

**THE ROLE OF LOW-DENSITY LIPOPROTEIN RECEPTOR-RELATED
PROTEIN 1 (LRP1) IN CARDIAC REPAIR AND REGENERATION-
GENERATING LRP1 KNOCKOUT ZEBRAFISH MODEL BY CRISPR-
CAS9 TECHNOLOGY**

BY

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DEANSHIP OF GRADUATE STUDIES

This thesis, written by **Hossam Mohammed Mohammed Ahmed** under the direction of his thesis advisor and approved by his thesis committee, has been presented and accepted by the Dean of Graduate Studies, in partial fulfillment of the requirements for the degree of **MASTER OF SCIENCE IN LIFE SCIENCES**.



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2024

Through perseverance and determination, the path to success unfolds with each step of relentless effort...

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First and foremost, Alhumdulillah for everything and for the countless blessings that have shaped my journey. I am deeply grateful to the Almighty for the opportunities and guidance that have led me to this point.

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LIST OF ABBREVIATIONS

MI: Myocardial Infarction

DEGs: Differentially Expressed Genes

GO: Gene Ontology

hpci: Hours Post-Cryoinjury

PCA: Principal Component Analysis

DAMPs: Damage-Associated Molecular Patterns

TLR: Toll-Like Receptor

IB4: Isolectin-B4

Mpx: Myeloid Peroxidase

CL: Clodronate Liposomes

CM: Cardiomyocyte

AFOG: Acid Fuchsin Orange G

ABSTRACT

Full Name : Hossam Mohammed Mohammed Ahmed

Thesis Title : THE ROLE OF LOW-DENSITY LIPOPROTEIN RECEPTOR-RELATED PROTEIN 1 (LRP1) IN CARDIAC REPAIR AND REGENERATION-GENERATING LRP1 KNOCKOUT ZEBRAFISH MODEL BY CRISPR-CAS9 TECHNOLOGY

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Heart failure, resulting from limited cardiac regeneration following myocardial infarction (MI), is a major cause of morbidity and mortality. In contrast to mammals, zebrafish exhibit a remarkable capacity for heart regeneration. This regenerative ability is associated with the resident macrophages expressing specific receptor Lrp1 (low-density lipoprotein receptor-related protein 1), which is mainly encoded by the *lrp1ab* gene based on our recently published scRNAseq profiling, while its function in heart regeneration is still unclear. To investigate the role of Lrp1, we generate large deletion and conditional KO alleles of *lrp1ab* via CRISPR-Cas9 technology. For large deletion, two guide RNAs were designed and synthesized to target the promoter and exon 1 and block the transcription of *lrp1ab* (“transcript-less” mutant), preventing potential genetic compensation from transcriptional adaptation. We have validated the deletion in injected embryos and raised potential founders. For conditional inactivation, we targeted insert a UFlip plasmid into the intron 2 of the *lrp1ab* locus. This plasmid contains loxp sites flanking a reporter cassette to hijack endogenous *lrp1ab* expression upon tissue-specific or temporal-specific Cre activity. We used both insertional reporter expression and junctional PCR analysis to confirm the precision integration of the UFlip cassette. In summary, we generated large deletion and conditional KO mutants to unravel the requirement and the molecular mechanisms underlying the potential contributions of Lrp1 in heart regeneration as well as its potential for enhancing heart regeneration in human patients as a potential therapeutic target.

ملخص الرسالة

الاسم الكامل: حسام محمد محمد احمد

عنوان الرسالة: دور البروتين الدهني منخفض الكثافة المرتبط بمستقبلات البروتين 1 (LRP1) في إصلاح القلب وتوليد التجديد LRP1 ZEBRAFISH MODEL بواسطة تقنية CRISPR-CAS9

التخصص: علوم الحياة

تاريخ الدرجة العلمية: 02/2024

يعد فشل القلب، الناتج عن تجديد القلب المحدود بعد احتشاء عضلة القلب (MI)، سبباً رئيسياً للمراضة والوفيات. وعلى النقيض من الثدييات، يُظهر سمك الزرد قدرة ملحوظة على تجديد القلب. ترتبط هذه القدرة على التجدد بالبلاعم المقيمة التي تعبر عن مستقبل محدد Lrp1 (البروتين المرتبط بمستقبلات البروتين الدهني منخفض الكثافة 1)، والذي يتم تشفيره بشكل أساسي بواسطة جين *lrp1ab* استناداً إلى ملف تعريف scRNAseq الذي تم نشره مؤخراً، في حين أن وظيفته في تجديد القلب لا تزال غير واضحة. . للتحقيق في دور Lrp1، نقوم بإنشاء حذف كبير وأليات KO المشروطة لتقنية *lrp1ab* مقابل تقنية CRISPR-Cas9. بالنسبة للحذف الكبير، تم تصميم وتوليف اثنين من RNAs الدليلي لاستهداف المروج و exon 1 ومنع نسخ *lrp1ab* (متحول "أقل نسخة")، مما يمنع التعويض الجيني المحتمل من التكيف النسخي. لقد تحققنا من صحة الحذف في الأجنة المحقونة وأثارنا المؤسسين المحتملين. من أجل التعطيل المشروط، استهدفنا إدخال بلازميد UFlip في الإنترون 2 في موضع *lrp1ab*. يحتوي هذا البلازميد على مواقع loxp التي تحيط بكاسيت مراسل لاختطاف تعبير *lrp1ab* الداخلي على نشاط Cre الخاص بالأنسجة أو الزماني. استخدمنا كلاً من تعبير مراسل الإدخال وتحليل PCR الوصلي لتأكيد التكامل الدقيق لشريط UFlip باختصار، قمنا بإنشاء حذف كبير وطفرة KO مشروطة لكشف المتطلبات والآليات الجزيئية الكامنة وراء المساهمات المحتملة لـ Lrp1 في تجديد القلب بالإضافة إلى قدرته على تعزيز تجديد القلب لدى المرضى من البشر كهدف علاجي محتمل.

CHAPTER 1

INTRODUCTION

1.1 Heart Failure

Heart failure (HF) affects over 23 million people globally and is a significant and expanding public health issue¹. The incidence and death rate of heart failure (HF) have not significantly decreased over the past few decades, despite a significant decline in the mortality rates linked to cardiovascular diseases such as hypertensive heart disease, acute coronary syndrome, congenital and valvular heart diseases^{2,3}, however the death rate of heart diseases have barely improved⁴⁻⁶. Heart failure (HF) is becoming more common, and the 5-year death rate for symptomatic HF is still over 50%. A key factor contributing to the resistant nature of HF is the very limited ability of the adult mammalian heart to repair following injury. Fibrotic tissue consequently replaces the injured myocardial, resulting in remodeling of the surrounding myocardium, worsening contractile function, and eventually heart failure and death. Interestingly, unlike adult mice and humans several vertebrates such as axolotls, newts and zebrafish exhibit a remarkable endogenous capacity to regenerate their hearts after various types of cardiac injuries^{7,8}. Zebrafish has a remarkable regenerative capacity after various cardiac insults^{4,5,7-9}. Out of all of them, cryoinjury most closely mimics the pathophysiology of clinical MI, which results in scarring and fibrosis during the first week the first week post cardiac injury¹⁰⁻¹². Recently it has been reported that neonatal mouse heart can fully regenerate after cryoinjury,

myocardial infraction (MI) or apical resection¹³⁻¹⁵. But the neonatal mouse heart's ability to regenerate itself is just transient, and by the time it is 7 days old, it has drastically decreased¹⁵. A recent study has shown that newborn humans may also possess the ability to fully regenerate their hearts after myocardial infraction¹⁶. Which suggest that mammals including human has the capacity to regenerate their hearts, despite this capacity is age-dependent and exist for a very short period after birth. It's interesting to note that current research has revealed that mature human¹⁷⁻¹⁹ and mouse²⁰⁻²⁴ cardiomyocytes (CMs) can still divide. This raises the possibility of creating therapeutic approaches to encourage CM proliferation and endogenous heart regeneration in order to restore myocardial function in HF patients.

1.2 Macrophage Dynamic Regulation Is Crucial for Homeostatic Heart Regeneration and Repair

Lai et al. conducted a comparative analysis between zebrafish and medaka to investigate the recruitment of macrophages following cryoinjury. The findings of their study demonstrated that medaka fish showed a diminished and delayed recruitment of macrophages in comparison to zebrafish. Notably, in zebrafish, delaying macrophage recruitment through the administration of clodronate liposome (CL) via intraperitoneal (IP) injection one day prior to cryoinjury led to compromised heart regeneration. However, despite the delayed recruitment, the number of infiltrating macrophages in zebrafish eventually recovered to control levels within the first week post-injury. This novel "macrophage-delayed model" in zebrafish treated with -1d_CL provides a unique opportunity to investigate and understand the molecular characteristics of regeneration-associated macrophages. These findings contribute to our understanding of tissue regeneration mechanisms by highlighting the distinct nature of late macrophages in zebrafish.

1.3 How inflammatory cells are activated repair in regenerative vs. non-regenerative conditions.

In their study, Lai et al. examined the global transcriptome changes in zebrafish hearts compared the regenerative and non-regenerative (resident macrophages deleted post cryo-injury) conditions. They conducted bulk RNAseq analysis and performed hierarchical clustering for all differentially expressed genes (DEGs) and gene ontology (GO) analysis (Figure 1). The findings revealed that the non-regenerative hearts exhibited delayed macrophage recruitment and prolonged inflammation compared to the regenerative PBS-treated hearts. Furthermore, they identified a broader range of phagocytic receptors and their ligands, such as integrin, calreticulin, and Lrp1, that were preferentially expressed in regeneration-associated macrophages. These results provide valuable insights into the molecular characteristics of macrophages involved in tissue regeneration. In Zebrafish Lrp1(low-density lipoprotein receptor-related protein 1), is mainly encoded by the *lrp1ab* gene based on Lai et al, recently published scRNAseq profiling, while its function in heart regeneration is still unclear. To investigate the role of Lrp1, we generated a large deletion and conditional KO alleles of *lrp1ab* vis CRISPR-Cas9 technology. In order to prevent potential genetic compensation from transcriptional adaptation, two guide RNAs were created and synthesized to target the promoter and exon 1 of the *lrp1ab* ("transcript-less" mutant) and block its transcription. The deletion has been validated in the injected embryos and raised potential founders.

For conditional inactivation, we targeted insert a UFlip plasmid²⁵ into the intron 2 of the *lrp1ab* locus. This plasmid contains loxp sites flanking a reporter cassette to hijack endogenous *lrp1ab* expression upon tissue-specific or temporal-specific Cre activity. We used both insertional reporter expression and junctional PCR analysis to confirm the precision integration of the UFlip cassette. In summary, we generated large deletion and conditional KO mutants to unravel the requirement and the molecular mechanisms underlying the potential contributions of Lrp1 in heart regeneration as well as its potential for enhancing heart regeneration in human patients as a potential therapeutic target.

