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Modeling and Drug Discovery of Clonal Hematopoiesis of Indeterminate Potential (CHIP)-driven Cardiovascular Disease in Self-organizing Cardiac Organoids (SCO)

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Ich erkläre ehrenwörtlich, dass ich die dem Fachbereich Medizin der Johann Wolfgang Goethe-Universität Frankfurt am Main zur Promotionsprüfung eingereichte Dissertation mit dem Titel

Modeling and Drug Discovery of Clonal Hematopoiesis of Indeterminate Potential (CHIP)-driven Cardiovascular Disease in Self-organizing Cardiac Organoids (SCO)

in der/dem Institut für Kardiovaskuläre Regeneration unter Betreuung und Anleitung von Prof. Dr. Jaya Krishnan ohne sonstige Hilfe selbst durchgeführt und bei der Abfassung der Arbeit keine anderen als die in der Dissertation angeführten Hilfsmittel benutzt habe. Darüber hinaus versichere ich, nicht die Hilfe einer kommerziellen Promotionsvermittlung in Anspruch genommen zu haben.

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Zusammenfassung in Deutsch

Hintergrund und Ziel: In genomweiten Assoziationsstudien wurde ein erheblicher Zusammenhang zwischen kardiovaskulären Erkrankungen (CVD) und klonaler Hämatopoese mit unbestimmtem Potenzial (CHIP) festgestellt, was darauf hindeutet, dass TET2 (Ten-Eleven-Translokation 2) ein Schwerpunkt für die CHIP-bezogene CVD-Forschung ist. Unser Labor hat selbstorganisierende Herzorganoide (SCO) entwickelt, die die Reaktionen des menschlichen Herzmuskels auf Stressstimulation nachahmen und die zelluläre Zusammensetzung und Struktur des natürlichen menschlichen Herzens aufweisen. Ziel dieser Studie ist es, festzustellen, ob SCOs ein wirksames CHIP-Modell sind, und mögliche Medikamente für die kardiovaskuläre CHIP-Behandlung zu identifizieren.

Methoden: Um TET2-mutierte kardiovaskuläre CHIP zu untersuchen, haben wir das TET2-Herz-CHIP-Modell geschaffen, indem wir TET2 in myeloischen Zellen, die in unser im Labor hergestelltes SCO eintraten, ausgeschaltet haben. Die TET2-defiziente myeloische Zelle Infiltration, die SCO-Fibrose und die Apoptose wurden mittels Immunfluoreszenz und qPCR untersucht. Sirius Red wurde zur Untersuchung der SCO-Fibrose verwendet, und die Kalziumflussanalyse diente zur Messung der Frequenz und Amplitude der Herzkontraktion. Schließlich wurden mittels RNAseq Transkriptomveränderungen in myeloiden THP-1-Zellen sowie in myeloid infiltrierten SCO-Ko-Kulturen untersucht.

Ergebnisse: Das TET2-Herz-CHIP-Modell verursachte eine deutlich verstärkte Entzündung im SCO sowie Fibrose und mehr gespaltene Caspase-3, was zum Absterben von Kardiomyozyten führte und die cTNT-Freisetzung erhöhte. Die ausgewählten Arzneimittel verringerten die Proliferation in TET2-KD-Myelozyten, senkten pro-

inflammatorische Zytokine und erhöhten die Apoptose. Darüber hinaus zeigte das TET2-KD-Herz-CHIP-Modell, das mit spezifischen Medikamenten behandelt wurde, einen signifikanten Rückgang der Infiltration von myeloischen TET2-KD-Zellen und der pro-inflammatorischen Zytokine, der Kardiomyozytenapoptose, der Fibrose und der verringerten cTNT-Spiegel, während die Medikamentenkontrollgruppen davon nicht betroffen waren. Des Weiteren erhöhte sich in den mit Medikamenten behandelten Gruppen die Frequenz und Amplitude der Herzschläge, wie mit dem Kalzium-Transienten-Assay gemessen. Die gleichen Schlussfolgerungen wurden auch durch RNAseq-Daten bestätigt.

Schlussfolgerungen und Diskussion: Unsere Ergebnisse deuten darauf hin, dass SCOs ein effektives präklinisches Modell für die Untersuchung und Validierung von CHIP-Genen und Medikamenteninteraktionen sind. TET2-defiziente myeloische Zelle dringt in SCO ein und sezerniert proinflammatorische Zytokine, die nach unseren Erkenntnissen den Tod von Kardiomyozyten und die Freisetzung von cTNT erhöhen. In dieser Hinsicht stimmt unser TET2 cardiac-CHIP-Modell mit dem Entzündungsprofil überein, das zuvor bei CHIP-Patienten beobachtet wurde. Die für diese Studie ausgewählten positiven Arzneimittelkandidaten Clopidogrel, R406 und Lanatosid C waren jedoch in der Lage, diesen Phänotyp umzukehren, was den enormen Nutzen unseres TET2-Herz-CHIP-Modells für das Arzneimittelscreening und präklinische Validierungsuntersuchungen unterstreicht. Außerdem entdeckten wir, dass Lancatosid C unter diesen drei pharmakologischen Kandidaten ein ungedecktes Potenzial für klinischen therapeutischen Bedarf aufwies, wie von der FDA/EMA nachgewiesen, was auf einen potenziellen Wert bei der Umwidmung von Lanatosid C für die Behandlung von TET2-mutiertem kardiovaskulären CHIP hindeutet.

Summary

Summary in English

Background and Aim: Genome-wide association studies revealed a strong association between cardiovascular diseases (CVD) and clonal hematopoiesis of indeterminate potential (CHIP), highlighting one of its most common CHIP-driving mutations-TET2 (ten-eleven translocation 2), as a target for CHIP related CVD research. Our lab has established the generation of self-organizing cardiac organoids (SCO), which demonstrate the cellular composition and organization of the native human heart, and mimics human myocardial responses to stress stimulation. This project aims to examine whether SCOs would be an appropriate CHIP model and decipher promising drugs for cardiovascular CHIP treatment.

Methods: To study TET2-mutant cardiovascular CHIP, we set up the TET2 cardiac-CHIP model through a knockdown (KD) of TET2 in myeloid cells that infiltrated our lab-made SCO. Immunofluorescence and qPCR were performed to ascertain TET2-KD myeloid cell infiltration, SCO fibrosis, and apoptosis assessments. SCO fibrosis was further analyzed by immunofluorescence staining, and cardiac contractile frequency and amplitude were determined by calcium flux analysis. Finally, RNAseq was performed to analyze transcriptomic changes in drug/vehicle-treated TET2-KD myeloid cells and the TET2 cardiac-CHIP model.

Results: The TET2 cardiac-CHIP model resulted in significantly increased inflammation in SCO, accompanied by fibrosis and more cleaved Caspase-3, causing cardiomyocytes apoptosis and promoting the release of cTNT. The shortlisted drugs revealed a reduction of proliferation in TET2-KD myeloid cells, decreased pro-inflammatory cytokines, and a higher apoptosis level. Furthermore, the TET2 cardiac-CHIP model treated with selected drugs showed a remarkable decline in TET2-KD myeloid cell infiltration and pro-

inflammation cytokines, cardiomyocyte apoptosis, fibrosis, and lowered cTNT levels, while drug control groups were not affected. Moreover, the drug treatment groups improved the heartbeat frequency and amplitude accessed by the calcium transient assay. RNAseq data also validated the above findings.

Conclusions & Discussion: Our results indicate that SCOs are an efficient pre-clinical model for studying and validating CHIP genes and drug interactions. Our data revealed that TET2-KD myeloid cells invade SCO and secrete pro-inflammatory cytokines, which promote apoptosis of cardiomyocytes and the release of cTNT. In this regard, our TET2 cardiac-CHIP model matches the inflammatory phenotype previously characterized in CHIP patients. Nevertheless, this phenotype could be rescued using positive drug candidates (Clopidogrel, R406, and Lanatoside C) selected by this project, emphasizing the significant value of our TET2 cardiac-CHIP model for drug screens and pre-clinical validation studies. Furthermore, among these three drug candidates, we found Lancatoside C, as proved by FDA/EMA, showed an unmet possibility for clinical therapeutic demand, insinuating potential benefit in repurposing Lanatoside C for the treatment of TET2-mutant cardiovascular CHIP.

List of abbreviation

Abbreviation

SCO: Self-organizing Cardiac Organoids

siRNA: small interfering RNA

siScr: scrambled siRNA (negative control)

siTET2: silenced TET2 gene

hiPSCs: human-induced-Pluripotent Stem Cell

CHIP: Clonal Hematopoiesis of Indeterminate Potential

TET2: Ten-eleven translocation 2

LDL: Low-density lipoprotein

DNA: Deoxyribonucleic acid

CHF: Chronic heart failure

AMI: Acute myocardial infarction

NLRP3: NLR family pyrin domain containing 3

HSCs: Hematopoietic stem cells

DNMT3A: DNA methyltransferase 3A

ASXL1: Additional sex combs like 1

CVD: Cardiovascular disease

VAF: Variant allele frequency

SMC: Smooth muscle cells

KD: Knockdown

FDA/EMA: Food and Drug Administration and European Medicines Agency

1. Introduction

1.1 Clonal hematopoiesis of indeterminate potential

Clonal hematopoiesis of indeterminate potential (CHIP) is defined by somatic mutations in hematopoietic stem cells (HSCs), which contribute to an aggressive survival and proliferative advantage over healthy HSCs and result in an expansion in the number of mutated HSCs, progenitors, and their mature progeny **(Figure 1.1)**[4-7]. In clinical practice, the presence of a somatic mutation in the peripheral blood with a variant allele frequency (VAF) of more than 2% links to the diagnosis of CHIP[7].

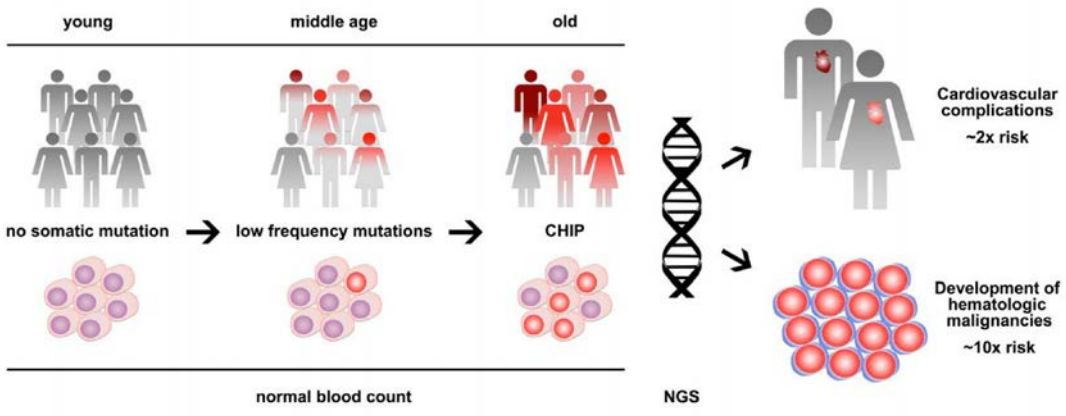


Figure 1.1 The definition of CHIP. Clonal hematopoiesis of indeterminate potential (CHIP) is a common age-related disease representing a clonal pre-phase of hematologic neoplasms with low progression risk and a significant risk factor for cardiovascular disease. Next-generation sequencing (NGS) can detect and monitor clonal hematopoiesis[8].

As a pre-stage of myelodysplastic syndromes and acute myeloid leukemia, CHIP has been proven to be associated with a grown risk of subsequent diagnosis of myeloid or lymphoid neoplasia **(Figure 1.2)**[9]. Each year, CHIP contributes a 0.5–1.0% risk of leukemia and thus increases all-cause mortality[10]. Nevertheless, in the initial CHIP stage, detecting any pathologic changes in the circulating hematopoietic cells is challenging, which causes a

more covert and progressive clonal-specific expansion of hematopoietic cells and an increased risk of hematological cancers[11, 12].

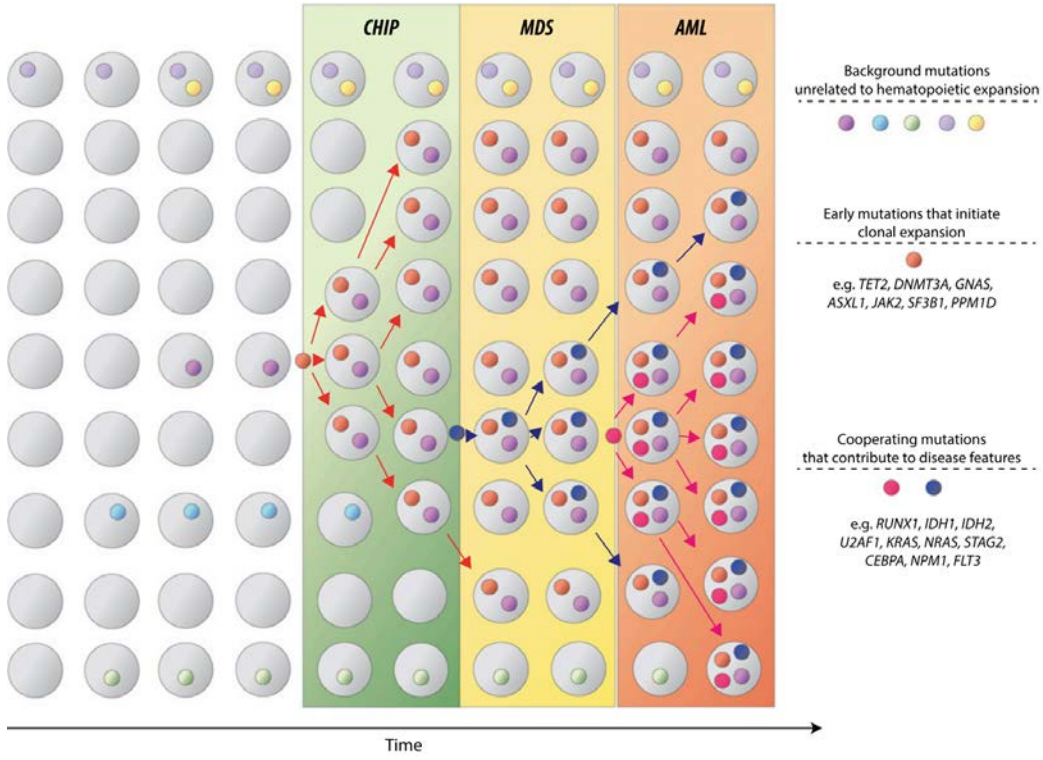


Figure 1.2 CHIP as a pre-stage for hematological neoplasms. This flow diagram shows the process from normal hematopoiesis to CHIP and then progresses to myelodysplastic syndromes (MDS) and acute myeloid leukemia (AML)[9].

DNA methyltransferase 3A (DNMT3A), ten-eleven translocation-2 (TET2), and additional sex combs like 1 (ASXL1) are the most common genetic mutations in CHIP[13]. TET2 is one of the various regularly mutated genes in CHIP and works on de-methylated DNA. It was the first gene described to manifest somatic mutations in the circulating blood cells in CHIP but without leukemia patients. More than 130 types of TET2 mutations have been found in non-cancer CHIP patients[4, 6, 11].

Introduction

Mutations of the TET2 encode an epigenetic regulator enzyme, which catalyzes the oxidation of 5-methylcytosine (5mc) to 5-hydroxymethylcytosine (5hmc) in DNA, modulating hematopoietic stem and progenitor cell self-renewal and additionally exerting non-catalytic functions **(Figure 1.3)**[14-17]. TET2 dysfunction tends to skew hematopoiesis towards the myeloid lineage[18]. Indeed, with the help of single-cell sequencing, Izzo and colleagues reported that TET2-KD HSCs enhanced monocytic clusters, marked by Ly6c2, Prtn2, and Lyz2, accompanied by a decrease of Car1 and Car2 expression in erythroid priming[18].

