

Ultrasound-mediated delivery of miR-1 for treatment of left-ventricle hypertrophy

Davindra Singh

A Thesis

In the Department of
Biology

Presented in Partial Fulfillment of the Requirements

For the Degree of
Doctor of Philosophy (Biology)

At Concordia University
Montreal, Quebec, Canada

October 2025

©Davindra Singh, 2025

CONCORDIA UNIVERSITY

School of Graduate Studies

This is to certify that the thesis

Prepared by: Davindra Singh

Entitled: Ultrasound-mediated delivery of miR-1 for treatment of left-ventricle hypertrophy
and submitted in partial fulfillment of the requirements for the degree of

Doctor of Philosophy (Biology)

complies with the regulations of the University and meets the accepted standards with respect to
to originality and quality.

Signed by the final Examining Committee:

_____	Chair
<i>Dr. Christopher Steele</i>	
_____	External Examiner
<i>Dr. Jonathan A. Kopechek</i>	
_____	Examiner
<i>Dr. Alisa Piekny</i>	
_____	Examiner
<i>Dr. Andreas Bergdahl</i>	
_____	Examiner
<i>Dr. Steve Shih</i>	
_____	Thesis Supervisor
<i>Dr. Brandon Helfield</i>	

Approved by:

Dr. Robert Weladji, Graduate Program Director

October 20th, 2025:

Dr. Pascale Sicotte, Dean of Faculty of Arts and Science

Abstract

Ultrasound-mediated delivery of miR-1 for treatment of left-ventricle hypertrophy

Davindra Singh, Ph.D.

Concordia University, 2025

Pathological left-ventricle hypertrophy (LVH) is a major contributor to heart failure, characterized by abnormal cardiomyocyte growth, which thickens the left ventricular wall and impairs cardiac contraction. Recent studies have demonstrated miR-1 downregulation in hypertrophic cardiomyocytes, establishing it as an early disease biomarker and a potential therapeutic target. Returning miR-1 to native levels presents a promising treatment strategy for LVH, however, safe and targeted delivery to the heart remains a significant challenge. Ultrasound-mediated delivery offers a non-invasive image-guided approach for cardiac-specific gene delivery.

In this thesis, I explore the use of ultrasound and microbubbles-mediated delivery of miR-1 for the treatment of LVH. First, using an *in-vitro* model, I optimized the delivery of miR-1 to diseased cardiomyocytes, ensuring a significant delivery while maintaining cell viability. These findings resulted in a reversion of hypertrophic phenotypes, including the expression of hypertrophic proteins, cytokines, and a restoration of cardiomyocyte size.

Building on these findings, an *in-vivo* delivery strategy was developed utilizing a clinical ultrasound system with clinically approved microbubbles. In healthy models, cardiac-specific delivery of miR-1 was observed with limited off-target effects. In hypertrophic models, a single treatment completely or partially reversed pathological phenotypes, including protein expression, serum cytokine levels, cardiomyocyte size, LV mass, heart function, mitochondrial respiration, and

aerobic capacity. This study was performed in both sexes and revealed a more significant recovery in the male models.

Overall, this thesis highlights the feasibility of ultrasound and microbubbles delivery of miR-1 to the heart and elucidates the therapeutic potential of miR-1 for pathological left-ventricle hypertrophy.

Acknowledgements

I would first like to thank my supervisor, Dr. Brandon Hefield, for his support and guidance throughout the completion of this thesis. I would not have been able to develop any of my skills as a researcher if not for your mentorship. It has truly been a privilege to learn from you, and I am grateful to for the opportunity to be a part of your lab. I would also like to thank my supervisory committee, Dr. Alisa Piekny and Dr. Andreas Bergdahl, for their feedback and their insight, without which this project would not have been possible.

I would like to thank Fiona Hui, Dr. Stephanie He, Dr. Hossein Yuseif, and Dr. Elahe Memari for their support and encouragement during the early stages of graduate school. And I would like to thank all of the current members of the Helfield lab. It has been a pleasure to work alongside all of you. I could not have asked for a better team. Finally, I would like to extend my special thanks to Dr. Stephanie He, Kyle Hazel, and Megan McManus for their time and effort. Your contributions to this work were truly invaluable.

Dedication

I like to dedicate this thesis to Deodat Sunny Sesaran and Daowkie Juliet Sohan.

I would also like to dedicate this thesis to my family. To my parents, Pursotam Singh and Banmatie Sesaran, for their sacrifices, unwavering support and the work ethic they instilled in me. This accomplishment would not have been possible without your guidance and encouragement. To my sister, Devina Singh, who has been a constant source of inspiration, thank you for your advice and for continually motivating me to do better.

Finally, to my partner, Yu Xiang Ren. I would not have been able to do any of this without you. Thank you for always being by my side.

Contribution of Authors

Chapter 1: Introduction and background

This section was written by Davindra Singh and edited by Dr. Brandon Helfield.

Chapter 2: Cardiac gene delivery using ultrasound: state of the field:

The published manuscript was written primarily by Davindra Singh, with small contributions from Dr. Elahe Memari, Dr. Stephanie He, Dr. Hossein Yusefi and Dr. Brandon Helfield. Final edits were performed by Davindra Singh and Dr. Brandon Helfield.

Chapter 3: Image-guided local delivery of microRNA-1 via a single treatment of microbubble-mediated clinical ultrasound ameliorates hypertrophic heart disease in a sex-dependent manner

This is a currently submitted manuscript. Dr. Brandon Helfield supervised the project and contributed to the writing and editing of the manuscript. For the *in-vitro* experiments, Dr. Stephanie He and Dr. Brandon Helfield assisted with the sample handling for the ultrasound experiment, as described in Chapter 3.7.2. Dr. Brandon Helfield also wrote the MATLAB software used to analyze the passive cavitation data, and provided Figure 3.1 N.

For the *in-vivo* experiments, Dr. Brandon Helfield assisted with the ultrasound treatment, as described in Chapter 3.7.3. Dr. Stephanie He and Megan McManus assisted with the animal handling during the ultrasound procedure and with the organ isolation. Additionally, Dr. Brandon Helfield helped perform the echocardiography imaging and analysis described in Chapter 3.7.13, contributing to Figures 3.5 H-L.

Dr. Andreas Bergdahl assisted in the histological analysis by providing the cryostat used for the sample preparation, as described in Chapter 3.7.12, and the Leica microscope used for imaging of the sliced cardiac sample, as shown in Figure 3.5 A. Dr. Andreas Bergahl also provided

the treadmill for the aerobic capacity experiment described in Chapter 3.7.14. Megan McManus and Kyle Hazel helped with the animal handling during this aerobic capacity experiment for Figure 3.6 D, E. Finally, Dr. Andreas Bergdahl performed the mitochondrial respirometry assays described in Chapter 3.7.14, contributing to Figures 3.6 A-C, and generated Figure 3.6 C.

Davindra Singh performed all remaining experiments, data analysis, manuscript writing and figures preparation.

Chapter 4: Conclusion and future directions

This section was written by Davindra Singh and edited by Dr. Brandon Helfield.

Table of contents

List of Figures.....	xiii
List of Tables.....	xiv
List of Abbreviations.....	xv
Chapter 1: Introduction and Background.....	1
1.1 Overview of left-ventricle hypertrophy.....	1
1.1.1 Characterization of LVH.....	1
1.1.2 Pathogenesis of pathological LVH	2
1.2 miR replacement therapy	7
1.2.1 miR biogenesis and transcription regulation.....	7
1.2.2 miR-1 role in cardiovascular biology and hypertrophy	8
1.2.3 Therapeutic application of miR.....	11
1.2.4 Current miR delivery strategies	11
1.4 Ultrasound and microbubbles	13
1.4.1 Fundamentals	13
1.4.2 Echocardiography	15
1.4.3 Microbubbles and contrast imaging.....	17
1.4.4 Ultrasound and microbubble-mediated delivery	18
1.4.5 Clinical and preclinical application of ultrasound therapy	21
1.5 Thesis objective and rationale.....	23
Chapter 2: Cardiac gene delivery using ultrasound: State of the field.....	25
2.1 Foreword:	25
2.2 Abstract.....	25
2.3 Introduction.....	25
2.4 Current Methods for Cardiac Gene Delivery.....	28
2.5 Brief Overview of The Salient Features of Microbubbles.....	30
2.6 Overview of Ultrasound-Assisted Gene Delivery.....	31
2.6.1 Microbubble-mediated influence on endothelial cells	32
2.7.1 Myocardial Infarction	37
2.7.2 Ischemia/Reperfusion Injury (I/R).....	39
2.7.3 Cardiomyopathies	40
2.8 Outlook and Future Directions.....	42

2.8.1 Global Summary	42
2.8.2 Molecularly Targeted Contrast Microbubbles	43
2.8.3 Microbubble-assisted vascular gene delivery	44
2.8.4 Microbubble-mediated CVD gene modulation without gene delivery	45
2.8.5 Perspectives.....	46
2.8.6 Concluding Remarks.....	48
2.9 Figures.....	Error! Bookmark not defined.
Chapter 3: Image-guided local delivery of microRNA-1 via a single treatment of microbubble-mediated clinical ultrasound ameliorates hypertrophic heart disease in a sex-dependent manner	
3.1 Foreword:	62
3.2 Abstract.....	62
3.2.1 Background and Aims:	62
3.2.2 Methods:	63
3.2.3 Results:.....	63
3.2.4 Conclusions:.....	63
3.2.5 Translational Perspective:	64
3.3 Introduction.....	64
3.4 Methods.....	67
3.4.1 Neonatal rat cardiomyocyte isolation and hypertrophy model	67
3.4.2 In-vivo hypertrophy model	67
3.5 Results	68
3.5.1 Microbubble-mediated US viably delivered miR-1 to neonatal cardiomyocytes in an acoustic pressure-dependent manner, and is positively correlated to microbubble signal.....	68
3.5.2 miR-1 replacement by US demonstrates complete reversion of the hypertrophy phenotype in-vitro	70
3.5.3 Targeted and site-specific functional miR-1 delivery to the heart can be achieved with clinical US and contrast agent microbubbles in vivo.....	73
3.5.4 A single in vivo treatment of clinical US-mediated cardiac therapy rescues miR-1 and restores the attenuation of pro-hypertrophic proteins.....	75
3.5.5 The efficiency of miR-1 replacement in reversing the hypertrophic phenotype is sex-dependent, with a more pronounced result in males and a more heterogeneous response in females.	77
3.6 Discussion.....	83
3.7 Supplementary Methods	89
3.7.1 Microbubble contrast agent.....	89
3.7.2 Ultrasound mediated delivery and cavitation detection in-vitro	89

3.7.3 Ultrasound mediated delivery in-vivo.....	90
3.7.4 Tissue extraction and processing	91
3.7.5 Flow cytometry and fluorescence microscopy.....	91
3.7.6 Real time quantitative PCR.....	92
3.7.7 miR blood degradation assay	92
3.7.8 Immunoblotting.....	93
3.7.9 Cell viability analysis.....	94
3.7.10 Cell size analysis.....	94
3.7.11 Cytokine/Chemokine profiling	94
3.7.12 Histological assessment of cell size	95
3.7.13 LV remodeling and function	95
3.7.14 Mitochondrial respiration measurements.....	96
3.7.15 Functional aerobic capacity	96
3.7.16 Statistics	97
3.8 Supplementary Figures	98
Chapter 4: Conclusion and future directions	102
4.1 Foreword:	102
4.2 Overview of thesis	102
4.3 Limitations and future directions.....	104
4.4 Conclusion	108
Appendix.....	109
5.1 Foreword.....	109
5.2 Abstract.....	109
5.3 Introduction.....	110
5.4 Overview of Physical Plasma Membrane Permeation Techniques	112
5.4.1 Microinjection.....	113
5.4.2 Sonoporation	113
5.4.3 Electroporation.....	116
5.4.4 Photoporation.....	118
5.5 Plasma Membrane Repair Mechanisms	120
5.5.1 Physical Intuition	121
5.5.2 Repair Triggers	122
5.5.3 Plasma Membrane Repair Hypotheses	123
5.6 Cytoskeletal Remodeling during Perforation.....	126

5.6.1 Initial Reaction: Deconstruction of Actin Network	127
5.6.2 Resealing and Remodeling: Actomyosin Contractile Ring	128
5.6.3 Resealing and Remodeling: S100A11-A2	129
5.6.4 Resealing and Remodeling: Repair Cap	130
5.6.5 Resealing and Remodeling: Exo/Endocytosis Events.....	131
5.7 Future Perspectives.....	132
5.8 Conclusions.....	135
References	136

List of Figures

Figure 1.1: Overview of pathological left-ventricular hypertrophy	4
Figure 1.2: miR-1 regulation in left-ventricle hypertrophy	10
Figure 1.3: Echocardiography overview.	16
Figure 1.4: Ultrasound and microbubble-mediated cell permeabilization.....	19
Figure 2.1: Schematic diagram depicting the concept of stable versus inertial cavitation and cardiac gene delivery.....	31
Figure 2.2: Ultrasound-enhancing agents vibrate in the vasculature and can give rise to local and reversible increases in vascular permeability and modulate intercellular signalling.....	33
Figure 2.3: Aggregate data from the studies reported in Table 3 depicting a measurement of “direct readout”, which is – when reported – a relative measure of the delivered gene product within the diseased heart of ultrasound treated versus control groups, compared to different aspects of the treatment protocol.	48
Figure 3.1: Ultrasound-mediated delivery of miR-1 to neonatal cardiomyocytes in-vitro is dependent on MI and is correlated with microbubble disruption	70
Figure 3.2: Recovery of hypertrophy phenotype via ultrasound-assisted miR-1 delivery in-vitro.	72
Figure 3.3: Single treatment using a clinical ultrasound system provides targeted and site-specific delivery of miR-1 to the heart in healthy male and female murine models in-vivo.....	74
Figure 3.4: miR-1 recovery and the attenuation of hypertrophic proteins after a single ultrasound treatment in male and female in-vivo hypertrophic models.	76
Figure 3.5: Sex dependent response to ultrasound-mediated miR-1 replacement therapy in reversing hypertrophic phenotypes	79
Figure 3.6: Mitochondrial and aerobic response to miR-1 replacement therapy in male and female hypertrophic murine model in-vivo.	82
Figure 3.7: Neonatal cardiomyocyte isolation	98
Figure 3.8: The experimental configurations for ultrasound-assisted miR-1 delivery	99
Figure 3.9: Real-time passive cavitation detection during in-vitro delivery of miR-1 to cardiomyocytes	100
Figure 3.10: Sex-dependent expression changes in mitochondrial VDAC protein levels.....	101
Figure 5.1. Summary of known plasma membrane resealing mechanisms	120