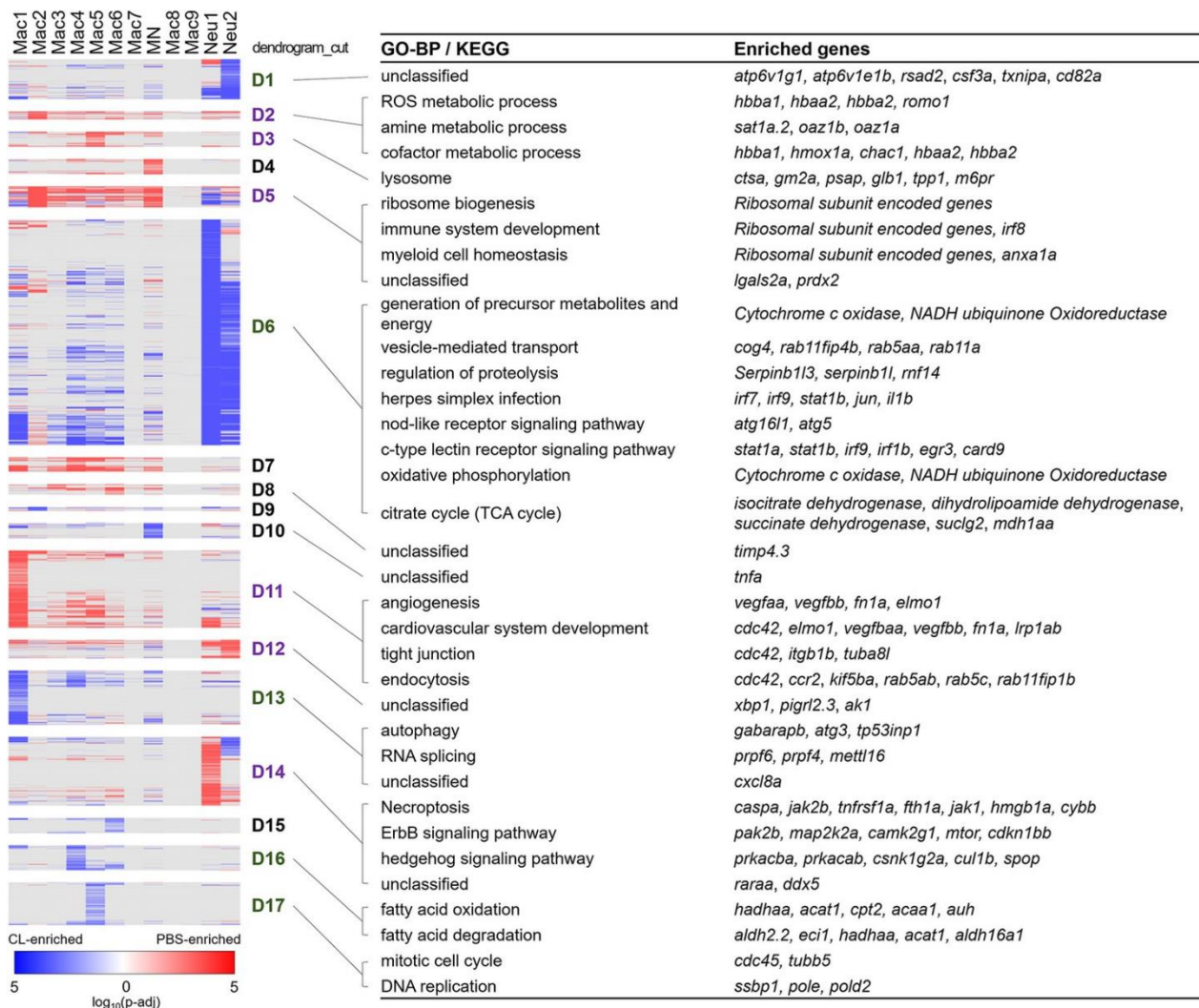


Figure 1. Differential gene expression patterns within inflammatory cell clusters are examined in the context of regenerative versus macrophage-delayed settings.³⁷

1.4 Problem

The problem addressed in this thesis is the differential expression of the low-density lipoprotein receptor-related protein 1 (LRP1) after cryo-injury in regenerative and non-regenerative zebrafish. Previous studies have shown that LRP1 is highly expressed in regenerative zebrafish compared to non-regenerative counterparts following cryo-injury. However, the specific role of LRP1 in the regenerative process and its interaction with macrophage M1, a distinct subset of resident macrophages, remain unclear. Therefore, the objective of this study is to investigate the role of LRP1, a phagocytic receptor highly expressed by macrophage M1, in the regenerative response of zebrafish. The study will employ experimental techniques such as cryo-injury induction, immunohistochemistry, and gene expression analysis to quantify LRP1 expression and evaluate tissue regeneration outcomes. By elucidating the functional implications of LRP1 and its association with macrophage M1, this research aims to enhance our understanding of tissue regeneration mechanisms in zebrafish. The findings may contribute to the development of regenerative therapies for non-regenerative organisms and advance the field of regenerative medicine.

1.5 Significance of this study

The significance of this study lies in the generation of a transgenic line of *lrp1ab* knockout zebrafish and the utilization of a conditional inactivation strategy using the UFlip plasmid containing loxP sites. This approach will enable a precise investigation of the role of the phagocytic receptor LRP1 in heart regeneration, scar resolution, and neutrophil recruitment.

By generating a transgenic line of *lrp1ab* knockout zebrafish, we can specifically target and disrupt the expression of this receptor. This allows us to study the functional consequences of LRP1 inactivation on heart regeneration and associated processes. The knockout model provides a valuable tool for understanding the specific contributions of LRP1 in tissue repair and regeneration, shedding light on its role as a potential therapeutic target.

Furthermore, the conditional inactivation of *lrp1ab* using the UFlip plasmid offers temporal control over gene inactivation. By activating the UFlip system just 48 hours prior to heart

injury, we can investigate the immediate effects of LRP1 inactivation during the critical regenerative period. This approach provides a precise and controlled means of studying the role of LRP1 in heart regeneration, scar resolution, and neutrophil recruitment.

The findings from this study will enhance our understanding of the intricate mechanisms underlying tissue regeneration and the involvement of LRP1 in these processes. Moreover, the results may have broader implications for regenerative medicine, as they can provide insights into potential therapeutic strategies for promoting efficient tissue repair and regeneration in humans.

In conclusion, the generation of a transgenic *lrp1ab* knockout zebrafish line and the utilization of a conditional inactivation system offer valuable tools to investigate the role of LRP1 in heart regeneration. This research has significant implications for understanding tissue repair mechanisms and may contribute to the development of novel therapeutic approaches in regenerative medicine.

1.6 Objective

The objective of this study is to investigate the role of the phagocytic receptor LRP1 in the process of heart regeneration, scar resolution, and neutrophil recruitment in zebrafish. Specifically, the study aims to achieve the following objectives:

1. Generate a transgenic line of *lrp1ab* knockout zebrafish: This objective involves creating a transgenic zebrafish line with a disrupted *lrp1ab* gene, allowing for the investigation of the functional consequences of LRP1 inactivation in heart regeneration. This will provide a valuable tool to study the specific role of LRP1 in tissue repair and regeneration.
2. Utilize conditional inactivation of *lrp1ab* using the UFlip plasmid: This objective aims to employ a conditional inactivation strategy by activating the UFlip system just 48 hours prior to heart injury. This approach will enable precise investigation of the immediate effects of LRP1 inactivation during the critical regenerative period, providing insights into the temporal role of LRP1 in heart regeneration, scar resolution, and neutrophil recruitment.

CHAPTER 2

LITERATURE REVIEW

1.1 Introduction

Heart failure is the leading cause of morbidity and mortality, partly because the human heart cannot regenerate damaged cardiomyocytes after a myocardial infarction. Unlike humans and adult mice Many vertebrates have the ability to regenerate their hearts, including some fish and amphibians. For example, zebrafish exhibit a remarkable capacity to regenerate their hearts following cryoinjury²⁶. Even mammals have the ability to repair their hearts throughout the embryonic and neonatal phases, albeit this ability soon disappears after birth, studies comparing between neonatal and adult mouse²⁶, as well as phylogenetically related species like medaka and zebrafish²⁶, have showed that the kind and degree of immune responses to cardiac injury²⁷. Rather than being exclusively dependent on genetic composition, environmental factors (such as hypoxia), or evolutionary complexity, the capacity for regeneration appears to depend on these factors and might significantly affect both the recovery and clinical prognosis following experimental MI²⁸

2.2 Comparative transcriptomic analyses are performed on the hearts of both zebrafish and medaka after injury to identify and compare gene expression patterns

To conduct comparative transcriptome studies, Lai et al. performed RNA extraction from the ventricles of regenerative zebrafish and non-regenerative medaka. Hierarchical clustering of more than 5 thousand differentially expressed genes (DEGs) provided a general overview of how genes expressed after cryo-injury, revealing similarities in the overall responses of zebrafish and medaka (Figure 2B). Distinct clusters with significant differences in gene expression variations between the two species were observed. For example, zebrafish exhibited increased expression of genes in clusters A1 and A2 after injury, while medaka showed an attenuated response, with elevated expression of genes in clusters B1 and B2. Compared to medaka zebrafish showed more dynamic response at 6 hours after heart injury, indicating a more acute gene response in zebrafish (Figure 2B). Principal Component Analysis (PCA) using normalized counts of all expressed genes revealed separation of samples by species in component 1, while component 2 showed temporal similarities in gene expression changes following cryoinjury, albeit with less dynamism in medaka (Figure 2C). Interestingly, medaka did not exhibit comparable reversion until three days after cryoinjury, whereas zebrafish showed substantial patterns of gene expression returning to the undisturbed condition after two days (Figure 2C). These

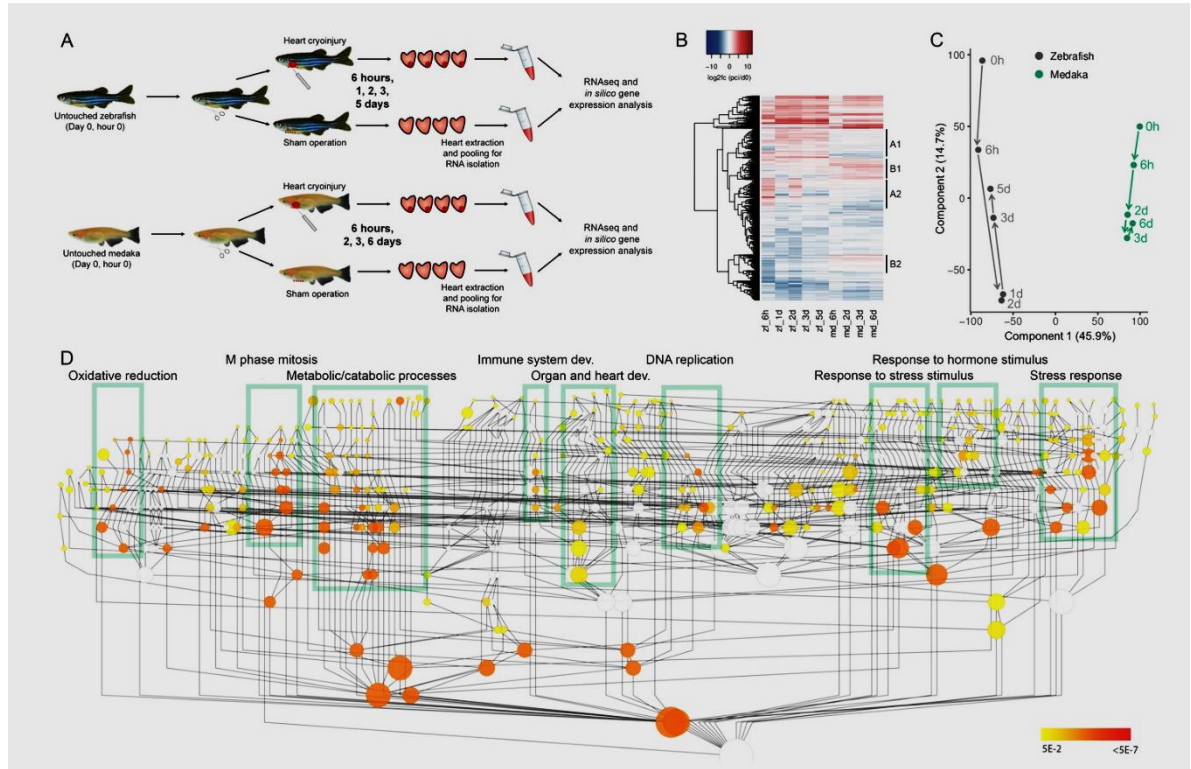


Figure 2. Comparative transcriptomic analysis between Zebrafish and medaka post cryoinjury²⁹

findings provide insights into the main gene expression responses of zebrafish and medaka following heart damage.