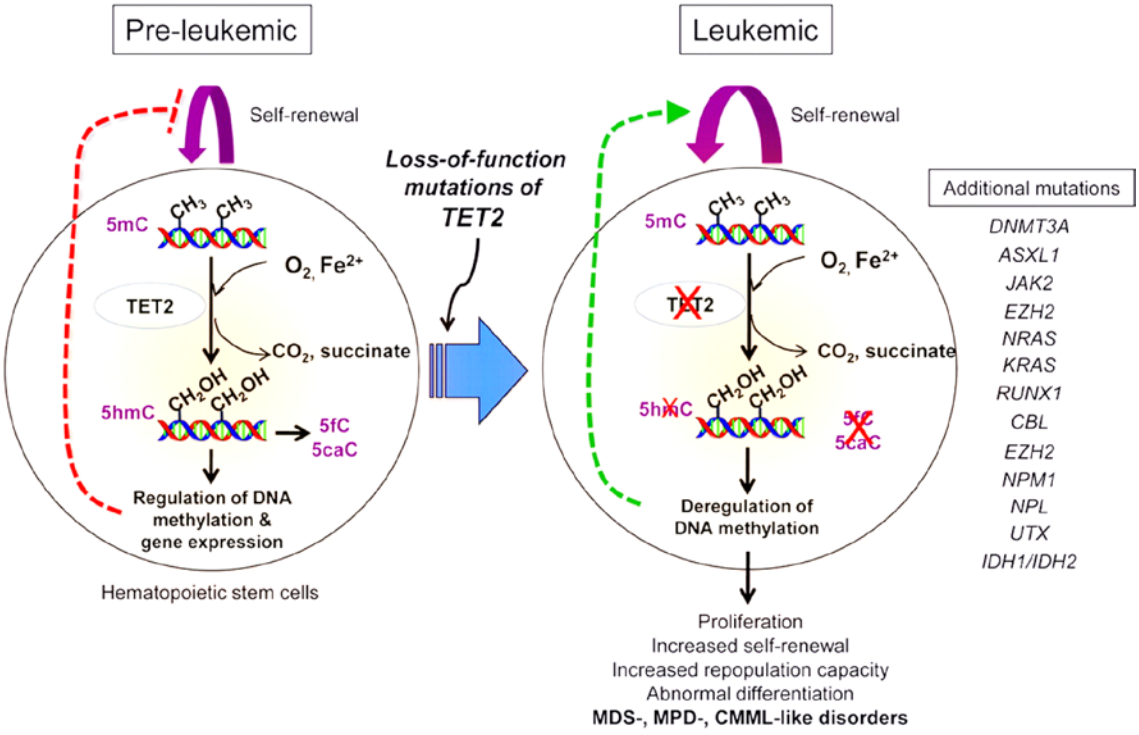


Figure 1.3 The function of TET2 and the consequence of loss-of-function TET2. TET2 responses for catalyzing the oxidation of 5mC to 5hmC, 5fC, and 5caC by regulating gene expression through DNA methylation[17].

1.2 Clonal hematopoiesis of indeterminate potential in cardiovascular disease

Cardiovascular disease and leukemia are both responsible for global mortality. However, the etiology of these two is considered to be different. The recent findings of CHIP contribute to genetic confirmation that there is an overlay between the two diseases [19, 20] and links to the development of cardiovascular diseases **(Figure 1.4)**[21-25].

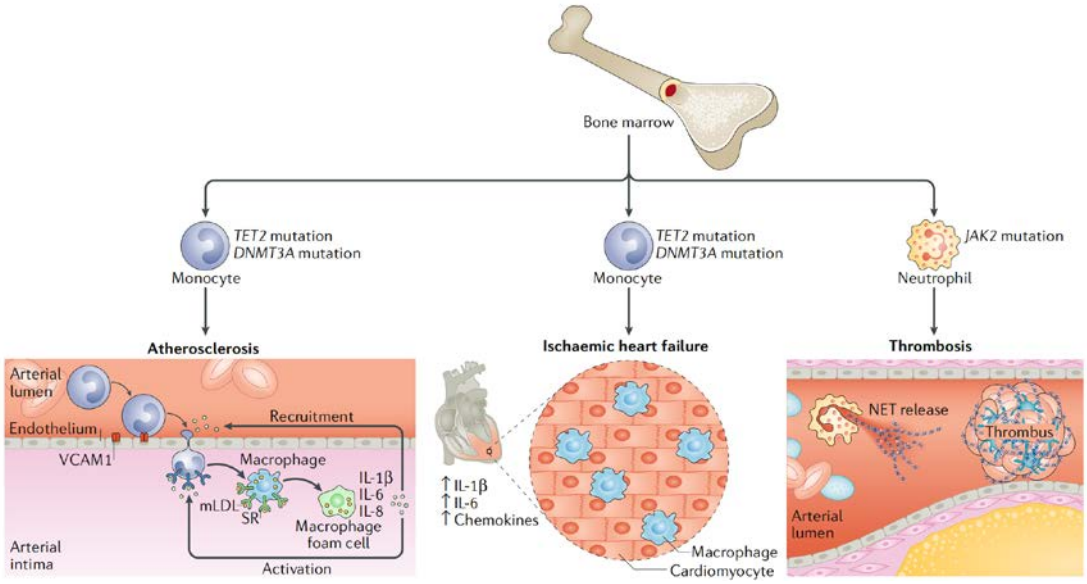


Figure 1.4 CHIP-related mutations linked with cardiovascular disease. Clonal hematopoiesis of indeterminate potential (CHIP) is associated with atherosclerosis, heart failure, and thrombosis. The mutations in TET2 accelerate the process of atherosclerosis, probably due to increased expression of inflammatory cytokines in lesional macrophages, which recruit immune cells to vessel walls, leading to the growth of the atherosclerotic plaque[17].

1.3 Modeling of clonal hematopoiesis of indeterminate potential *in vitro* and *in vivo*

1.3.1 Current status of modeling cardiovascular CHIP in mouse

Studies of experimental atherosclerosis in TET2-KD mice indicate that this condition and gene contribute to accelerated atherosclerosis **(Figure 1.5)**[21, 26, 27]. The loss of TET2 in macrophages exposed to native LDL increased the expression of inflammatory chemokines[26, 28]. Additionally, mice with hematopoietic TET2 deficiency showed

augmented IL-1β and inflammasome activation[26]. As such, the epigenetic regulatory enzyme TET2 acts as a negative transcriptional regulator of pro-inflammatory responses in macrophages, where a loss of function exacerbates NLRP3-mediated IL-1β production, accelerating atherosclerosis in the context of clonal hematopoiesis[26, 29-31]. Furthermore, TET2 deficiency worsens cardiac remodeling and function in mouse models of chronic ischemia and pressure overload hypertrophy[11, 24].

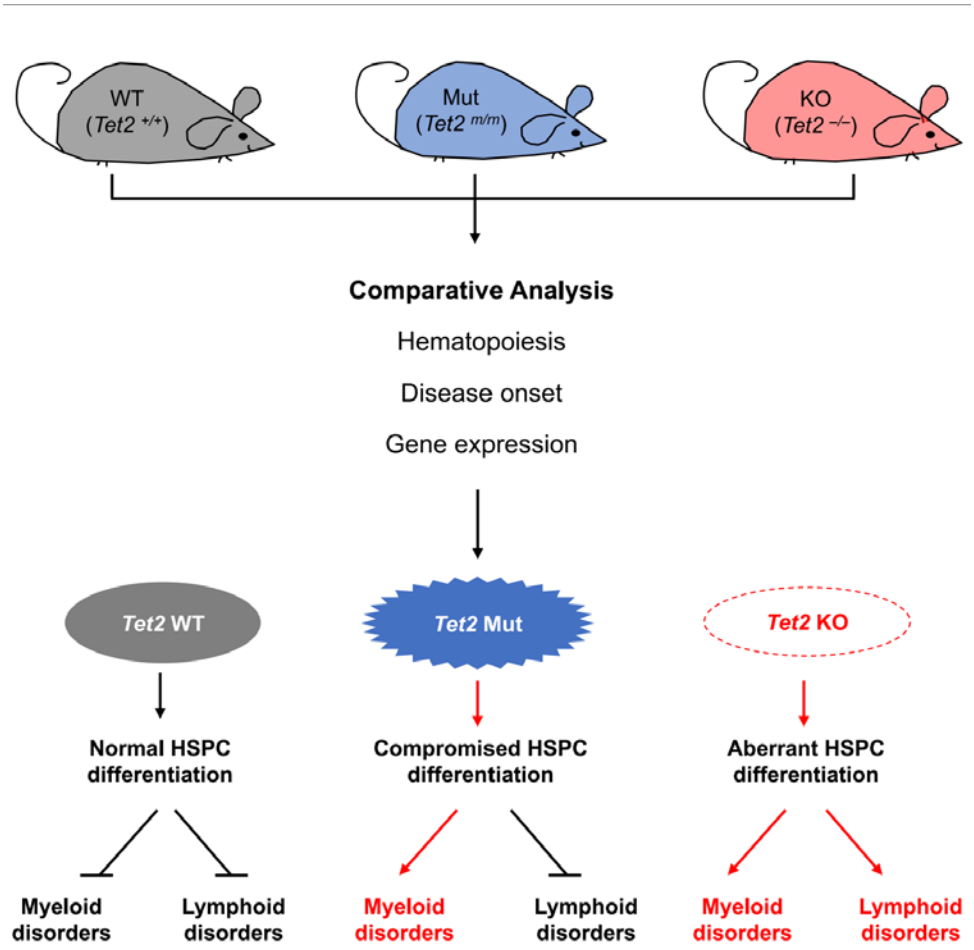


Figure 1.5 Overview of the experimental platform using TET2 WT, Mut, and KO mice. As DNA demethylase, the mutation of TET2 commonly exists in hematological disorders. However, the significance of TET2 enzymatic versus nonenzymatic roles in hematopoiesis remains undefined. Therefore, scientists generated TET2 catalytic mutant and knockout mice to study the role of TET2. As a result, the activity of the TET2 enzyme is critical for Myelopoiesis, whereas aberrant lymphopoiesis links with the total loss of TET2[27].

However, using animal models for CHIP study not only costs scientists a tremendous amount of time for ethical review and defense, as well as countless research funding for purchasing or generating models but also goes against The Three Rs principle (Replace, Reduce, and Refine) aiming to make scientific research a more effective and humane process[32].

In this regard, we set up the TET2 cardiac-CHIP model through a knockdown (KD) of TET2 in myeloid cells based on self-organizing cardiac organoids introduced from human-induced pluripotent stem cells (hiPSCs).

1.3.2 Modeling of clonal hematopoiesis of indeterminate potential in TET2-KD myeloid cells

We set up TET2-KD myeloid cells by using small interfering RNA (siRNA) to silence the TET2 gene (siTET2) in THP-1 (myeloid cells) and using non-silencing scrambled control (siScr) as the negative control **(Figure 1.6)**.

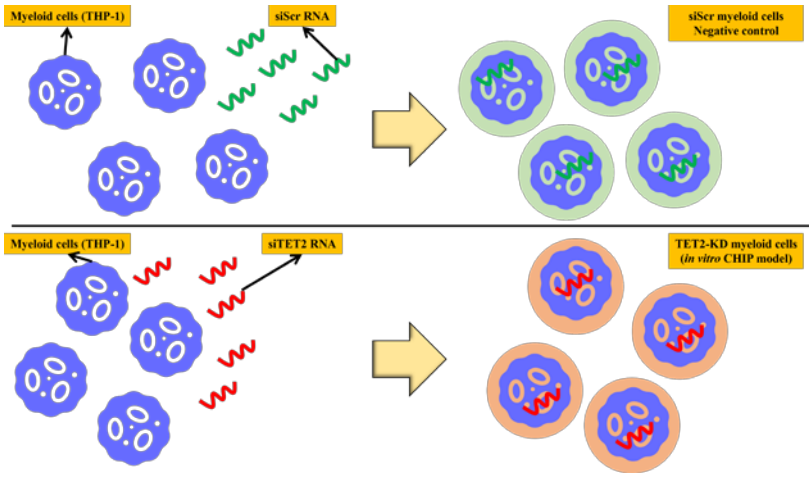


Figure 1.6 The schematic diagram for generating TET2-KD myeloid cells. We use small interfering RNA (siRNA) to silence the TET2 gene (siTET2) in THP-1 (myeloid cells) and use non-silencing scrambled control (siScr) as the negative control.

1.3.3 Introduction of hiPSCs-induced self-organizing cardiac organoids (SCO)

Thomson and colleagues successfully developed embryonic stem cells (ESCs) in 1998, which comprise all three embryonic germ layers, including smooth muscle cells, endothelial cells, cardiomyocytes, neural cells, and other cell types[33, 34]. Due to the limitation of ethics, in 2006, Yamanaka and Takahashi introduced human-induced pluripotent stem cells produced from adult somatic tissue without the requirement of embryos with the methods of overexpression Sox2, Klf4, Oct3/4, and c-Myc[35].

For decades, animal models have been the most broadly considered strategy to study various cardiac diseases. However, these models show disadvantages in species specificity differences in physiology, metabolism, and genetics, which result in inaccurate, inconstant, and unpredictable drug safety and efficacy results. Therefore, to conquer these difficulties, scientists established hiPSCs-cardiomyocytes in cardiac disease modeling,

cell therapy, and drug discovery in traditional two-dimensional monoculture (hiPSCs-derived cardiomyocytes) and three-dimensional organoids **(Figure 1.7)**[36-38].

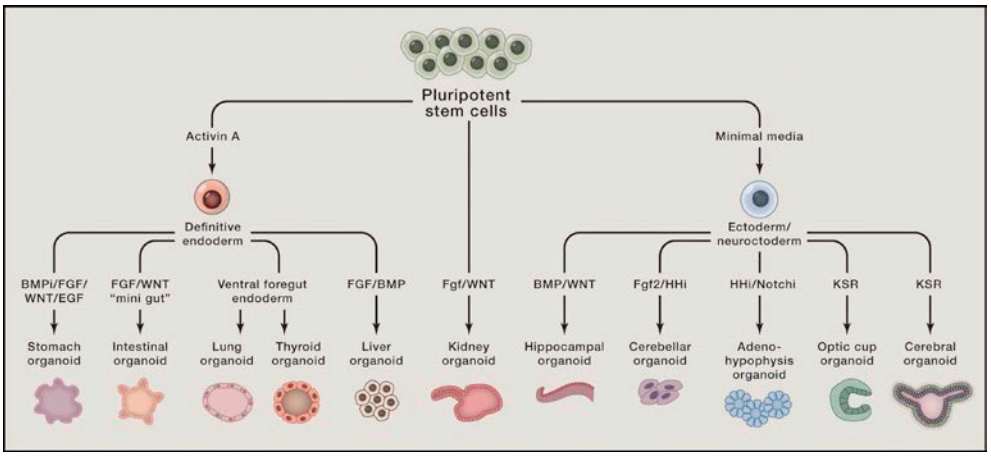


Figure 1.7 Schematic diagram of various organoids grown from pluripotent stem cells[38].

The current state of stem cell research has enabled scientists to generate self-organized tissue structures from patient-derived induced pluripotent stem cells (iPSC), termed organoids[1]. Various organoid modalities have been established for physiological and disease modeling and cell therapy in different tissues[2]. However, an unavoidable obstacle to this 3D tissue structure is the lack of a microcirculatory system, limiting the size of 100μm, and undergoing necrosis due to hypoxia when organoids exceed the limit. Although researchers have attempted to circumvent these issues with several approaches, the problem remains a stumbling block for optimal tissue modeling and mimicry[3,4].

Therefore, we have established the generation of self-organized cardiac organoids, which contain a developed and mature vascular network, and have confirmed its structural, cellular, and molecular recapitulation of the native human adult heart[39].

1.3.4 Generating TET2 cardiac-CHIP model based on hiPSCs-induced self-organizing cardiac organoids (SCO)

We set up the TET2 cardiac-CHIP model using built-up TET2-KD myeloid cells infiltrating SCO **(Method 2.2)** and regarded the non-silencing scrambled control (siScr) as the negative control **(Figure 1.8)**.

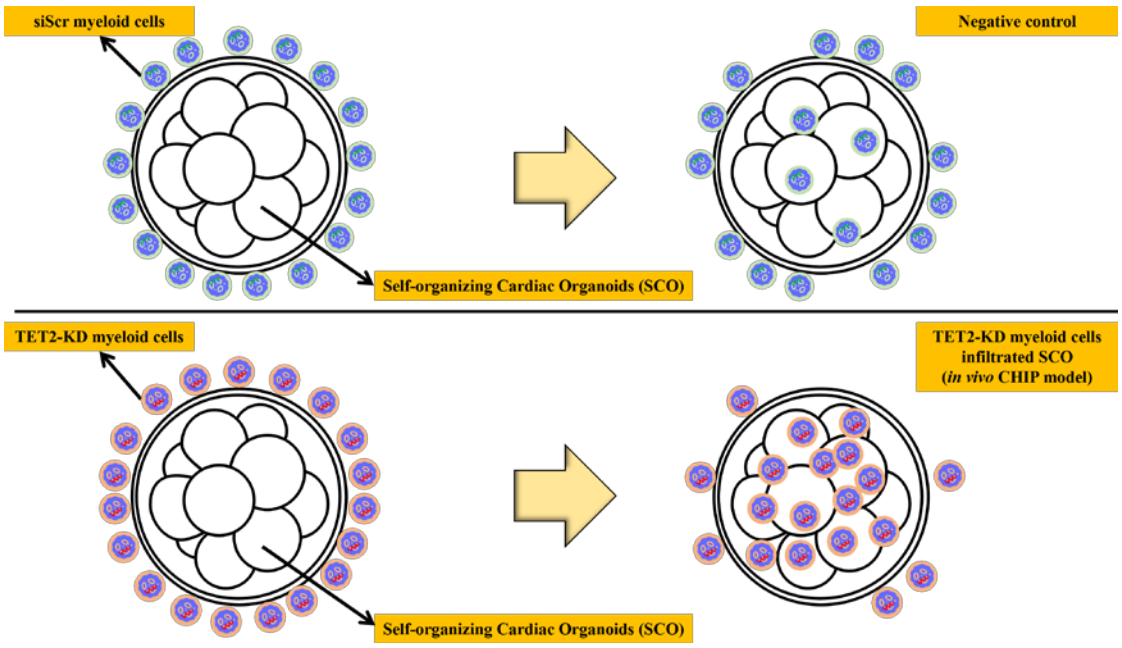


Figure 1.8 The schematic diagram for generating the TET2 cardiac-CHIP model. We use the TET2-KD myeloid cells to infiltrate SCO and use non-silencing scrambled control (siScr) as the negative control.