List of Tables

Table 1: Summary of vectors, along with select examples, that have been used in conjunction with ultrasound for targeted delivery in the context of CVD.	27
Table 2: Pillars of gene therapy targets for CVD.	37
Table 3: Summary of ultrasound-assisted gene delivery to the heart in pre-clinical CVD models	50
Table 4: Overview of experimental outcomes of ultrasound treatment of hypertrophic in-vitro neonatal cardiomyocyte model and in-vivo male and female murine models.....	88
Table 5. A summary of the salient features of the main physical methods used to generate plasma membrane perforations	112
Table 6. A summary of the key proteins involved in plasma membrane repair.	121
Table 7. A summary of the involvement of cytoskeletal remodeling post-pore formation.	127

List of Abbreviations

r_p	Pore radius
η_0	Cytosolic viscosity
ΔP	Pressure difference across the membrane
AAV	Adeno-associated virus
AC6	Adenylyl cyclase 6
ACE	Angiotensin-converting enzyme
ADP	Adenosine diphosphate
AGO	Argonaut
ALIX	ALG-2 interacting protein X
AM	Adrenomedullin
Ang II	Angiotensin II
Ang1	Angiopoietin-1
ANGPTL8	Angiopoietin-like protein 8
ARB	Angiotensin receptor blockers
ARNI	Angiotensin receptor–neprilysin inhibitors
Arp2/3	Actin-Related Protein 2/3 complex
ASM	Acid sphingomyelinase
ATP	Adenosine triphosphate
BAPTA-AM	1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid
Bcl-2	B-cell lymphoma 2
BIN1	Bridging integrator-1
B-mode	Brightness mode
BMSC	Bone mesenchymal stem cells
BSA	Bovine serum albumin
BW	Body weight
CACNA1C	Calcium voltage-gated channel subunit alpha 1 C
Cav3	Caveolin-3
CCB	Calcium channel blocker
Cdc42	Cell division cycle 42
cDNA	Complementary DNA
CEUS	Contrast-enhanced ultrasound
CM	Cardiomyopathy
CNP	C-type natriuretic protein
CNS	Central nervous system
CO	Cardiac output
CVD	Cardiovascular disease
CX43	Connexin-43
DC	Duty cycle
DGCR8	DiGeorge syndrome critical region 8
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
dsRNA	Double-stranded RNA
E/A	Mitral ratio of peak early to late diastolic filling velocity

EB1	Microtubule-associated protein 1
EDTA	Ethylenediaminetetraacetic acid
E _{ext}	External E-field
E-field	Electric field
EGF	Epidermal Growth Factor
EHD1/2	Eps homology domain
ESCRT	Endosomal sorting complex required for transportation
ET-1	Endothelin-1
ETS	Electron transport system
EV	Extracellular vesicle
F-actin	Filamentous actin
FCCP	Carbonyl cyanide p-(trifluoromethoxy) phenylhydrazone
FDA	Food and Drug Administration
FFT	Fourier fast transform
FGF	Fibroblast growth factor
FITC	Fluorescein isothiocyanate
Gal-3	Galectin-3
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
G-CSF	Granulocyte colony-stimulating factor
GFP	Green fluorescent protein
GLP-1	Glucagon-like peptide
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GOI,	Gene of interest
GPCR	G-protein-coupled receptor
GRO/KC	Growth-related oncogene/keratinocyte chemoattractant
GTPases	Guanosine triphosphatase
HFpEF	Heart failure with preserved ejection fraction
HFrEF	Heart failure with reduced ejection fraction
HGF	Hepatocyte growth factor
HO-1	Heme oxygenase-1
HRP	Horseradish peroxidase
HUVEC	Human umbilical vein endothelial cell
HW	Heart weight
Hz	Hertz
I/R	Ischemia/reperfusion
ICAM-1	Intercellular adhesion molecule-1
IFN γ	Interferon gamma
IL	Interleukin
INF2	Inverted formin 2
IP-10	Interferon gamma-induced protein 10
IRE	Irreversible electroporation
IV	Intravenous
KCND2	Potassium voltage-gated channel subfamily D member 2
KCNJ2	Potassium inwardly rectifying channel subfamily J member 2
kDa	Kilodalton
LAD	Left-anterior descending artery

LIX	LPS-induced CXC chemokine
LNP	Lipid nanoparticles
LVAWd,	LV anterior wall thickness in diastole
LVEDP,	LV end-diastolic pressure
LVEDV	LV end-diastolic volume
LVEF	LV ejection fraction
LVESP	LV end-systolic pressure
LVFS	Fractional shortening
LVH	Left-ventricle hypertrophy
LVIDd	Left ventricular internal dimension-diastole
LVIDs	Left ventricular internal dimension-systole
LVPWd	Left ventricular posterior wall -diastole
LVPWs	Left ventricular posterior wall -systole
MaFGF	Mutant acidic fibroblast growth factor
MAPK	Mitogen-activated protein kinase
MB	Microbubble
MCP-1	Monocyte chemoattractant protein-1
MG53	Mitsugumin 53
MHC- β	Beta-myosin heavy chain
MHz	Megahertz
MI	Mechanical index
MIP	Macrophage inflammatory protein
miR	microRNA
MLCK	Myosin light chain kinase
M-mode	Motion-mode
MRI	Magnetic resonance imaging
mRNA	Messenger RNA
MSC	Mesenchymal stem cells
MTT	3-(4,5-di methyl thiazol-2-yl)-2,5-diphenyltetrazolium bromide
mV	Milivolts
MVD	Microvascular density
MYBPC3	Myosin-binding protein C, cardiac type
MYH7	Myosin heavy chain 7
n.s.	Non-significant
NFAT	Nuclear factor of activated T-cell
N-WASP	Neural Wiskott-Aldrich Syndrome Protein
ODN	Oligodeoxynucleotides
OXPHOS	Oxidative phosphorylation
Pa	Pascal
PBS	Phosphate-Buffered Saline
PCR	Polymerase chain reaction
PE	Phenylphrine
PHD2	Prolyl hydroxylase-2
PKC	Protein kinase C
PN	Product number
PRF	Pulse repetition frequency

PRI,	Pulse repetition interval
pri-miR	Primary microRNA
P-selectin	Platelet selectin
$\tilde{Q}$	Initial cell volume
R_0	Equilibrium radius
RANTES	Regulated on activation, normal T-cell expressed and secreted
R_c	Radius of cell membrane
RE	Reversible electroporation
RF	Radiofrequency
RhoA	Ras (rat sarcoma virus) homolog family member A
RIPA	Radioimmunoprecipitation assay
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
RNase	Ribonuclease
ROK	Rho-associated kinase
ROS	Reactive oxygen species
RT-qPCR	Reverse transcription PCR
RyR2	Ryanodine receptor 2
S100A6	S100 calcium-binding protein A6
SCF	Stem cell factor
SDF-1	Stromal-cell-derived factor 1.
SDS	Sodium dodecyl sulfate
SEM	Standard error mean
SERCA2a	Sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase
siRNA	Short interfering RNA
Sirt3	Sirtuin-3
SNARE	Soluble N-ethylmaleimide-sensitive factor Attachment protein receptor
STM1	Stomatin-like protein 1
SYT7	Synaptotagmin 7
TAT	Transactivating transcriptional activator
TBS	Tris buffer saline
TBST	Tris buffer saline with Tween-20
TIMP3	TIMP metalloproteinase inhibitor 3
TNF- α	Tissue necrosis factor-alpha
TRAF3IP2	TRAF interaction protein 2
TRP	Transient receptor potential channel
TWF1	Twinfilin actin-binding protein 1
US	Ultrasound
VCAM-1	Vascular cell adhesion molecule 1
VDAC	Voltage-dependent anion channel
VEGF	Vascular endothelial growth factor
ZO-1	Zonula Occludens-1
$\Delta\Psi_m$	Transmembrane potential
λ	Wavelength

Chapter 1: Introduction and Background

1.1 Overview of left-ventricle hypertrophy

1.1.1 Characterization of LVH

The principal function of the heart is to ensure the continuous flow of blood throughout the body. Under certain forms of stress, the heart can undergo remodeling to preserve cardiac output and maintain adequate blood flow. One particular type of remodeling includes left-ventricle hypertrophy (LVH), a cardiovascular condition that affects approximately 15-20% of the global population¹. LVH is characterized by an increased thickness of the left ventricular wall as a compensatory response to chronic hemodynamic stress or increased contractile workload. In accordance with Laplace's law, this increase in left-ventricle wall thickness attenuates the rise in wall pressure, thereby helping maintain contractile force². The increased thickness of the ventricle wall is primarily due to the hypertrophic growth of the cardiomyocyte, rather than hyperplastic growth³. Within cardiomyocytes, the increase in size is a result of sarcomereogenesis, the production of new sarcomeres, which are contractile units responsible for force generation in cardiac cells. The addition of the sarcomere unit enhances the contractile strength of the heart, thereby supporting cardiac output.

The development of cardiac remodeling is dependent on the type of stress. When the heart is subjected to increased preload (volume overload), both wall thickness and chamber size increase, resulting in ventricular dilation (eccentric hypertrophy). Alternatively, an increase in afterload (pressure overload) results in thickening of the ventricular wall with no change or decrease in chamber size (concentric hypertrophy). However, depending on the stimulus and the

signaling pathway involved, the remodeling can either be classified as physiological or pathological⁴.

Physiological hypertrophy occurs during postnatal growth of the heart and in response to conditions that increase cardiac workload, such as endurance training, pregnancy (eccentric) or during strength training (concentric). This form of hypertrophy is recognized as a beneficial adaptation due to its ability to maintain cardiac functions and its reversibility upon removal of the stimulus.^{5,6} Pathological hypertrophy develops as a result of either extrinsic factors, including chronic hypertension and aortic stenosis, or intrinsic factors, such as the genetic mutations associated with hypertrophic cardiomyopathy^{7,8}. Under these conditions, the heart undergoes concentric remodeling, initiated through a distinct molecular pathway from their physiological counterpart.

1.1.2 Pathogenesis of pathological LVH

Pathological hypertrophy develops in response to stress signals released from cardiac and non-cardiac cells which initiate molecular pathways responsible for driving maladaptive remodeling of the heart. These signalling molecules can include the release of peptide hormone (e.g. Ang II, ET-1)^{9,10}, catecholamines (e.g. norepinephrine)¹¹, growth factors (e.g. FGF2)¹⁰ and pro-inflammatory cytokines (e.g. IL-6)⁴. Hemodynamic stress from the wall pressure can further contribute to remodeling through the activation of mechanosensitive channels on cardiomyocytes (e.g. TRP, STIM1)^{12,13} or indirectly through the extracellular matrix integrin interactions¹⁴. This mechanical stress can also stimulate surrounding cells, such as cardiac fibroblasts and endothelial cells, to promote the release of paracrine signaling molecules, which can further induce hypertrophic signaling in cardiomyocytes¹⁵.

One of the most prominent receptors in hypertrophy induction is the activation of G-protein-coupled receptors (GPCRs) by catecholamine and peptide hormones. Upon activation, a myriad of signalling pathways are initiated, including MAPK and PKC pathways^{16–18}. GPCR activation results in an increase in intracellular calcium, which activates the calcineurin/NFAT pathway, promoting the nuclear localization of NFAT and interaction of transcription cofactors such as MEF2A. This results in the induction of hypertrophic-associated genes, including those of the fetal gene program¹⁹. Normally downregulated after birth, the fetal gene program encompasses a series of genes that are essential for the development of the heart at the fetal stage. The re-expression of these genes under a pressure-overload environment results in a variety of non-adaptive changes, including the metabolic shift from fatty acid oxidation to less efficient glycolysis and the re-emergence of fetal sarcomere isoform (MHC- β isoform), which reduces contractile efficiency and power output^{20–23}.

Other cellular changes initiated by hypertrophic stimuli include reduced contractility due to impairment in calcium homeostasis, due to downregulation of calcium channels such as SERCA2 and RYR2^{24,25}, as well as disorganization of critical gap junction proteins, such as Cx43^{26,27}. Impaired sarcomere shortening has also been shown to occur as a result of cytoskeletal remodeling, which has been shown to increase microtubule and F-actin accumulation as a result of dysregulation in the expression of regulator proteins (e.g. TWF1)^{28,29,30}. Furthermore, a decrease in mitochondrial oxidative capacity³¹, increased ROS production³², and enhanced cardiomyocyte apoptosis are also present^{33–35}. Collectively, these maladaptive changes directly impact the cardiac morphology and function, resulting in diastolic dysfunction and heart failure with preserved ejection fraction (HFpEF)³⁶, as illustrated in Figure 1.1. Unlike in physiological hypertrophy, the

cellular and morphological changes associated with pathological hypertrophy are less easily reversible^{37,38}.

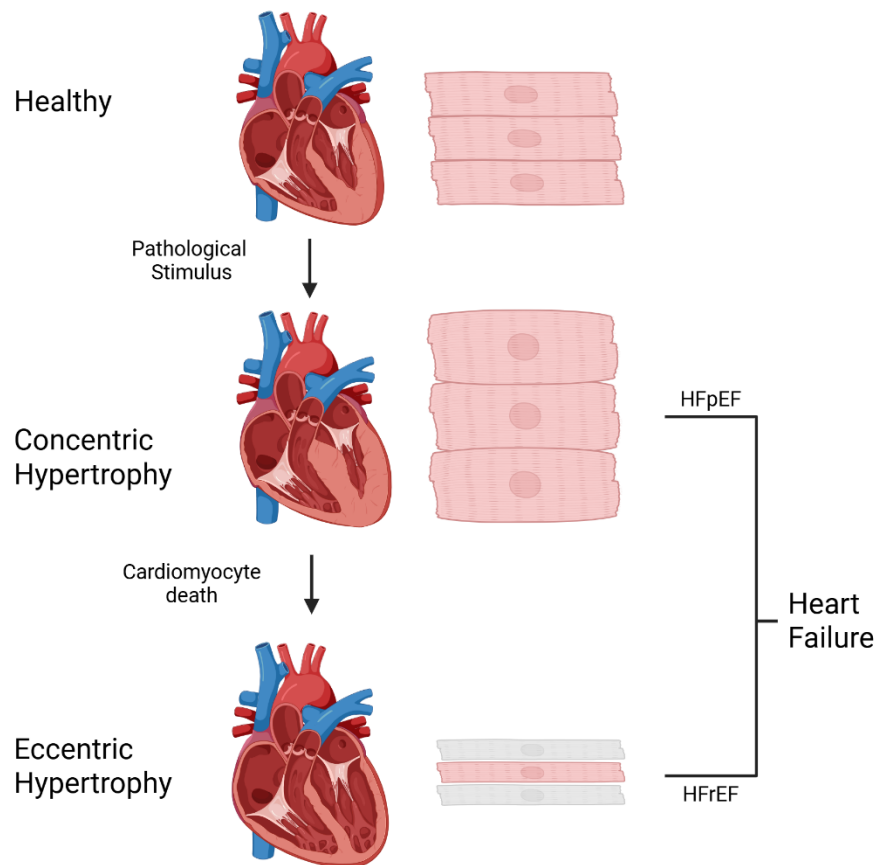


Figure 1.1: Overview of pathological left-ventricular hypertrophy. Under pathological stimulus, cardiomyocytes become hypertrophic, leading to the thickening of the left ventricular wall. This stage is associated with diastolic dysfunction and the development of heart failure with preserved ejection fraction (HFpEF). Under chronic stimulation, cardiomyocyte death and fibrosis can occur, resulting in the dilation of the ventricular chamber and eccentric remodeling. This change can impair systolic functions, eventually leading to heart failure with restricted ejection fraction (HFrEF). Figure created on Biorender.com.