2.3 Comparing the medaka heart to the zebrafish heart, macrophage recruitment is both reduced and delayed after injury

Innate immune cells recognize and react to DAMPs generated at the site of tissue damage, in part due to TLR signaling. Therefore, at various stages after damage, we looked at and contrasted leukocyte recruitment in the hearts of zebrafish and medaka. We labeled macrophages and neutrophils in both organisms using isolectin-B4 (IB4) and a myeloid-specific peroxidase (Mpx) antibody, respectively, for direct comparison. In post-infarction adult hearts and embryonic mouse tissues, macrophages are labeled with IB4^{30, 31}. Both

neutrophils and macrophages were seen in the damaged zebrafish heart at 6 hpci. At this point, neutrophils were more prevalent than macrophages, which is in line with findings from the fin amputation model³². They noticed a notable decrease at 7 dpci and a peak in neutrophil recruitment at 2 dpci, suggesting that neutrophil clearance happens in zebrafish between 2 and 7 dpci. Conversely, in zebrafish, macrophage counts rose by 7 dpci within the damaged region after starting out low at 6 hpci. However, at 7 dpci, macrophage recruitment in medaka was considerably lower and occurred more slowly than in zebrafish²⁹. Additionally, in medaka neutrophil recruitment peaked at 2 dpci and resembled that of zebrafish at 6 hpci; however, by 7 dpci, clearance was less effective, and the number of neutrophils was higher. The prolonged neutrophils in the damaged area may be a factor in the medaka hearts increased fibrotic reaction following injury³³. As a whole the processes of neutrophil clearance and macrophages recruitment are delayed in medaka.

2.4 The regenerative capacity of zebrafish hearts is hindered when there is a delay in the recruitment of macrophages

Lai et al., pre-depleted macrophage recruitment one day before injury using injections of clodronate liposomes (CL) in order to investigate the significance of prompt macrophage recruitment in zebrafish cardiac regeneration. An established technique for reducing macrophages in mice, salamanders, and zebrafish is the administration of CL³⁴. In fact, at 1 dpci, we observed wounded areas devoid of macrophages, in contrast to control hearts where macrophages (both mpeg1:EGFP+ and IB4+) were clearly present in the injured area. This recruitment was drastically reduced in hearts subjected to CL. However, the numbers of neutrophils in the hearts of the control and CL-exposed groups were comparable. When compared to controls, the hearts subjected to CL showed a rise in

macrophage numbers by 7 dpci, suggesting that the therapy just postponed the recruitment and invasion of macrophages into the damaged area. Regarding neovascularization in these hearts, they found that, at 7 dpci, vessel development was clearly lower in hearts exposed to CL than in controls, even if angiogenic sprouting was still somewhat unaffected. At 7 dpci, CM proliferation was also considerably lower in hearts exposed to CL than in controls, which corresponded with decreased neovascularization ²⁹. In hearts subjected to CL, a few arteries were visible at 7 dpci; however, the scar tissue was entirely avascular and unresolved by 1 mpci, indicating that the few vessels were insufficiently stable to revascularize the wounded area. Moreover, scar tissue in hearts exposed to CL had a fibrin and collagen composition comparable to controls, but it was noticeably bigger and unresolved at 1mpci, according to Acid Fuchsin Orange G (AFOG) staining. In contrast, the coronary network was restored and there was little evidence of scarring in the control hearts. According to Lai et al., hearts exposed to CL had impaired neutrophil clearance. The presence of Mpx⁺ structures within IB4⁺ macrophages at 7 days post-cardiac injury also indicated that macrophages play a role in clearing neutrophils. This suggests that macrophages partially remove neutrophils through the process of phagocytosis. However, the recruitment of neutrophils to the damaged area did not seem to be affected in hearts exposed to CL at 6 hours post-cardiac injury. Ongoing recruitment of neutrophils was still observed at 2 days post-cardiac injury, potentially due to the involvement of coronaries. However, in hearts exposed to CL, the number of neutrophils in the damaged area was significantly higher compared to control hearts at 7 days post-cardiac injury. Additionally, at 1-month post-cardiac injury, there was still a substantial presence of neutrophils in CL-exposed hearts, whereas in control hearts, neutrophil clearance occurred between 2 and 7

days post-cardiac injury. In CL-exposed hearts, the clearance of neutrophils by macrophages consistently occurred at 1-month post-cardiac injury rather than at 7 days post-cardiac injury, as observed in control hearts. These findings suggest that in the process of zebrafish heart regeneration, the prompt recruitment of macrophages is crucial for coordinating neovascularization, neutrophil clearance, cardiomyocyte proliferation, and resolution of scar tissue.²⁶

2.5 Heart regeneration in zebrafish relies on the presence of distinct resident cardiac macrophages that play a crucial role in the regenerative process

The study conducted by Wei et al. focused on investigating the impact of macrophage pre-depletion and analyzing the characteristics of macrophages and neutrophils in regenerative (–1d_PBS) and non-regenerative (–1d_CL) zebrafish hearts using bulk and single-cell transcriptomic profiling. They generated split UMAPs to examine the dynamic changes in inflammatory cell clusters under different conditions and time points. In the undamaged heart/naïve state, Mac 2 and 3 were identified as the main resident macrophage clusters, followed by Mac 1, 4, and 8. In the regenerative PBS-control hearts, Mac 2 and 3 expanded significantly during the first week after injury, while Mac 1, 4, and 5 showed rapid growth at 1 day post-cardiac injury (dpci) and then gradually decreased to a steady state at 7 dpci.

In contrast, in the -1d_CL-treated hearts, Mac 2 dramatically decreased, while Mac 1, 4, 5, and 6 were retained over time after injury.

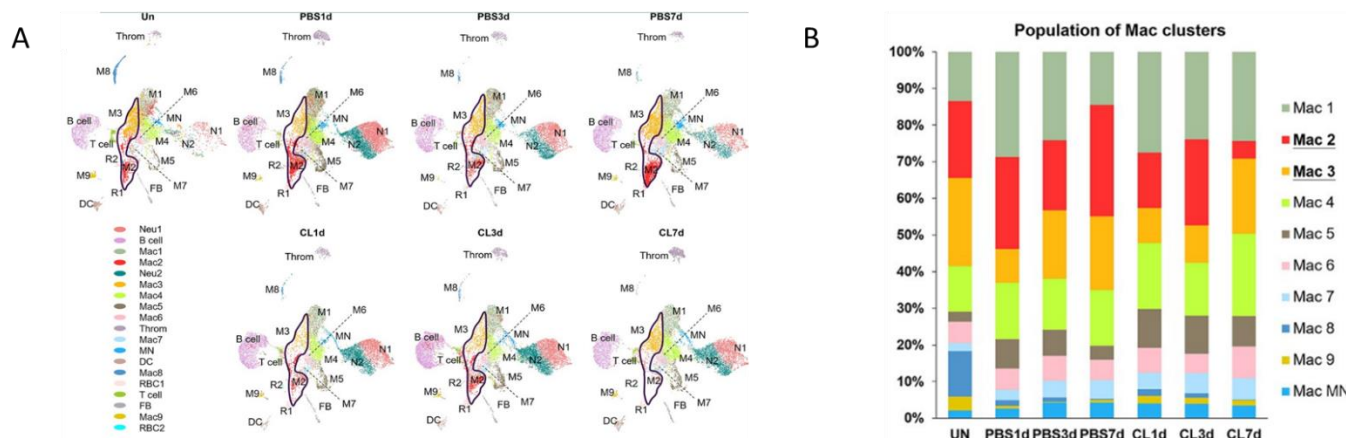


Figure 3. Analysis of the temporal cell percentage of inflammatory cells revealed resident macrophage clusters that were more abundant under regenerative circumstances³⁷.

2.6 Lrp 1 is highly expressed by Mac 1 post cryoinjury

In zebrafish Lrp1 has four paralogs but it is mainly encoded by *lrp1ab* (Figure 4A), scRNA profiling done by Ke-Hsuan Wei et al., shows stronger upregulation of *lrp1ab* in resident macrophages (M1) after cryoinjury (Figure 4B)³⁷ When combined, these findings

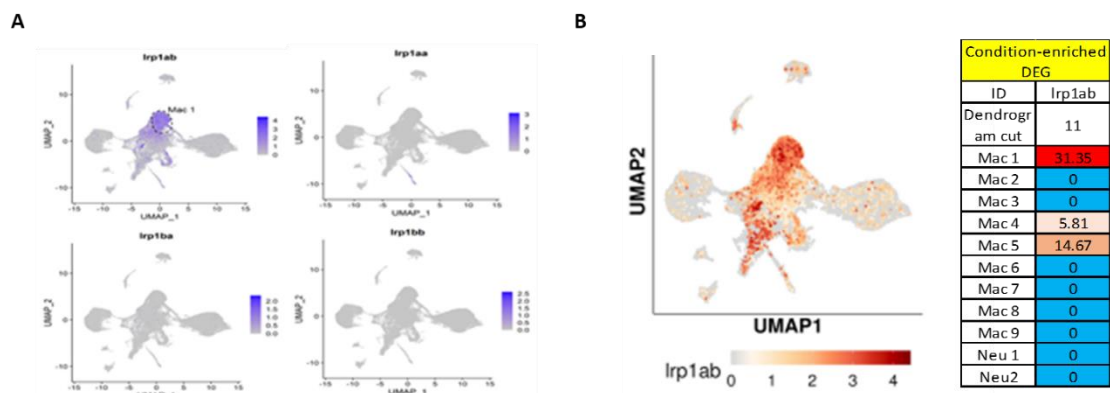


Figure 4. Lrp1 is highly expressed by *lrp1ab* by distinct resident macrophage following cryoinjury.

(A) UMAP comparison between lrp1 paralogs expression after cryoinjury in zebrafish as only lrp1 is highly expressed by lrp1ab following heart injury. (B) lrp1 is highly expressed by Mac 1

point to a critical but hitherto unknown involvement for these receptor-expressing macrophages in the regeneration of the injured heart.