1.4 Synthetic lethal screen

The synthetic lethal screen is a strategy for separating novel mutants whose survival is subordinate to the quality of intrigue[40]. Combining the Alamar blue color-based test with a synthetic lethal screen offers an implication to outwardly distinguish a mutant that depends on a cell for survival[41, 42].

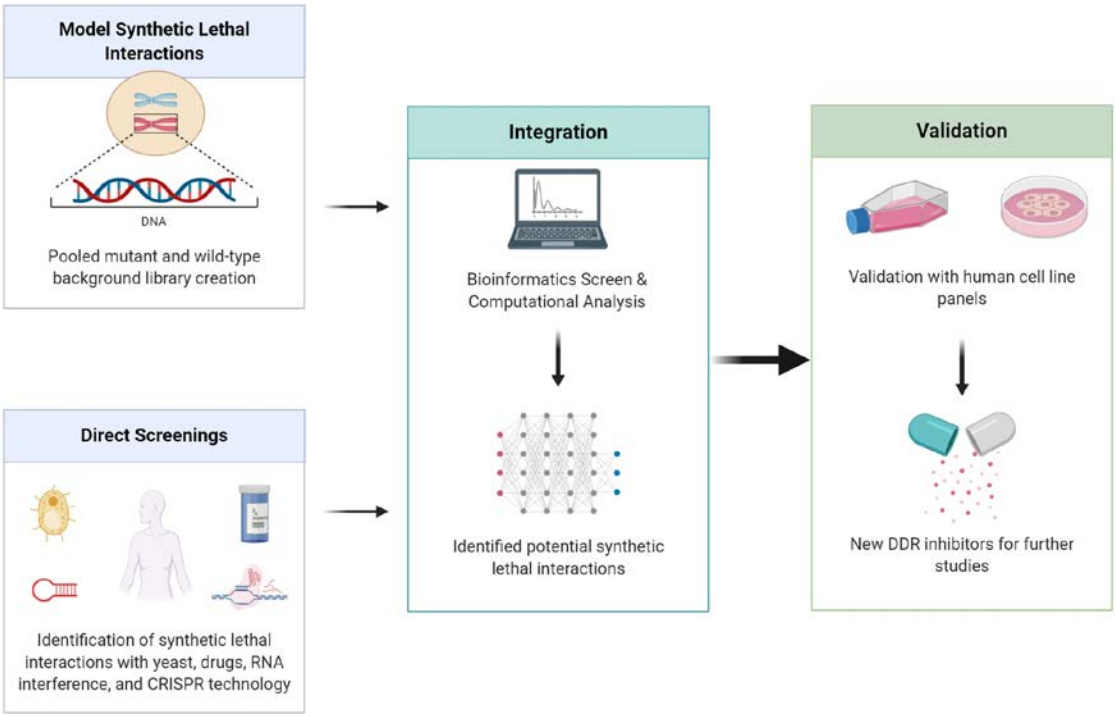


Figure 1-9. Example schematic diagram for Synthetic lethal screen[8]. The potential synthetic lethal interaction data are derived from the TET2-KD myeloid cells using the drug library and Alamar blue as a direct screening method. Finally, the discovered drug candidate will be applied to the TET2 cardiac-CHIP model.

1.5 Summary

Since CHIP is associated with a doubling risk of atherosclerosis and a poor prognosis in chronic heart failure (CHF) of ischemic origin and acute myocardial infarction (AMI), we sought to examine whether SCOs would be an appropriate study model[21-24, 43-45]. CHIP occurs due to modifications in transcriptional regulators of hematopoietic stem cells, namely in TET2 – where the loss of function promotes activation of the IL-1β/NLRP3 inflammasome in macrophages[26, 46, 47]. Our results showed that TET2-KD myeloid cells could invade, cluster, and promote cardiomyocyte apoptosis in SCOs.

2. Method

2.1 Cell culture

Human myeloid cells (THP-1) were purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen (ACC16, DSMZ) and were cultured in the medium: RPMI-1640 (Gbico, A1049101) +10% FCS (Gbico, 10270-106), 1% Penicillin-Streptomycin (Sigma-Aldrich, P4333-100ml) and L-Glutamine (Sigma-Aldrich, G7513). Subculture when cell density reaches around $8*10^5$ cells/ml and adjusting cell concentration to $2\text{-}4*10^5$/ml and splitting them from 1:3 to 1:4 every 2-3 days. iPSC cell line was bought from Takara (Y00325, Takara) using the cardiac differentiation kit (05010, Stemcell). Self-organizing Cardiac Organoids were generated by using the Medium M, Medium M plus, and Medium from Genome Biologics.

2.2 Establish TET2-KD myeloid cells and TET2 cardiac-CHIP model

The human TET2 siRNA (siTET2) and Non-targeting Control Pool (siScr) were brought from Horizon (L-013776-03-0020 and D-001810-10-20). siRNA were made by mixing 500μL OptiMEM (Gibico, 31985062) + 2μL siRNA (100pmol) + 6.25μL LTX PLUS reagent (Invitrogen, 15338100). Then mix well and wait 15 minutes at room temperature. Next, add 6.25μl Lipofectamine LTX (Invitrogen, 15338100), then well and wait 30 minutes at room temperature. At last, add 500μL to the target 10^5 myeloid cells containing 2mL culture medium and put the cells in an incubator for 18 hours at 37℃. Finally, we can get TET2-KD myeloid cells. For sequences of siRNA used in this project, please see **Table 1**. To set up the TET2 cardiac-CHIP model, 5000 described above TET2-KD myeloid cells co-culture with SCO for 48 hours, we can have TET2 cardiac-CHIP model.

2.3 RNA isolation and real-time PCR

The total RNA of SCOs was extracted using Trizol reagent (Invitrogen, 15596026), and myeloid cells were extracted using miRNeasy Mini Kit (Qiagen, 217004), both following the manufacturer's instructions. cDNA was synthesized from 1 μg and was performed using an RNA to cDNA EcoDry™ Premix (Takara, 639545). According to cDNA sequences, the primers **(Table 2)** were synthesized by Metabion (Germany). Real-time PCR was carried out in the Applied Biosystems StepOnePlus Real-Time PCR System using SYBR Green detection reagent (Applied Biosystems, 4385612). The relative mRNA expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method and normalized to the siScr vehicle HPRT[48].

2.4 Synthetic Lethality Screening

2500 THP-1 were seeded in 384-well plates (Greiner bio, 781186) co-culture with each Anti-Cardiovascular Disease Compound Library (Targetmol, L5400) at 10μM. The vehicle group was added 1% DMSO (Carlroth, A994.2). At the end of the screening, cell viability was measured using AlamarBlue (Bio-rad, BUF012B) and normalized to the siScr vehicle.

2.5 Qtacker labeling and SCO infiltration assay

Human myeloid cells were labeled with Qtracker (Invitrogen, Q25021MP) and followed the manufacturer's instructions. After 5000 labeled myeloid cells are co-cultured with one SCO for 48 hours, these can be used for further experiments.

2.6 Calcium transient assay

SCO's amplification and heartbeat frequency were measured by Cal-520®, AM (AAT bioquest, 21130), following the manufacturer's instructions. Each well should contain 100µl medium + 1µl mixed reagent (4% Pluronic and 200µM Dye). At the end of the assay, amplification and heartbeat frequency were measured using a plate reader and normalized to the siScr vehicle.

2.7 Trypan blue assay

To detect cell viability, using 0.4% Trypan blue solution (Gibco, 15250061) mixed with equal volumes of myeloid cells, counting the cells under the microscope and normalized to the siScr vehicle. This experiment is based on the concept that viable cells do not take up membrane-impermeable dyes (like Trypan Blue), but dead cells are permeable and take up the dye[49].

2.8 RNAseq data analysis

The isolated RNA was sent to Novogene (Novogene, UK) then the sequence data were analyzed by PartekFlow (Partek, US) and aligned with STAR.

2.9 SCO dissociation

Add 1mL 2.5% Trypsin (Merck, 59427C-100ML) to 40 SCOs to a 1.5ml tube, then put the tube to Eppendorf Thermomixer Compact at 1400rpm for 15 minutes at 37°C. Next, pipette up and down several times till most SCOs dissolved and continued for another 5 minutes. Last, add 1ml culture medium to stop the dissociation.

2.10 Immunofluorescence analysis

The cells on the tissue slide were fixed in 4% paraformaldehyde for 10 minutes (overnight for SCO) and processed for immunofluorescence. SCO needs 45 minutes in 1% TritonX-100 in PBS. The slides were blocked by 10% horse serum (Gibco, 16050130) for 1 hour and stained with primary antibody **(Table 3)** overnight at 4 °C. Then, washed the slides 3 times in PBS (6 times for SCO) and incubated with secondary antibodies **(Table 2)** for 1 hour (4 hours for SCO) at room temperature. Afterward, the slides were washed 3 times in PBS (6 times for SCO). Slides were mounted with an antifade mounting medium (Invitrogen, P36934) and analyzed using Leica SP8 confocal microscope. Data analysis by ImageJ.

2.11 Human Cardiac Troponin T quantitative measurement

Cell culture media were collected and then diluted to 1:3. The cTNT in diluted media was evaluated by the cTNT Elisa kit (Abcam, ab223860) and followed the manufacturer's instructions.

2.12 Cell proliferation assay

Each well in 384-well plates was seeded by 2500 cells. Proliferation was detected by the Cell Proliferation Assay (Promega, G3852) and followed the manufacturer's instructions.

2.13 Statistical analysis

All data were analyzed using GraphPad Software (GraphPad 8.0, US). Two-way ANOVA determined the significance of the difference for group comparisons and student's t-test

for pairwise comparisons between two groups. $P < 0.05$ was considered statistically significant.

Method

Table 1. siRNA sequences

Name	Sequences
ON-TARGETplus Non-targeting Control Pool	UGGUUUACAUGUCGACUAA
	UGGUUUACAUGUUUUCUGA
	UGGUUUACAUGUUGUGUGA
	UGGUUUACAUGUUUUCCUA
ON-TARGETplus Human TET2 siRNA	ACAAGAAAGUAGAGGGUAU
	ACACCUAGUUUCAGAGAAU
	CCUCAGAAUAAUUGUGUGA
	CAGCAAAGGUACUUGAUAC

Table 2. Primer sequences for qPCR

Name	Forward	Reverse
TET2	GTGAGGCTGCAGTGATTGTG	GATTGGTGAGCGTGCCGTAT
IL1β	AGCTACGAATCTCCGACCAC	CGTTATCCCATGTGTCGAAGAA
IL18	TCTTCATTGACCAAGGAAATCGG	TCCGGGGTGCATTATCTCTAC
CCL5	GGAAAGATTTTGTTGTTGTT	AGTCACCTACAATTCAAGAC
CXCL2	GGGCAGAAAGCTTGTCTCAA	GCTTCCTCCTTCCTTCTGGT
CD68	CTTCTCTCATTCCCCTATGGACA	GAAGGACACATTGTACTCCACC
MERTK	GTGCAGCGTTCAGACAATGG	TCGATGTAGATGGGATCAGACAC
NPPA	CAACGCAGACCTGATGGATTT	AGCCCCCGCTTCTTCATTC
NPPB	TGGAAACGTCCGGGTTACAG	CTGATCCGGTCCATCTTCCT
Col8a1	AGGAAGCCGTACCCAAGAAAGG	GGTATCCCATGACCTGGCAAAC
FSP1	CAGAACTAAAGGAGCTGCTGACC	CTTGGAAGTCCACCTCGTTGTC
HPRT	CCTGGCGTCGTGATTAGTGAT	AGACGTTCAGTCCTGTCCATAA
CASP8	GACAGAGCTTCTTCGAGACAC	CCTGAGTGAGTCTGATCCACAC
CASP10	AGAAACCTGCTCTACGAACTGT	GGGAAGCGAGTCTTTCAGAAG
TNFRSF1A	TCACCGCTTCAGAAAACCACC	GGTCCACTGTGCAAGAAGAGA
BAX	CCCGAGAGGTCTTTTTCCGAG	CCAGCCCATGATGGTTCTGAT
FADD	GTGGCTGACCTGGTACAAGAG	GGTAGATGCGTCTGAGTTCCAT

BAK	ATGGTCACCTTACCTCTGCAA	TCATAGCGTCGGTTGATGTCG
GADD45A	GAGAGCAGAAGACCGAAAGGA	CAGTGATCGTGCGCTGACT
GADD45B	TGCTGTGACAACGACATCAAC	GTGAGGGTTCGTGACCAGG
GADD45G	CAGATCCATTTTACGCTGATCCA	TCCTCGCAAAACAGGCTGAG
BCL2	AGGACTATTCGGCAGAGTACAT	TGATTCCAGGGTATTCCTCCTC
BIRC3	TTTCCGTGGCTCTTATTCAAACT	GCACAGTGGTAGGAACTTCTCAT

Table 3. Antibody list

Name	Company	Ref.	in cells	in SCO
anti-α-Actinin	Sigma-Aldrich	A7811-.2ML	1 to 1000	1 to 200
Anti-cleaved Caspase-3	Abcam	Ab32042	\	1 to 50
Anti-PCM1	Sigma-Aldrich	HPA023374	1 to 100	\
Anti-CD68	Invitrogen	MA1-80133	1 to 100	\
Anti-CD68	Abcam	ab233172	1 to 200	1 to 100
Pan neuronal marker	Merck	MAB2300	1 to 25	\
VE-Cadherin	Cell Signaling	#2500	1 to 100	\
DAPI	Abcam	ab285390	1 to 1000	1 to 1000
Anti-alpha smooth muscle Actin	Abcam	ab202295	1 to 100	\
AlexaFlour 488	Invitrogen	A11017	1 to 2000	1 to 500
AlexaFlour 555	Invitrogen	A21425	1 to 2000	1 to 500
Phalloidin 647	Abcam	ab176759	1 to 500	1 to 50
Phalloidin 488	Abcam	ab176753	1 to 500	1 to 50
Anti-COL1A1 antibody	Sigma-Aldrich	HPA011795	\	1 to 100

3. Result

3.1 Self-organizing cardiac organoids (SCO) have a similar composition and organization to the native human heart

Our lab has established the generation of self-organizing cardiac organoids (SCOs). To demonstrate that our SCOs own the property of the cellular composition and organization of the native human heart, we isolated SCOs into single cells and stained the single cell with different markers.

We regarded 40 mature SCOs (on Day 35) as a group, isolated six groups of SCOs into single cells separately, then used the fluorescence marker to stain smooth muscle actin, cardiomyocytes, neurons, fibroblast, endothelial, fibroblast, and macrophages, and count each kind of cells. After three independent replicate experiments, we had the cellular composition of our SCO: 3.97±0.16% smooth muscle cells, 54±3.21% cardiomyocytes, 5.67±0.52% endothelial, 2.07±0.43% of neurons, 5.86±1.93% of fibroblasts, and 6.88±1.96% macrophages **(Figure 3.1 A-B)** which highlighted the similarity with the adult human heart[39].