Beyond the cardiomyocytes, there are other impacts to the cardiac tissue, such as the decrease in myocardial capillary density, reducing blood flow to the myocardium, and inducing ischemia^{39,40}. Furthermore, there is also an increase in inflammation due to the release of pro-inflammatory cytokines by cardiomyocytes, fibroblasts, immune cells, and endothelial cells⁴¹. The

impacts of myocardial inflammation and cardiac death also initiate pro-fibrotic pathways⁴². In response to hypertrophic changes, multiple cell types (e.g., macrophage, mast cells, cardiomyocyte) release pro-fibrotic mediators (e.g., TNF- α)^{43,44} which induce fibroblast proliferation and collagen deposition. As this persists over time, it can result in cardiac fibrosis, which further develops into heart failure through impaired cardiac contraction and impaired electrical conduction^{16,45}. With continued cardiomyocyte death and replacement with fibrosis, the ventricular wall becomes thinner and stiffer, undergoing eccentric remodelling^{16,46}. The consequence of this remodeling is the inability of the ventricular chamber to fully contract at systole, resulting in heart failure with reduced ejection fraction (HFrEF)⁴⁷, as demonstrated in Fig. 1.1. At this stage, referred to as the decompensated phase of cardiac hypertrophy, patients have a higher rate of mortality⁴⁸.

1.1.3 Current treatment strategies

The current clinical treatment options for LVH are dependent on the stage at which the pathology is discovered, as well as the underlying stimulus responsible for the initial stress. As such, treatment can vary from lifestyle modifications to surgical interventions. However, because of the asymptomatic nature of hypertrophy during the early stages, it is most common for treatment to be initiated once the disease has progressed to more advanced forms^{49,50}. Among the available clinical treatment options, pharmacological interventions are the most common and affordable treatment approach. Given the intricate signaling cascade involved in inducing hypertrophy, some pharmaceuticals aim to inhibit mediators responsible for activating hypertrophic pathways. Some studies have demonstrated the feasibility of impairing the signaling molecules from activating hypertrophic pathways with pharmaceuticals. Examples include angiotensin receptor blockers (ARB), which prevent the binding of AngII to its target receptor⁴⁶, and angiotensin-converting

enzyme (ACE) inhibitors, which avoid the formation of angiotensin^{51,52}. Beta-blockers are used to reduce heart rate, decrease blood pressure, and act as a competitive antagonist to catecholamines, preventing pathological signalling. Calcium channel blockers (CCB) are also utilized to maintain calcium homeostasis by blocking L-type channels in myocytes⁵³. Though these interventions have some success in reducing LVH remodeling, their efficacy is limited⁵⁴. Some of the main limitations of a pharmaceutical approach include systemic administration, which can lead to possible adverse side effects and increased risks in cases of comorbidity⁵⁵. Furthermore, the majority of current pharmaceuticals for LVH target the stimulus for hypertrophy, but it does not treat the myocytes that have already undergone disease remodeling.

A more recent therapeutic development is the use of Mavacamten. This pharmacological agent reduces the number of myosin heads that interact with actin, thereby decreasing hypercontractility in cases of hypertrophic cardiomyopathy (HCM). While this represents a significant advance in the management of HCM, there are still limitations. Some of these include side effects such as reduced systolic contraction and cases of atrial fibrillation, and stress cardiomyopathy⁵⁵. Like other systemic drugs, it also does not directly reverse established molecular remodeling within cardiomyocytes.

At later stages of hypertrophy, when wall thickness severely impacts cardiac function and patients are at risk for decompensation, a more invasive approach is utilized to remove the excess cardiac tissue. These methods are either septal myectomy or alcohol septal ablation, both of which help restore cardiac functions. Although effective, this form of treatment is highly invasive and does not address the pathological changes already made to the cardiomyocytes.

These limitations highlight the need for cardiac-specific therapy that can reverse the pathological remodeling at a cellular level. Given these limitations, there is a growing interest in

gene-based therapies that can directly target maladaptive remodeling at the cellular level. Among these, RNA-based therapeutics—particularly microRNAs (miRs)—have emerged as promising candidates.

1.2 miR replacement therapy

1.2.1 miR biogenesis and transcription regulation

miRs are small non-coding RNA strand (~22nt) that are involved in post-transcriptional regulation. They are synthesized in the nucleus and are transcribed by RNA Polymerase II into primary miR (pri-miR), a larger RNA strand with at least one hairpin structure⁵⁶. Drosha and DGCR8 complex are then used to cleaved into precursor miR (pre-miR) and are then exported to the cytoplasm through protein exportin 5⁵⁷. Once in the cytoplasm, Dicer, an RNA enzyme, is used to cleave the miR and remove the hairpin loop. This produces a double stranded miR duplex (~18-25 nt) consisting of a guide strand and a passenger strand⁵⁸. The dsRNA is then loaded into Argonaut (AGO) protein, which drives the unwinding and removal of the passenger strand⁵⁹. The remaining guiding strand with the AGO generate the RNA induced silencing (RISC) complex, which will be used to bind to mRNA⁶⁰. Recognition of mRNA target occurs by binding to guiding miR (nucleotide site 2-8) within the 3'-untranslated region^{61,62}. This results in the degradation of the miR target through decapping and deadenylation^{63,64}. miRs are unique due to their capability of targeting a diverse array of genes, thereby regulating multiple proteins simultaneously. For this reason, miR replacement therapy has gained a lot of interest, as it has a broader range of impact.

1.2.2 miR-1 role in cardiovascular biology and hypertrophy

In the heart, there are multiple miRs that contribute to the regulation of cardiac function. Among them, miR-1 is the most abundant miR, consisting of almost 40% of total cardiac miR⁶⁵. This miR is involved in diverse gene regulatory networks, essential for cardiovascular development and adult cardiac physiology^{66–68}.

The expression of miR-1 begins as early as embryonic day 8.5 and rises sharply during the postnatal stages^{69,70}. During the developmental stages, miR-1 is essential for the differentiation of embryonic stem cells to cardiomyocytes⁷¹, and for regulating the proliferation of cardiac progenitor cells^{70,72}. Studies have emphasised the overall importance of miR-1 levels at this stage of development, as miR-1 knockout mice models resulted in cardiac defects and high pre-weaning lethality⁷³. Conversely, overexpressed miR-1 induces premature differentiation and early cell cycle withdrawal, leading to cardiac malformation⁶⁸. In early postnatal development, the miR-1 expression levels continue to rise, and are closely associated with decreasing proliferation rates through cell cycle arrest⁷⁴. This developmental upregulation contributes to the transition of cardiomyocytes toward a terminally differentiated, non-proliferative state.

Beyond its developmental role, miR-1 is essential for maintaining cardiac electrophysiology through regulating several key ion channels⁷⁵. These include L-type calcium channels (CACNA1C) during depolarization⁷⁶, outward potassium channels for repolarization (KCND2)⁶⁸ and inward potassium channels for stabilization of membrane potential (KCNJ2)⁷⁷. The loss of miR-1 in the heart can disrupt the expression of these channels, increasing the susceptibility of arrhythmia and impaired cardiac contraction⁷⁸. Similarly, miR-1 regulates intracellular cardiac conduction through CX43, one of the most abundant gap junction channels in

the heart⁷⁹. In miR-1 downregulated models, CX43 activity is impaired, resulting in an increased risk of ventricular tachyarrhythmias²⁵.

Cardiac contraction is also regulated by miR-1. Intercellular calcium levels are essential in the excitation-contraction coupling of the myofilament, and the sarcoplasmic reticulum plays a critical role in calcium homeostasis through calcium channel proteins such as RyR2⁸⁰. The dysregulation of miR-1 expression is shown to impair these channels, resulting in an increase potential for arrhythmia⁸¹. In addition, miR-1 directly regulates myosin light chain kinase (MLCK), a modulator in cardiac contraction and sarcomere organization⁸². In miR-1 knockout models, MLCK upregulation resulted in impaired contraction and defective sarcomeres in cardiomyocytes⁷³. Finally, miR-1 is shown to have a role in cytoskeletal regulation, as it is known to directly target twinfilin-1 (TWF1), an actin monomer binding protein. In the absence of miR-1, TWF1 is upregulated and resulting in pathological LVH⁸³.

The alteration of miR-1 expression has also been associated with a number of cardiac disorders, such as pathological LVH⁸⁴. Under cardiac hypertrophy, miR-1 has been demonstrated to be significantly decreased, even as early as during the asymptomatic stages of hypertrophy, making it not only a therapeutic target but an early biomarker for the disease^{85–87}. This decrease of miR-1 has also been shown to increase expression of hypertrophic associated gene and fetal genes through the fetal gene program, resulting in the continued stimulation of pathological LVH (Figure 1.2a)⁸⁸.

Collectively, these studies demonstrate that the dysregulation or removal of miR-1 can result in a myriad of structural, electrophysiological, and metabolic abnormalities in the heart. Therefore, given the importance of miR-1 in cardiac biology and its prominent role in the

development of hypertrophy, the return of miR-1 to its native levels poses a potential cardiomyocyte-specific therapeutic strategy for hypertrophy^{88,89}

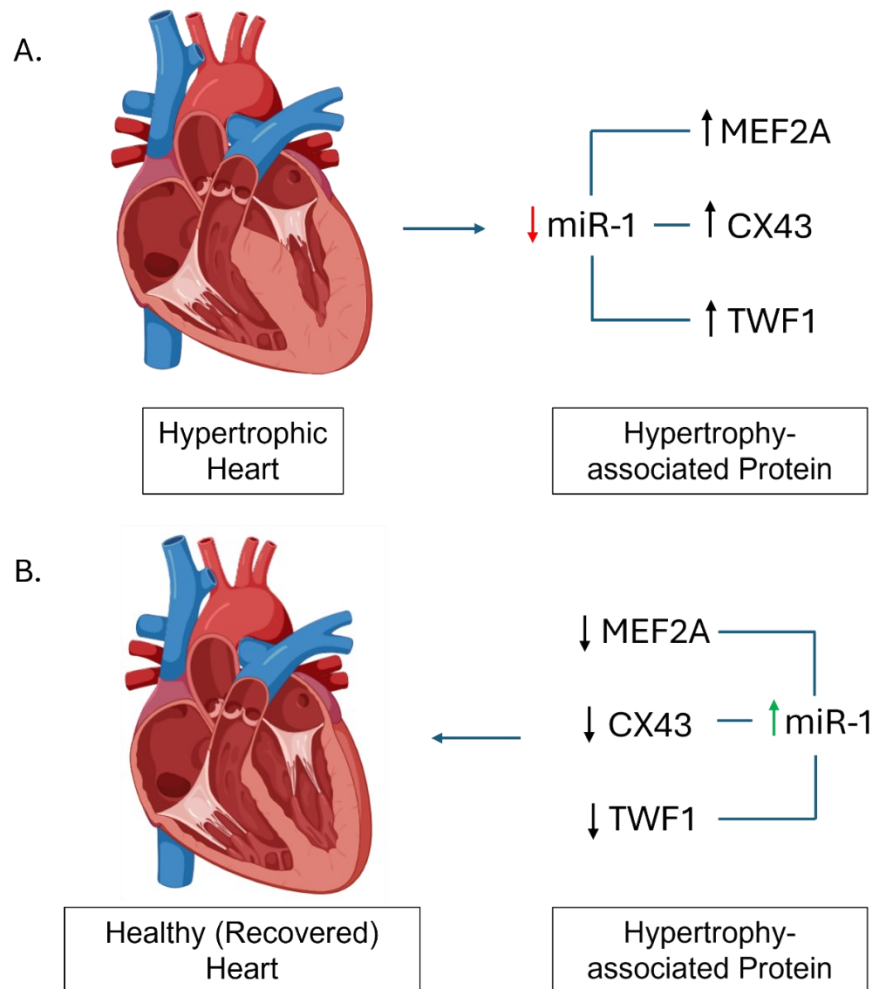


Figure 1.2: miR-1 regulation in left-ventricle hypertrophy. (A) During cardiac hypertrophy, miR-1 expression is downregulated, resulting in the upregulation of hypertrophic-associated proteins (e.g., MEF2A, TWF1, CX43). (B) Delivery of miR-1 to cardiac tissue targets hypertrophic-associated proteins resulting in the recovery of hypertrophy in the heart. Figure created on Biorender.com

1.2.3 Therapeutic application of miR

The application of miR as a therapeutic strategy has been demonstrated in many other studies, predominantly in the context of oncology. With respect to cardiac therapy, the first clinical study of miR-based therapeutics was with the Thum group in 2019, through intravenous administration of miR-132 for treatment of heart failure (NCT04045405). Though the treatment efficacy was limited, it highlighted the safety and feasibility of this treatment strategy⁹⁰.

In recent years, there have been many pre-clinical studies that explored the concept of miR therapy. In the case of miR-1 for treatment of hypertrophy, there has been some *in-vitro* and *in-vivo* experiments that have demonstrated its feasibility. Studies have shown in hypertrophic animal models the feasibility of miR-1 attenuation of pro-hypertrophic gene expression and reverting hypertrophic phenotypes (Figure 1.2b)^{25,83,91,92}. However, one of the main obstacles in the application of miR replacement therapy is its targeted delivery in a safe and efficient manner.

