Developing an Optimized Cardiac Reprogramming Cocktail for Gene Therapy in Humans

Chetan Srinath[1], Laura M. Lombardi[1], Tawny Neal Qureshi[1], Elena C. Leon[1], Timothy Hoey[1], Kathryn N. Ivey[1], Huanyu Zhou[1]

1. Tenaya Therapeutics, Inc., 171 Oyster Point Blvd, 500, South San Francisco, CA

TENAYA THERAPEUTICS

Abstract

Heart failure affects an estimated 38 million people worldwide and is typically caused by cardiomyocyte loss or dysfunction. Due to the limited ability of cardiomyocytes to regenerate, *in vivo* reprogramming of non-myocytes into functioning myocardial cells using a cocktail of genes has great potential to treat heart failure. However, current human reprogramming cocktails are complex and include several different combinations of transcription factors, epigenetic regulators, kinases, microRNAs and/or small molecules, making it challenging to develop a reprogramming gene therapy for clinical application.

We have developed a novel human cardiac reprogramming cocktail consisting of only two transcription factors. This new cocktail robustly reprograms cardiac fibroblasts *in vitro,* generating cells that exhibit calcium transients and express numerous cardiomyocyte-specific genes. Importantly, these reprogramming factors can be engineered into a single AAV cassette of ~4.7kb in size. We have also identified several microRNAs that further improve the reprogramming efficiency of this cocktail and can fit into one AAV cassette together with the two transcription factors. We are currently advancing cardiac reprogramming for clinical use by developing a novel AAV variant to deliver the reprogramming factors to target cardiac cells and evaluating safety and efficacy in animal MI models.

Introduction

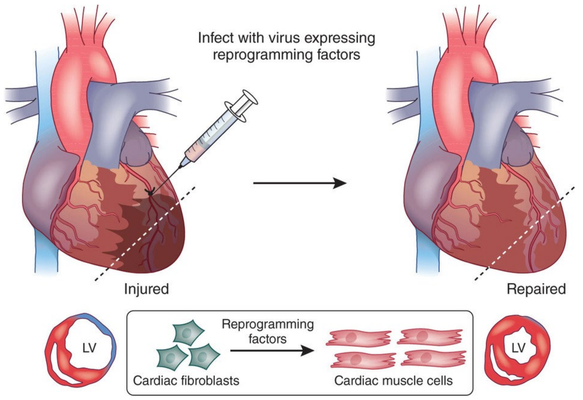


Fig 1. Overview of heart repair by *in vivo* reprogramming of cardiac fibroblasts into cardiomyocyte-like cells. Figure taken from Nam, Y. J., Song, K. & Olson, E. N. Heart repair by cardiac reprogramming. *Nat. Med.* **19**, 413–415 (2013).

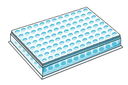
A challenge in translating cardiac reprogramming into a clinical gene therapy has been that reported human cardiac reprogramming cocktails have consisted of 3-7 genes and are thus too large to package into a single AAV. We sought to identify a minimal cocktail selected to efficiently reprogram human cardiac fibroblasts into cardiomyocytes. MYOCD is a common, required component of other multi-factor human reprogramming cocktails. We screened a human ORF library curated to contain known transcriptional regulators, as well as a human microRNA library to identify factors that combine with MYOCD to efficiently reprogram human cardiac fibroblasts and are small enough to package into a single AAV.

csrinath@tenayathera.com
chetan.srinath@gmail.com
hzhou@tenayathera.com

Methods

Isolation of primary adult human cardiac fibroblasts. For isolation of adult human cardiac fibroblasts (HCFs) adult human left ventricles were minced into small pieces and digested in cardiac fibroblast digestion medium. After digestion, the cells were filtered, pelleted and plated in fibroblast growth medium. Four days later, HCFs were frozen or re-plated for viral transduction.

Cellular reprogramming. For *in vitro* cardiac reprogramming, HCFs were seeded into culture plates in fibroblast growth medium. One day after plating cells, growth media was replaced with virus medium. One day after viral transduction, virus media was replaced by iCM media and cells were cultured in iCM media for 21 days.