Result

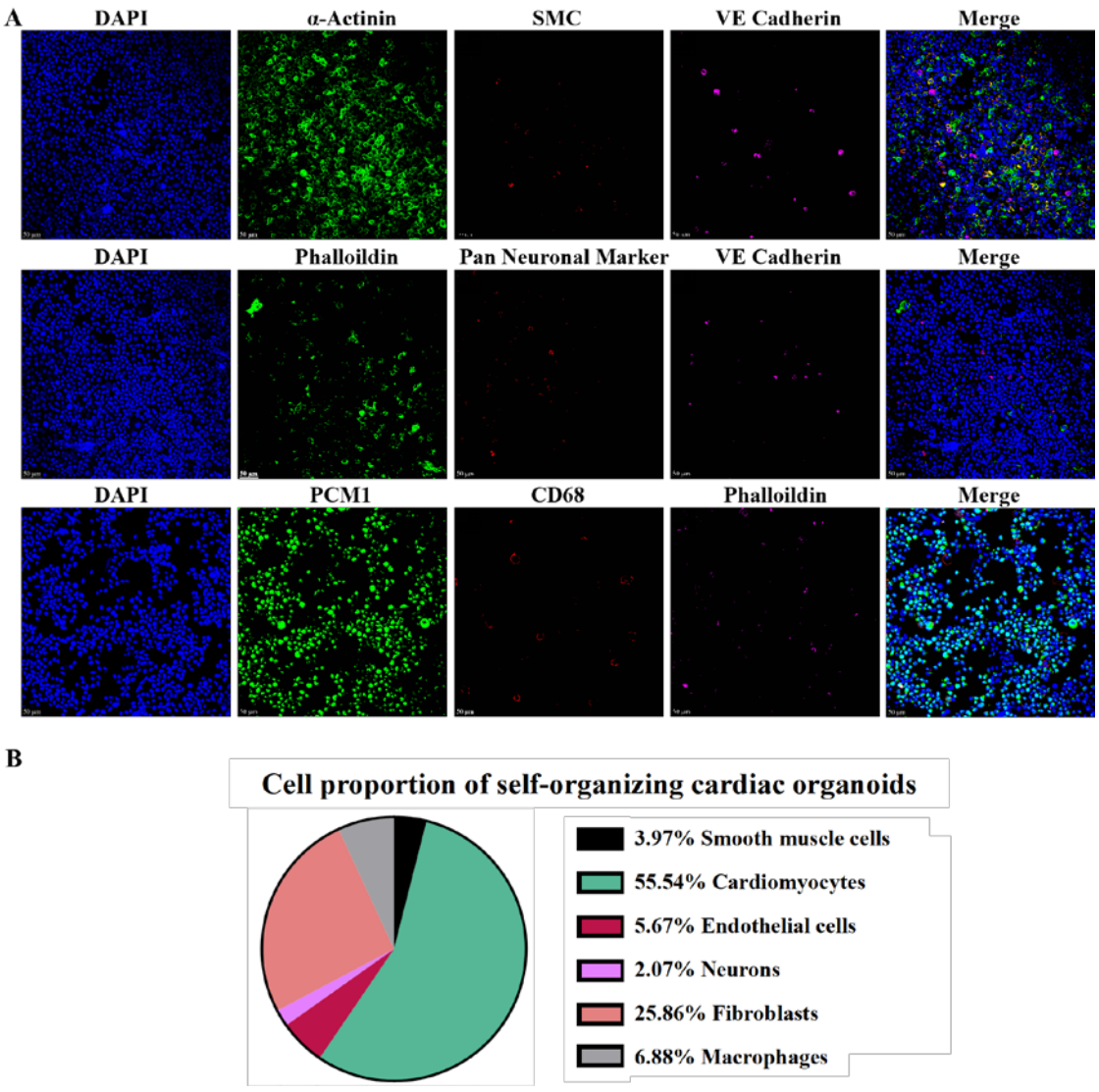


Figure 3.1 Cell proportion of self-organizing cardiac organoids (SCO).
A. Representative images of SCO cell proportion. **B.** Pie chart of SCO cell proportion. Our SCO contains 3.97±0.13% Smooth muscle cells, 54±2.62% cardiomyocytes, 5.67±0.52% endothelial cells, 2.07±0.35% neurons, 5.86±1.58% fibroblasts, and 6.88±1.60% macrophages matching the adult human heart. DAPI stands for nucleus, α-Actinin stands for cardiomyocytes, SMC stands for smooth muscle cells, VE Cadherin stands for endothelial cells, Phalloidin stands for fibroblasts, Pan Neuronal Marker stands for neurons, PCM1 stands for cardiomyocytes and CD68 stands for macrophages. N=720 using mean ± SD.

Result

3.2 Establish the TET2-KD myeloid cells based on human myeloid cells

Human myeloid cells (THP-1) were first transfected with Human Non-targeting Control Pool scrambled siRNAs (siScr) or TET2 siRNA (siTET2) to imitate the loss-of-function effects of clinically-relevant TET2 mutations[50]. Then we performed a time-course knockdown (KD) kinetics of the siTET2 experiment **(Figure 3.2 A)**, and the data indicated that after 72 hours of post-transfection, the expression of TET2 dropped to 37±3.14%, notably lower than at 48 and 96 hours (68±7.14% and 77±7.11% respectively) after post-transfection. Therefore, to have a similar TET2-KD proportion in TET2-mutant cardiovascular CHIP patients[51], we used the TET2-KD myeloid cells from 72 hours after post-transfection in the whole experiment.

In order to validate TET2-KD myeloid cells, we performed qPCR for inflammation-related markers. The results indicated that inflammatory marker levels (IL-1B, CCL5, CXCL2, IL18) and macrophages makers (MerTK) increased in TET2-KD myeloid cells **(Figure 3.2 B)**, and these results were also observed in cardiovascular CHIP patients[21, 22, 52, 53]. Consequently, all above proved that we successfully set up the TET2-KD myeloid cell*s*. Later, we will use these TET2-KD myeloid cells to establish the TET2 cardiac-CHIP model.

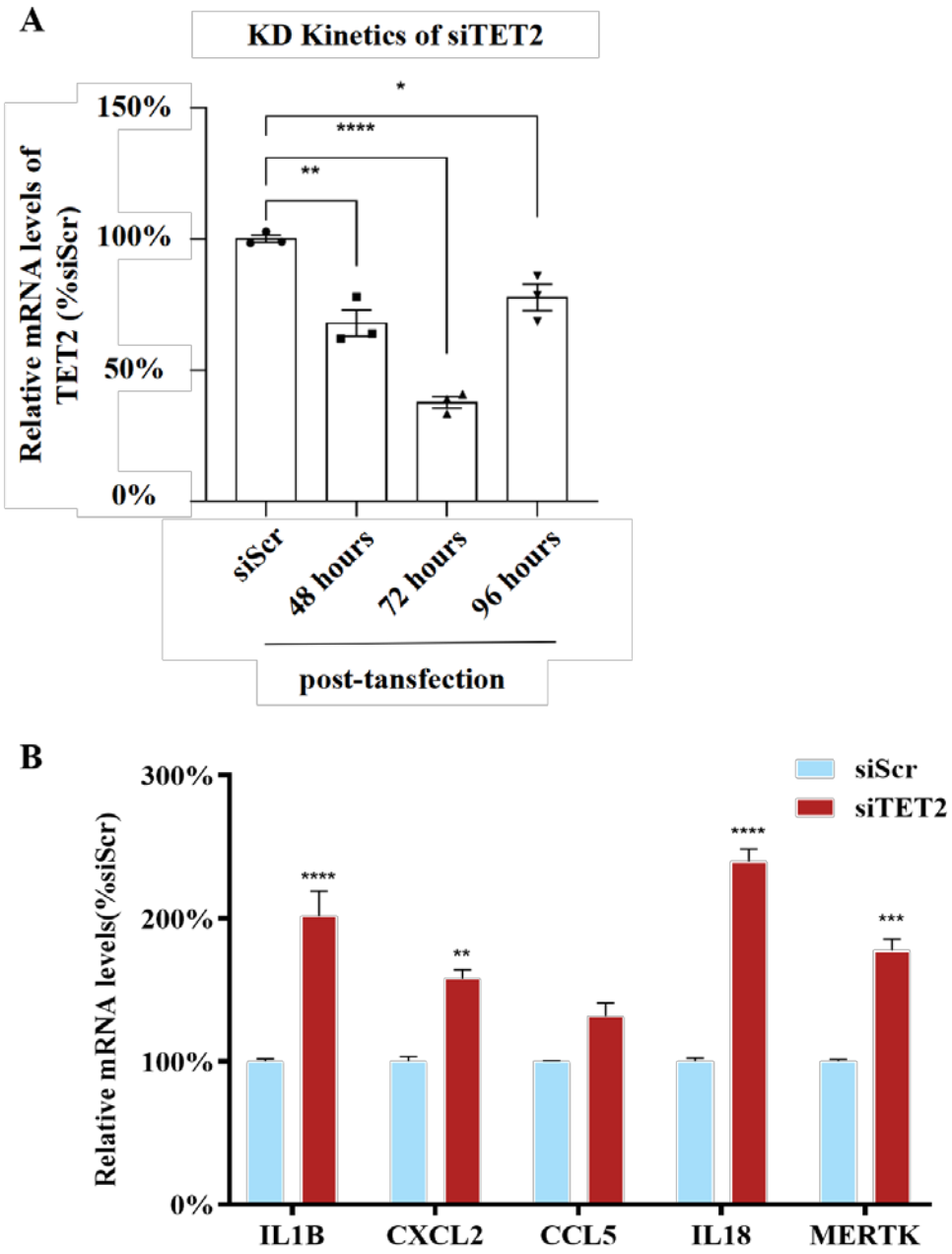


Figure 3.2 TET2-KD myeloid cells set up in human myeloid cells.

A. Time-course experiment of KD kinetics of siTET2. The expression of TET2 genes dropped to 68±7.14% (at 48 hours), 37±3.14% (at 72 hours) and 77±7.11% (at 96 hours). N=3 using Stundent's t-test, siTET2 vs. siScr. All bar graphs show mean ± SEM. **B.** qPCR measurements showed increased inflammatory marker levels (IL-1B, CCL5, CXCL2, IL18) and macrophages makers in TET2-KD myeloid cells compared to siScr control, demonstrating increased inflammation in the TET2-KD myeloid cells model, matching the inflammation phenotype in CHIP patient. N=4 using unpaired t-test, siTET2 vs. siScr. All bar graphs show mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

Result

3.3 Establish TET2 cardiac-CHIP model based on human iPSCs-derived SCOs

With the established mature SCO model **(Result 3.1)**, we aimed to explore whether it could be transformed and applied to model clinically-relevant cardiovascular CHIP. Therefore, we co-cultured SCOs with TET2-KD myeloid cells to evaluate tissue invasion. Qtracker, which can mark exogenous myeloid cells specifically, enabled us to monitor and analyze TET2-KD and siScr control myeloid cell infiltration into the SCOs. As the infiltration data shown **(Figure 3.3 A)**, we can find an increased infiltrated area of TET2-KD myeloid cells compared to siScr control, replicating the increased invasion observed in CHIP patients[21, 22, 52, 53]. Furthermore, we observed that TET2-KD myeloid cells cluster in certain tissue areas **(Figure 3.3 B)**. Moreover, quantifying the infiltration through immunofluorescence suggested an increased TET2-KD myeloid cells covered area *vs.* siScr control **(Figure 3.3 E-F)**.

Additionally, we analyzed SCOs apoptosis due to pathologic stress stimulation, using cleaved Caspase-3 as readout. Co-localization and quantification of cardiomyocytes and cleaved-Caspase-3 indicated that cardiomyocytes undergo apoptosis more in TET2-KD myeloid cells **(Figure 3.3 C-D, G)**. Similarly, the increased cardiomyocyte death was confirmed by a surge in the prognostic cardiac biomarker cTNT in the TET2-KD myeloid cells group **(Figure 3.3 H)**.

Besides, qPCR measurements of macrophages makers (CD68, MerTK), inflammatory markers (IL-1B, CCL5, CXCL2), heart failure (NPPA, NPPB), and fibrosis markers (Col8A1, FSP1) raised in SCO co-cultured with TET2-KD myeloid cells *vs.* siScr control

(Figure 3.3 I). These results demonstrate a grown inflammation in the TET2-KD myeloid cells, matching the inflammatory phenotype previously characterized in CHIP patients[5, 13, 21, 46, 54-56], validating our TET2 cardiac-CHIP model.

In conclusion, we have successfully developed a human TET2 cardiac-CHIP model comprising a co-culture of human myeloid cells and induced pluripotent stem cells (iPSCs)-derived self-organized human cardiac organoids. Furthermore, we utilized this model to demonstrate the causal role of TET2-KD in myeloid cells in driving disease development augmentation of myeloid cell filtration and cardiac inflammatory program induction culminating in cardiomyocyte apoptosis and tissue fibrosis. Our TET2 cardiac-CHIP model can be considered an efficacious pre-clinical research model comparable with clinical cardiovascular CHIP patients[57, 58].

Result

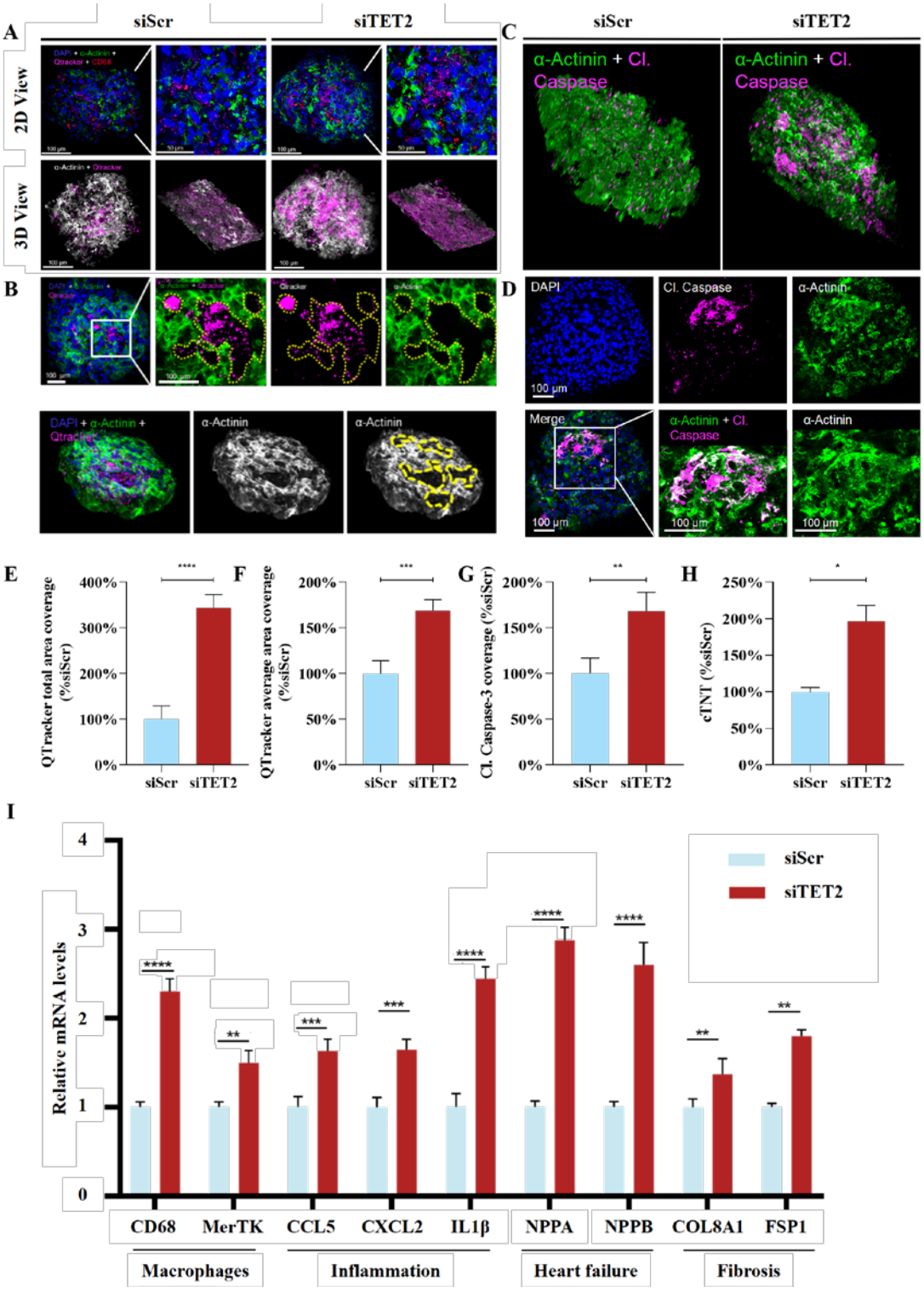

A
siScr
siTET2
2D View
3D View
B
C
α-Actinin + Cl. Caspase
D
DAPI
Cl. Caspase
α-Actinin
Merge
100 µm
E
QTracker total area coverage (%siScr)
F
QTracker average area coverage (%siScr)
G
Cl. Caspase-3 coverage (%siScr)
H
cTNT (%siScr)
I
Relative mRNA levels
siScr
siTET2
CD68
MerTK
CCL5
CXCL2
IL1β
NPPA
NPPB
COL8A1
FSP1
Macrophages
Inflammation
Heart failure
Fibrosis

Figure 3.3 Modeling Clonal Hematopoiesis of Indeterminate Potential in self-organized cardiac organoids (SCOs).

A. The staining and subsequent tracking of myloid cells (Qtracker, magenta) enabled monitoring and analysis of their infiltration into SCO. The result showed increased infiltration of TET2-KD myeloid cells compared to the siScr control group (replicating the increased myeloid cells infiltration observed in CHIP patients). **B.** 3D rendering of organoids with labeled infiltrating myeloid cells. The absence of cardiomyocytes from infiltration areas further confirmed effective macrophage staining and tracking. Additionally, macrophage clustering was demonstrated. **C-D.** Evident colocalization of cardiomyocytes and cleaved Caspase-3 apoptosis staining demonstrates that it is the cardiomyocytes that undergo apoptosis. **E-F.** Quantification of myeloid cells infiltration in organoids showed the increased organoid area covered with myeloid cells in organoids co-cultured with TET2-KD myeloid cells compared to the siScr control group. **G.** Quantification of cleaved Caspase-3 staining showed increased apoptosis in the TET2-KD myeloid cells group compared to the siScr control group. **H.** The increased cardiomyocyte death was further confirmed by increasing the prognostic cardiac biomarker cTNT in the TET2-KD myeloid cells group. **I.** qPCR measurements showed increased macrophages makers (CD68, MerTK), inflammatory marker levels (IL-1B, CCL5, CXCL2), heart failure (NPPA, NPPB), and fibrosis markers (Col8A1, FSP1) in TET2-KD myeloid cells compared to siScr control group, demonstrating increased inflammation in TET2-KD myeloid cells model, matching the inflammation phenotype in CHIP patients. α-Actinin stands for cardiomyocytes, Qtracker stands for infiltrated myeloid cells, Cl. Caspase-3 stands for apoptosis, and double positive of α-Actinin and Cl. Caspase-3 stands for apoptosis of cardiomyocytes. E-H, N=8 using unpaired t-test. I, N=4 using two-way ANOVA. Each drug-treated siTET2 *vs.* siScr. All bar graphs show mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

Result

3.4 Identification of repositioned drugs for CHIP from Cardiovascular and FDA/EMA-approved drug libraries

To identify drugs that can selectively reduce TET2-KD myeloid cell viability, we performed Synthetic Lethality screening with compounds from the Cardiovascular and FDA/EMA-approved drug libraries **(Figure 3.4 A)**. Candidate drugs (Enoxolone, Miltefosine, Ticlopidine hydrochloride, Clopidogrel, Gabapentin, Pravastatin sodium, R406, NU2058, and Lanatoside C) from the Synthetic Lethality screening showed selectively inhibit TET2-KD myeloid cells *vs.* drug control group.