1.2.4 Current miR delivery strategies

Though miR therapy shows a lot of promise, the optimal method of delivery needs to be determined. The most direct method of delivery for most cardiac gene therapeutics has been intramyocardial delivery. This method delivers the therapeutic either through direct injection into the cardiac tissue or through a catheter-based transendocardial approach⁹³. Intramyocardial approach ensures direct and spatially targeted delivery into the myocardium, resulting in high payload concentrations^{94,95}. Studies have shown the efficacy of this method in addition to the ability to treat cardiovascular disorders, an example of which is the EPICURE clinical trial, which delivered VEGF modRNA for myocardial infarction (NCT03370887)⁹⁶. However, the main disadvantage is the invasiveness of this technique, which has an increased risk of cardiac

perforation⁹⁷, acute inflammation⁹⁵, ventricular arrhythmia⁹⁸ and an increase in fibrosis⁹⁹, which can be especially dangerous when treating a pathological hypertrophic heart. These limitations also become an obstacle in the case where repeated delivery is required. As well, some studies have shown that intramyocardial delivery is generally limited to the site of the injection, preventing a homogenous expression of the therapeutic throughout the myocardium^{100–102}.

An alternative approach with decreased invasiveness and reduced cardiac damage is intravascular delivery. However, the main limitations of intravascular delivery of RNA therapeutics are RNA degradation from endogenous RNase, low cellular uptake, and an increased chance of off-target delivery^{103,104}. For this reason, delivery carriers may be utilized to improve miR delivery to the heart. An example includes the use of liposomal delivery, in which therapeutics are encapsulated within a lipid vesicle, resulting in a non-invasive method that increases the half-life of the gene therapeutic¹⁰⁵. However, some overall limitations of this delivery strategy include a limited loading capacity, limited intracellular delivery, and low targeting capabilities^{105,106}. Some studies have developed targeted liposomes using cardiac-specific antibodies on the surface of the liposomes to improve targeting¹⁰⁷. Other groups have utilized liposomal nanoparticles as a delivery vehicle to enhance cellular internalization¹⁰⁸. However, in both these cases, there are still significant off-target effects, with the accumulation of therapeutic agents in different organs. Recently, extracellular vesicles (EV) have also shown considerable potential as a delivery carrier. These vesicles are secreted by cells in the body and are responsible for transferring signalling molecules (proteins, nucleic acids, lipids) essential for intercellular communication¹⁰⁹. They also have roles in cardiovascular disease, such as the increase in circulation of miR (miR-1, miR-208, and miR-499) in response to myocardial infarction to initiate a cardiovascular repair¹¹⁰. There are many benefits in the use of EVs as a delivery carrier, such as improved cellular uptake, increased

half-life of miR cargo, and endogenous targeting. Studies have also suggested that EVs may be more efficient than liposomal-based delivery techniques¹¹¹. Recently, Gu et al. demonstrated the use of a cardiac-specific engineered exosome loaded with miR-302 to reduce ischemic cardiac injury¹¹². There are even ongoing clinical studies determining the feasibility of EV for the delivery of RNA therapeutics (NCT005774509). However, for the majority of EV-mediated delivery, there is still a significant uptake of EV in off-target organs such as the liver, spleen, and lungs¹⁰⁹.

Finally, one of the most utilized methods of delivery, particularly *in-vivo*, is through the use of viral vectors. The benefit of these vectors includes the high delivery efficiency and the capability, in principle, of directly targeting the target tissue. There are many ongoing clinical studies examining viral gene therapy for cardiovascular disorders, though the majority still use an intramyocardial approach¹¹³. However, there are currently ongoing pre-clinical studies that have shown the feasibility of viral miR delivery to cardiac tissue using non-intramyocardial injections^{114,115}. Despite this, the use of viral vectors does possess the major limitation of potentially inducing an immunogenic response, increased cellular toxicity, and a relatively limited carrying capacity¹¹⁶. It is for these reasons that alternative targeted cardiac delivery strategies are under investigation.

1.4 Ultrasound and microbubbles

1.4.1 Fundamentals

Biomedical ultrasound imaging is the use of high-frequency sound waves to generate an image. This is achieved by the use of a transducer containing a piezoelectric element, which converts an electrical signal to mechanical sound waves, thus generating ultrasound. These sound waves contain alternating regions of high and low pressures, creating regions of expansion and

rarefaction. Ultrasound waves emitted from a transducer travel through different mediums, such as tissue and fluids. As the waves reach an interface between mediums of different acoustic impedance (e.g. density), they are partially reflected back to the probe as echoes, while the remainder of the wave energy continues to propagate. The returning echoes are converted to an electrical signal by the same transducer, which are then processed and transformed into a digital depth image based on time-of-flight information. One of the primary uses of medical ultrasound is as a diagnostic tool in clinical settings due to its capability of providing rapid, real-time imaging in a non-invasive manner, such as in the fields of cardiology, obstetrics, and vascular diagnostics. However, differences in tissue type, density, and location can attenuate or refract the ultrasound wave, reducing the image quality. Therefore, adjustments are made to the ultrasound parameters to optimize image quality; the salient of which are briefly described here.

The frequency of the wave is represented as the number of ultrasound cycles per second (Hz). Clinical ultrasound frequencies generally range from 1-10 MHz, where higher frequencies have an improved image resolution at the expense of lower ultrasound penetration and thus are more suitable for superficial imaging. For visualization of structures deeper in the body (e.g. liver, pancreas), lower frequencies have greater penetration but decreased image resolution¹¹⁷. In clinical settings, the mechanical index (MI) is used to denote the intensity of the wave, which is calculated as the peak-negative pressure (i.e., amplitude) divided by the square root of the frequency. In instances where the MI is too high, there a potential risk of unwanted bioeffects, such as increased heating, as a result of the absorption of the wave energy by the tissue¹¹⁸. It is for this reason that the maximum MI for human use, recommended by the Food and Drug Administration (FDA), is 1.9¹¹⁹.

Finally, ultrasound can also be outputted as a continuous wave or a pulsed wave. During a pulsed wave, the ultrasound is emitted as bursts with periods of rest between the ultrasound waves. The pulse repetition interval (PRI) provides the time from the beginning of a pulse to the beginning of the next pulse. The duty cycle is the percentage of time that the pulse is on throughout the pulse repetition interval. Together, along with the frequency, these parameters can determine the number of acoustic cycles within a pulse interval.

1.4.2 Echocardiography

One of the most common applications of ultrasound imaging is echocardiography, which is the assessment of the cardiac structure and function. Due to the ability to provide a quick, real-time visualization of the heart in a non-invasive manner, it is considered as the first line of imaging modality for cardiology.

When performing echocardiography, there are different modes that can be utilized depending on the diagnostic objective. The most common is B-mode (brightness mode), which produces a two-dimensional real-time image of the heart. The echocardiographic view obtained is dependent on the positioning of the transducer (parasternal, apical, subcostal, suprasternal) and the imaging plane selected (long axis, short axis, four-chamber). Some of the most common views include the parasternal long axis, which provides a longitudinal view of the heart, and the parasternal short axis, which provides a circular cross-section of the heart⁵³. This mode can be used to identify cardiac structures, as well as measure cardiac function. M-mode (Motion mode), guided with B-mode, provides a one-dimensional image along a scan line with a high temporal resolution. This method allows for the measurement of motion over time, which enables the precise measurement of chamber dimensions, wall thickness, and valve movement during the cardiac cycle.

Beyond qualitative structural observation, B-mode and M-mode can also provide quantitative information for cardiac assessment. These include measurements of the left-ventricular size, mass, and wall thickness. Left-ventricular systolic function can be determined from the calculation of the ejection fraction, defined as the relative amount of blood pumped by the left ventricle during contraction, using B-mode assessment of ventricular volume. Fractional shortening can also be used to determine left-ventricular systolic activity, using M-mode to measure the difference in left-ventricular diameter during systole and diastole¹²⁰. An example of an echocardiogram illustrating B-mode and M-mode can be shown in Figure 1.3. This figure demonstrates an example measurement of the left-ventricular internal diameter at systole and diastole (LVIDs and LVIDd), and the left-ventricular posterior wall thickness at systole and diastole (LVPWs, LVPWd).

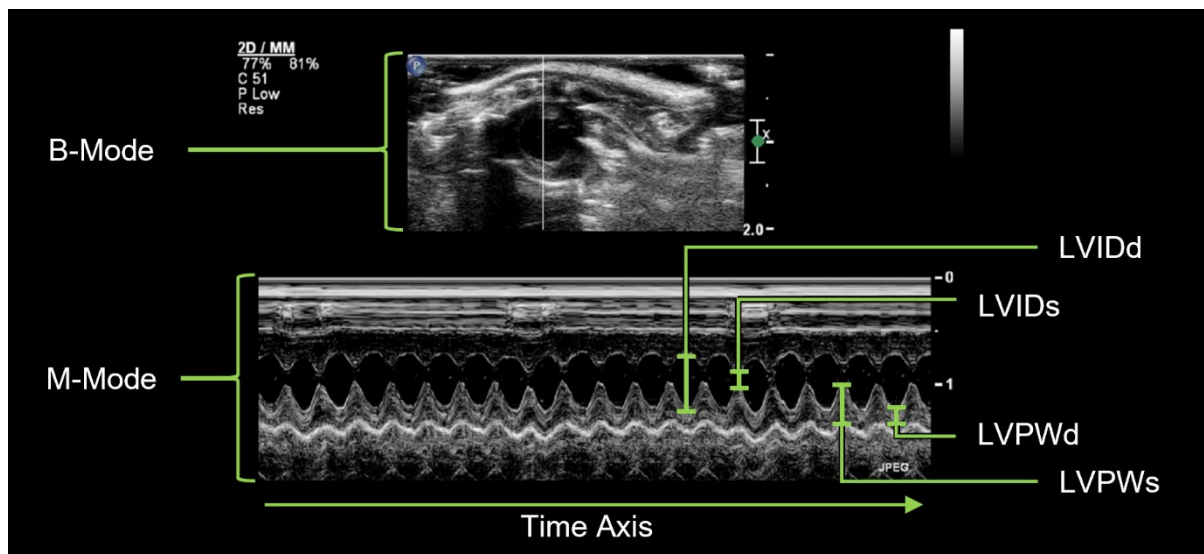


Figure 1.3: Echocardiography overview. Parasternal short-axis view of rat heart, with B-mode and M-mode. The left-ventricular internal diameter at systole (LVIDs) and diastole (LVIDd) are highlighted. These parameters are used in the calculation of the fractional shortening (FS). The left-ventricular posterior wall thickness at systole (LVPWs) and diastole (LVPWd) are also identified and used to measure the ventricular wall thickness. Image acquired by Davindra Singh using a Philips IU22 ultrasound system.

Overall, these techniques can provide a sufficient amount of data to help diagnose a number of cardiac diseases. One example includes hypertrophic cardiomyopathy, which can be diagnosed by the wall thickness, left-ventricular mass, and ejection fraction. While B-mode and M-mode ultrasound can provide a good visualization of soft tissues, the ability to visualize microvasculature is limited due to the low echogenicity of blood. To overcome this, contrast agents have been utilized to enhance the imaging of the smaller vasculature.

1.4.3 Microbubbles and contrast imaging

Ultrasound contrast agents (microbubbles) are micrometer-sized (1-10 μ m) gas-filled bubbles that are intravascularly administered and used to enhance ultrasound contrast. They have an insoluble gas core encapsulated by a thin layer of lipid, protein, or polymer¹²¹. Due to their size, they can easily travel throughout the capillary blood vessels without extravasation beyond the endovascular border¹²². In the presence of an ultrasound wave, microbubbles undergo volumetric oscillation. This occurs due to the alternating compression and rarefaction of the gas core in response to the high- and low-pressure phases of the ultrasound wave, resulting in the contraction and expansion of the microbubbles along their equilibrium radius (R_0)¹²³. The type of oscillation is dependent on multiple ultrasound parameters, one of which is the MI. Under lower intensity ultrasound (MI<0.1), the microbubbles undergo stable cavitation, which results in a sustained oscillation along their equilibrium volume¹²⁴. At higher intensities (MI> 0.4), microbubbles undergo inertial cavitation, resulting in increased asymmetrical oscillation, often leading to the destruction of the bubble. These cavitations can also be defined as nonlinear oscillations, as they are capable of producing backscatter echoes that not only reflect the fundamental frequency of the transmitted ultrasound signal, but also at harmonic (integer multiple of transmit frequency) and

subharmonics (half-order multiple of transmit frequency)^{125,126}. Non-linear signals are utilized in most ultrasound systems for contrast imaging, as tissue can only reflect fundamental frequencies; therefore, this method enhances the bubble-to-tissue backscatter signal ratio^{127,128}.

In clinical settings, contrast-enhanced ultrasound imaging is most commonly used to visualize blood flow, due to the ability of microbubbles to travel throughout the capillary network and clearly be delineated from the anechoic blood. In myocardial perfusion studies, contrast-enhanced imaging is essential for the assessment of microvascular integrity and myocardial viability in cases of myocardial infarction, ischemia, and coronary artery disease¹²⁶. As well, contrast imaging is used for assessment of cardiac structure, as the use of microbubbles provides a clear distinction of the endocardial border of left-ventricle cavity, allowing for the visualization of wall motion abnormalities, left-ventricle thrombus, as well as improved assessment of ejection fraction¹²⁹. In more recent applications, ultrasound and microbubbles have enabled super-resolution ultrasound imaging, allowing for the visualization of microvascular structures, such as in the brain¹³⁰. Recently, many studies have demonstrated the utility of microbubbles beyond an imaging agent, capable of targeted therapeutic action.

1.4.4 Ultrasound and microbubble-mediated delivery

Aside from imaging, ultrasound-mediated microbubble activity has been utilized for targeted delivery of therapeutic molecules. This application arose from studies on the interaction of a cavitating microbubble near an endothelial cell, which demonstrated a transient increase in membrane permeability, in a process known as sonoporation¹³¹. The pores generated through this mechanism can range from 10-1200 nm in size and typically reseal within approximately 2 minutes of treatment, depending on ultrasound conditions applied^{132,133}.

There are many mechanisms involved in microbubble-mediated perforation of cell membranes, as illustrated in Figure 1.4. The exact mechanism of pore formation is strongly dependent on the type of cavitation. Under stable cavitation, oscillating microbubbles exert a mechanical force on nearby cell membranes, either through a “pulling and pushing” mechanism¹³⁴ or the generation fluid flow (microstreaming)¹³⁵. Alternatively, during inertial cavitation, the violent collapse of microbubbles initiates membrane permeabilization through shockwaves or high velocity microjets^{136–138}.