Retroviral Libraries. Human ORF cDNA Library used for screening consists of approximately 1000 different transcription factors, cytokines, epigenetic regulators and nuclear receptors [ref]. The microRNA Library used was cloned from human genomic DNA and consists of approximately 400 curated human microRNAs.

Human ORF Screen Identifies ASCL1 as a Novel Cardiac Reprogramming Factor

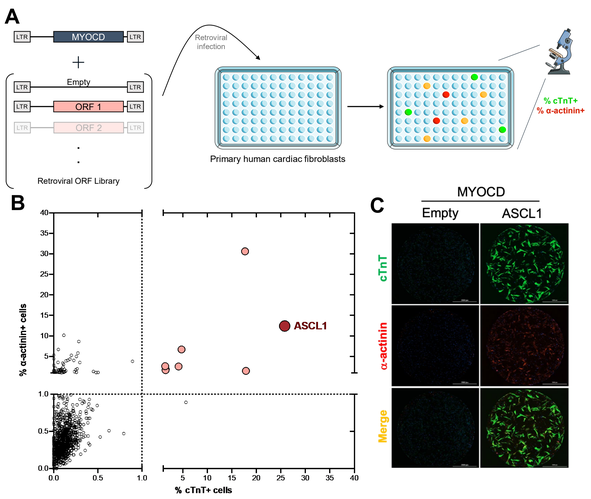


Fig 2. Identification of Activators of MYOCD-mediated Cardiac Reprogramming from a Human ORF Library screen. (A) Schematic diagram of the screening strategy for a novel human cardiac reprogramming cocktail. The strategy involved adding individual human open reading frames (ORF) representing mostly transcription factors, on top of a key human cardiac reprogramming factor, MYOCD, to identify a two-factor cocktail that can reprogram human cardiac fibroblasts (hCFs) to induced cardiomyocytes (iCMs). **(B-C)** Cardiomyocyte marker immunofluorescence of cardiac fibroblasts, 21 days after retroviral infection. MYOCD and ASCL1 efficiently induce the expression of cardiomyocyte marker proteins, α-actinin and cTnT.

MYOCD+ASCL1 (MyA) is a Novel Cardiac Reprogramming Cocktail

Fig 5. Identification of microRNA Enhancers of MyA-mediated Cardiac Reprogramming. (A) Schematic diagram of the screen strategy for microRNA enhancers of MYOCD-2A-ASCL1 (MyA) mediated human cardiac reprogramming. The small size of miRNAs allow them to fit into one AAV vector alongside MyA. **(B-D)** Cardiomyocyte marker immunofluorescence (B, C) and qPCR analysis (D) of human cardiac fibroblasts, 21 days after retroviral infection. Adding miR-133 further induces the expression of many cardiomyocyte marker genes, indicating enhanced cardiac reprogramming.

