we can reduce Collagen I upregulation in the microenvironment of HCC. Because collagen type I has been proven to be upregulated in HCC, inhibition of the COL1A1 gene will provide positive results in reducing proliferation and migration levels of HepG2 cells (Wang et al., 2020).

Expected Outcomes. The overall outcome of this aim is to establish that the depletion of COL1A1 reduces the proliferation and migration rates of HepG2 cells. This knowledge will be used for further testing involving the upregulation of TGF-β1.

Potential Pitfalls and Alternative Strategies. We expect COL1A1 mRNA levels might not entirely decrease through siRNA knockdown, as siRNA delivery may fall below a threshold required for activity. Using siRNA, a different part of the exon responsible for collagen

production can be targeted. In case of inadequate performance, COL1A1 targets can be replaced with COL2A1 or COL4A1, as both create elevated expression of type 2 and 4 collagens, respectively.

Specific Aim #2: Assess the role of TGF-β1 in COL1A1 upregulation in HCC by treating HepG2 cells with TGF-β1.

Justification and Feasibility. By treating the HepG2 cells with TGF-β1, the results will convey that TGF-β1 increases the expression of *COL1A1*. TGF-β1 promotes type I collagen secretion in many organs and cellular models, conveying that TGF-β1 may upregulate *COL1A1* (Ezzoukhry et al., 2016). Based on a study conducted by Ezzoukhry et al., 2016, mRNA expression levels of *COL1A1* were upregulated in Huh7 cells 48, 72, and 96 hours after TGF-β1 treatment (Fig. 2). Therefore, similar results can be expected when treating HepG2 cells with TGF-β1 treatment and garnering *in vitro* results for the relation of TGF-β1 with HCC progression.

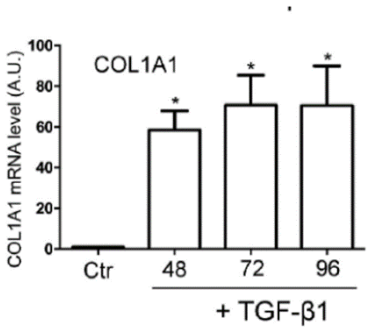

Figure 2: Huh7 cells were treated with 3 ng/ml TGF-β1. After 48, 72 or 96 h, COL1A mRNA was analyzed by qRT-PCR.

Expected Outcomes. The outcome of this aim is to draw a correlation between the upregulation of TGF-β1 and the upregulation of COL1A1. When HepG2 cells are treated with TGF-β1, COL1A1 levels will increase simultaneously.

Potential Pitfalls and Alternative Strategies. If targets and inhibitors are too toxic, focus will shift to downstream targets.

Specific Aim #3: Reduce HCC cell migration through siRNA-induced knockdown of Smad pathways to silence COL1A1 expression. The silencing of the COL1A1 gene will decrease levels of collagen I within the HCC cell microenvironment.

Justification and Feasibility. By conducting a wound healing assay to compare the knock down of downstream TGF-β targets against a control group of normal HCC cells, the results will convey that the reduction of overexpressed COL1A1 decreases carcinoma migration and potentially metastasis. To test the role downstream targets of TGF-β play in HCC, Smad 4 will be knocked down to test the effects on *COL1A1*. A study conducted by Sysa et al. (2007) in hepatic fibrosis conveyed that siRNA mediated silencing of Smad4 expression abolished the upregulation of the α1 collagen promoter (Fig. 3), reducing collagen type I at the mRNA level. Therefore, similar results can be expected in the knockdown of Smad2 and 4 in HCC to stop collagen I expression from the COL1A1 gene. The knockdown of collagen type I will help reduce HCC cell migration.

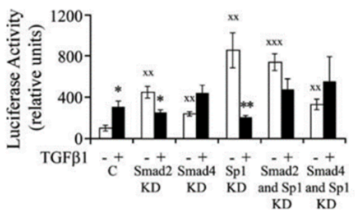

Figure 3: Effects of selective inhibition of Sp1, Smad2, Smad3, and Smad4 by siRNA on the activity of the contrasfected pGL3-2.3 a1 wt collagen promoter in LX-2 cells treated or not treated with TGFb1

Expected Outcomes. The outcome of this aim is to reduce HCC cell migration by silencing the downstream targets of TGF-β, specifically Smad4. This knowledge will be used to analyze the potential of the TGF-β1 pathway targets against COL1A1 in HCC.

Potential Pitfalls and Alternative Strategies. If targets and inhibitors are too toxic, focus will shift to downstream targets.

Section III: Resources/Equipment

The material and equipment used in these assays are:

- HepG2 cell line
- Dulbecco's Modified Eagle Medium (DMEM), supplemented with GlutaMAX™, 10% Fetal Bovine Serum (FBS), and 1% Penicillin-Streptomycin
- 1X Phosphate Buffered Saline (PBS)
- Dulbecco's Phosphate Buffered Saline (DPBS)
- 0.25% Trypsin-EDTA, phenol red
- COL1A1 siRNA
- TGF-β1
- PCR Machine
- Microscopy and imaging
- Electronic cell counter
- Centrifuge
- Plate absorbance reader
- Nanometer
- Cell imaging softwar**e**

Section V: Ethical Considerations

This project does not require working with any recombinant DNA products that would permanently integrate in the genome. All lab procedures conducted for the project will adhere to UMass Chan's protocol when dealing with cells and media.

Section VI: Timeline

Name: Charuvi Singh
Working Title of Project: Targeting TGFβ1 pathways and COL1A1 Expression in Hepatocellular Carcinoma through siRNA silencing

❑ Phase 1: Literature Research
- Background research
- Complete preliminary research
  - Read 10+ articles (October 16th)
- Identify research gaps and genes target
- Identify possible methods to gather data
- Create research question
- Identify labs that pertain to my project

❑ Phase 2: Communication
- Meet with professors and get feedback on project + what can be changed/added
- Lab training
- Gather preliminary data (in silico and in vitro)
- Conduct preliminary research
- Create methodology
- Grant writing

November - Early December

❑ Phase 3: Research and Testing
- December fair
- Conduct research and gather data needed to support hypothesis
  - Proliferation Assay
  - Migration Assay

December - January

Phase 4: Analysis + Presentation preparation
- Data and image analysis
- Prepare for February fair
  - Practice + prepare
- Figure out additional research to continue project over summer

Section VII: Appendix

Section VIII: References

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