2.7 CRISPR Cas-9 technology

Genome editing technology allows for the introduction of specific changes in the DNA sequence through nucleotide substitutions, insertions, and deletions. Its primary purpose is to prevent the emergence of genetic diseases by targeting and modifying specific genes. Many diseases are associated with alterations in gene expression, particularly hereditary disorders caused by mutations in a single gene. CRISPR technology, in particular, has gained significant popularity due to its efficiency and versatility compared to ZFNs and TALENs. By modifying a short guide RNA segment's base sequence, CRISPR directs Cas proteins to a specific genomic location. This is different from ZFNs and TALENs, which use proteins to target DNA strands. The use of CRISPR/Cas9 as a gene-editing technique has become prevalent in the scientific community. The CRISPR/Cas9 system naturally evolved in bacteria and archaea as a defense mechanism against phage invasion and plasmid transfer. When these microorganisms are exposed to exogenous phage or plasmid DNA, they incorporate a portion of the foreign DNA into the CRISPR spacer region. Upon reinfection with homologous DNA, the CRISPR region is transcribed, and a single guide RNA (sgRNA) is produced through maturation and processing steps. The sgRNA guides Cas9 to cleave the DNA strand at the complementary spacer region. Recognition of the sgRNA requires short guanine-enriched sequences called protospacer-adjacent motifs (PAMs), with the prevalent NGG sequence preferred by *Streptococcus pyogenes* Cas9 (SpCas9). This preference facilitates the application of CRISPR technology in various fields, including biomedicine, plant science, and animal science. CRISPR/Cas9 allows

precise targeting of almost any desired genomic location to correct disease-causing mutations or silence genes associated with disease onset by modifying the nucleotide sequence of a specific guide RNA segment. However, it may face challenges in accessing highly chromatinized regions of the genome. Promising applications of CRISPR/Cas9 include potential treatments for cancer, heart conditions, sickle cell anemia, and neurological disorders.

2.7.1 Conditional inactivation using CRISPR Cas-9 targeted integration

To investigate the role of cell type-specific genes in various developmental stages, postembryonic processes, and disease models, it is essential to regulate gene activity spatially and temporally. The Cre/lox system, facilitated by the bacteriophage Cre recombinase, allows for precise insertion of loxP sites into target genes, enabling conditional gene inactivation. In mice, the Cre/lox resource has been developed using embryonic stem cells and homologous recombination to create transgenic lines expressing Cre and modify genes with loxP sequences. However, in zebrafish, alternative methods have been employed because of the absence of technology that allow homology arms insertion in embryos at very early stage. Tol2 transposon transgenesis in combination with defined promoters or BAC recombineering has been used to generate Cre lines in zebrafish. Random Tol2 transposon enhancer trap screens have yielded numerous zebrafish cell-type-specific Cre drivers. Previously, zebrafish only had Cre/lox-mediated conditional gene regulation through floxed/FRT gene traps, this trap can be reversed through Cre activation that led to inversion or reversion of loxP sites. Recent advancements in zebrafish gene editing techniques, particularly using CRISPR-Cas9 and TALENs, have simplified the isolation of Cre drivers and the production of actual conditional alleles regulated by loxP sites through targeted integration. Integration of a floxed cassette with a connected reporter in a single event offers advantages such as fluorescence genotyping and streamlined targeting. Various approaches, including homology-based strategies and Zwitch cassette inversion, have been utilized to generate conditional alleles and achieve efficient gene knockdown or knockout.

CHAPTER 3

MATERIALS AND METHODS

3.1 Equipment and Consumables Utilized in the Research Study

The following equipment and consumables were utilized in the research study:

Micro-loader Tips (World Precision Instrument)

Borosilicate Glass Capillaries (World Precision Instrument)

Polystyrene Petri Dishes (Thermo Fisher)

NEB Stable Competent E. coli (New England BioLab)

ECOS X Bacteria (Thermo Fisher)

Thermo Scientific PCR Tubes (Thermo Fisher)

Pierce™ Microcentrifuge Tubes, 1.5 mL (Thermo Fisher)

Sutter Instruments Micromanipulator (Fisher Scientific)

myGel™ Mini Electrophoresis System (Thermo Fisher)

Eppendorf Thermal Cycler Gradient (Eppendorf)

iBright™ FL1500 Imaging System (Thermo Fisher)

Sorvall™ Legend™ Micro 21R Microcentrifuge (Thermo Fisher)

NanoDrop™ 2000/2000c Spectrophotometers (Thermo Fisher)

Nikon SMZ 25 (Fluorescence Stereo Microscope) (Nikon)

Modular Stereo Microscope for Fluorescent Imaging Leica MZ10 F (Thermo Fisher)

Fisherbrand™ Isotemp™ Ultra Low Temperature Freezer (Fisher Scientific)

The specified equipment and consumables were selected based on their established reliability and suitability for the experimental procedures conducted in this research.

3.2 Incorporation of Ethical Guidelines and Compliance in Zebrafish Research

The utilization of zebrafish for research purposes adhered to the approved protocols by the Iowa State University Animal Care and Use Committee, specifically IACUC-18-279 and IACUC-058. The research procedures involving zebrafish were conducted in accordance with the Guidelines for Ethical Conduct in the Care and Use of Animals (APA, 1986). Furthermore, all zebrafish-related procedures strictly followed the ethical criteria outlined by the American Veterinary Medical Association (2020), ARRIVE⁶⁰, and NIH guidelines for the humane use of animals in research.

3.3 Chemicals

Chemicals and Vendors

Tricaine (Sigma-Aldrich) - Merck

DreamTaq Green PCR Master Mix (2X) - Thermo Fisher

TBE Buffer (Tris-borate-EDTA) (10X) - Thermo Fisher

Agarose I (Molecular Biology Grade) - Thermo Fisher

Ethanol, Absolute (200 Proof), Molecular Biology Grade - Fisher Scientific

LB Broth - Fisher Scientific

Select Agar™, powder - Thermo Fisher

Kanamycin Sulfate (White Powder), Fisher BioReagents - Fisher Scientific

DNA ladder, 100 bp - Thermo Fisher

(6X) GeneRuler 1 kb DNA Ladder - Thermo Fisher

(6X) Orange DNA Loading Dye - Thermo Fisher

SYBR™ Green II RNA Gel Stain, 10,000X concentrate in DMSO - Thermo Fisher

Diethyl pyrocarbonate - Sigma-Aldrich/Merck

TE Buffer - Thermo Fisher

UltraPure™ DNase/RNase-Free Distilled Water - Thermo Fisher

Remel™ Phenol Red Solution - Thermo Fisher

Glycerol for Molecular Biology $\geq 99.0\%$ - Sigma-Aldrich/Merck

S.O.C. Medium - Thermo Fisher

NEBuffer™ Set (r1.1, r2.1, r3.1, and rCutSmart™) - New England Biolab

Phenol Red - Thermo Fisher

Please note that the ordering is for the purpose of clarity and does not imply any specific grouping or categorization of the reagents.

3.4 Microinjection

Prior to magnification, fine-tuning the resolution is recommended.

ENTREPRISE OF EMBRYOS, Next, you must arrange the embryo so that the cytoplasmic cell membrane of the first cell is perpendicular to the needle.

INJECTION: Press the injection pedal while carefully extending the needle through the yolk and chorion.

- The injection solution won't disperse right away if the needle is inside the yolk.
- The phenol red will diffuse right away if the needle is outside the embryo after passing through the yolk.
- If the injection is successful, the yolk or cytoplasm will have a red patch.
- You need two labeled plates filled with egg water: one for collecting eggs and the other for the embryo following microinjections.
- After two hours following microinjection, the injected embryo should be placed at 28 C° and its water changed, or the dead embryo removed.

3.5 Kits and Enzymes

Various enzymes and kits from different vendors were employed in the experimental procedures. The specific enzymes and kits used, along with their respective vendors, are detailed below:

Quick Plasmid Miniprep Kit: The QIAGEN Quick Plasmid Miniprep Kit (Vendor) was used for the rapid isolation and purification of plasmid DNA from bacterial cultures (QIAGEN).

Quick Gel Extraction and PCR Purification Kit: The purification of PCR products and gel extraction were accomplished using the QIAGEN Quick Gel Extraction and PCR Purification Kit (Vendor). This kit allows for efficient recovery of DNA fragments from agarose gels and PCR reactions (QIAGEN).

T4 DNA Ligase: T4 DNA Ligase from Thermo Fisher (Vendor) was employed for DNA ligation reactions. This enzyme facilitates the formation of phosphodiester bonds between DNA fragments (Thermo Fisher).

MEGAscript™ T7 Transcription Kit: The Thermo Fisher MEGAscript™ T7 Transcription Kit (Vendor) was used for in vitro transcription of RNA. This kit enables the synthesis of large quantities of RNA (Thermo Fisher).

MEGAscript™ SP6 Transcription Kit: The Thermo Fisher MEGAscript™ SP6 Transcription Kit (Vendor) was utilized for in vitro transcription of RNA. This kit allows for the generation of RNA transcripts using SP6 RNA polymerase (Thermo Fisher).

QIAquick Nucleotide Removal Kit: The QIAGEN QIAquick Nucleotide Removal Kit (Vendor) was employed for the purification of nucleotides. This kit effectively eliminates unincorporated nucleotides from reaction mixtures (QIAGEN).

T4 DNA Polymerase: T4 DNA Polymerase from Thermo Fisher (Vendor) was used for DNA end repair and polishing reactions. This enzyme possesses 3' to 5' exonuclease activity and terminal transferase activity (Thermo Fisher).

dNTP Mix (10 mM each): The Thermo Fisher dNTP mix, containing 10 mM each of deoxyribonucleoside triphosphates, served as a source of nucleotides for DNA synthesis (Thermo Fisher)

EcoRV: The New England Biolabs EcoRV restriction enzyme (Vendor) was employed for DNA digestion. EcoRV recognizes and cleaves the DNA sequence (New England Biolabs, Catalog Number).

SpeI: DNA digestion was also performed using the New England Biolabs SpeI restriction enzyme (Vendor). SpeI recognizes and cleaves the DNA sequence (New England Biolabs).

KAPPA PCR: PCR amplifications were conducted using the Sigma Aldrich KAPPA PCR master mix (Vendor). This master mix provides high fidelity and efficient amplification of DNA targets (Sigma Aldrich).

In-Fusion HD Cloning Kit: The Takara Bio In-Fusion HD Cloning Kit (Vendor) was utilized for DNA cloning procedures. This kit enables seamless cloning of DNA fragments into vectors (Takara Bio)

3.6 Primers and gRNAs

Oligonucleotides	Sequences (5' 3')
Promoter new gRNA F.primers	Cagattgcgtttcagagggttg
Exon1 reverse primer	Agacttaattactagttcttcacatcaagctgtggcaggg
Forward primer to check after KO	Ggtgggtttcagacggagac
Exon 1 reverse primer to check after KO	Cactattaccaggccatgtctg
Intron 2 forward primer	Aagtttgcattggcggtatgtac
Intron 2 reverse primer	Ctgggggtgatttgactgggtaaa
2 nd 5' for junctional PCR	Cgcacacacacacagacctaatt
2 nd 3' for junctional PCR	CAAGTGTTCCCTCATATGAAGG
3 rd 5' for junctional PCR	Tctgcacctctcttcacatctg
3 rd 3' for junctional PCR	CAAGTGTTCCCTCATATGAAGG

3.7 Preparation of LB-Agar Plates

1. To prepare 20 agar plates, I combined 10 g of LB (Luria-Bertani) and 6 g of agar A and added them to a 400 ml solution. This mixture was then poured into a 1-liter bottle.
2. I applied autoclave tape to the bottle and placed it inside the autoclave machine. The autoclave was set to run for 30 minutes.
3. After completing the autoclaving process, I transferred the bottle to a water bath to allow it to cool down to room temperature.
4. Once the solution had cooled, I added 400 µl of kanamycin and gently swirled the bottle to ensure proper mixing.
5. Prior to pouring the media, I sterilized 36 petri dishes in a Hepa filter under UV lights.

