Analysis of Yeast TIMP-3 Library

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Abstract

The extracellular matrix (ECM) is composed of a complex network of components and allows the stabilization of the cell and tissue structure. Matrix metalloproteinases (MMPs) are proteases that are involved in the breakdown of the extracellular matrix and play an important role in cell structure regulation. Alteration of the MMP gene expression can lead to diseases. MMP activity is regulated in part by tissue inhibitors of metalloproteinases (TIMPs) (Dewing et al., 2020). The aim of this study is to investigate genetic mutations that would optimize TIMP-3 activity. A yeast library (containing TIMP-3 mutations) and bacteria (containing TIMP-3 wild-types) samples were obtained from the Dr. Maryam Raesszadeh-Sarmazdeh laboratory at the University of Nevada, Reno. Synthetic dextrose plus casein amino acids (SDCAA) media plates were made to culture the yeast and to evaluate the integrity of the samples. A miniprep was used to extract the wild-type TIMP-3 DNA from bacteria, PCR was performed to amplify the DNA. Gel electrophoresis was used to verify the wild-type TIMP-3 gene. The wild-type TIMP-3 gene from the bacteria will be used as a positive control. The TIMP-3 mutation was extracted by Zymoprep in yeast. The TIMP-3 mutation was PCR'd and a gel was used to verify the gene. The next step of this study is to sequence the TIMP-3 gene in yeast with possible mutations of interest.

Introduction

The extracellular matrix (ECM) is composed of a complex network that contains components such as collegns, enzymes and glycoproteins (Paolilo and Schinelli, 2019). These components allow cells to be stabilized and form tissue structure. The ECM plays an important role in cellular function such as adhesion, proliferation, migration, and differentiation (Paolilo and Schinelli, 2019). When there is cellular damage or cellular senescence the ECM goes through remodeling where ECM components are degraded or modified. The ECM remodeling is facilitated by proteinases such as matrix metalloproteinase (MMP) (Walker et al., 2018). MMPs play an important role in degradation of ECM components, which allows cellular regeneration, migration, and differentiation to occur. Alteration of MMP gene expression can lead to abnormal degradation of the ECM causing diseases such as metastatic cancer. The tissue inhibitor of metalloproteinase (TIMP) is a regulator protein that inhibits activation of MMP (Cabral-Pacheco et al., 2020). Genetic mutations of the TIMP genes are still poorly understood. The aim of this study is to investigate genetic mutations that would enhance TIMP-3 activity.

Materials and Methods

Samples of bacterial colonies were picked and placed into Luria broth with 50 µg/mL solution of ampicillin. The bacteria were incubated for a minimum of 15 hours. The bacteria was centrifuged at 5000 rpm for 10 minutes. The supernatant was discarded and the pelleted bacteria were stored at -20℃.

A miniprep was used to extract the plasmid DNA from bacteria. The pellets were resuspended in 250 µL of P1 Buffer and pippetted to a microcentrifuge tube. Then 250 µL of P2 Buffer was added to the tube and inverted to mix. After, 350 µL of N3 Buffer was added and centrifuged at 13,000 rpm for 10 minutes. The supernatant was pipetted to a QIAprep 2.0 spin column and centrifuged for 60 seconds and flowthrough discarded. The spin column was then washed with 0.5 mL Buffer PB and centrifuged with flowthrough discarded. Spin column washed with 0.75 mL Buffer PE and centrifuged, flowthrough discarded, and centrifuged additionally for 1 minute. Then 50 µL of DNAse water was added to elute DNA, centrifuged and stored at -20℃.

To amplify the DNA isolated from the miniprep, a PCR procedure was performed. The pCHA Forward primer was reconstituted with nuclease free water. The pCHA Forward primer was diluted to make 10 µM solution. The 10 µM pCHA Reverse primer was removed from storage and thawed. The Forward and Reverse primers are shown in Figure 1. A sample size of five was used for the calculation of the MasterMix. Then 62.5 µL of 2X Q5 MasterMix was added to a microcentrifuge tube. After which 6.25 µL of both pCHA Forward and Reverse primers were pipetted into the tube, 35 µL of sterile water was added to the tube making a final volume of 110 µL. Then 22 µL of the MasterMix was pipetted into four PCR tubes followed by 3 µL of DNA and PCR was performed.