Then we narrowed down our selection through a proliferation test. The result demonstrated that Clopidogrel, R406, and Lanatoside C were identified as top candidates due to their selective activity in reducing the viability and proliferation of TET2-KD myeloid cells but not targeting control myeloid cells **(Figure 3.4 B-C)**.

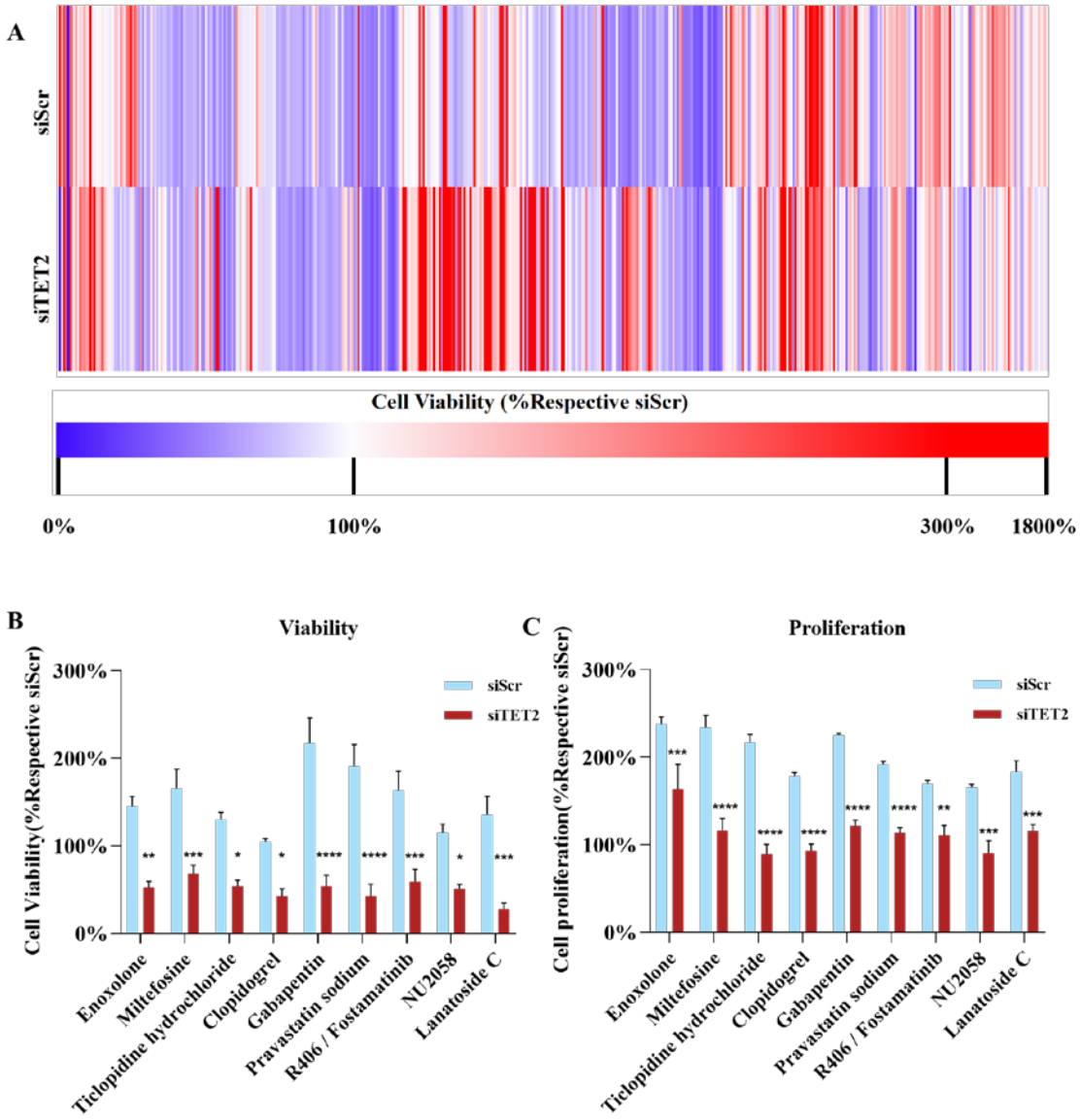


Figure 3.4 Synthetic lethality screening in TET2-KD myeloid cells identified FDA/EMA-approved drugs for repositioning in TET2 cardiac-CHIP therapy

A. Heatmap of Synthetic lethality screening in TET2-KD and the control myeloid cells, the drugs were selected from Anti-Cardiovascular Disease Compound Library **(Method 2.5)**. Cell viability was calculated by each drug-treated siTET2 vs. siScr vehicle. N=4 using unpaired t-test. **B-C.** Quantitation data of viability and proliferation. The candidate drugs chosen from synthetic lethality screening further tested viability and proliferation. Clopidogrel, R406, and Lanatoside C showed adequate results in lower TET2-KD myeloid cells among the ten drugs. N=4 using 2way ANOVA. Each drug-treated siTET2 *vs.* siScr. All bar graphs show mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

Result

3.5 Clopidogrel, R406, and Lanatoside C decreased the expression of pro-inflammation genes in the TET2-KD myeloid cells

To evaluate the drug potency, we added each drug into TET2-KD myeloid cells for 48 hours (after post-transfection 72hours) and tested pro-inflammatory genes qPCR.

The result of qPCR demonstrated the candidate drugs that could ameliorate the inflammation induced by TET2-KD myeloid cells. The levels of macrophage markers **(Figure 3.5 A-C)**, inflammatory markers **(Figure 3.5 D-F)**, and chemokines **(Figure 3.5 G-H)** lowered in TET2-KD myeloid cells **(Figure 3.5 A-B)**.

Besides, the decreased expression of monocyte/macrophage markers **(Figure 3.5 A-C)** indicated that the TET2-KD myeloid cells could undergo cell death. In this regard, we next plan to verify the above conjecture and investigate whether the candidate drugs could selectively induce the cell death of TET2-KD myeloid cells.

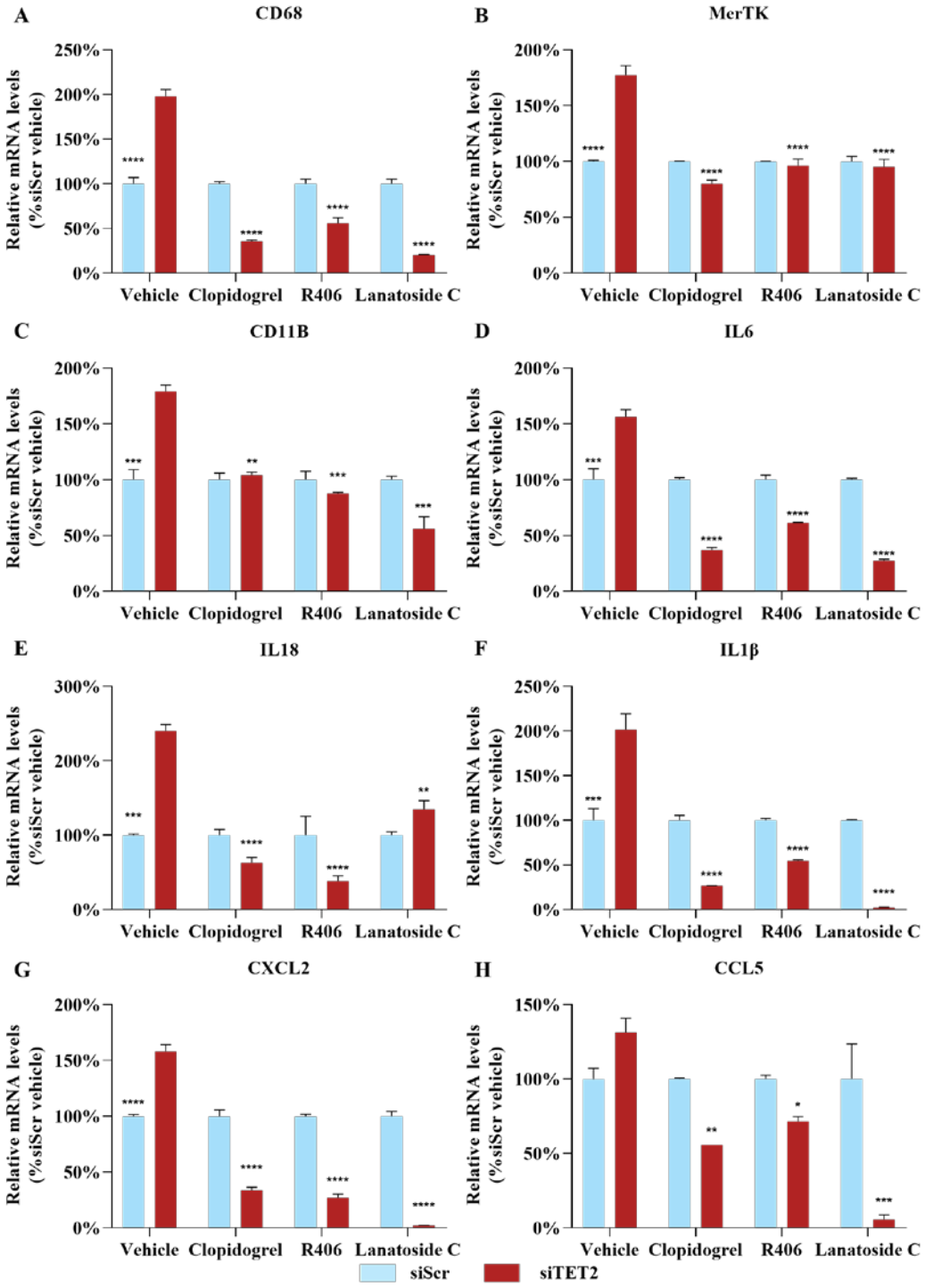


Figure 3.5 Real-time PCR assessment of drug treatment in TET2-KD myeloid cells. **A-C.** CD68, MerTK, and CD11B are monocyte/macrophage markers. **D-F.** IL6, IL18 and IL1β are inflammation makers. **G-H.** CXCL2 and CCL5 are chemokines which involved in many immune responses. Compared to the drug control group (siTET2 vehicle), qPCR results showed the anti-inflammation effect of drug treatment. N=4 using two-way ANOVA. All groups were normalized to siScr vehicle and each drug treatment group vs. siTET2 vehicle. All bar graphs show mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

Result

3.6 Clopidogrel, R406, and Lanatoside C selectively declined cell viability and induced apoptosis in TET2-KD myeloid cells

Due to the result of lower monocyte/macrophage markers expression in TET2-KD myeloid cells, we supposed whether these drugs could particularly suppress TET2-KD myeloid cells. On this matter, we then performed the cell viability test and cell death by Trypan blue and cleaved Caspase-3.

The result indicated that Clopidogrel, R406, and Lanatoside C could selectively reduce the cell viability in TET2-KD myeloid cells and induce their apoptosis but not the drug control group, as proved by cleaved Caspase-3 staining **(Figure 3.6 A-B)** and Trypan blue assay **(Figure 3.6 C)**.

Lanatioside C-treated TET2-KD myeloid cells showed significantly higher apoptosis values among all three drugs. Therefore, and combined with reduced secretion cytokines **(Figure 3.5 F-H)**, Lanatioside C could be the outstanding drug from Clopidogrel and R406. Hence, we prepared drug/vehicle-treated samples for RNA sequencing to validate our hypothesis and verify the data.

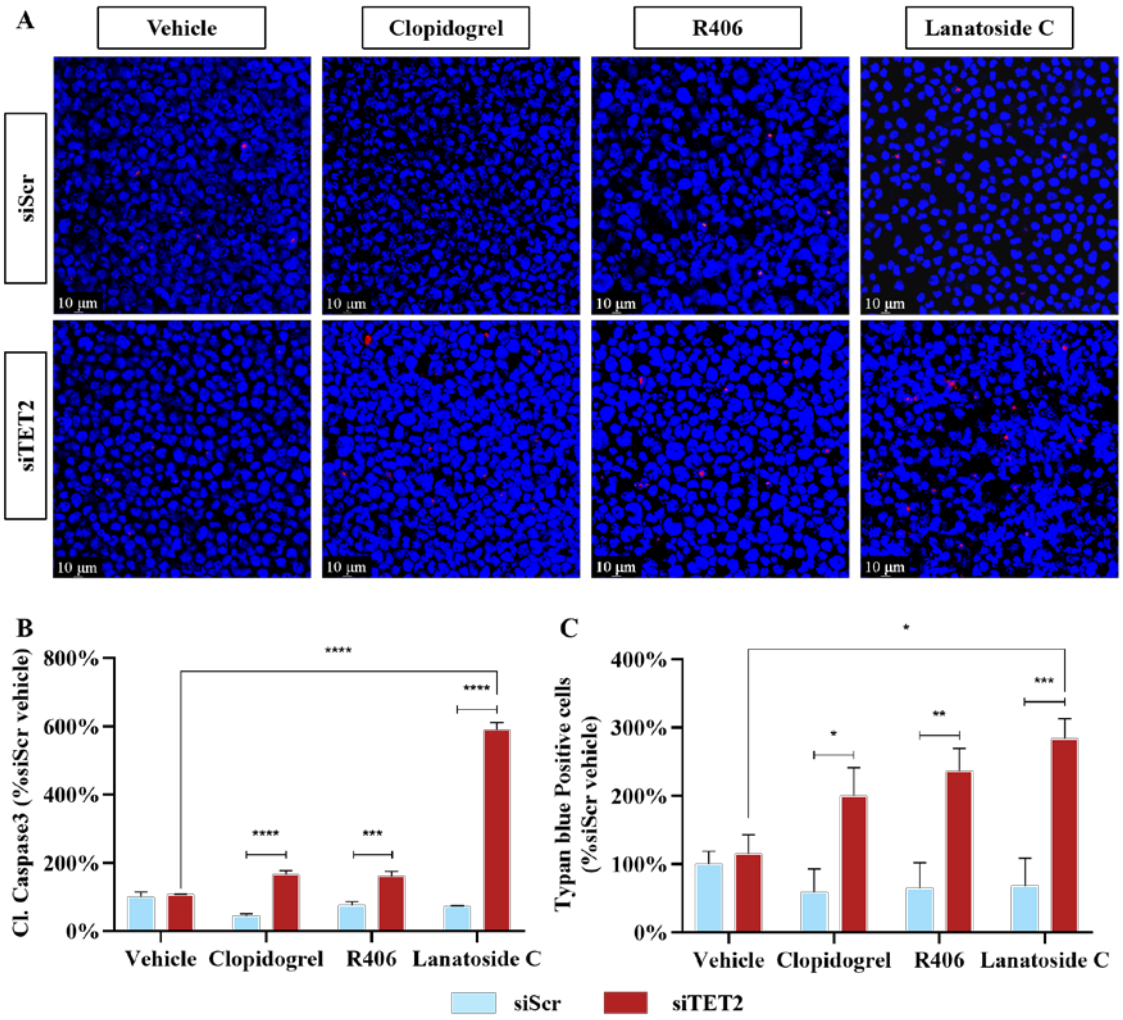


Figure 3.6 Measuring cell viability and apoptosis after drug treatment in TET2-KD myeloid cells.

A. Representative images of cleaved Caspase-3 staining. **B.** cleaved Caspase-3 aimed to detect apoptosis. The result indicated that the drug treatment group could increase cell death in TET2-KD myeloid cells other than the drug control group. **C.** Trypan blue was performed to detect cell viability. The result indicated drug treatment group reduced cell viability in TET2-KD myeloid cells but not the drug control group. DAPI stands for myeloid cell nucleus, Cl. Caspase-3 stands for apoptosis, and double positive of DAPI and Cl. Caspase-3 stands for apoptosis of myeloid cells. N=4 using two-way ANOVA. All groups were normalized to siScr vehicle and each drug treatment group vs. siTET2 vehicle. All bar graphs show mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$. Scale bar: 10µm.

Result

3.7 Transcriptomic changes of TET2-KD myeloid cells affected by Clopidogrel, R406, and Lanatoside C

To further understand the mechanism and gene changes, we performed RNAseq in the drug/vehicle-treated TET2-KD/scramble-controlled myeloid cell groups **(Figure 3.7 A-C)**. The analysis indicated that the Clopidogrel, R406, and Lanatoside C influenced the anti-inflammatory genes **(Figure 3.7 A)**, proliferation genes **(Figure 3.7 B)**, and pro-apoptosis genes **(Figure 3.7 C)**.