6. Next, I carefully poured the prepared media into approximately one-third of each petri dish.
7. The plates were then placed in a designated area for 20 to 40 minutes to allow the agar to solidify.
8. After solidification, the agar plates were ready for bacterial growth.

It is worth mentioning that the LB medium was initially prepared by adding 15 g/L agar before autoclaving. Once autoclaved, the solution was cooled to approximately 55 °C. Antibiotics, if required, were added before pouring the media into the Petri dishes. After solidification, the plates were flipped and stored at +4 °C. In the case of tetracycline, storage was done in the dark.

3.8 Zebrafish strains and their upkeep and management

Zebrafish (*Danio rerio*) were housed in a controlled environment using an Aquaneering aquaculture system, maintaining a constant temperature of 26 °C. A lighting schedule of 14 hours of light and 10 hours of darkness was implemented to simulate natural diurnal cycles.

For the generation of the *lrp1ab* large deletion knockout, we utilized a transgenic zebrafish line expressing *fli1:EGFP* and *myl7:DsRed2-NLS*. This transgenic line allowed for the visualization of endothelial cells (*fli1:EGFP*) and cardiomyocytes (*myl7:DsRed2-NLS*) through the expression of fluorescent proteins.

In the case of the *lrp1ab* conditional inactivation, we employed a transgenic zebrafish line expressing *mpeg1.1-T4:2A-CreERT2*. This transgenic line facilitated the controlled induction of Cre recombinase activity in macrophages upon treatment with tamoxifen, enabling conditional gene inactivation of *lrp1ab*.

These zebrafish strains and transgenic lines were carefully selected and maintained to ensure the successful implementation of our experimental approach in studying the role of *lrp1ab* in our research on coral regeneration.

3.9 Transformation via Cloning using E. Coli E-CosX Cells

- a. vial containing 50 µl of E. Coli E-CosX competent cells should be allowed to thaw on ice for around five minutes.
- b. Label microcentrifuge tubes for each ligation and set them on ice while the cells thaw.
- c. To transform the E-CosX competent cells, add 2 µl of the ligation reaction. Make sure that the amount of ligation reaction added does not exceed 5% of the total volume of the cells that are competent.
- d. Use the pipette tip to gently swirl the contents or tap the tube several times to gently mix them. Steer clear of pipetting during mixing as this could lyse the cells.
- e. Heat shock the cells: For one minute, immerse the cell-containing section of the tube in a water bath at 42°C.
- f. Give the cells two minutes of ice-soaking time.

g. For every transformation, add 125 μ l of room-temperature LB or SOC media.

h. Shake the incubator and incubate the cells for one to one and a half hours at 37°C.

As the cells undergo recovery, distribute 40 μ l of X-Gal solution onto LB Kanamycin selection plates. Because X-Gal is deadly to cells when it's wet, it's best to label the plates first, then let them dry in an incubator set at 37°C.

i. Transfer 150 μ l of every transformation to the appropriate, labeled plate after the recovery period and once the X-Gal is dry.

j. Keep the plates in an overnight 37°C incubator.

3.10 Electrophoresis

DNA fragments are separated by electrophoresis in order to verify their size and purity. Based on the anticipated size of the DNA fragments, agarose gels were made with different concentrations ranging from 1.0% to 2.0%. Samples are accompanied by loading and fluorescence dyes prior to loading, and 1xTAE electrophoresis buffer is used for the electrophoresis, with an electric field intensity of 3–5 V/cm. To ascertain the dimensions and quantity of the DNA-laden samples, a suitable DNA marker (DNA ladder, NEB) was loaded concurrently.

3.11 DNA fragmentation

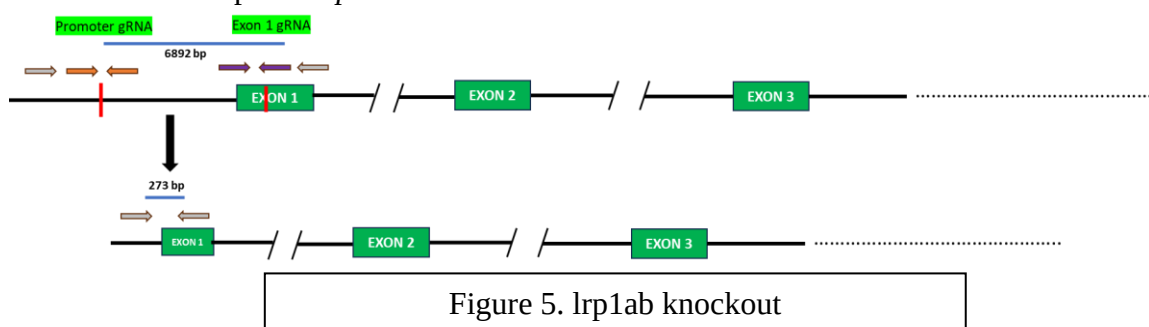
To digest the DNA, 1 to 10 μ g of DNA, 10 μ l of buffer, 2 μ l of restriction enzymes, and 50 or 100 μ l of water are added one at a time. For one to two hours (depending on the needs of the experiment), all reactions were incubated at 37 °C. One to two microliters of the digestion solution placed onto a gel electrophoresis for examination (using loading dye).

CHAPTER 4

RESULTS

4.1: Generate *lrp1ab* knockout line.

Firstly, in order to generate *lrp1ab* knockout line, I designed two gRNAs the first one upstream promoter (to make sure that we removed the promoter and potential co-promoter) and the second one within exon 1 (Figure 5). The length between these two gRNA is relatively long 6982 bp. This large deletion will help to stop the transcription process and result in transcriptless *lrp1ab*.



4.1.1 Select gRNA sequence.

To design the gRNAs within the target regions we sequenced the region that we intend to design the gRNAs within in it to make sure from the sequence of these regions and to find the unique sequences that can we design the gRNAs within it. So, we designed primers for the flanking regions of the target sites in order to amplify them then we sent them for sequencing.

Primers to amplify the region that flanks exon 1 gRNA.

Left primer: acacttactacgacagagccaag

Length: 23 bp Tm: 60.1 B: 96.616 GC: 47.8 % Any: 0.0 End: 0.0
TB: 7.0 HP:0.03' Stab: 3.6
Penalty: 0.057

Right primer: ttactcaccttcaatggctctgg

Length: 23 bp Tm: 60.3 C B: 96.756 % GC: 47.8 % Any: 0.0
End: 0.0 TB: 8.0 HP: 0.0 3' Stab: 3.9 Penalty:
0.308.

It has secondary structure at 9.2 C°.

Target between promoter and exon							
Genomic Location	Overlapping Gene(s)	Orientation	Query start	Query end	Length	Score	E-val
CHR_ALT_CTG23_1_27-25686839-25687557 (Sequence)		Forward	17	735	719 (Sequence)	1421	0.0
23,25686839-25687557 (Sequence)	lrr1ab	Forward	17	735	719 (Sequence)	1421	0.0
chr10:181,351,924-181,351,924 (Sequence)		Forward	414	499	22 (Sequence)	44.0	2.2
8,26495082-26495119 (Sequence)		Forward	447	484	38 (Sequence)	44.0	2.2
3,30764508-30764529 (Sequence)	shank1	Forward	474	495	22 (Sequence)	44.0	2.2
CHR_ALT_CTG6_3_4-33616155-33616175 (Sequence)		Forward	227	247	21 (Sequence)	42.0	8.6
25,35108173-35108193 (Sequence)		Reverse	675	695	21 (Sequence)	42.0	8.6
19,12897891-12897915 (Sequence)		Forward	465	489	25 (Sequence)	42.0	8.6
6,33614691-33614711 (Sequence)		Forward	227	247	21 (Sequence)	42.0	8.6

Figure 6. BLAST result of the sequence of the larvae DNA and the sequence from Ensembl database

We found that the sequence that we got is almost the same sequence that is present on ensemble database.

4.1.2 new promoter and exon 1 gRNAs synthesis

After we confirmed the sequence of our target regions, we synthesized new promoter and exon 1 gRNAs as following:

1- gRNAs synthesis

DNA template synthesis of promoter, exon 1, and intron 2 gRNAs.

1. Annealing of oligonucleotide by PCR:

PCR mix

Component	Volume
sgRNA oligo (100 μ M)	1.0
Constant oligo (100 μ M)	1.0
ddH ₂ O	8.0
Total volume (μL)	10.0

PCR condition:

95 °C	5 min
95 °C $\rightarrow$ 85 °C	-2 °C/s
85 °C $\rightarrow$ 25 °C	-0.1 °C/s
4 °C	Hold

2. T4 DNA polymerase addition for gap filling:

Component	Volume
dNTP (10 mM)	2.5
10X NEB buffer 2.1	2.0
ddH ₂ O	5.0
T4 NEB DNA polymerase	0.5
Total volume (μL)	10.0

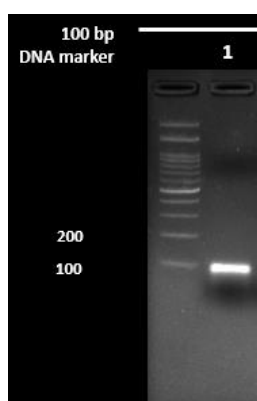
Table 1. gRNA synthesis reagents and conditions

-Incubation for 20 minutes at 12 °C and hold at 4 °C.

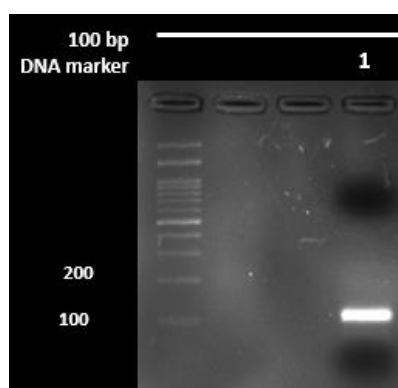
Sample ID	Nucleic Acid Conc.	Unit	A260	A280	260/280	260/230	Sample Type
<i>lrplab promoter gRNA</i> <i>DNA template</i>	210.6	ng/μl	8.573	4.836	1.85	2.27	DNA
<i>lrplab exon1 gRNA</i> <i>DNA template 1</i>	195.6	ng/μl	3.912	2.053	1.91	2.35	DNA

Table 2. gRNA DNA template concentrations using nanodrop for new promoter and exon 1 gRNAs

Electrophoresis



Promoter gRNA DNA template



Exon 1 gRNA DNA template

In vitro

Transcription of promoter, exon 1, and intron 2 gRNAs.

I. Assembly of component:

Component	T7 RNA polymerase (per rxn)
ATP	1.0
GTP	1.0
UTP	1.0
CTP	1.0
10X buffer	1.0
T7/SP6 enzyme mix	1.0
RNase inhibitor (ArrowTec)	1.0
Template (1 - 2 μg)	3.0
Total reaction volume	10.0

Table 3. In vitro Transcription

- Incubate at 37°C for 4 hours.
- II. Add 9 µL of nuclease-free water 1.0 µL Turbo DNase and incubate for another 30 mins.
 - III. Add 10 µL of 5M Ammonium Acetate, 60 µL 100% Ethanol.
 - IV. Mix well and freeze the tube at -80°C for overnight.
 - V. Centrifuge at 4°C for 30 min at max speed (17,000 X g).
 - VI. Remove the supernatant carefully and wash the precipitate with 1 mL of 75% Ethanol.
 - VII. Centrifuge at 4°C for 15 min at max speed (17,000 X g).
 - VIII. Carefully remove the supernatant and dissolve the RNA pellet using 30 µL of nuclease-free water warmed at 50 °C.
 - IX. Quantify RNA and run gel electrophoresis to confirm.