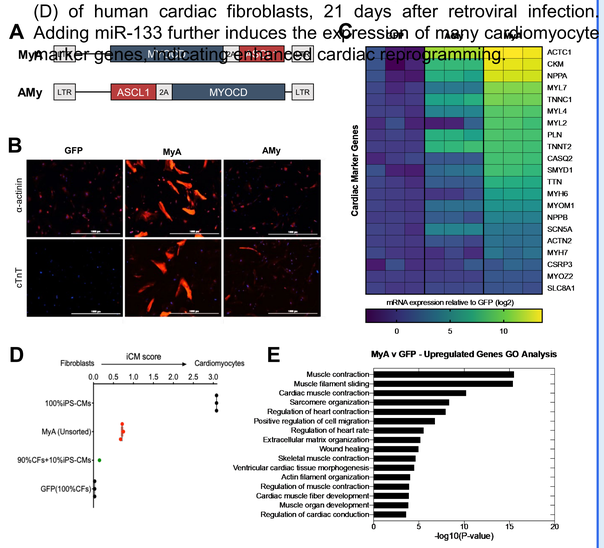


Fig 3. MYOCD and ASCL1 Robustly Reprogram Human Cardiac Fibroblasts to Induced Cardiomyocytes. (A) Bicistronic retroviral vectors designed to express MYOCD and ASCL1 using a self-cleaving 2A peptide. MYOCD and ASCL1 have the potential to fit into one AAV vector (CDS size: MYOCD 3.0kb, ASCL1: 0.7kb). **(B-C)** MYOCD-2A-ASCL1 (MyA) efficiently induces the expression of cardiomyocyte marker genes, as determined by immunofluorescence (B) and qPCR (C). **(D-E)** Whole transcriptome RNA-seq analysis of primary cardiac fibroblasts, iPSC-CMs, and MyA-reprogrammed fibroblasts. MYOCD and ASCL1 robustly induces cardiomyocyte gene expression, as determined by an iCM Score generated by linear discriminant analysis (D) and through gene ontology analysis (E).

MyA Can Fit Into One AAV Vector Capable of Cardiac Reprogramming

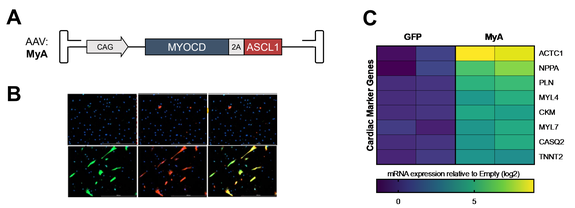
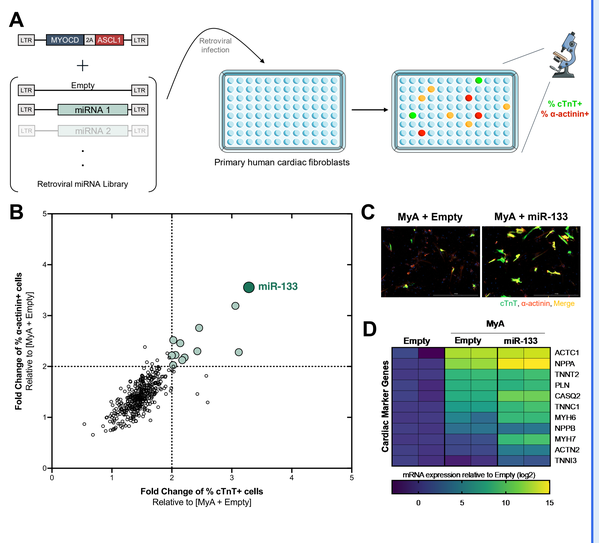


Fig 4. MYOCD and ASCL1 Can Fit into One AAV vector. (A) Bicistronic AAV vector designed to express MYOCD and ASCL1 using a self-cleaving 2A peptide. This vector is approximately 5kb in size is capable of packaging by AAV. **(B-C)** Cardiomyocyte marker immunofluorescence (B) and qPCR analysis (C) of human cardiac fibroblasts, 21 days after AAV infection. AAV:MyA induces the expression of MYOCD, ASCL1, and multiple cardiomyocyte marker genes.

miR-133 is an Enhancer of MyA-mediated Cardiac Reprogramming



Developing a Clinical Cardiac Reprogramming Gene Therapy

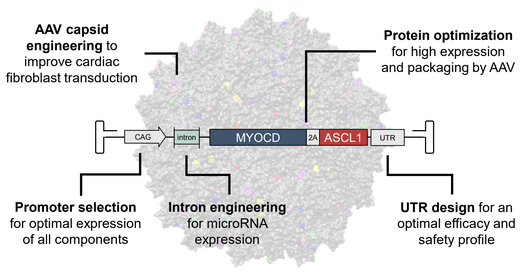


Fig 6. Developing AAV:MyA into a Clinical Cardiac Reprogramming Gene Therapy. Ongoing studies at Tenaya Therapeutics to develop AAV:MyA into a clinical gene therapy vector, and evaluate its safety and efficacy in animal models of heart disease.

Please check out Laura L. Lombardi's poster titled **'Cardiac Direct Reprogramming Gene Therapy for Ischemic Injury'** for more information on AAV engineering and animal studies. (Poster #XXX, AAV Vectors - Preclinical and Proof-of-Concept Studies).