Lancatoside C had the most substantial effect on anti-inflammation, down-regulated proliferation genes, and apoptogenic function in these three drugs. Similarly, Clopidogrel could up-regulate anti-inflammatory genes and pro-apoptosis genes. Moreover, R406 relied on pro-apoptosis genes and anti-proliferation to decrease TET2-KD myeloid cell viability. RNAseq data verified the anti-inflammation function **(Figure 3.5)** and apoptogenic function **(Figure 3.6)** we previously tested.

In a short conclusion, we identified drugs that can selectively reduce TET2-KD myeloid cell viability and performed Synthetic Lethality screening with compounds from the Anti-Cardiovascular Disease Compound Library **(Figure 3.4)**. Clopidogrel, R406, and Lanatoside C were identified as top candidates due to their selective activity in reducing the viability of TET2-KD myeloid cells (but not control myeloid cells). These compounds function, in part, through the Induction of anti-inflammatory pathways, proliferative inhibition, and apoptosis induction in TET2-KD myeloid cells **(Figure 3.7)**.

Result

Clopidogrel, R406, and Lanatoside C collectively showed the capacity to target TET2-KD myeloid cells selectively. However, Lanatoside C stands out from the others due to its significantly reduced cell viability and inflammation markers and surprisingly induced TET2-KD myeloid cell death **(Figure 3.5-6)**. Besides, Lanatoside C is approved both by the European Medicines Agency (EMA) and the United States of America (USA) Food and Drug Administration (FDA), suggesting we could repurpose Lanatoside C as an efficacious treatment method for TET2-mutant cardiovascular CHIP patients. Therefore, to further validate Clopidogrel, R406, and Lanatoside C, we decided to experiment with these drugs in our established TET2 cardiac-CHIP model **(Figure 3.2)**.

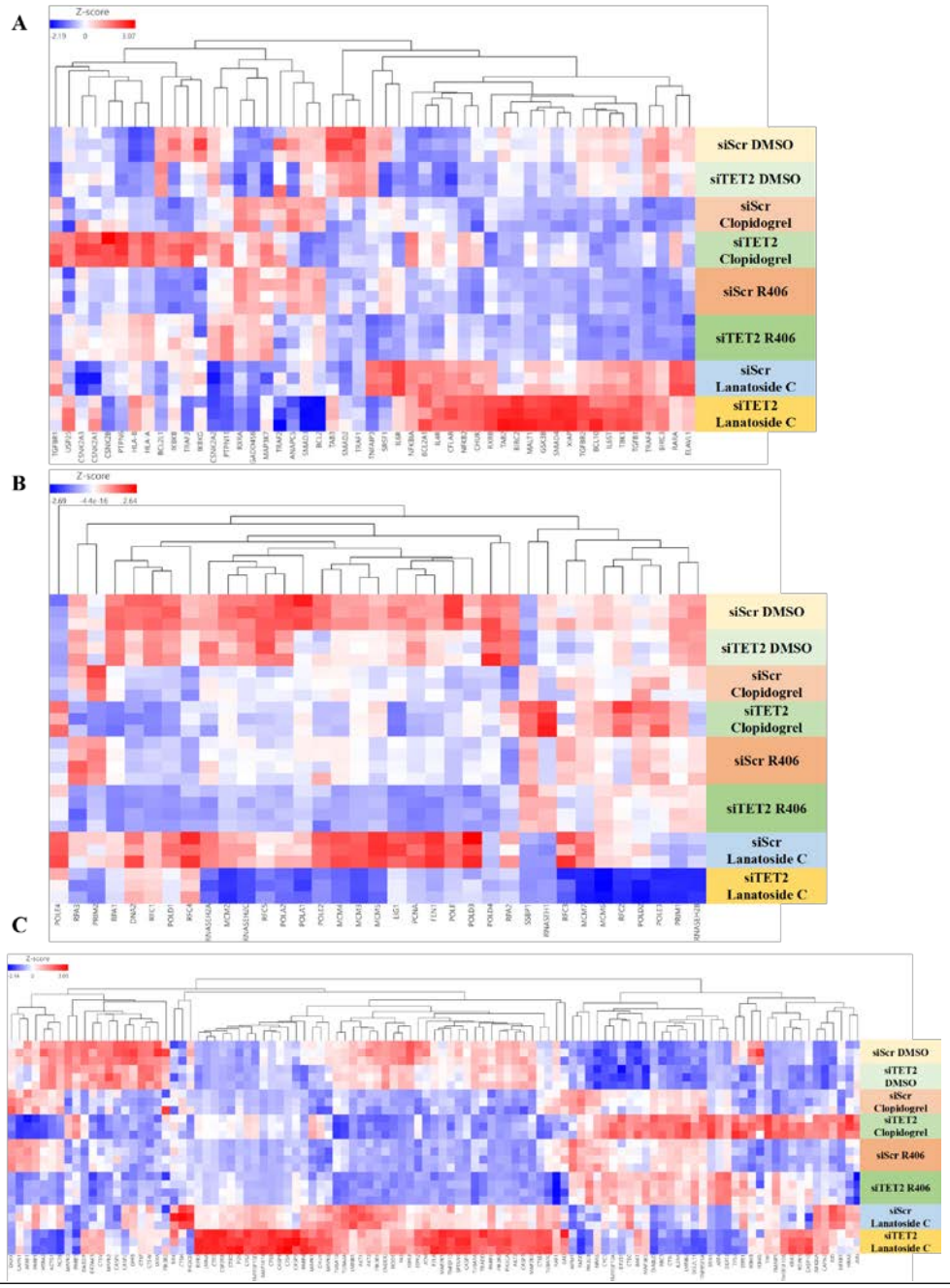


Figure 3.7 RNAseq data of Clopidogrel, R406, and Lanatoside C treatment in TET2 cardiac-CHIP model.

A. Hierarchical clustering and heatmap of anti-inflammatory genes. Clopidogrel and Lanatoside C showed up-regulation of anti-inflammatory genes. **B.** Hierarchical clustering and heatmap of proliferation genes. R406 and Lanatoside C revealed anti-proliferation function to TET2-KD myeloid cells. **C.** Hierarchical clustering and heatmap of pro-apoptosis genes. Clopidogrel, R406, and Lanatoside C all could enhance TET2-KD myeloid cell apoptosis. Each row stands for one independent sample from the depicted drug/vehicle group. Expression values are shown as the Z-scores for each gene.

3.8 Decrease in TET2-KD myeloid cells infiltration in SCO upon drug treatment

After validating the TET2 cardiac-CHIP model **(Figure 3.2)** and the selected drugs (Clopidogrel, R406, and Lanatoside C) from previous Synthetic Lethality screening, we aimed to test the candidate drugs in this model to determine whether these drugs could alleviate infiltration and apoptosis due to pathologic stress stimulation caused by TET2-KD myeloid cells.

We marked myeloid cells by QTracker, which notably targeted exogenous cells, enabling us to monitor and analyze the infiltrated TET2-KD and siScr control myeloid cells in SCOs **(Method 2.6)**. The quantification data of myeloid cell infiltration through immunofluorescence showed a meaningful decrease in TET2-KD myeloid cell invasion after each drug treatment compared with the siTET2 vehicle **(Figure 3.8 A-B)**.

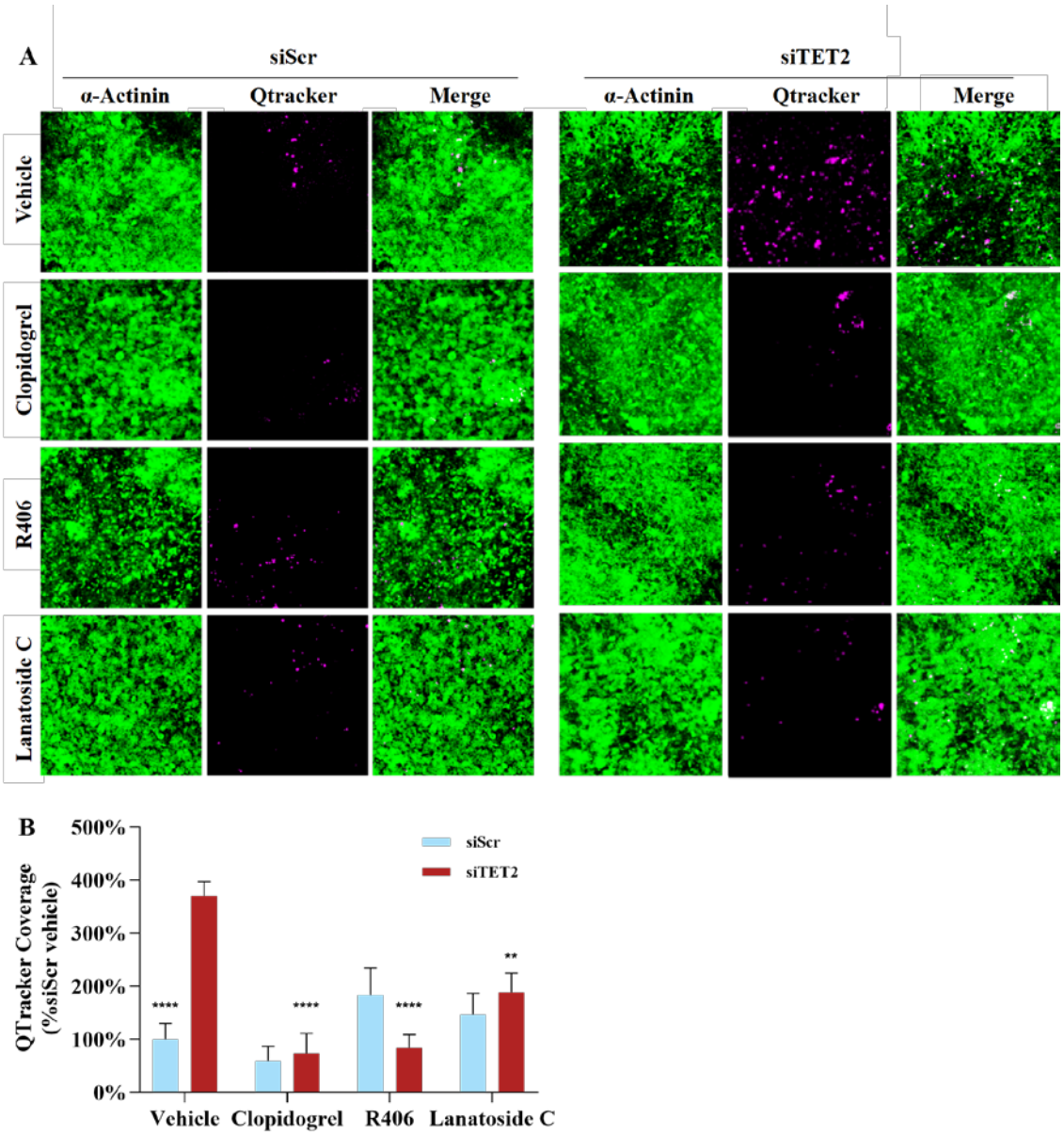


Figure 3.8 Infiltration assessment in TET2-KD myeloid cells infiltrated SCO after drug treatment.

A. Representative images of quantification of myeloid cell infiltration. α-Actinin stands for cardiomyocytes, and Qtracker stands for marked myeloid cells. **B.** Quantification data of myeloid cell infiltration in organoids showed the increased organoid area covered with myeloid cells in organoids co-cultured with TET2-KD myeloid cells, but the infiltration area decreased after drug treatment. α-Actinin stands for cardiomyocytes, and Qtracker stands for infiltrated myeloid cells. N=4 using two-way ANOVA. All groups were normalized to siScr vehicle and each drug treatment group *vs.* siTET2 vehicle. All bar graphs show mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$. Scale bar: 50μm.

Result

3.9 Clopidogrel, R406, and Lanatoside C decreased the expression of infiltration, inflammation, fibrosis, and heart failure genes in TET2-KD myeloid cells infiltrated SCO

Next, we assessed whether Clopidogrel, R406, and Lanatoside C could ameliorate the inflammation and fibrosis exacerbated by TET2-KD myeloid cells induced pathologic stress stimulation in in TET2-KD myeloid cells infiltrated SCO. qPCR measurements demonstrated that monocytes/macrophages makers (CD68, MerTK), inflammatory markers (IL-1B, CCL5, CXCL2), heart failure (NPPA, NPPB), and fibrosis markers (Col1A1, Col4A1, Col8A1, TGFβ2, POSTN, THBS1, FSP1, FN1) could lower in drug-treated TET2 cardiac-CHIP model group *vs.* drug control group (**Figure 3.9 A-O**). The data is consistent and cross-validation with the previous infiltration **(Figure 3.8)**.

Among the three drugs, Lanatoside C displayed a significantly reduced in the expression of monocyte/macrophage markers **(CD68 and MerTK, Figure 3.9 A-B)** without inducing the cytokine **(IL1β, Figure 3.9 C)** and chemokines **(CCL5 and CXCL2, Figure 3.9 D-E)** in the siScr drug control group (siScr + Clopidogrel and siScr + R406) therefore broadly decrease the expression of heart damage-related genes **(NPPA and NPPB, Figure 3.9 F-G)**. Besides, except for lowing the fibrosis gene expression in the TET2-KD myeloid cells infiltrated SCO, Lanatoside C could also decline the markers in drug-control (siScr + Lanatoside C) and drug-treated (siTET2 + Lanatoside C) groups. Collectively, Lanatoside C is outstanding from Clopidogrel and R406.

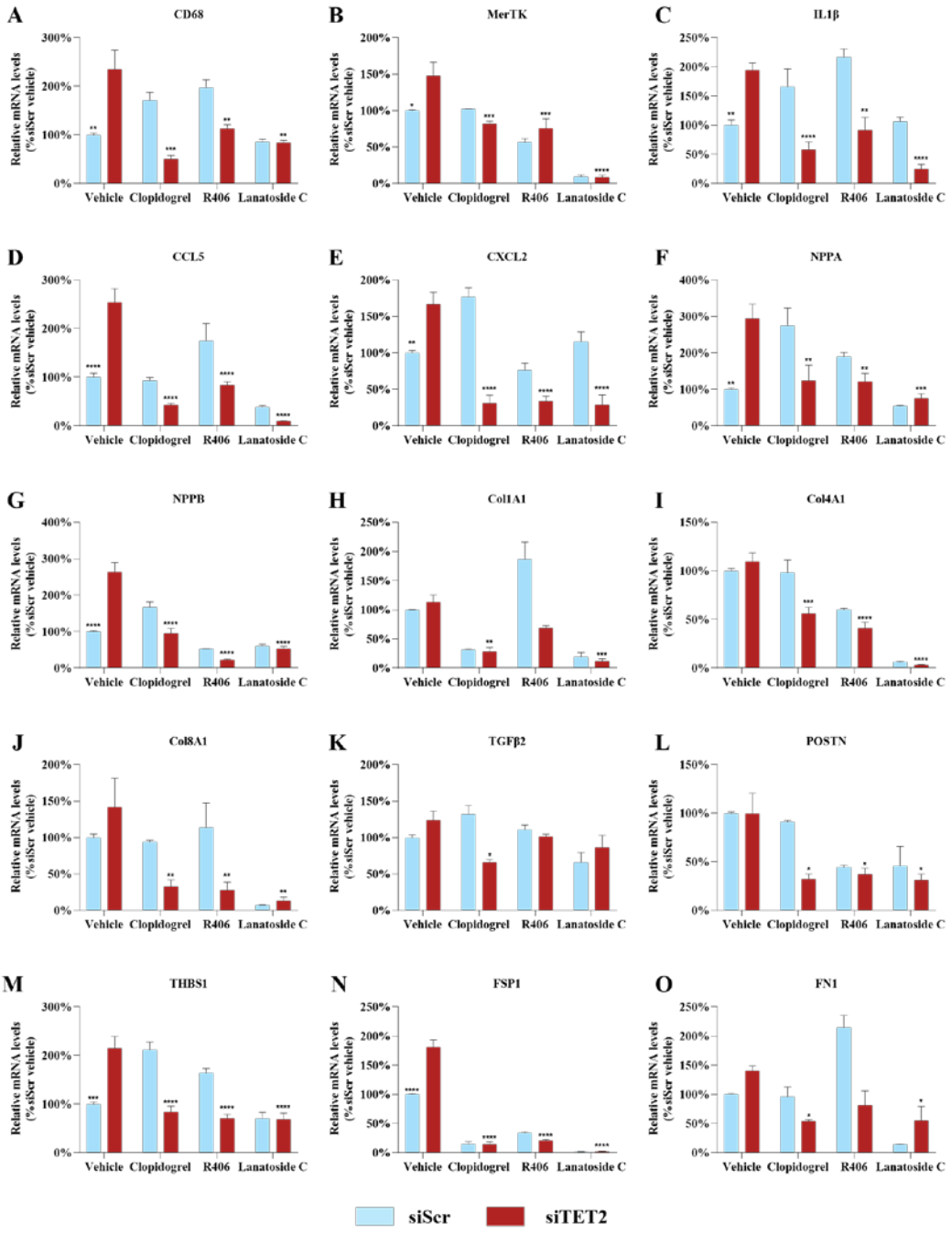


Figure 3.9 qPCR measurements of infiltration, inflammtion, fibrosis and heart failure genes in TET2-KD myeloid cells infiltrated SCO after drug treatment.