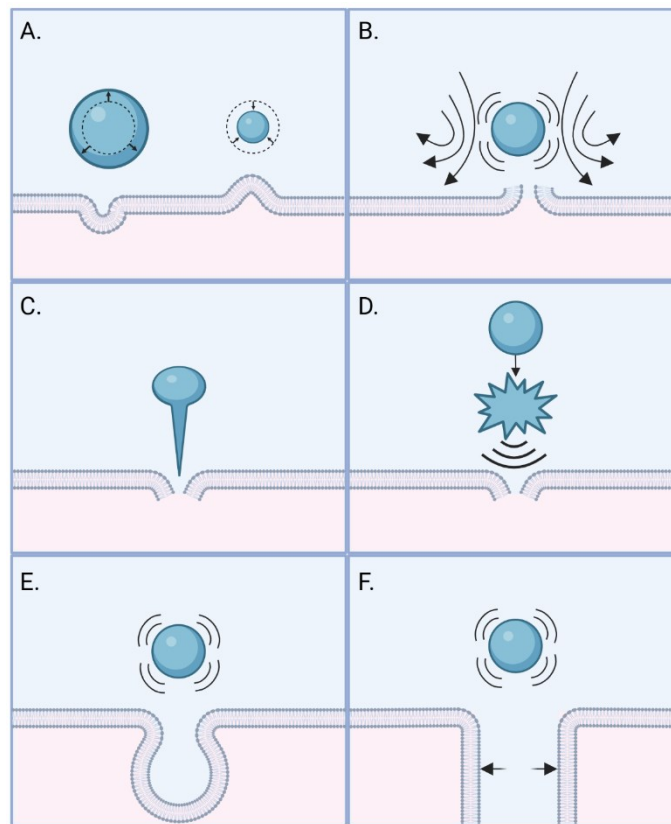


Figure 1.4: Ultrasound and microbubble-mediated cell permeabilization. The pore formation occurs when microbubbles cavitate near the surface of a cell membrane. During stable cavitation, the microbubble can create pores through a (a) “pulling and pushing” force or (b) microstreaming. Under inertial cavitation, collapsing microbubbles can generate pores through (c) micro-jets or through (d) shock wave formation. Cavitating microbubbles are also shown to (e) enhance endocytosis and (f) allow for paracellular transport through the opening of tight junctions. Figure created on Biorender.com

In addition to direct perforation of the cellular membranes, studies have also demonstrated the capability of ultrasound and microbubbles to enhance delivery through other mechanisms. Some studies have demonstrated the ability of ultrasound and microbubbles to induce the transient opening of intercellular tight junctions, which can be utilized to allow for a paracellular route of therapeutic agents¹³⁹. Furthermore, the increase in endocytotic activity in response to ultrasound stimulation is suggested to play a role in the increase of transcellular delivery¹⁴⁰. It should be noted that beyond cellular delivery, a number of studies have elucidated intracellular bioeffects associated with microbubble cavitation. Some of these include the influx of calcium ions¹⁴¹ and the release of ATP¹⁴², which are associated with changes in cellular and vascular activity.

Overall, these mechanisms make the use of ultrasound and microbubbles a versatile technique for inducing viable site-directed cellular permeabilization for delivery. Numerous studies have demonstrated this concept through the delivery of a wide spectrum of therapeutic payloads. Some studies have demonstrated the capability of delivering pharmacological agents, such as the delivery of chemotherapeutics for cancer therapy¹⁴³. Studies have also utilized ultrasound and microbubbles to deliver a wide variety of gene therapeutics, including siRNA¹⁴⁴, miRNA¹⁴⁵, mRNA¹⁴⁶ and plasmid DNA¹⁴⁷.

One of the main techniques for such a paradigm is the development of a modified microbubble agent capable of carrying therapeutics directly on the surface. Some strategies include the encapsulation of therapeutics in liposomes, which are then attached to the surface of the microbubbles through a bioconjugation technique, e.g., biotin-avidin interaction¹⁴⁸. For gene therapy, the common method includes the use of a cationic lipid-encapsulated microbubble, creating a charge-interaction with the therapeutic nucleic acids¹⁴⁹. Upon ultrasound-stimulated cavitation, the therapeutic is released from the surface of the microbubble, providing spatio-

temporal control over the release, and ensuring that cargo remains near the permeated cell. This method also protects the payload from endogenous nucleases, thereby increasing the half-life in circulation¹⁵⁰. Studies such as Vannan et al., have demonstrated the improved targeting efficiency of plasmid DNA-loaded microbubbles in delivery to the myocardium *in-vivo*¹⁵¹. However, this method has several caveats, including a limitation on the total payload delivery amount based on the surface area of a bubble and the requirement for the newly formulated microbubble to undergo review and approval before clinical use. Another approach for microbubble-assisted therapeutic delivery is through the co-injection of microbubbles and the therapeutic agent. The microbubbles used for these strategies are often clinical agents such as DefinityTM (Lantheus Medical Imaging), OptisonTM (GE Healthcare) and SonovueTM (Bracco). In principle, the use of clinically approved agents facilitates the translation from preclinical to clinical studies.

1.4.5 Clinical and preclinical application of ultrasound therapy

Focused ultrasound has two main bioeffects on surrounding tissue: those of thermal and mechanical origin. Thermal effects are generated under high-intensity focused ultrasound. During this process, the surrounding tissue absorbs the acoustic energy from the ultrasound waves and converts it to thermal energy, drastically increasing the heat in a specific region. More specifically, this thermal increase can be localized to an area as small as 1x1.5mm. A common application is within the field of oncology, through thermal ablation of tumors, prostate cancer¹⁵² and bone metastasis¹⁵³, and as a therapeutic strategy in neurological disorders, such as the treatment of essential tremors¹⁵⁴; both of which have received FDA approval. For cardiovascular applications, several preclinical studies have demonstrated the therapeutic potential of focused ultrasound. Some of these include the thermal ablation of arrhythmogenic tissue in atrial fibrillation¹⁵⁵ and enhanced perfusion for stroke recovery¹⁵⁶. Alternatively, focused ultrasound can also provide

therapeutic benefit via mechanical mechanisms through a process known as histotripsy. This application utilizes short repeats of high intensity focused ultrasound in order to break down tissue without heating¹⁵⁷. Histotripsy has been approved in clinical practice for the treatment of liver tumors¹⁵⁶. Additionally, there are ongoing clinical studies such as in the treatment of calcified aortic stenosis by breaking up calcium deposit to restoring mobility to the valve (NCT03779620), and destruction of enlarged prostate in prostatic hyperplasia (NCT01896973).

In terms of ultrasound mediated delivery of an exogenous biologic, many ongoing pre-clinical and clinical studies have demonstrated the feasibility of this technique. Over the past years, ultrasound-mediated cargo delivery to the central nervous system is perhaps the most clinically advanced application. This is in part due the ability of focused ultrasound to reversibly open the blood brain barrier, allowing for the localized delivery drugs and gene therapeutics. This has led to many clinical trials, such as for the treatment of neurological conditions such as Alzheimer's (NCT03739905), Parkinson's (NCT03608553) and obsessive-compulsive disorder (NCT019862961). Additionally, focused ultrasound is also used for the treatment of cancers, though the delivery of chemotherapeutics for the treatment of glioblastomas (NCT03626896). In the field of cardiovascular therapy, ultrasound-mediated delivery remains less advanced relative to the progress achieved in neurological applications. Nevertheless, numerous ongoing preclinical studies continue to demonstrate the feasibility of delivering various forms of gene-based therapeutics for the treatment of cardiovascular disease. More specifically, these studies demonstrate miR delivery to the heart, highlighting not only the feasibility of ultrasound delivery to cardiovascular tissue, but also miR-based therapy for cardiovascular disorders 150–154.

1.5 Thesis objective and rationale

Ultrasound and microbubble-mediated delivery have shown a lot of promise as a non-invasive, site-directed, and image-guided method of delivery. In parallel, miR-1 replacement therapy presents a viable therapeutic option for pathological left-ventricle hypertrophy. However, to date, there are no studies that have established the feasibility of delivering miR-1 to the heart using an image-guided, non-viral approach. Therefore, the goal of this thesis is to demonstrate the feasibility of ultrasound-mediated delivery of miR-1 as a therapeutic strategy for pathological left-ventricle hypertrophy in an *in-vitro* and *in-vivo* model.

In Chapter 2 of the thesis, I present a comprehensive overview of all of the ultrasound-mediated cardiac delivery studies performed *in-vivo* to date. This review highlights the therapeutic payloads along with the ultrasound parameters and treatment regimens that were utilized in these studies, thereby establishing a methodological foundation for future works that follow.

In Chapter 3, I begin by demonstrating microbubble-based delivery of miR-1 to hypertrophic neonatal cardiomyocytes *in-vitro*. This study aims to identify the optimal ultrasound conditions for efficient delivery while maintaining cell viability, and to evaluate the capability of delivered miR-1 in reversing the hypertrophic phenotype. In order to better understand the feasibility of this technique in a more physiologically relevant model, our next aim would be to deliver to an *in-vivo* murine model using a clinical ultrasound system and clinically used microbubbles. These studies are also performed in both male and female models to evaluate sex-dependent delivery efficiency and therapeutic recovery response. I first begin by optimizing acoustic parameters and treatment regimens in a healthy rat model to maximize delivery efficiency. Upon successful delivery, I then moved on to a hypertrophic model in order to determine the therapeutic potential of miR-1 in reversing hypertrophic-associated phenotypes.

Finally, in Chapter 4, I provide an overview of the work presented in this thesis. This chapter summarizes the main findings and discusses the key limitations of these studies. In addition, I outline the potential future directions of this thesis, highlighting specific areas of investigation to advance the research of ultrasound and microbubble-mediated delivery of miR to the heart towards clinical application.

Overall, the goal of this work is to establish the feasibility of focused ultrasound as a method for cardiovascular delivery and to demonstrate the therapeutic efficiency of miR-based therapies for cardiac disorders such as hypertrophy. With the completion of this thesis, I aim to advance the understanding of ultrasound-based cardiovascular therapeutics and highlight miR-1 as a promising candidate for future clinical translation.

Chapter 2: Cardiac gene delivery using ultrasound: State of the field

Manuscript published as: Singh, D., Memari, E., He, S., Yusefi, H., & Helfield, B. (2024).

Cardiac gene delivery using ultrasound: State of the field. *Molecular therapy. Methods & clinical development*, 32(3), 101277. <https://doi.org/10.1016/j.omtm.2024.101277>

2.1 Foreword:

In this chapter, we provide a literary review of the recent studies performed using ultrasound delivery for cardiovascular disease *in-vivo*. This review provides a rational and insight for the work presented in Chapter 3, in which we perform our novel investigations into microbubble-assisted cardiac delivery of miR-1.

2.2 Abstract

Over the past two decades, there has been tremendous and exciting progress towards extending the use of medical ultrasound beyond a traditional imaging tool. Ultrasound contrast agents, typically used for improved visualization of blood flow, have been explored as novel, non-viral gene delivery vectors for cardiovascular therapy. Given this adaptation to ultrasound contrast-enhancing agents, this presents as an image-guided and site-specific gene delivery technique with potential for multi-gene and repeatable delivery protocols – overcoming some of the limitations of alternative gene therapy approaches. In this review, we provide an overview of the studies to date that employ this technique towards cardiac gene therapy using cardiovascular disease (CVD) animal models and summarize their key findings.

2.3 Introduction

Ultrasound is one of the most widely used imaging modalities to assess and diagnose conditions of the heart, in part due to its real-time, non-ionizing nature. Ultrasound contrast agents,

which consist of a suspension of small, encapsulated bubbles between 1-5 μm in diameter, are strong reflectors of ultrasound and enhance the signal from the blood pool to which they are confined, adding diagnostic utility in the assessment of blood flow and perfusion¹⁶³. Leveraging the excellent safety profile of medical ultrasound and the near global approval of microbubble contrast agents, recent work has explored the possibility of extending its use towards image-guided therapy for a range of disease conditions¹⁶⁴. Perhaps one of the most notable approaches is using these microbubbles for the purpose of targeted nucleic acid therapeutics. Either through co-injection or via attachment of a genetic payload, ultrasound-triggered microbubbles can be made to locally deposit molecular therapeutics within a target diseased tissue. Currently, the most clinically advanced application of this technique is brain therapy via the reversible opening of the blood-brain-barrier under MRI guidance (*e.g.* Alzheimer's disease¹⁶⁵).

Cardiovascular disease (CVD), however, has long been envisioned as a candidate for such a gene therapy which, despite advancements in pharmacological and imaging approaches, remains a major cause of morbidity and mortality in North America and worldwide¹⁶⁶. Despite initial trials showing neutral or modest results, significant advancements in the development of gene delivery vehicles, specifically non-viral techniques, have brought it back into the spotlight^{167,168}. As such, ultrasound-mediated gene delivery within the context of CVD holds tremendous promise since this approach¹⁶⁹ is *i)* non-viral, which avoids potential immunogenic responses and dose-limiting side effects; *ii)* inherently image-guided, as these bubbles still function as ultrasound-enhancing agents; *iii)* non-invasive, which opens the opportunity for protracted, repeated therapy; *iv)* integrates nicely within the clinical arena, as echocardiography is a common approach towards the initial diagnosis of many CVDs. In fact, this technique has been shown feasible for the delivery of many types of molecular therapeutics (Table 1).

Table 1: Summary of vectors, along with select examples, that have been used in conjunction with ultrasound for targeted delivery in the context of CVD.

Vector	Example Gene
Plasmids	VEGF ¹⁷⁰ , S100A6 ¹⁷¹ , Ang1 ¹⁷²
siRNAs	ICAM-1 ¹⁷³ , galectin-7 ¹⁷⁴
miR	miR-21 ¹⁶² , miR-126 ¹⁷⁵
Anti-miR	antimiR-23a ¹⁵⁹ , antimir-155 ¹⁷⁶
Piggybac	GLP-1 ¹⁷⁷ , ANGPTL8 ¹⁷⁸
Minicircle DNA	VEGF ¹⁷⁹

VEGF, vascular endothelial growth factor; *S100A6*, *S100* calcium binding protein A6; *Ang1*, angiopoietin-1; *ICAM-1*, intercellular adhesion molecule-1; *GLP-1*, glucagon-like peptide; *miR*, microRNA; *ANGPTL8*, angiopoietin like protein 8.

In this review, we provide an overview of ultrasound-mediated gene delivery to the heart. We acknowledge that this is a subset of the applications for which microbubble-mediated therapies have been demonstrated, both within CVD (*e.g.* vascular disease¹⁷⁰) and outside of it (*e.g.* cancer¹⁴⁴, blood-brain-barrier-based approaches¹⁶⁵). We do touch upon some of these topics but refer the reader to excellent, recent reviews on those subject matters¹⁸⁰. Here, we begin with setting the context for this review by a brief survey of current gene therapy approaches for CVD, followed by an overview of microbubble dynamics within an ultrasound field to provide context for their use in targeted CVD gene therapy; and continue with a summary of postulated microbubble-based bioeffects and bubble agents that are employed for ultrasound-assisted gene therapy. Next, we provide an overview of the preclinical *in-vivo* studies to date that specifically target the heart. Finally, we provide an outlook on this exciting inherently image-guided approach to targeted cardiac gene delivery.