Sample ID (number)	Nucleic Acid Conc.	Unit	A260	A280	260/280	260/230	Type
<i>lrp1ab promoter sgRNA</i>	730	ng/µl	18.272	9.293	1.97	2.62	RNA
<i>lrp1ab Exon 1 sgRNA</i>	134.9	ng/µl	3.483	1.600	2.18	3.19	RNA

Table 4. New promoter and exon 1 gRNAs concentrations

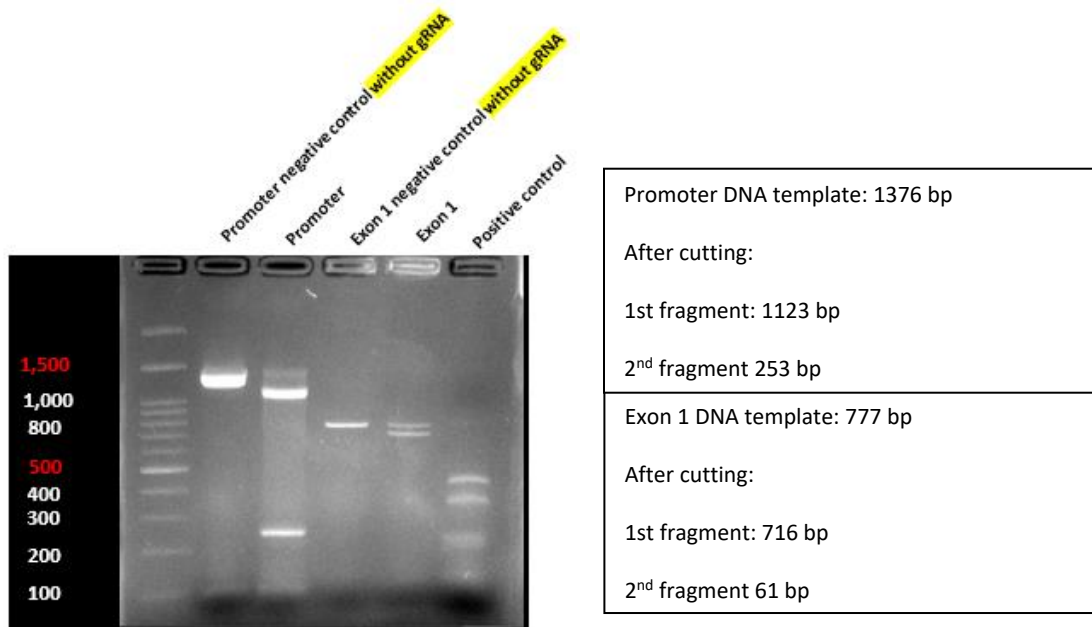
4.1.3 In vitro cleavage

In order to check the efficiency of these gRNAs prior to microinjection, we did in vitro cleavage assay as following.

Components	Required volume	Final conc. (ng/μl)	Final Conc. (Ref: NEB)
Nuclease free water (DEPC treated ddH ₂ O)	add to total volume minus volume of buffer, sgRNA, Cas 9	-	-
10X NEB r3.1 buffer	1.0	1X	
Exon1 sgRNA	3.3	40	30 nM
Cas9 protein (1000 ng/ μl)	0.3	20	30 nM
Assemble these components in one PCR tube and incubate for 10 minutes at 37 C°			
Target DNA template	3.4	5	3 nM
DEPC	--	-	-
10X NEB r3.1 buffer	0.5	1X	
Incubate for 2 hours at 37 C°			
Total volume	15 μl		

Table 5. In vitro cleavage assay

Electrophoresis



From these results we can confirm that both primers work efficiently (can cut at their target very well).

4.1.4 Microinjection

I did microinjection following the protocol that I highlight in Table 6.

Injection composition	mix	Initial Conc. (ng/ μ L)	Volume required (μ L)	Final Conc. (ng/ μ L)
Cas9 mRNA		2000	1	150
exon 1 gRNA		155	8.5	65.875
New promoter gRNA		136.4	8.5	57.97
Molecular grade water		-	0	-
Phenol red		-	2	-
Total volume			20.0	

Table 6. Microinjection for knockout

4.1.5 PCR to check the knockout

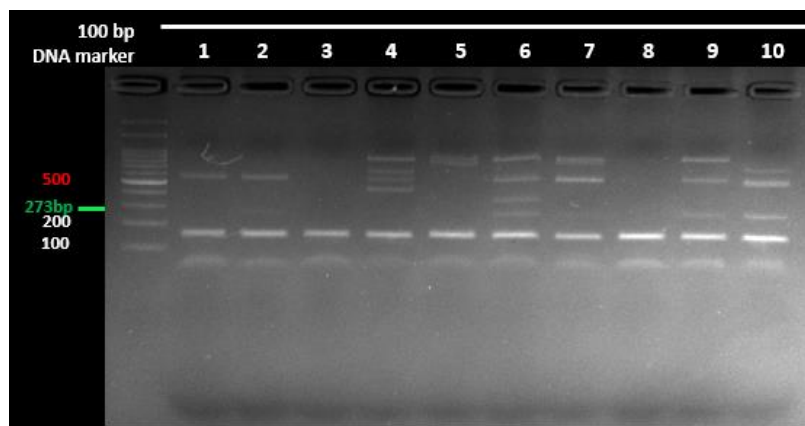
1- PCR to check cut.

Components (per reaction)	Volume (μ L)	Final Conc
Morre Taq	5.0	1x
Forward primer	0.5	0.5 μ L
Reverse Primer	0.5	0.5 μ L
ddH ₂ O	3.0	
Genomic DNA	1.0	
Total	10	

PCR conditions	
95 C°	5 mins
95 C°	15 s
50-70 C°	30 s
72 C° (extension time)	30 s
72 C°	5 mins
4 C°	Infinite

Table 7. PCR reagents concentrations and conditions to check the knockout

Electrophoresis



We found the expected size 273 bp on the gel which means that the knockout happened successfully.

4.1.6 Gel extraction and DNA sequencing

I did gel extraction for the target band, and I sent it for sequencing. When I received the results of sequencing, I found that the sequence of this band is similar to the expected sequence after knockout.

Score	Expect	Identities	Gaps	Strand
420 bits(227)	2e-122	232/234(99%)	2/234(0%)	Plus/Minus
Query 11	CTCTTG-ATGCATGCATGGAGAAAATAACACGTTTGATCGCGGTTACTCACCTTCAATGG	69		
Sbjct 234	CTCTTGAATGCATGCATGGAGAAAATAACACGTTTGATCGCGGTTACTCACCTTCAATGG	175		
Query 70	CTCTGGTGGGATCTACAACCTCCAGTGCAAGAACTAATCCCAGGT-ATTGGGGTATAAAT	128		
Sbjct 174	CTCTGGTGGGATCTACAACCTCCAGTGCAAGAACTAATCCCAGGTCATTGGGGTATAAAT	115		
Query 129	CCAACAAGCTCTTCTTGACACAGCATGACAACCATGCATGCTTAATGACCTCCTTccc	188		
Sbjct 114	CCAACAAGCTCTTCTTGACACAGCATGACAACCATGCATGCTTAATGACCTCCTTCCC	55		
Query 189	ccccAGGCATCTGTGACAATCTGTGCATTGACTGTCTCCGTCTGAAACCCACC	242		
Sbjct 54	CCCCAGGCATCTGTGACAATCTGTGCATTGACTGTCTCCGTCTGAAACCCACC	1		

Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
420	420	95%	2e-122	99.15%	273	Query_51689

Figure 7. Sequence after knockout

4.2 *lrp1ab* conditional inactivation using UFlip plasmid.

I designed a RNA target sequence within intron 2 in order to insert UFlip cassette within intron 2, so, after Cre activation through tamoxifen injection it will result in lox P sites inversion which led to *lrp1ab* inactivation just 48 hours prior to cryo-injury (Figure 8) which can be considered a precise tool to identify or investigate the role of this interesting gene in the process of heart regeneration.

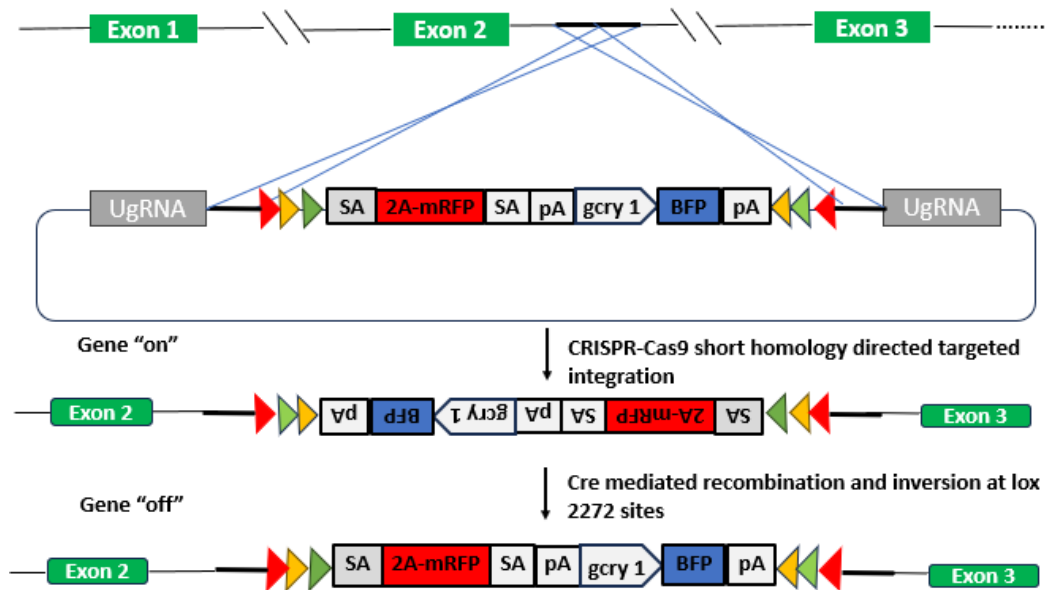


Figure 8. Proposed methodology for *lrp1ab* conditional inactivation

4.2.1 Sequence gRNA target region

- A. Intron 2 forward primer: aagtttgcattggcggttatgtagc
B. Intron 2 reverse primer: ctgggggtgattttgactgggtaaa

Length: 781 bp.

The sequence was the same as that present in database without any change in the target region.

4.2.2 gRNA synthesis

DNA template generation

3. Annealing of oligonucleotide by PCR:

PCR mix

Component	Volume
sgRNA oligo (100 μ M)	1.0
Constant oligo (100 μ M)	1.0
DEPC treated water	8.0
Total volume (μL)	10.0

PCR condition:

95 °C	5 min
95 °C $\rightarrow$ 85 °C	-2 °C/s
85 °C $\rightarrow$ 25 °C	-0.1 °C/s
4 °C	Hold

4. T4 DNA polymerase addition for gap filling:

Component	Volume
dNTP (10 mM)	2.5
10X NEB buffer 2.1	2.0
DEPC treated water	5.0
T4 NEB DNA polymerase	0.5
Total volume (μL)	10.0

Table 8. Intron 2 gRNA synthesis conditions

- Incubation for 20 minutes at 12 °C and hold at 4 °C.
- Purification using nucleotide removal kit.
- Quantify the concentration using nano drop machine.