Materials and Methods Continued

To determine the amplification of the wild-type TIMP-3 DNA fragment isolated from bacteria, a 1% TAE agarose gel was made. The agarose gel was loaded with 10 µL of PCR samples and was run at 100V for approximately 45 minutes see Figure. 2.

A 1000 mL synthetic dextrose plus casein amino acids (SDCAA) media was made and autoclaved. The media was then poured into Petri dishes and stored at 4°C. The SDCAA Petri dishes were used to re-culture the yeast samples, which were incubated at 30°C for 24-48 hours. Then a 1000 mL yeast extract peptone dextrose (YPD) media was made and autoclaved. Three yeast samples were picked with sterile pipette tips from the Petri dishes and placed in sterile glass tubes with 1 mL of YPD media. The samples were then placed in a incubator shaker at 25°C at 160 RPM for 24 hours. To extract the TIMP-3 mutation DNA from yeast a Zymoprep was used. Briefly, the samples were then transferred to 1.5 mL tubes and centrifuge for 2 minutes leaving a pellet. The supernatant from each sample were discard. The pellets were then resuspended in 200 µL of Solution 1 Buffer and 3 µL of Zymolyase. The tubes were vortexed to mix.

The samples were then placed in an incubator at 37°C for at least an hour. After incubation, 200 µL of Solution 2 were added into the tubes and vortexed to mix. Then 400 µL of Solution 3 were added to the tubes and vortexed to mix. The tubes were then centrifuge at max speed for 3 minutes. The supernatant from each tube were transferred to Zymo-Spin columns. The columns were centrifuged at max speed for 30 secs. The flow through were discarded. Then 550 µL of Wash Buffer were added to the tubes and centrifuge at max speed for 2 minutes. The columns were then transferred to a sterile 1.5 mL tubes. Then 25 µL of DNase free were added to the column and incubated for 5 minutes. The columns were centrifuged to elute the DNA. DNA amplification was carried out using the same PCR parameters described previously. The PCR products were analyzed by gel electrophoresis using a 1% TAE agarose gel. The agarose gel was loaded with 10 µL of PCR samples and was run at 100V for approximately 45 minutes see Figure. 4.

Seq Name	Seq 5' to 3'
pCHA Forward	GCCTTAGCTCAACCG
pCHA Reverse	GATTTGCTCGCATATAG

Figure. 1: The Forward and Reverse PCR Primers for TIMP-3 DNA.

Above shows the forward and reverse PCR primers used to amplify TIMP-3 DNA.

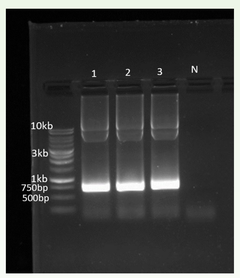

Figure. 2: Positive Control Gel Electrophoresis

Above shows the PCR amplification of the wild-type TIMP-3 DNA from bacteria. The bands are between the 750bp and 500bp marker as expected.

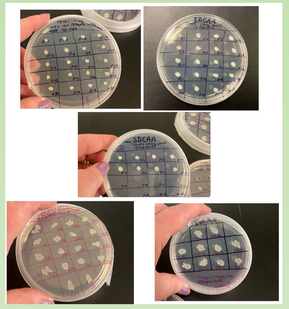

Figure. 3: Yeast Cultures containing TIMP-3 library.

Above shows 5 SDCAA plates containing yeast cultures from the TIMP-3 library.

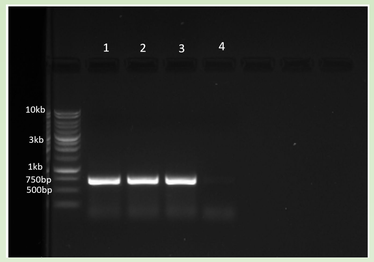

Figure. 4: TIMP-3 DNA Gel Electrophoresis

Above shows the PCR amplification of the TIMP-3 mutation DNA isolated from yeast. The bands are between the 750bp and 500bp marker, which match to the positive control in Figure. 2. Indicating the yeast samples have the TIMP-3 mutation DNA.