2.4 Current Methods for Cardiac Gene Delivery

Perhaps the most direct and earliest approach is the naked delivery of molecular therapeutics via surgical intervention^{181,182}. Recently, the clinical study EPICCURE (NCT03370887) was the first to report direct epicardial injection of naked mRNA encoding VEGF-A¹⁸³. This trial demonstrated an excellent safety profile with no severe adverse effects, yet the relatively small sample size rendered interpretation of the efficiency challenging. While very promising, naked delivery is typically characterized with sub-optimal gene uptake¹⁸⁴, and direct needle injection into the target poses challenges in achieving homogeneous tissue distribution, potential localized damage from the needle, and clinical implementation is limited by the impracticality of repetitive needle injections into sites which may be difficult to access^{185,186}.

The most recognized vehicle-based method of cardiac gene therapy to date is the use of viral vectors. Robust preclinical research in both small and large animal models have revealed two viral vector systems that yield effective gene transfer -adenovirus and adeno-associated virus (AAV). Earlier works with adenovirus vectors have advanced to clinical trials for heart failure¹⁸⁷, however this type of vector still retains many adenoviral genes and results in strong inflammatory cascades that raise safety concerns. The gold-standard viral approach is the use of AAVs¹⁸⁸, which despite their proven efficiency, are still met with limitations including packaging capacity, tissue specificity, and immune evasion – all areas of active research¹⁸⁹. Additionally, unlike adenovirus methods in which transgene expression returns to baseline over the time-course of a few weeks¹⁹⁰, AAV methods result in permanent transgene expression. While this presents safety concerns in terms of off-target effects, it may also not be desirable from a pathophysiology viewpoint¹⁹¹. The strategy for delivery for these viral vectors remains relatively invasive, with one of the more common approaches the direct intramyocardial injection either through surgical access or through intraventricular delivery via percutaneous catheter.

Attempts to address these concerns has led to more recent work exploring the use of non-viral approaches, one example of which is lipid nanoparticles (LNP) as a delivery vehicle. Indeed, LNP formulations of small molecule drugs represent a relatively mature technology¹⁰⁶, and considerable research is ongoing to expand their application to gene delivery¹⁹². The first such application of LNP-mediated gene delivery with significant *in-vivo* efficiency was demonstrated in 2006, carrying siRNA to silence genes in hepatocytes¹⁹³. While there is much investigation into the lipid composition and overall design of gene-bearing LNPs, this approach is still limited in the context of CVD; including the resulting immune response to LNP formulations of RNA and DNA, and challenged by delivery to non-hepatocyte tissues, in part due to a lack of mechanistic understanding of their systemic biodistribution¹⁹⁴. Beyond LNPs, extracellular vesicles (EVs) – which are cell-derived lipid nanoparticles typically involved in cell-to-cell communication - have gained a lot of traction in recent years as a non-viral gene delivery vectors¹⁹⁵. Intrinsic benefits of EVs include their stability, increased propensity for cellular uptake, and their ability to avoid interaction with the immune system¹⁹⁶. Recent studies have demonstrated the potential for EV-based gene therapy for CVD^{197–200}, and while such studies highlight the exciting promise of this methodology, several challenges need to be addressed; including the development of techniques for the characterization of EV cargo, design and integration of platforms to monitor the vesicles *in-vivo*, and the development of strategies to modify EVs to improve their targeted accumulation in specific tissues²⁰¹. To this end, recent work has demonstrated improved cardiac retention of intramyocardial delivered EVs by using genetic modifications to incorporate cardiac binding peptides on their surface²⁰².

Either alone or in combination with the current approaches listed here, ultrasound-assisted cardiac gene delivery using contrast agent microbubbles has many advantages, including its non-viral nature, the inherent image-guidance, and excellent gene localization potential.

2.5 Brief Overview of The Salient Features of Microbubbles

Owing to the compressibility of the gas core, microbubbles vibrate as they pass through the acoustic beam; expanding and contracting about their resting, equilibrium radius R_0 . Indeed, microbubble agents are polydisperse and exhibit resonance; the specific frequency at which a given bubble vibrates with maximum amplitude is inversely related to its size²⁰³. Microbubble vibrations can be broadly subdivided into two categories (Fig. 2.1). Under low acoustic intensity, microbubbles undergo periodic oscillations about their equilibrium size resulting in echoes that possess a rich resonant structure, exhibiting energy at harmonic frequencies (integer multiples of the transmit frequency) and often sub-harmonic frequencies (half-order multiples of the transmit frequency)²⁰⁴. This type of vibration is often termed stable oscillation, which is desired in routine contrast imaging. In this vibration regime, microbubbles generate local shear forces on neighboring cells and tissue, as well as local fluid streaming (termed microstreaming)²⁰⁵. Under higher intensities, microbubbles can be made to disrupt due to rapid expansion and subsequent violent bubble collapse. This vibration regime, termed inertial cavitation, can generate highly localized increases in pressure and temperature, which may result in shock wave production or the formation of high-speed liquid jets²⁰⁶.

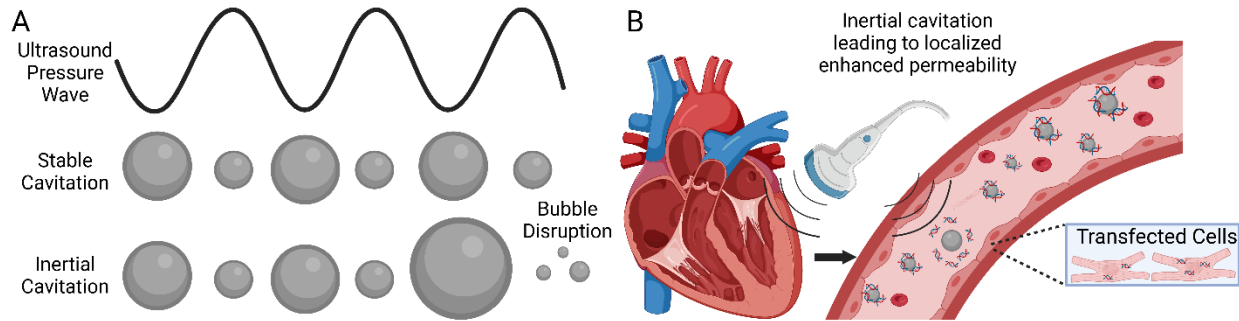


Figure 2.2: Schematic diagram depicting the concept of stable versus inertial cavitation and cardiac gene delivery. A) Stable cavitation consists of periodic vibrations, whereby the bubble gets bigger during rarefaction and smaller during the compressional phase. Inertial cavitation is the process whereby large bubble expansion results in subsequent bubble disruption. B) Externally applied ultrasound to the heart can result in local bubble cavitation and/or molecular therapeutic release. Enhanced cellular and vascular permeability results in transfection of target cells. Note that two strategies exist: co-injection of standard microbubbles with a molecular therapeutic, and gene-bearing microbubbles (shown here).

The propensity for microbubbles to initiate sustained stable cavitation or inertial cavitation is defined by a confluence of factors, not the least of which is the mechanical index (MI) – defined as the acoustic pressure scaled by the square root of the frequency²⁰⁷.

2.6 Overview of Ultrasound-Assisted Gene Delivery

It was first observed in the late 1990s that ultrasound-mediated microbubble activity can result in vessel permeability^{208,209}. These studies spurred the onset of many mechanistic physical and biological investigations, as well as application-driven research aimed at harnessing this bioeffect for therapeutic purposes. With respect to gene delivery, the concept is to promote such transient cellular and vascular permeability while simultaneously depositing a nucleic acid payload to the target tissue of interest. As microbubbles remain intravascular, successful gene delivery to the heart first requires interaction between them and endothelial cells. In this section, we briefly describe microbubble-induced endothelial permeabilization and outline some of the approaches towards ultrasound gene delivery.

2.6.1 Microbubble-mediated influence on endothelial cells

In one of the earliest mechanistic, real-time studies conducted in the context of microbubble-endothelial cell interactions¹³¹, it was observed that vibrating bubbles in contact with an endothelial cell result in plasma membrane deformation due to the local fluid motion (*i.e.* a pushing and pulling effect). It was later determined that bubble vibration amplitude, itself a function of intensity and other intrinsic factors, is a threshold indicator for localized, resealable endothelial membrane perforation and the loosening of intercellular junctions²¹⁰ (Fig. 2.2). The shear-stress induced by the vibrating microbubbles on the neighboring endothelial cells triggers calcium ion influx^{211,212} and local ATP release¹⁴², which is responsible for many downstream cellular and vascular processes including flow augmentation and vasoactivity²¹³. Even without direct endothelial cell permeability, the mechanical stress generated by microbubble oscillations has been shown to stretch the blood vessel wall²¹⁴, activate mechanosensitive ion channels and open up otherwise tight intra-endothelial junctional contact^{215,216} providing a paracellular entry route for gene delivery. Indeed, given the enhanced local permeability of the vasculature, one of the advantages of this approach is that it does not in principle require activation of endocytic pathways and thus payloads do not require release from early endosomes to be effective. However, there is mounting evidence that locally vibrating microbubbles do have an influence on endothelial endocytosis^{140,217}, which may play either a primary or an ancillary role in transcellular delivery routes.

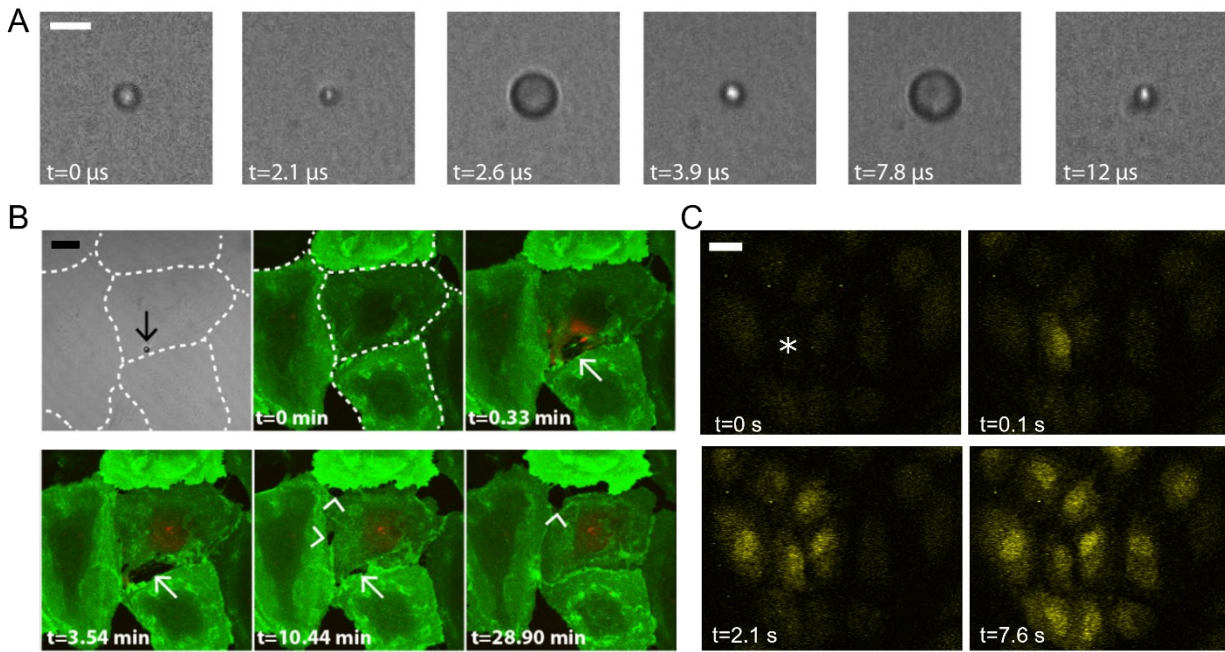


Figure 2.3: Ultrasound-enhancing agents vibrate in the vasculature and can give rise to local and reversible increases in vascular permeability and modulate intercellular signalling. A) Ultrafast-frame camera recording of a single gas-filled microbubble contrast agent oscillating under 1 MHz ultrasound (MI=0.8). This vibration generates local shear stress on neighboring tissue. Scale bar is 5 μm . B) An individual microbubble (black arrow) can reversibly perforate endothelial cell plasma membrane (green) to locally delivery a therapeutic (red). White-dotted lines demarcated each endothelial cell. The white arrow depicts the resealable perforation caused by the single microbubble and the white arrowheads denotes intercellular gaps between adjacent endothelial cells. MI=0.8. Scale bar is 25 μm . Modified from Helfield et al. PNAS 2016 with permission. C) Individual microbubble oscillation (white asterisk) causes a direct increase in intracellular Ca^{2+} (yellow) as well as intercellular Ca^{2+} signaling to neighboring, non-treated cells. Scale bar is 25 μm . Modified from Helfield et al. Ultrasound Med Biol. 2020 with permission.

There are many investigations confirming microbubble-mediated transient vascular permeability enhancement without evidence of vascular rupture²¹⁸. These studies, broadly speaking, are conducted using pulsed ultrasound (~ 0.1 -10 ms) and low duty cycles, resulting in restoration of native endothelial permeability on the order of 1-2 minutes²¹⁹, and vascular barrier function from minutes to hours²²⁰ – depending on the pulsing scheme and tissue type. However, it is important to note that large-magnitude microbubble vibration may cause local damage to neighboring endothelial cells and tissue. Excessive driving force (*e.g.* MI, burst length, duty cycle,

exposure duration) will result, all else equal, in large microbubble excursions and increased probably of violent bubble collapse. Prolonged perforation – whether it be due to very large perforations or those that do not reseal in a timely manner – have been shown to cause endothelial cell death^{221,222}. Indeed, aside from bubble vibration characteristics, there is a limited set of experimental data that highlights that perforation time-course is also a function of biological regulatory mechanisms (*e.g.* availability of calcium ions²²³, plasma membrane constituents²²²). On the vascular level, treatment with exceedingly large MI (~1.3-2.0) under certain acoustic schemes can cause vascular rupture and hemorrhage^{147,224,225}, although the specific damage thresholds will depend on a myriad of acoustic (*e.g.* pulse scheme, microbubble composition and concentration) and non-acoustic (*e.g.* vessel properties, tissue type) variables^{226,227}.

2.6.2 Microbubble gene carrier agents

With respect to the delivery approach, there are two main strategies. The first is via a co-injection of both microbubble agent and the molecular therapeutic. While this can be done with in-house synthesized agents, the microbubbles used here are similar to those commonly clinically used in medical imaging. In fact, clinical agent DefinityTM, OptisonTM and SonoVueTM have all been used for this purpose. Combining an FDA/Health Canada approved microbubble agent and an approved molecular therapeutic is likely advantageous for easier entry into clinical trials.