A-B. qPCR measurements of macrophage markers. **C-E**. qPCR measurements of inflammation. **F-G**. qPCR measurements of heart failure markers. **H-O**. qPCR measurements of fibrosis markers. The above results indicate that Clopidogrel, R406, and Lanatoside C could decrease the expression of infiltration, inflammation, fibrosis, and heart failure genes. N=4 using two-way ANOVA. All groups were normalized to siScr vehicle and each drug treatment group *vs.* siTET2 vehicle. All bar graphs show mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

Result

3.10 Decrease in cardiomyocytes apoptosis in TET2-KD myeloid cells infiltrated SCO upon drug treatment

To evaluate whether the Clopidogrel, R406, and Lanatoside C could reduce cardiomyocyte apoptosis due to pathologic stress stimulation caused by infiltrated TET2-KD myeloid cells. cTNT Elisa assay and cleaved Caspase-3 staining have been performed to detect cardiomyocyte apoptosis in the drug/vehicle treatment in TET2-KD myeloid cells infiltrated SCO.

The quantification data of cleaved Caspase-3 indicated that the drugs substantially reduced the apoptosis after each drug treatment compared with the siTET2 vehicle **(Figure 3.10 A-B)**. Furthermore, the decreased cardiomyocyte death was also proved by a surge in the prognostic cardiac biomarker cTNT in the TET2-KD myeloid cells infiltrated SCO **(Figure 3.10 C)**. Generally, Clopidogrel, R406, and Lanatoside C all displayed the same significant effect on a decrease in cardiomyocyte apoptosis in TET2-KD myeloid cells infiltrated SCO.

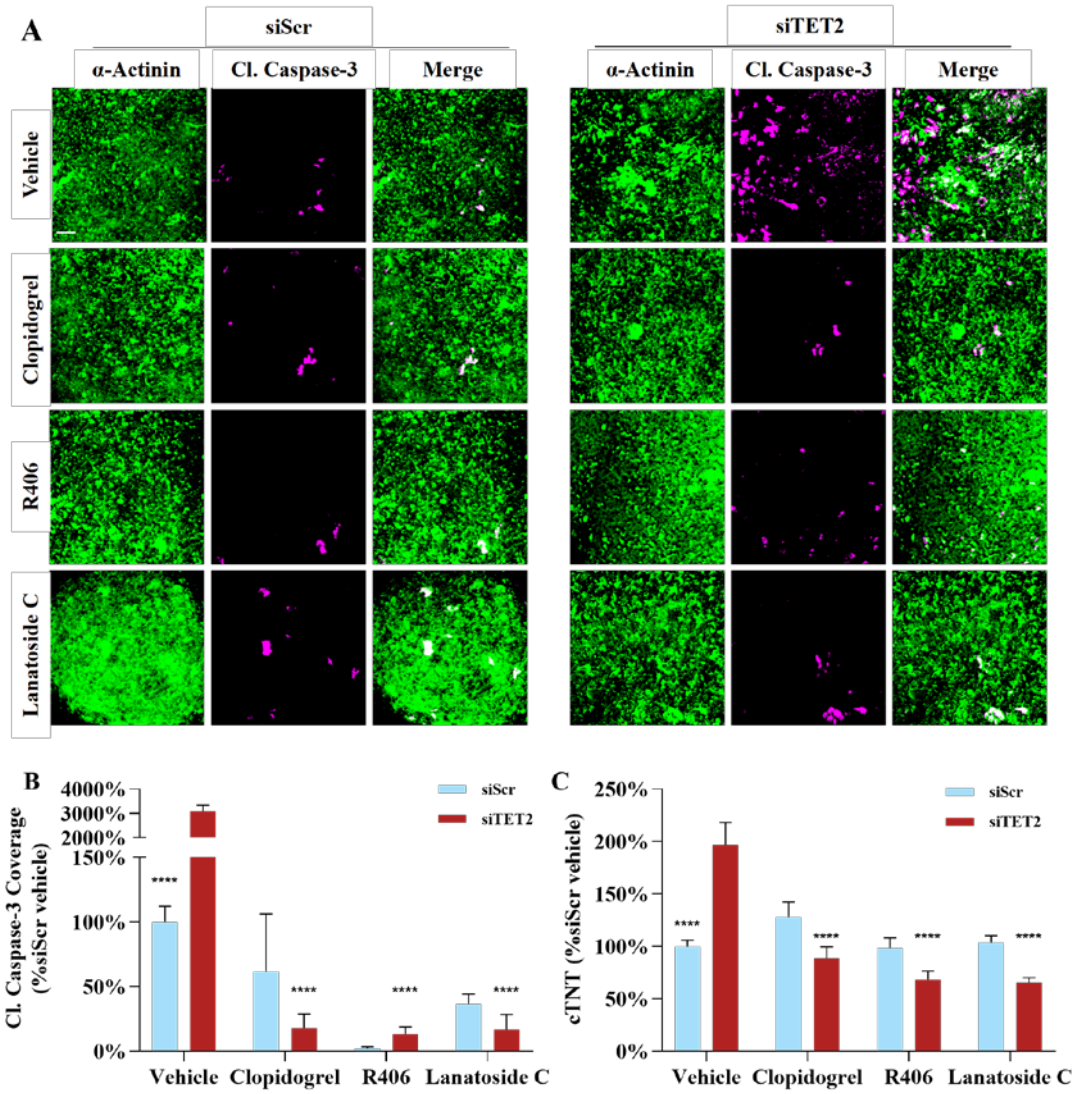


Figure 3.10 Detection of cardiomyocytes apoptosis in TET2-KD myeloid cells infiltrated SCO after drug treatment.

A. Representative images of cleaved Caspase-3 staining. **B.** Quantification of cleaved Caspase-3 staining showed decreased apoptosis in the siTET2 drug treatment group compared to the siTET2 vehicle. **C.** The decreased cardiomyocyte death was further confirmed by declining the prognostic cardiac biomarker cTNT after drug treatment. α-Actinin stands for cardiomyocytes, Cl. Caspase-3 stands for apoptosis, and double positive of α-Actinin and Cl. Caspase-3 stands for apoptosis of cardiomyocytes. N=4 using two-way ANOVA. All groups were normalized to siScr vehicle and each drug treatment group *vs.* siTET2 vehicle. All bar graphs show mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$. Scale bar: 50μm.

3.11 Clopidogrel, R406, and Lanatoside C affected the expression of pro-and anti-apoptosis genes in TET2-KD myeloid cells infiltrated SCO.

Next, we evaluated whether Clopidogrel, R406, and Lanatoside C could ameliorate the cell death induced by infiltrated TET2-KD myeloid cells in SCO. qPCR measurements demonstrated that all three compounds could reduce pro-apoptosis genes **(Figure 3.11 A-F)**, consistent and cross-validation with cleaved caspase-3 staining results mentioned in **Figure 3.10**.

However, in these three compounds, only Lanatoside C showed an unexpected increase in the expression of anti-apoptosis genes **(Figure 3.11 G-K)**. Furthermore, the BCL2/BAX ratio **(Figure 3.11 L)**, which stands for a positive value of prognostic marker[1-3], indicates Lanatoside C owns potentially clinical treatment value for TET2-mutant CHIP cardiovascular disease patients.

Result

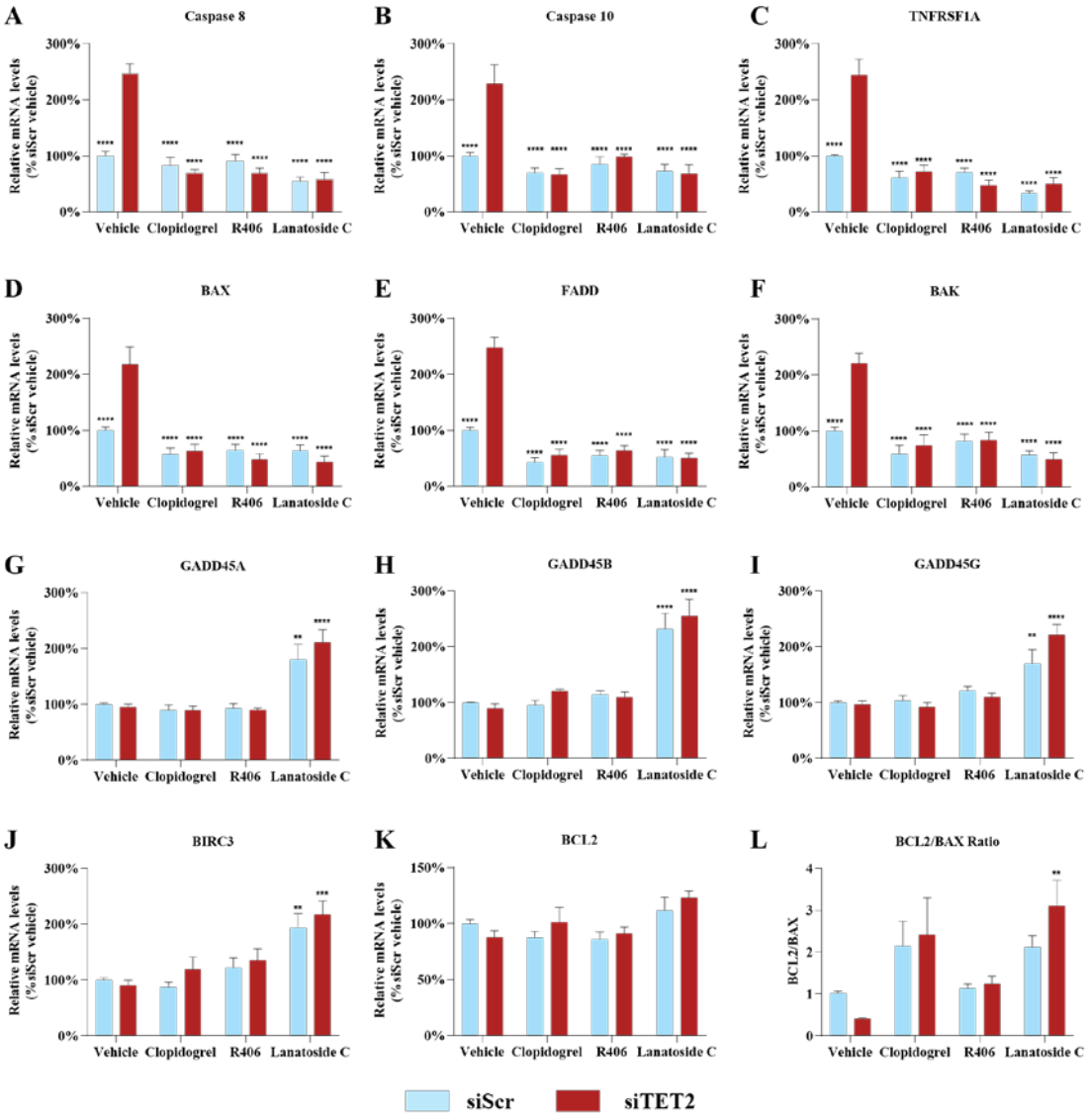


Figure 3.11 qPCR measurements of apoptosis genes in TET2-KD myeloid cells infiltrated SCO after drug treatment.

A-F. qPCR measurements of pro-apoptosis genes. **G-K.** qPCR measurements of anti-apoptosis genes. **L.** BCL2/BAX ratio is a positive value of the prognostic marker[1-3]. The above results indicate that Clopidogrel, R406, and Lanatoside C could decrease the expression of pro-apoptotic genes. However, other than Clopidogrel and R406, Lanatoside C showed an anti-apoptosis effect and potentially clinical treatment value for TET2 cardiac-CHIP patients. N=3 using two-way ANOVA. All groups were normalized to siScr vehicle and each drug treatment group *vs.* siTET2 vehicle. All bar graphs show mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

Result

3.12 Clopidogrel, R406, and Lanatoside C could decrease the heartbeat but enhance SCO contractility in TET2-KD myeloid cells infiltrated SCO

We next performed the Calcium Transient Assay to determine the contractile amplitude and frequency in response to drug treatment **(Figure 3.12 A-C)**. **Figure 3.12 A** showed that the contractile amplitude considerably dropped in TET2-KD myeloid cells infiltrated SCO. However, the contractile amplitude improved after the drug treatment in both siTET-drug and siScr-drug groups, revealing a potential capacity to enhance the contractility of Clopidogrel, R406, and Lanatoside C.

Correspondingly, in **Figure 3.12 B**, all three drugs demonstrated the same significant effect in reducing the heartbeat frequency compared to the TET2-KD myeloid cells infiltrated SCO, consistent with the qPCR results of NPP1 and NPPB expression **(Figure 3.9 F-G)** and apoptosis detection in TET2-KD myeloid cells infiltrated SCO **(Figure 3.10).** Mentionedforahead highlights and points out the potential therapy for heart failure. Furthermore, the decreased frequency-to-amplitude ratio also verified the effectiveness of the treatment **(Figure 3.12 C)**.

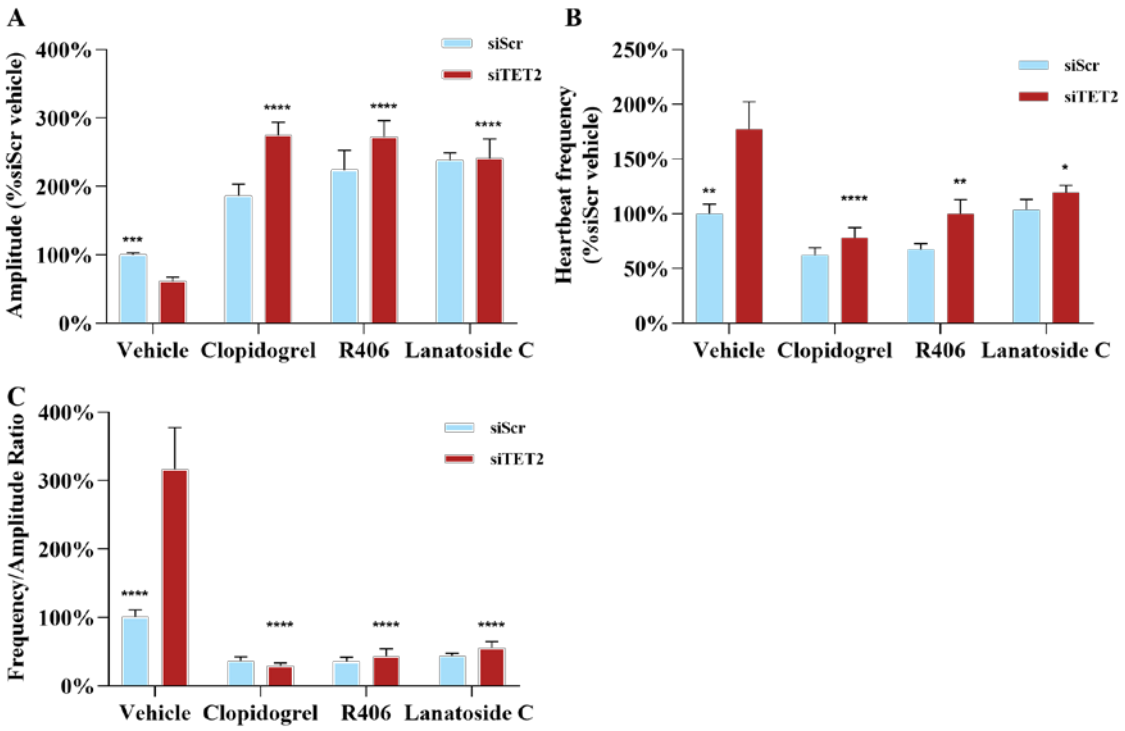


Figure 3.12 Detect the contractile and heartbeat frequency by calcium transient assay in TET2-KD myeloid cells infiltrated SCO.