Results

A miniprep was used to extract the wild-type TIMP-3 DNA from bacteria. A nanodrop mass spectrometer was used to determine DNA concentration and purity. The sample 1 concentration was 382.6 ng/µl, sample 2 concentration was 428.4 ng/µl and the sample 3 concentration was 460.9 ng/µl. All samples had an AD 260/280 read of approximately 1.85, revealing high quality DNA. After the wild-type TIMP-3 DNA was extracted, the DNA was amplified by PCR. The amplified wild-type TIMP-3 DNA was verified using gel electrophoresis as shown in Figure 2.

The SDCAA culture of the yeast confirmed yeast viability, shown in Figure 3. The Zymoprep was used to extract the TIMP-3 mutation DNA from yeast. A nanodrop mass spectrometer was used to determine DNA concentration and purity. The sample 1 concentration was 7.9 ng/µL, sample 2 concentration was 13.8 ng/µL, and the sample 3 concentration was 25 ng/µL. All samples had an AD 260/280 average of 1.93 indicating DNA of significant purity. The TIMP-3 mutation DNA was amplified by PCR. The amplified TIMP-3 mutation DNA were verified using gel electrophoresis shown in Figure 4.

Discussion

The aim of this study is to investigate genetic mutations that would optimize TIMP-3 activity. A yeast model was chosen due to its unique surface display. Yeast surface display (YSD) can be used to engineer proteins and antibodies, making it suitable for designing multidomain proteins (Raeeszadeh-Sarmazdeh et al., 2019). A bacterial plasmid containing the wild-type TIMP-3 gene is used as a positive control. We were able to confirm that our bacterial samples contained the positive control. The gel shows the sample bands between the 750bp and 500bp mark on the ladder shown in Figure 2. Once yeast viability was confirmed (Figure. 3), three yeast samples were picked to determine TIMP-3 mutation DNA uptake. The three sample bands were between the 750bp and 500bp mark on the ladder shown in Figure 4. The TIMP-3 mutation sample bands matched the wild-type TIMP-3 sample bands confirming yeast uptake.

Future Works

TIMPs play an important role in the regulation of MMP. MMP alteration may lead to diseases and may exacerbate existing diseases such as cancer. The MMP protein allows the cells to have enough room to proliferate and migrate in the ECM. Alteration to MMP may allow cancer cells to metastasize (Paolilo and Schinelli, 2019). TIMP-3 has been studied as an effective tumor suppressor in malignant melanoma (Das et al., 2016).

TIMP-3 has shown to be an anti-inflammatory mediator and induced by proinflammatory agents such as phorbol myristate acetate (PMA) and anti-inflammatory cytokine transforming growth factor beta (TGF-ß) (Smookler et al., 2006). Additionally, TIMP-3 was found to inhibit cell migration and cell invasion which are both inherent in tumor metastasis (Das et al., 2016). Studies show the TIMP N-terminal domain binds and inhibits the MMP catalytic domain; however, the TIMP C-terminal domain is poorly understood in MMP inhibition (Raeeszadeh-Sarmazdeh et al., 2019).

The next step in this study is to isolate potential TIMP-3 mutation genes from the yeast library. Possible mutations will then be sequenced and compared to the wild-type TIMP-3 gene sequence. Once the TIMP-3 mutation is sequenced and a genomic library is obtained, protein function will be evaluated. Additional experiments such as SDS-PAGE, Western blotting, and ELISA could be used to determine the function of the TIMP-3 mutation. SDS-PAGE and Western blotting would allow detection of the protein and protein concentrations. An ELISA would allow detection and actions of certain proinflammatory cytokines or anti-inflammatory mediators. Protein kinetics can be used to examine the catalytic rate. More work needs to be done to translate the knowledge of TIMP-3 mutation behavior in the laboratory setting to a viable therapy in a clinical setting.

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