Sample ID	Nucleic Acid Conc.	Unit	A260	A280	260/280	260/230	Sample Type
<i>Lrp1ab</i> promoter gRNA DNA template	210.6	ng/μl	8.573	4.836	1.85	2.27	DNA

Table 9. Intron 2 gRNA DNA template concentration using nanodrop

Electrophoresis

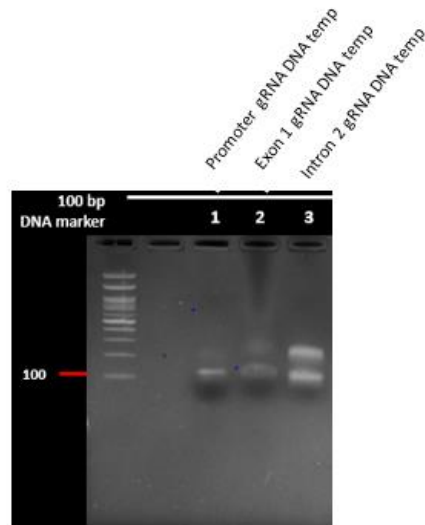


Figure 9. Intron 2 gRNA DNA template

In vitro Transcription

Component	T7 RNA polymerase (per rxn)
ATP	1.0
GTP	1.0
UTP	1.0
CTP	1.0
10X buffer	1.0
T7/SP6 enzyme mix	1.0
RNase inhibitor (ArrowTec)	1.0
Template (1 - 2 µg)	3.0
Total reaction volume	10.0

Table 10. Intron 2 gRNA in vitro transcription

Incubate at 37°C for 4 hours.

- I. Add 9 µL of nuclease-free water 1.0 µL Turbo DNase and incubate for another 30 mins.
- II. Add 10 µL of 5M Ammonium Acetate, 60 µL 100% Ethanol.
- III. Mix well and freeze the tube at -80°C for overnight.

Component	T7 RNA polymerase (per rxn)
ATP	1.0
GTP	1.0
UTP	1.0
CTP	1.0
10X buffer	1.0
T7/SP6 enzyme mix	1.0
RNase inhibitor (ArrowTec)	1.0
Template (1 - 2 µg)	3.0
Total reaction volume	10.0

Incubate at 37°C for 4 hours.

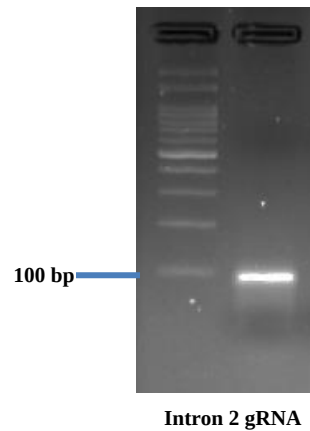
- IV. Add 9 µL of nuclease-free water 1.0 µL Turbo DNase and incubate for another 30 mins.
- V. Add 10 µL of 5M Ammonium Acetate, 60 µL 100% Ethanol.

VI. Mix well and freeze the tube at -80°C for overnight.

- Quantify the concentration using nano drop machine.

Sample ID (number)	Nucleic Acid Conc.	Unit	A260	A280	260/280	260/230	Type
<i>lrp1ab</i> intron 2 sgRNA	652.4	ng/μl	16.310	8.182	1.99	2.20	RNA

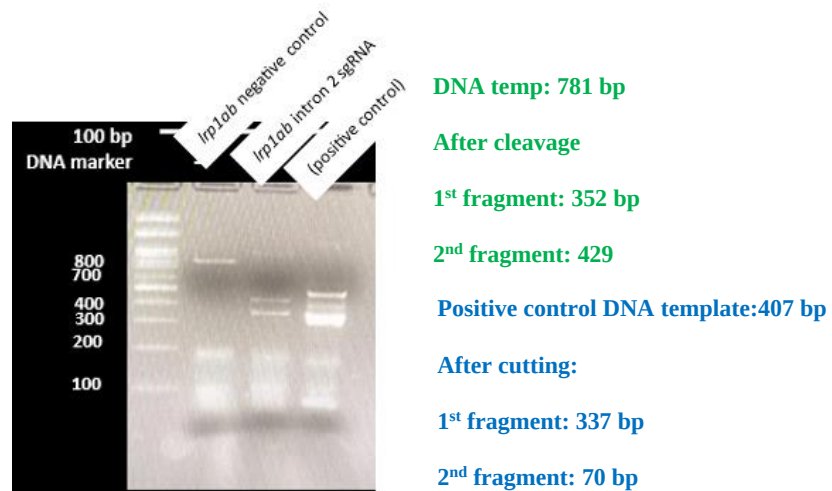
Electrophoresis



4.2.3 I vitro cleavage

Components	Required volume	Final conc. (ng/μl)	Final Conc. (Ref: NEB)
Nuclease free water (DEPC treated ddH ₂ O)	7.4	-	-
10X NEB r3.1 buffer	1.0	1X	
sgRNA	1.3	40	30 nM
Cas9 protein (1000 ng/ μl)	0.3	20	30 nM
Assemble these components in one PCR tube and incubate for 10 minutes at 37 C°			
Target DNA template (30ng/ μl)	2.5	5	3 nM
DEPC treated water	2	-	-
10X NEB r3.1 buffer	0.5	1X	
Incubate for 2 hours at 37 C°			
Total volume		15 μl	

Electrophoresis



4.2.4 Homology arms design

5'HA oligo A

5'GCGG CCT

TAAGTTGCTATGTCTGTTCTCCTGCTGGACAGTAAAGCACTACCCAAA3'

5'HA oligo B

5'GAAGTTTGGGTAGTGCTTTACTGTCCAGCAGGAGAACAGACATAGCAA

GTTA AGG 3'

3'HA oligo A

5'AAGTGCTGATGGAGGAACCATCATGGCCGCCTGCTGGGAGTCTGTCTGG

CT GTT3'

3'HA oligo B

5'CGGAACAGCCAGACAGACTCCCAGCAGGCGGCCATGATGGTTCCTCCA

TCAGCA3'

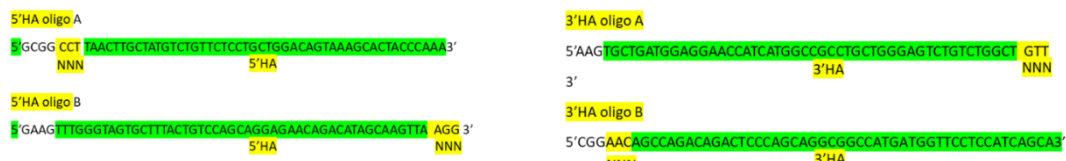


Figure 10. Annealed 5' and 3' homology arms

4.2.5 One Pot Cloning of homology arms into UFlip vector.

Homology arms annealing

	per rxn (Down)	per rxn (Up)
Downstream homology oligo A (10 μ M)	4.5	-
Downstream homology oligo B (10 μ M)	4.5	-
Upstream homology oligo A (10 μ M)	-	4.5
Upstream homology oligo B (10 μ M)	-	4.5
10 $\times$ NEB3.1 buffer	4.0	4.0
H ₂ O	27.0	27.0
Total	40.0	40.0

Use a thermal cycler and the following program to anneal the oligos: stage 1 involves incubation at 98 °C for 5 minutes, then incubating at 97 °C for 45 seconds; repeat Step 2 for 90 cycles, lowering the temperature by 1°C each cycle; the last stage involves holding the temperature at 4 °C.

1-Pot Digest

Reagents	per rxn (UFlip)
UFlip-2ARFP_gcryBFP-1 (73.2 ng/ μ l)	2.0
10 $\times$ NEB3.1 buffer	1.0
BfuAI (5 U/ μ l)	0.5
BspQI (10 U/ μ l)	0.5
H ₂ O	6.0
Total	10.0

Table 11. One pot digest reagents and conditions

Incubate 50 °C for 1 hour.

Incubate at 25 °C, 30 min.

Ligation

Reagents	per rxn (UFlip)
1-Pot Digest	10.0
Upstream annealed homology arm	1.0
Downstream annealed homology arm	1.0
T4 DNA Ligase	0.5
2X Rapid Ligase buffer	12.5
Total	25.0

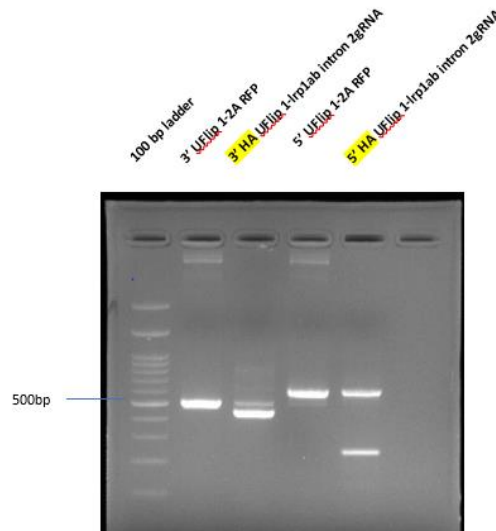
Incubate at 25 °C, 30 min.

-KAPA: PCR of ligation mix

Reagents	5' HA	3' HA
	Volume (μL)	Volume (μL)
2X KAPA	10	10
5'HA-ColonyPCR-F	1	-
5'HA-Check-R	1	-
3'HA-Check-F	-	1
3'HA-ColonyPCR-R	-	1
dH2O	6	6
Total	9 ul/Rxn x 4	9 ul/Rxn x 4
Ligation mix	1	1
Stock UFLIP-1	1	1
Total	10ul/Rxn	10ul/Rxn
Thermal Profile		
Initial Denaturation		95 °C-3mins
35 cycles	Denaturation	95 °C-30s
	Annealing	60 °C-30s
	Extension	72 °C-30s
Final Extension		72 °C-10mins
Pause		10 °C

Table 12. PCR for ligation mix conditions and reagents

Electrophoresis



Outcome: The objective was to get the ligated 5' and 3'HA amplified at the designated sites before transformation. We got positive confirmation and can proceed to top10 transformation.