The second strategy is through the design of newly constructed gene-bearing agents. While many design types exist (see Lentacker et al.²²⁸ for a nice overview), the most common approach used in pre-clinical cardiac delivery work is through the development of a cationic lipid-encapsulated agent, whereby nucleic acid payloads are charge-coupled to the surface of the bubbles^{159,170}. In principle, this ensures that the microbubble activity and local nucleic acid concentration are spatially-temporally coherent – that is to say that the presence of the molecular therapeutic remains

in close proximity to the ultrasound induced permeability-enhancing effect - and that it protects the nucleic acid payload from degradation. However, this brings along new considerations as to whether the same ultrasound conditions that ‘shake’ off the drug from the bubble (which will be a function of how strongly bound the polyanion is to the cationic lipid) are the same conditions that result in safe, repeatable vascular and cellular permeability. Additionally, incorporation of the therapeutic within the bubble design can also limit the payload size.

2.6.3 Non-cardiac Implementation of Ultrasound-assisted Gene Delivery

Currently, the most advanced application of ultrasound-assisted gene therapy is within the context of delivery to the central nervous system (CNS)²²⁹. Over the past 12 years, MRI-guided focused ultrasound has demonstrated successful gene delivery in preclinical models (*e.g.* mice, rats, non-human primates)²³⁰. This progress is largely owed to the expansive research spanning decades into the localized and reversible opening of the blood-brain-barrier via focused ultrasound^{231–235}. Indeed, MRI-guided microbubble-mediated opening of the blood-brain-barrier is a rapidly expanding technology currently in clinical trials within the context of oncology (*e.g.* NCT03626896), Alzheimer’s (*e.g.* NCT03739905) and Parkinson’s disease (NCT03608553). In neuro-oncology patients, which is the most clinically advanced sub-area, promising pilot clinical studies have confirmed feasibility of this technique for the targeted delivery of chemotherapeutics (*e.g.* temozolomide²³⁶, carboplatin²³⁷) and immunotherapies (*e.g.* monoclonal antibodies²³⁸). Simultaneously to this, there is active research on utilizing the acoustic feedback of the microbubble vibrations to control the acoustic beam in order to localize the intended bioeffects and minimize unintended damage^{239–241}. Indeed, these treatments are performed with specifically designed transducer systems, either transcranial (*e.g.* ExAblate Neuro; Insightec, Haifa, Israel) or

implanted (*e.g.* SonoCloud, CarThera, Paris, France), to achieve the desired treatment and/or acoustic monitoring function²⁴².

Aside from this, there is ongoing preclinical research on ultrasound-assisted gene therapy to extracranial applications, which for the most part utilize single-element or commercial array transducers - including ocular disease²⁴³, cancer^{144,244} and diabetes²⁴⁵. A subset of this work employs separate, co-aligned transducers to provide some indication of microbubble activity throughout the treatment.

2.7 Applications of Ultrasound-assisted Gene Delivery to the Heart

Recent advances in identifying key molecular pathways and genes involved in the initiation and evolution of CVD have enabled the concept of targeted molecular therapeutics. Indeed, the genes that are selected as a target molecular therapeutic for CVD broadly fall into several categories¹⁶⁷, summarized in Table 2. There is a wide array of pre-clinical studies in animal models investigating CVD gene therapy, using a myriad of target genes highlighting successful and promising results. This has led to several clinical trials examining this technology towards translation to human patients, but despite their careful design, many of these trials show either neutral or modest benefit²⁴⁶. This highlights the need to examine the details of these approaches, from the initial gene selection, vector dosing, frequency of administration and the mode of delivery – all in relation to the target disease process and its stage. When considering the mode of delivery, the typically employed method of choice is viral vectors. Despite the fact that currently available vectors have demonstrated promising transduction to cardiac tissue, undesired side effects, including non-specific uptake in non-target tissue and the presence of neutralizing antibodies against the gene products may be limitations moving forward¹⁶⁷.

Table 2: Pillars of gene therapy targets for CVD.

Gene Therapy Pathway Targets for CVD	Example Genes
1. Promoting angiogenesis	VEGF-165 ²⁴⁷ , FGF-4 ²⁴⁸
2. Modifying CM Contractility	SERCA2a ²⁴⁹ , AC6 ²⁵⁰
3. Apoptosis Inhibition	AM ²⁵¹ , Bcl-2 ²⁵²
4. Superseding Cell Checkpoints to Promote CM Proliferation	Wnt11 ²⁵³
5. Increasing Cytoprotective Mechanisms	VEGF-B167 ²⁵⁴ , HO-1 ²⁵⁵
6. Exogenous cardiac stem cells or progenitors	SDF-1 ^{256,257}

CVD, cardiovascular disease; FGF-4, fibroblast growth factor-4; SERCA2a, sarcoplasmic/endoplasmic reticulum Ca²⁺ ATPase; AC6, adenylyl cyclase 6; AM, adrenomedullin; Bcl-2, B-cell lymphoma 2; HO-1, heme oxygenase-1; SDF-1, stromal-cell-derived factor 1.

In recent years, ultrasound-assisted gene therapy has been gaining traction as a non-invasive, non-viral tool to tackle some of the limitations described above. The ‘controllability’ of this approach, granted by spatial targeting of the ultrasound beam, along with its inherent image-guided nature, make it an attractive option for cardiac gene delivery. Indeed, the first application of this approach was demonstrated over two decades ago, in which healthy rodents were subjected to ultrasound in conjunction with albumin microbubbles linked to β –galactosidase and exhibited a myocardial expression over 10-fold more than untreated controls four-days post-treatment²⁵⁸. As an exhaustive summary of all ultrasound-assisted CVD gene therapies is out of the purview of this review, the following sections summarize applications in which ultrasound-assisted gene therapy targeted towards the heart (as opposed to peripheral tissue¹⁷⁰) has been demonstrated in diseased animal models (as opposed to pure proof-of-principle studies in healthy models²⁵⁹); the summary of which is presented in Table 3.

2.7.1 Myocardial Infarction

Acute myocardial infarction (MI) is one of the more severe manifestations of coronary heart disease, which is attributed to over 40% of all deaths due to CVD in the USA¹⁶⁶. Perhaps

one of the first applications of functional gene delivery to the heart in an acute MI model was demonstrated by Kondo and colleagues²⁶⁰ via the delivery of hepatocyte growth factor (HGF) in a rat model. HGF plays a role in preventing myocyte death due to oxidative stress and cardiomyocyte apoptosis during reperfusion. In fact, it has been demonstrated through viral approaches that gene delivery of HGF before MI attenuates reperfusion injury, and induces angiogenesis in infarcted myocardium²⁶¹. Starting 2 hours after coronary ligation, plasmid HGF (1.5 mg) was infused into the LV via catheter for 1.5 minutes while simultaneous clinical contrast agent Optison™ was injected in the femoral vein at 0.2ml/min for 5 min²⁶⁰. In the treatment groups, echocardiography was administered using a Philips Sonos 5500 system (S3 probe) operating in ultraharmonic mode at 1.3 MHz and an MI=1.8 for 4 minutes, triggered every 8th end-systole. A week post-surgery, HGF was quantified using immunohistochemistry, revealing homogenous expression in the treated groups compared to patchy or non-existent expression in HGF alone and negative control groups respectively. At three weeks post treatment, serial echocardiography measurements confirmed that LV mass was decreased, and histology data revealed reduced scar size, and capillary/arterial density was much improved in the treated groups compared to controls.

More recently, studies are beginning to explore the synergistic effects of gene therapy in conjunction with stem cell therapies. Wang and colleagues²⁶² explored using microbubble-assisted to simultaneously deliver two genes: SERCA2a, which regulates cardiac contraction and relaxation by controlling intracellular Ca²⁺ handling; and Cx43, well-known to mediate electrical coupling between cardiomyocytes via gap junctional intracellular communication. Dual-gene therapy was performed in a rat model of acute MI both with and without pre-transplantation of bone mesenchymal stem cells (BMSCs) 4 weeks post ligation surgery. Their motivation here was the hypothesis that BMSC transplantation would amplify the effect of gene transfection by

providing better preconditioning in the border zone. Four weeks after treatment, they observed about a factor of 2x increase in SERCA2a and Cx43 protein expression relative to non-treated controls in both the infarct and border zones, with no difference in remote cardiac tissue and no change in infarct size. Further, echo measurements confirm greater LVEF and LVFS compared to control groups, and that LV function was improved slightly in treated model rats that were preconditioned with BMSCs (~4-5% increase in LVEF and LVFS).

2.7.2 Ischemia/Reperfusion Injury (I/R)

Early work by Erikson and colleagues²⁶³ investigated the use of antisense oligodeoxyribonucleotides (ODNs) as a gene therapy strategy to attenuate expression of TNF- α in a rat model of I/R injury. Using a 1 MHz treatment protocol for 15 minutes, they compared their treatment approach using albumin-coated bubbles carrying the ODN in two different scenarios: 24 hours before I/R, and treatment during the first 15 minutes of reperfusion. With either a single treatment or a multiple treatment strategy (3 treatments daily for three consecutive days), the authors found an approximate 40% decrease in protein levels of TNF- α while observing no significant change in mRNA expression with treatment 24 hrs before I/R injury. With similar findings in each scenario (before or after I/R injury), the authors found a much stronger therapeutic effect with direct injection of ODN-bound bubbles within the LV, observing decreases on the order of 70-75% in this manner. Importantly, the decreased expression of TNF- α significantly inhibited other post-ischemic inflammatory mediators on both the protein and mRNA level, including ICAM-1 and IL-1 β .

More recently, robust work by Mofid et al.¹⁷¹ confirmed successful functional delivery of S100A6 plasmid in a rodent model of I/R, highlighting that cardiac overexpression of this gene leads to improved myocardial perfusion injury and LV function. Indeed, S100A6 is a key Ca²⁺-

binding protein that plays a role in many cellular processes, including cell proliferation, differentiation, cardiomyocyte contractility, hypertrophy and apoptosis. In this work, the authors charge-coupled either S100A6 plasmid or an empty plasmid (as a control) to cationic lipid microbubbles at a dose of 500 μg per 10^9 bubbles. With a 5 minute injection of the DNA-microbubble complexes via the jugular vein, high-powered ultrasound was delivered via a Sonos 5500 Philips system using an S12 transducer at a frequency of 5 MHz placed along the short axis of the LV. The probe was slowly moved from the base to the apex of the heart to increase dose deposition, and the total treatment duration was thirty minutes. Treatment was performed 48 hours before I/R injury, and the data highlights increased expression of S100A6 in the anterior LV of S100A6-treated rats up to 28 days after I/R induction, with an 8-fold increase in expression at day 1 that decreased over time. Additionally, LV ejection fraction and fractional area assessed through echocardiography progressively improved in treated rats compared to control up to day 28, with markedly smaller scar tissue regions (approximately 2-fold less).

2.7.3 Cardiomyopathies

Cardiomyopathy (CM) refers to a heterogenous group of diseases that cause abnormalities in the myocardium resulting in impaired cardiac activity²⁶⁴ – including dilated CM, hypertrophic CM, restrictive CM and CM caused as a side-effect of known cancer therapeutics (*e.g.* doxorubicin-induced CM). An early example of using ultrasound-mediated cardiac gene delivery for this application was shown in a study by Lee et al²⁶⁵, in which a survivin gene plasmid was delivered to a doxorubicin induced cardiomyopathic rodent model. Survivin is a gene recognized for its ability to inhibit apoptosis, and the motivation for its targeted delivery is that this would help prevent disease related cell death and thus LV systolic dysfunction. The study utilized a cationic microbubble formulation bound with a survivin plasmid to perform a single delivery three

weeks after beginning doxorubicin injection. Treatment was conducted using an S12 transducer from a Philips Sonos 5500 ultrasound system, positioned transversely over the heart at the mid-papillary level while slowly sweeping. A frequency of 5 MHz and 120 V power was utilized for 5 minutes with a pulsing interval of 10 cardiac cycles during infusion of the plasmid bound bubbles. This therapeutic gene was confirmed to successfully reach the cardiomyocytes and significantly increased expression 1 week post-delivery (4 weeks after beginning doxorubicin). The cardiovascular therapeutic benefit was clearly demonstrated with decreased apoptosis and fibrosis observed 1 week post-delivery, and improved cardiac function (LVEDV and LVFS) 3 weeks post-delivery.

In comparison, one of the more recent examples of this technique being used for cardiomyopathy is shown in a study by Qin and colleagues²⁶⁶. The gene of focus is Sirt3, which has been shown to have anti-oxidative activity and regulate hypertrophy. A Sirt3 plasmid bound to a cationic bubble formulation was employed and delivered to a pathological hypertrophy porcine model. Here, the treatment protocol takes advantage of the non-invasive nature of ultrasound mediated delivery to perform multiple treatments. This therapeutic gene is delivered three times with one day intervals, and treatment was done using a S5-1 transducer on a Philips EPIQ5 ultrasound system, with a MI of 1.2-1.35 triggered every four diastolic cardiac cycles for a 30-minute period throughout the infusion of the plasmid-microbubble solution. Repression of hypertrophic phenotypes was observed at 7 days and as long as 2 months after final treatment, including inhibition of disease related conditions such as cardiac enlargement, fibrosis, and apoptotic activity. This study also analysed organs such as kidneys, livers and lungs for any potential damages associated with the treatments. However, no impact was shown, thereby highlighting the spatial specificity and safety from off-target effects.

Though both studies look at different therapeutic genes and different forms of cardiomyopathy, they both provide robust evidence of the efficiency and capability of ultrasound mediated delivery to this class of disease. These examples highlight success with plasmids, but other therapeutics have been used with success, including miR and even small proteins (Table 3).