A. Improved contractile amplitude both in the drug treatment and control groups, revealing the drugs' potential capacity to enhance the contractility. **B.** Decreased heartbeat frequency after the drug treatment compared to the siTET2 vehicle, suggesting the potential therapy for heart failure. **C.** Frequency to amplitude ratio verified the effectiveness of the treatment. N=8 using two-way ANOVA. All groups were normalized to siScr vehicle and each drug treatment group *vs.* siTET2 vehicle. All bar graphs show mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

Result

3.13 Transcriptomic changes of TET2-KD myeloid cells infiltrated SCO affected by Clopidogrel, R406, and Lanatoside C

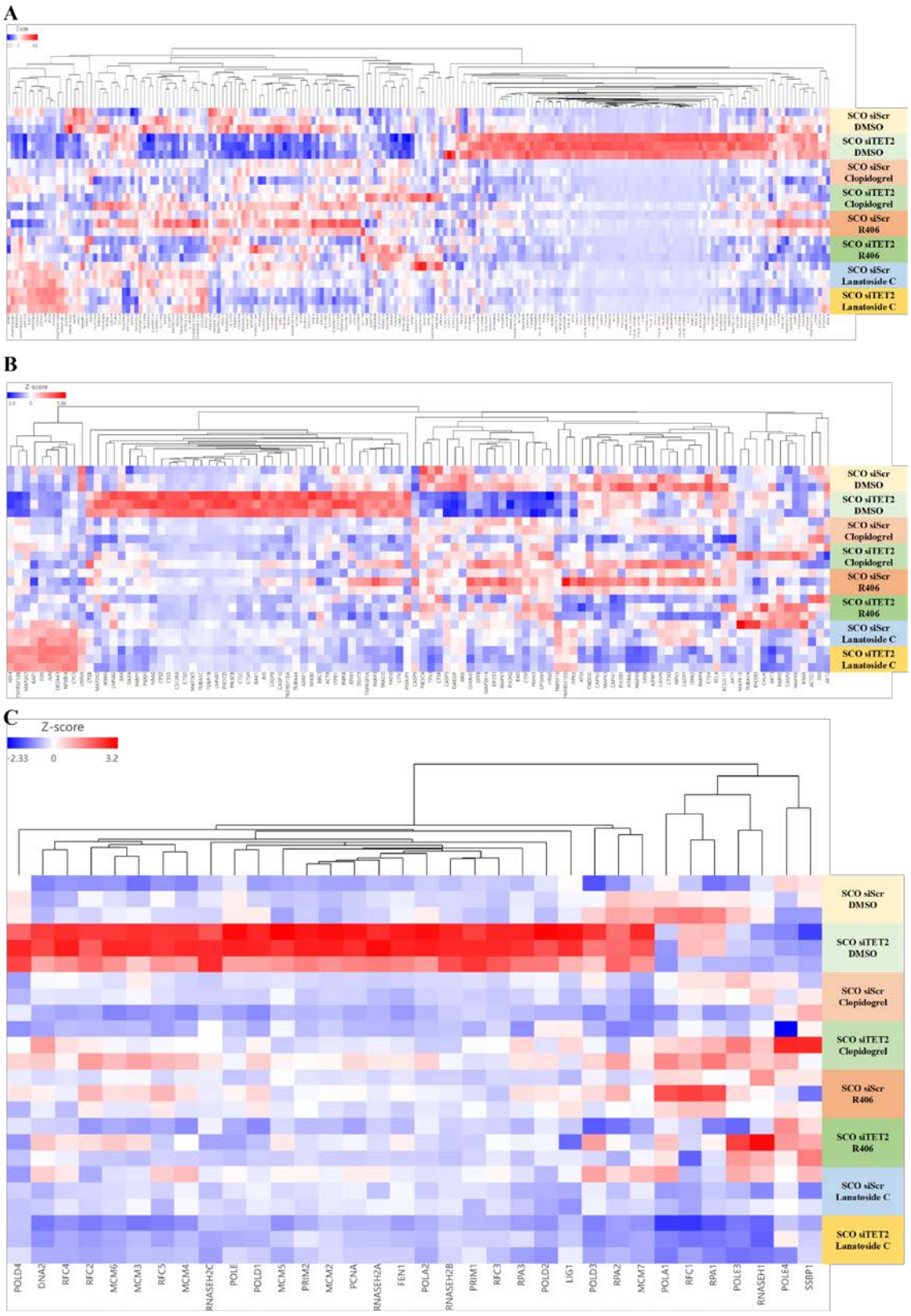
To further comprehend the mechanism and gene changes, we achieved RNAseq in the drug/vehicle-treated SCO infiltrated by TET2-KD/scramble-controlled myeloid cells **(Figure 3.13 A-E)**.

The analysis proved that Clopidogrel, R406, and Lanatoside C moderated TET2-KD myeloid cells induced inflammation, apoptosis, and proliferation by suppressing pro-inflammatory, pro-apoptosis, and proliferation genes **(Figure 3.13 A-C, respectively)**, which are consistent and cross-validated with previous results **(Figure 3.9 & 11)**. Similarly, the down-regulated expression of myeloid cell markers and collagen genes suggested that all three compounds could reduce the TET2-KD myeloid cell infiltration and their induced collagen **(Figure 3.13 D-E)**, which also co-responded with our previous data **(Figure 3.9 H-O)**.

Lanatoside C, other than Clopidogrel and R406, as the compound approved by FDA/EMA, presented a more robust suppression than Clopidogrel and R406 on the TET2-KD myeloid cells induced gene expression in SCO **(Figure 3.9),** indicating that Lanatoside C could own a potential preventive effect on myocardial fibrosis, which further could cause heart failure[59, 60]. Besides, Lanatoside C exhibited selectively reduced pro-apoptotic gene expression and strengthened anti-apoptotic gene expression in TET2-KD myeloid cells infiltrated SCO **(Figure 3.10)**. Therefore, the data above suggests we could

re-locate the usage of Lanatoside C as an effectual therapy for TET2-mutant CHIP patients in cardiovascular diseases.

Result


A
SCO siScr DMSO
SCO siTET2 DMSO
SCO siScr Clopidogrel
SCO siTET2 Clopidogrel
SCO siScr R406
SCO siTET2 R406
SCO siScr Lanatoside C
SCO siTET2 Lanatoside C
B
Z-score
SCO siScr DMSO
SCO siTET2 DMSO
SCO siScr Clopidogrel
SCO siTET2 Clopidogrel
SCO siScr R406
SCO siTET2 R406
SCO siScr Lanatoside C
SCO siTET2 Lanatoside C
C
Z-score
-2.33
0
3.2
SCO siScr DMSO
SCO siTET2 DMSO
SCO siScr Clopidogrel
SCO siTET2 Clopidogrel
SCO siScr R406
SCO siTET2 R406
SCO siScr Lanatoside C
SCO siTET2 Lanatoside C
POLD4
DNA2
RFC4
RFC2
MCM6
MCM3
RFC5
MCM4
RNASEH2C
POLE
POLD1
MCM5
PRIM2
MCM2
PCNA
RNASEH2A
FEN1
POLA2
RNASEH2B
PRIM1
RFC3
RPA3
POLD2
LIG1
POLD3
RPA2
MCM7
POLA1
RFC1
RPA1
POLE3
RNASEH1
POLE4
SSBP1

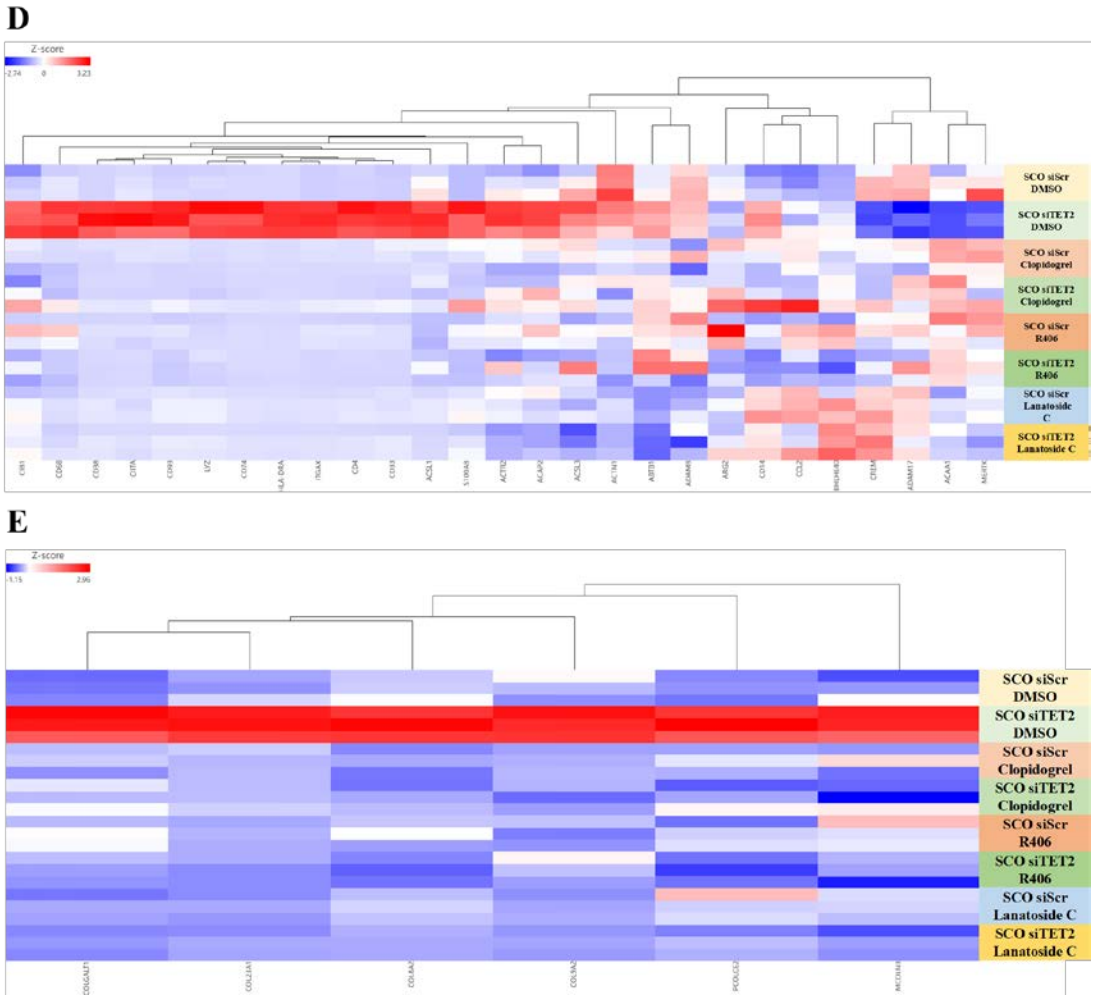


Figure 3.13 RNAseq data of Clopidogrel, R406, and Lanatoside C treatment in TET2-KD myeloid cells infiltrated SCO.

A. Hierarchical clustering and heatmap of pro-inflammatory genes. Clopidogrel, R406 and Lanatoside C showed down-regulation of pro-inflammatory genes. **B.** Hierarchical clustering and heatmap of pro-apoptosis. A lower expression of pro-apoptosis genes suggested a cardiac production effect after drug treatment in SCO infiltrated by TET2-KD myeloid cells. **C.** Hierarchical clustering and heatmap of proliferation genes. Clopidogrel, R406, and Lanatoside C could decelerate the proliferation rate in siTET2 vehicle SCO. **D.** Hierarchical clustering and heatmap of myeloid cell markers. The data indicated that all three compounds could decrease the infiltration of TET2-KD myeloid cells into SCO. **E.** Hierarchical clustering and heatmap of collagen genes. The data demonstrated that all three compounds could reduce the collagen formation induced by infiltrated TET2-KD myeloid cells. Each row stands for one independent sample from the depicted drug/vehicle group. Expression values are shown as the Z-scores for each gene.

4. Conclusion

To recap briefly, we first successfully built human self-organizing cardiac organoids compressing complete cell types of the human heart[39] **(Figure 3.1)**, then established the TET2 cardiac-CHIP model based on TET2-KD myeloid cells infiltrated SCO **(Figure 3.2-3)** and deliberated on the mechanism of the Clopidogrel, R406, and Lanatoside. Furthermore, the shortlisted drugs revealed a reduction in TET2-KD myeloid cell proliferation, decreased pro-inflammatory cytokines, and increased apoptosis levels **(Figure 3.4-7)**. Eventually, the TET2 cardiac-CHIP model treated with selected drugs showed a remarkable decline in TET2-KD myeloid cell infiltration and pro-inflammation cytokines, cardiomyocytes apoptosis, and fibrosis, and lowered cTNT levels **(Figure 3.8-13)**, while drug control groups were not affected. Cooperatively, the candidate drugs Clopidogrel, R406, and Lanatoside C revealed a positive clinical treatment value for TET2-mutant cardiovascular CHIP patients.

Our results indicate that SCOs can be an efficient pre-clinical model to study and validate CHIP genes and drug interactions due to their high tissue maturation and consistency but low variability. Furthermore, SCOs exhibit the possibility to mimic mature human myocardial responses to stress stimulation at the molecular, biochemical, and physiological levels. When used to evaluate CHIP-heart disease associations, our data revealed that TET2-KD myeloid cell invades SCO vasculature, cluster, and secret pro-inflammatory cytokines (IL-1β, IL-6, CCL-5, IL-18), which promote the release of cardiac Troponin T (cTNT) and cleaved Caspase-3 causing cardiomyocytes apoptosis. This phenotype could be rescued using positive drug candidates selected by Synthetic Lethality screening, emphasizing the significant value of our TET2 cardiac-CHIP model

for pre-clinical validation studies. Furthermore, the candidate drugs could be potentially applied to TET2-mutant CHIP cardiovascular disease patients.

Collectively, our results demonstrated that Clopidogrel, R406, and Lanatoside C treatment protected SCO from TET2-KD myeloid cells. However, Lanatoside C, as proved by both FDA and EMA, exhibited a robust protective effect in our drug-treated/controlled groups and targeted a potential medical therapy value for TET2-mutant CHIP patients in cardiovascular diseases. Lanatoside C targets Na^+-K^+ adenosine triphosphatases (Na^+-K^+ ATPases) in plasma membrane [61, 62], while Clopidogrel purinergic receptor P2Y12[63] [64, 65] and R406 (Tamatinib) primarily target spleen tyrosine kinase (SYK)[66, 67].

We identified Lanatoside C, which selectively targets and induces apoptosis in TET2-KD myeloid cells and attenuates cardiac inflammatory processes resulting in the following infiltration and inflammation. Besides, Lanatoside C displayed a desirable result for the substantial protective effect and potential medical value for TET2 mutant CHIP patients in cardiovascular diseases. Our data deliver a foundation and rationale for the clinical repositioning usage of Lanatoside C for treating TET2-mutant cardiovascular CHIP patients.

5. Discussion

The human body contains over 200 cell types[68], and many model systems have been established to study the intercommunications between multiple cell types and systemic[68]. Nevertheless, animal models somehow show weaknesses in connecting the human condition because of species-specific differences in immunology, metabolism, and genetics[69, 70]. These differences would lead to inaccurate drug safety or efficacy test results sequentially to drug failure during clinical trials. Thus, a new generation of multiple cell lineage platforms has been developed by scientists that can reliably and safely assess interplays between different cell types in the situation of human disease research and drug discovery.

State-of-the-art cardiac organoid technology exhibits an innovative biomedical model and tool to study tissue regeneration and disease modeling. Disease modeling based on organoids progressed drug screening platforms, compound development, and regulatory approval but lowered animal usage. Furthermore, organoid models describe an advanced in vitro tool, contributing to more physiologically related experiments that cannot be achieved in animals or humans[71]. The 3D organoid cultures provide multilevel external manipulations, allowing us to obtain and observe organ-like behaviors.

Moreover, the recent discovery of CHIP confirms that even before circulating leukocyte levels start to change, these mutations in CHIP induce HSC priming and lead to a harmful transformation of hematopoietic cells[11, 12]. However, there is no current therapy for patients diagnosed with CHIP[13]. Future work should identify what factors can accelerate or suppress mutated clonal growth. The existing literature suggests that patients with low

Discussion

VAFs have a low risk of developing CHIP complications[5]. Thus future therapeutics should inhibit mutated HSCs growth and limit the expansion of CHIP-related comorbidities. Additionally, the acquisition of CHIP mutations has been associated with malignant alterations and worsened CVD, and therefore patients should also be assessed for possible cardiovascular complications.

Regarding the future research direction of CHIP, our RNAseq data indicated the metabolism changes in our TET2-KD myeloid cells (data not shown). Coincidentally, Lanatoside C, as the most valued among our drug candidates, targets Na^+-K^+ ATPases in the plasma membrane[61, 62], which is also involved in metabolism. In this regard, interchanging metabolism drugs with CHIP could be a future drug target and research interest. As for Clopidogrel and R406, researchers can investigate the connection between CHIP with P2Y12 and SYK[63, 65-67], as these two drugs target, respectively.

Lanatoside C, other than Clopidogrel and R406, as the FDA and EMA proved, would jump from *in vitro* and *in vivo* tests but directly go to phase one clinical trial, saving much time for scientists. Furthermore, patients could benefit from the shortened drug research and development time, making profits for scientists and patients. As for Clopidogrel and R406, we can have them as a backup plan and, in parallel, apply them for FDA and EMA for the repurposed usage of cardiac-CHIP.

We linked our lab-built human iPSCs-induced self-organizing cardiac organoids (SCO) to the adult human heart[39]. As a result, our SCOs showed better *in vivo* human disease

modeling and served as an alternative to *in vivo* rodent models to understand human disease. Hence, our SCO platform potentially facilitates a reduction in animal use, which saves scientists a tremendous amount of time for ethical review and defense and reduces numerous research grants for purchasing or generating animal models. Furthermore, our lab-built SCO platform makes our experiment more humanitarian and environmental-friendly, which fits The Three Rs principle (Replace, Reduce, and Refine), aiming to make it more effective and humane[32].

In the future, human cardiac organoids will play an essential role in optimizing new drug discovery by developing cardiac toxicity prediction and providing mechanical knowledge of human heart physiology in high throughput screening. Furthermore, future collaboration will be broadly established between different scientific fields, including pathology, cell biology, physiology, pharmacology, animal medicine, and toxicology, ideally presenting an even more notable impact of this innovative technology.

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