4.2.6 Transformation and colony PCR

1- Colony PCR

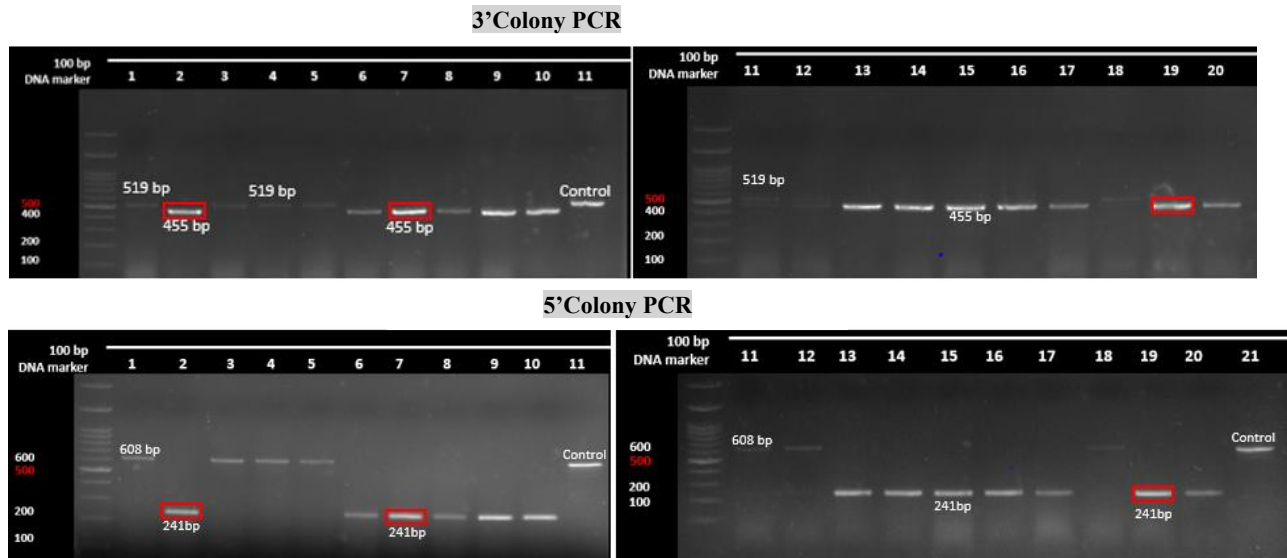
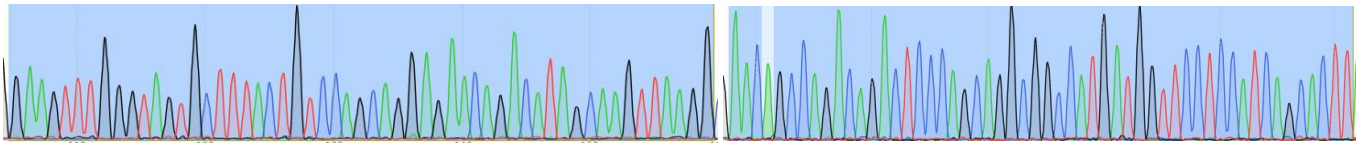


Figure 11. Colony PCR

I sent colony no 2, 7, and 19 for sequencing.

Colony 2, 7, and 19 (5' HA)

Colony 2, 7, and 19 (3' HA)



All colonies have the 3' and 5' HAs sequence in the right place within the plasmid.

4.2.7 Microinjection

I did microinjection as following.

Injection mix composition	Initial Conc. (ng/ μ L)	Volume required (μ L)	Final Conc. (ng/ μ L)
Cas9 protein (Synthego)	20 μM	0.20	0.2 μM
Lrp1ab intron 2 gRNA	1100	0.45	25
UgRNA (Synthego)	750	0.66	25
1X TE buffer (Synthego)	10 mM	5.00	-
<i>Incubate at 37°C for 15 min to allow sgRNA-Cas9 ribonucleoprotein (RNP) complex formation, tap to mix after that</i>			
DEPC water	-	10.7	-
Phenol Red	-	2.00	-
UFLIP Lrp1ab 3'+5' (Donor plasmid)	195 (according to the Eppendorf)	1	10
Total volume		20	

Figure 12. Microinjection Protocol

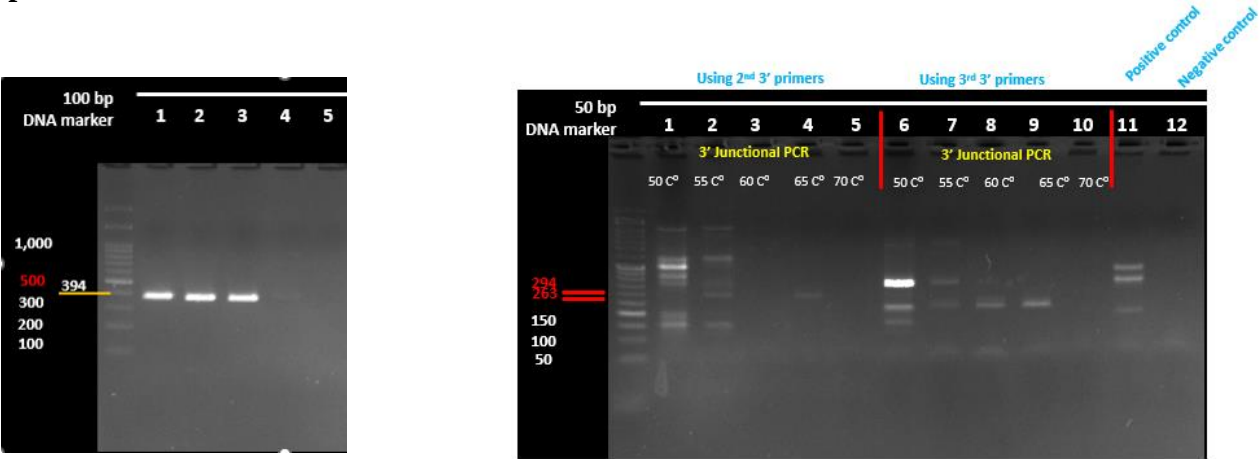
4.2.8 Gradient junctional PCR using 3rd couple of primers for

Gradient junctional PCR for 5' and 3'.

Components (per reaction)	Volume (μ L)	Final Conc
Kappa Taq	5.0	1x
Forward primer	0.5	0.5 μ L
Reverse Primer	0.5	0.5 μ L
ddH ₂ O	3.0	
Genomic DNA	1.0	
Total	10	

Table 13. Gradient junctional PCR conditions and reagents

Electrophoresis



4.2.9 Sequence of 5’ and 3’ of junctional PCR products

I sent both 5’ and 3’ fragments after I did gel extraction for sequencing.

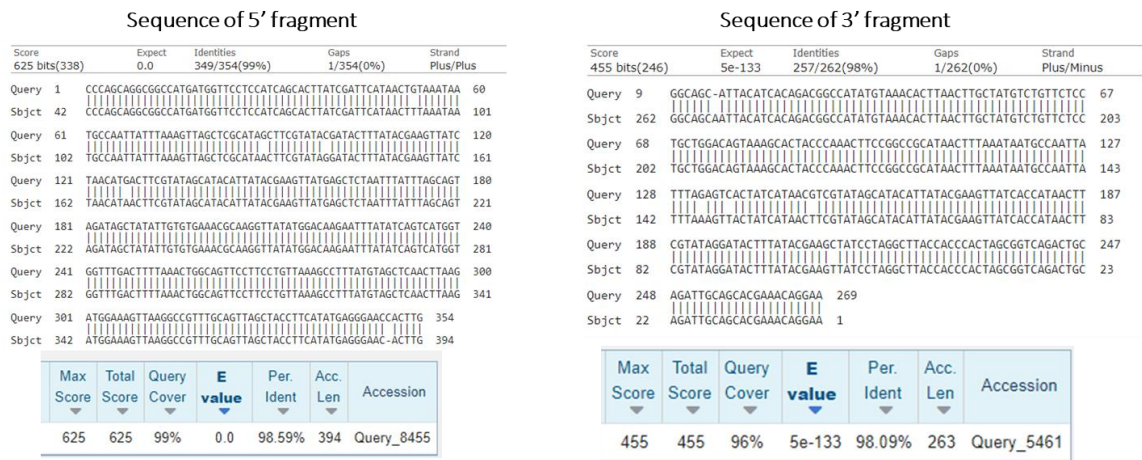


Figure 13. Junctional PCR 5’ and 3’ fragments sequences

Overall, these results confirmed that both strategies (lrp1ab knockout and lrp1ab conditional inactivation) were succeeded.

CHAPTER 5

Discussion

Heart failure (HF) remains a significant global health issue, with increasing incidence and mortality rates. Despite advancements in the treatment of cardiovascular diseases, the limited regenerative capacity of the adult mammalian heart contributes to the resistance of HF to repair following injury. In contrast, several non-mammalian species, including zebrafish, exhibit remarkable regenerative abilities in their hearts. Comparative analysis of these regenerative models can provide valuable insights into cardiac regeneration mechanisms.

Through bulk RNAseq analysis of zebrafish hearts at different time and comparing between regenerative zebrafish and non-regenerative zebrafish (resident macrophages deleted before cryo-injury by CL). The hierarchical clustering analysis of differentially expressed genes (DEGs) uncovered a delayed recruitment of macrophages and prolonged inflammatory response in the non-regenerative condition. Interestingly, we observed a wider range of phagocytic receptors and ligands that were predominantly expressed in macrophages associated with regeneration. This included integrin, calreticulin, and Lrp1, indicating their potential involvement in the regenerative processes.

To investigate the role of Lrp1 (low-density lipoprotein receptor-related protein 1) in heart regeneration, we generated large deletion and conditional knockout (KO) alleles of the *lrp1ab* gene using CRISPR-Cas9 technology. To prevent potential genetic compensation, we targeted the promoter and exon 1 of *lrp1ab* to create a "transcript-less" mutant. Additionally, we employed the UFlip plasmid, which contains loxp sites flanking a reporter cassette, to achieve tissue-specific or temporal-specific Cre-mediated regulation of endogenous *lrp1ab* expression.

By integrating the UFlip cassette into the intron 2 of the *lrp1ab* locus, we confirmed precise integration through insertional reporter expression and junctional PCR analysis. This novel

approach allows us to investigate the requirement and molecular mechanisms underlying the potential contributions of Lrp1 in heart regeneration. Understanding the role of Lrp1 in zebrafish heart regeneration may shed light on its therapeutic potential for enhancing heart regeneration in human patients.

In conclusion, our study highlights the crucial role of macrophages in cardiac regeneration and repair. Comparative analysis of regenerative and non-regenerative conditions provides insights into the activation of inflammatory cells during cardiac repair. Additionally, our investigation of Lrp1 through large deletion and conditional KO mutants opens up new avenues to unravel the molecular mechanisms underlying heart regeneration and explore potential therapeutic targets for enhancing myocardial function in HF patients.

CHAPTER 6

FUTURE WORK AND RECOMMENDATIONS

This study provides a basis for future research to explore the role of the *lrp1ab* gene in cardiac angiogenesis, cardiomyocyte proliferation, scar resolution, and neutrophil removal. The work will be continued by other lab members in Dr. Ben Lai's lab, focusing on generating F1 generations of two transgenic lines: Tg(fli1:EGFP;myl7:DsRed2-NLS) *lrp1ab* knockout line and Tg(mpeg1.1-T4:2ACreERT2:gcry:EGFP;lrp1ab-intron2:UFlip) line.

The generated F1 generations will be utilized to investigate the impact of *lrp1ab* gene on key aspects of cardiac regeneration following cryoinjury. Specifically, the role of *lrp1ab* gene in cardiac angiogenesis, cardiomyocyte proliferation, scar resolution, and neutrophil removal will be assessed using these lines. This approach will provide valuable insights into the contribution of *lrp1ab* gene in these processes.

To further explore the functional significance of *lrp1ab* gene, conditional inactivation of its specific expression will be achieved using the Tg(mpeg1.1-T4:2ACreERT2:gcry:EGFP;lrp1ab-intron2:UFlip) line. This will allow for tissue-specific regulation of *lrp1ab* gene expression, enabling a more comprehensive understanding of its role in distinct resident macrophages, particularly the M1 subtype, after cryoinjury.

This future work will shed light on the involvement of phagocytic receptors, such as *lrp1ab*, which are known to be highly expressed by distinct resident macrophages (M1) following cryoinjury. Investigating the role of *lrp1ab* gene in cardiac regeneration will contribute to our understanding of the molecular mechanisms underlying the functions of these phagocytic receptors.

By elucidating the specific contributions of the *lrp1ab* gene in cardiac angiogenesis, cardiomyocyte proliferation, scar resolution, and neutrophil removal, this work will provide valuable insights into the complex processes involved in cardiac regeneration. The

findings may open new avenues for therapeutic interventions aimed at enhancing cardiac repair and improving outcomes for individuals with heart failure.

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