2.8 Outlook and Future Directions

2.8.1 Global Summary

The wide array of treatment parameters, in terms of ultrasound (*e.g.* system, sequences, duration), dosing (*e.g.* microbubble concentration and injection details, gene concentration), readouts (*e.g.* mRNA, protein, physiological) and disease model make comparisons between all studies (Table 3) a non-trivial matter. However, some general trends within the field of cardiac gene delivery using ultrasound can be proposed, as we have summarized in Figure 2.3. This figure attempts to quantify the effectiveness of gene delivery through the metric of ‘direct readout’, which is – when reported – a direct measure within the diseased heart of the genetic material delivered via ultrasound and microbubbles as compared to a control, untreated diseased cohort (*i.e.* the negative controls within the respective study), here demarked as a fold-change. Indirect measures of therapeutic efficiency, including physiological improvements of the diseased heart, and downstream cytokine or protein expression, are not possible to compare due to the distinct differences in model disease and heterogeneity/sparsity of their measurements. As shown in Fig. 2.3A, there is a general trend of increasing delivery efficiency in larger pre-clinical models, with a median fold-change in delivery of 1.7, 2.0, 3.8 and 4.5 in mice, rats, canines, and pigs, respectively ($p < 0.02$ between pigs versus rats, and $p < 0.02$ for pigs versus mice). Indeed, many of these studies use clinical systems with clinical frequencies, and thus the acoustic beam is more compatible with human-sized anatomy. This suggests that as this technique scales up to the clinical

area, it will only serve to strengthen the direct and targeted therapeutic delivery efficiency. There is also a slight increase in gene delivery efficiency reported in studies using in-house, usually lipid-based bubbles compared to commercially available, clinical agent (2.0 versus 1.33; $p < 0.02$) – Fig. 2.3B. Indeed, in the few studies that directly compared these two types of agent, gene-loaded in-house agents yielded slightly higher efficiencies²⁶⁷. There is however a strong correlation with transmit frequency, with studies using at or below 1 MHz ($f \leq 1$) yielding more effective cardiac gene delivery than those above ($f > 1$) – 2.7 versus 1.7 ($p = 0.008$) – Fig. 2.3C. While higher transmit frequencies would bring imaging resolution more in line with anatomical length scale in the smaller pre-clinical models, microbubble response (and thus gene delivery potential) does not scale in the same manner – owing to their nonlinear and resonance response^{203,268,269}. It is important here to note that, while not evident within this aggregate data summary, studies in which multiple, repeated treatments were performed do suggest prolonged gene expression as compared to single treatment paradigms²⁷⁰.

2.8.2 Molecularly Targeted Contrast Microbubbles

While conventional microbubbles are used clinically for routine contrast imaging, there is another class of contrast microbubbles that are designed to adhere to specific molecules or cells within the vascular system. Typically, these are synthesized via conjugation of disease-targeting ligands (*e.g.* antibodies, peptides) to the surface of the bubbles. The disease-specific retention of contrast bubbles – which ultimately provides a disease-specific contrast-enhanced echo - has many applications from assessing spatial/temporal patterns of expression and disease progression, to evaluating and optimizing new therapies, to early diagnosis of disease, and to monitor disease progression. Indeed, robust research using these molecularly targeted microbubbles in atherosclerosis and ischemia-based imaging pre-clinical applications have demonstrated selective

imaging of VCAM-1²⁷¹, ICAM-1^{272,273} and P-selectin^{274,275}. Recently, the first targeted microbubble agent selective for VEGFR has been translated into humans for cancer detection, the initial results of which were published in 2010²⁷⁶. With respect to cardiovascular applications, there is an ongoing trial (NCT03009266) investigating a commercially-available agent and its use towards selective imaging of ischemic memory²⁷⁷.

There is some pre-clinical evidence that molecularly-targeted microbubbles can further be made to carry a genetic payload and used to improve gene delivery as compared to more traditional, non-targeted agent. Within the context of CVD, Xie and colleagues¹⁴⁷ were perhaps one of the first to show that endothelial targeting (via P-selectin and ICAM-1) of gene-carrying microbubbles was feasible, and in fact increased the gene efficiency by 5-fold at a modest MI of 0.6 in a murine model of hindlimb ischemia compared to non-targeted control bubbles. Zhou et al.²⁷⁸ demonstrated a similar result, using ICAM-1 targeted microbubbles to deliver Ang1 in a rabbit model of myocardial infarction, which resulted in an approximate 3-fold increase in delivery efficiency compared to non-targeted bubbles using a Philips iE33 system in contrast mode at 1.7 MHz and an MI of 1.3.

2.8.3 Microbubble-assisted vascular gene delivery

While this review focuses on delivery to the heart, it is important to note that there has been exciting progress in ultrasound-assisted gene delivery for vascular therapy in peripheral tissues. In one of the earlier studies in this area, Takeuchi et al.²⁷⁹ investigated ultrasound-mediated potentiation of C-type natriuretic peptide (CNP) in a rat carotid artery balloon injury model to prevent neointimal hyperplasia. They employed a co-infusion approach of CNP with commercially-available OptisonTM agent, with 10 min. long treatments repeated 5 times at 3 min. intervals using a clinical probe at 1.8 MHz and an MI of 1.0. Their results indicated that ultrasound-

mediated delivery of CNP markedly reduced the intima/media ratio to 18% of that of the control rats which persisted for 28 days post-injury, and resulted in a drastic decrease in the dose requirement of CNP (10x decrease).

Direct gene delivery of an otherwise impermeable plasmid was first demonstrated in a rodent model of hindlimb ischemia by Leong-Poi and colleagues¹⁷⁰, through administration of a cationic microbubble formulation coupled to VEGF165 plasmid continuously infused for 10 minutes. Using a Philips Sonos 5500 system at 1.3 MHz, treatment was performed for 20 minutes while gradually sweeping the probe along the length of the proximal hindlimb muscles. In this work, the authors convincingly demonstrate through contrast ultrasound imaging and confocal microscopy that VEGF treated hindlimb exhibited increased muscle perfusion and vascular density at 2 weeks post treatment (4 weeks post surgery) with partial regression at 6 weeks. Further, they showed using GFP plasmid that gene delivery was localized within the vascular endothelium of both arterioles and capillaries, as well as evidence of delivery to adjacent cardiomyocytes. Since then, there have been a handful of other studies investigating ultrasound-assisted delivery of plasmids to peripheral tissues for vascular gene therapy, including VEGF^{179,280}, Ang-1²⁸¹, SDF-1²⁸², and microRNA¹⁷⁵.

2.8.4 Microbubble-mediated CVD gene modulation without gene delivery

As previously mentioned, it has long been observed that contrast agent microbubbles under specific acoustic conditions are able to permeate blood vessels²⁰⁹. It was hypothesized soon thereafter that this local blood vessel rupture might stimulate the growth and remodeling of neovessels via natural repair mechanisms^{283,284}, without the need to deliver an exogenous compound. In these studies, standard ultrasound-enhancing microbubbles were used in conjunction with a single-element transducer at 1 MHz. Treatment consisted of 0.1 s of ultrasound

every 5 seconds to either healthy or occluded rat skeletal muscle at an MI ranging from 0.5-0.75 for 1 minute. The results highlight that, compared to non-treated controls, stimulating the muscle with ultrasound and microbubbles resulted in increased lateral hyperemia blood flow and density of smooth muscle cell α -actin positive vessels up to 14 days post-treatment. Subsequent work investigating the molecular underpinnings of this approach using a mouse model of limb ischemia revealed increased levels of VEGF and recruitment of leukocytes (*e.g.* macrophages and T cells)²⁸⁵.

With the concept of microbubble-induced angiogenesis as a basis, more recent work has demonstrated this technique towards reversing cardiac dysfunction in an animal model of diabetic cardiomyopathy^{286,287}. Using clinical SonoVueTM agent a commercial scanner (VINNO 70, 4 MHz, 8-36 cycles, DC=60%, 20 min. duration) aimed directly at the heart, improved reparative neovascularization and increased cardiac perfusion was observed in a rat model of this disease²⁸⁶.

2.8.5 Perspectives

Targeted delivery of molecular therapeutics using ultrasound has tremendous potential for cardiac gene delivery. While met with exciting pre-clinical success, it is important to acknowledge its limitations. Firstly, the results summarized here demonstrate modest transfection efficiency, notably in comparison to viral methods. Given the non-invasive and localization of this method, this challenge can be mitigated by repeat injections, as well as the development of more efficient molecular therapeutics, *e.g.* in combination with CRISPR/Cas9 approaches²⁸⁸. Secondly, the mechanisms of this approach are not fully elucidated, particularly the way in which intravascular microbubbles deliver the payload to extravascular target tissue. To this end, there are many ongoing investigations exploring this concept in both cardiac and oncology contexts^{219,222,289}. Indeed, comparisons of gene quantification using this natively IV approach versus direct

intramyocardial injection has not been thoroughly addressed but would be a useful measure to appreciate the efficiency of ultrasound-assisted cardiac gene delivery. Further elucidation of this concept will drive modulation of the approach itself (*e.g.* pulse parameters, microbubble concentration) towards improving the delivery efficiency.

With a view towards advancing the technique of cardiac gene delivery using ultrasound there are numerous factors which need optimization. Firstly, the summarized data in Fig. 2.3b suggests that, generally speaking, incorporation of the gene construct with a cationic bubble agent result in increased transfection compared to co-injection. What is less clear, however, is the amount of gene dosing in these studies. Quantification of the mass of molecular therapeutic that remains bound to the microbubble solution post-synthesis (*i.e.* how efficient the loading process is) is not often reported. Further, synthesis of in-house cationic agent is sensitive to agent handling, and the resulting effects on microbubble formulation and size distribution play a large role in the efficiency of gene-loading and overall gene capacity. Indeed, even if the same dose of microbubble-bound therapeutic is administered, slight differences in bubble size distribution and *in-vivo* stability will change the available concentration of gene construct to be deposited. Herein lies the major advantage of using a co-injection strategy with clinical agent, whereby the dose of injected gene construct, along with the bubble agent characteristics and resulting acoustic physics are well documented and more reproducible.

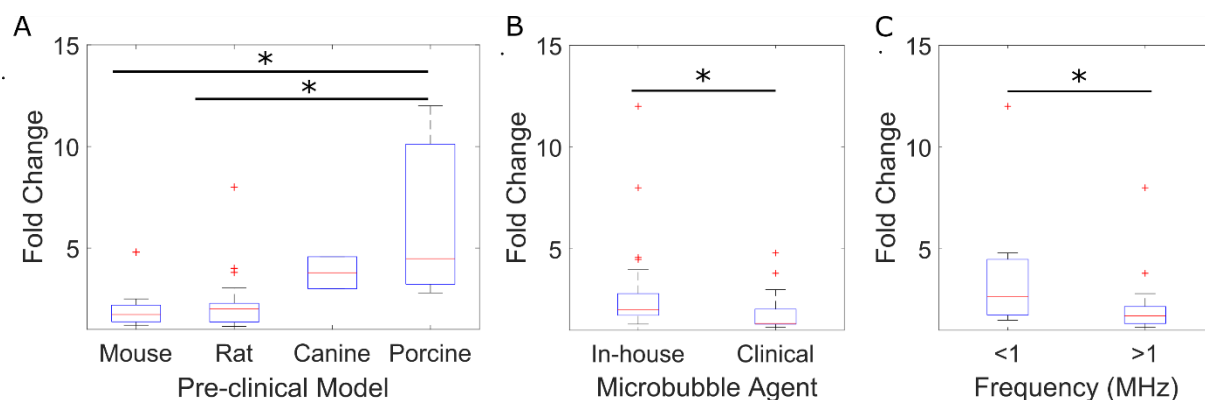


Figure 2.4: Aggregate data from the studies reported in Table 3 depicting a measurement of “direct readout”, which is – when reported – a relative measure of the delivered gene product within the diseased heart of ultrasound treated versus control groups, compared to different aspects of the treatment protocol. The central red line within each box marks the median, and the bottom and top edges mark the 25th and 75th percentile, respectively. The whiskers extend to the most extreme data points not considered outliers, while the red plus signs indicate data outliers. There is a notable trend of increasing cardiac gene delivery efficiency with A) increasing size of the pre-clinical model ($p<0.02$), B) when custom, in-house microbubbles were employed compared to clinical ones ($p<0.02$), and C) when the transmit frequency was kept at or below 1 MHz ($p=0.008$).

In terms of ultrasound transmit conditions, perhaps the most direct approach is the use of clinical scanners around 1-2 MHz. Here, image-guided delivery is possible via standard contrast-enhanced modes, and parameters for the disruption-replenishment techniques including acoustic power and number of burst frames can be modified. The real-time visualization of contrast perfusion helps confirm agent reproducibility, and treatment pulses can be triggered either manually or externally to allow microbubbles to replenish the target cardiac tissue. Given the imaging nature of these scanners, a drawback of this technique is the inability to modify the pulse length itself, which are fixed to diagnostic pulses. However, recent technical advancements have been employed to modify commercial transducer probes to incorporate longer duration pulses (up to 20 μ s)²⁹⁰. Further, a subset of the studies using clinical probes in Table 3 moved the probe position during therapy to ensure complete treatment of the heart. While this may not be necessary in applications in which the focal volume of the acoustic beam is size-matched to the region of the interest within the heart, it will likely be required for translation within the clinical arena.

2.8.6 Concluding Remarks

With the recent clinical successes of gene therapy, including seven FDA approved products and several hundred more in the final stages of the clinical approval pipeline, research into cardiac

gene therapy is on the rise¹⁶⁸. Given that the gene delivery approach plays a critical role in gene therapy efficiency, ultrasound-mediated gene delivery - which uses ultrasound-stimulated microbubbles - is an exciting and emerging methodology. Indeed, this technique is an inherently image-guided approach that can be performed using both clinical scanners and clinical agents – facilitating its translation into clinical practice – notably in diagnostic situations in which echocardiography is the standard of care. While there is still more fundamental work to be explored, including optimal therapeutic protocols (*e.g.* injection timing, relevant dose, high MI parameters), selection of molecular therapeutic, and perhaps the design of new therapeutic transducers or non-viral acoustically-sensitive vectors, the tremendous advancements that have been demonstrated in pre-clinical models to date reinforce the idea that translation of contrast echocardiography-mediated localized cardiac gene delivery is a feasible near-term objective.

Table 3: Summary of ultrasound-assisted gene delivery to the heart in pre-clinical CVD models

Author	GOI / Model / Targeted Pathwaya / Injection Route	US settings	Summary
Ischemic Heart Disease (MI)			
Wang et al. 2022 ²⁶²	Cx43 and SERCA2a/ Rat/#2/ tail vein (IV)	US system: Kunlun 7 (Mindray) Probe/Frequency: L11-3 U Pressure/Power: MI=1.0 Pulse length: Single flash frame Interval: Triggered manually every 3-5 s Total duration: 10 min. MB: Single treatment with in-house gene-carrying lipid agent (@4x10 ⁹ MB/ml); injection volume of 100 µl consisting of bubble-bound to Ad-encoding of either Cx43, SERCA2a or both (MOI of 500); infused via tail vein at 15 ml/h.	Direct readout: About a 2-fold increase in protein levels of SERCA2a and Cx43 using dual gene-carrying microbubbles at four weeks post treatment in the infarct zone; similar result for each protein using the single-gene loaded bubble. Other details: BMSCs were transplanted by myocardial injection 4 weeks post ligation surgery. Modest improvements in LVEF and LVFS in treated groups with BMSC pre-treatment, but no change in infarct size.
Cao et al. 2021 ¹⁷²	Ang1/Canine/#1/ elbow vein (IV)	US system: Single-element Probe/Frequency: 0.3 MHz Pressure/Power: 2W/cm ² Pulse length: 10s Interval: PRI of 10s Total duration: 20 min. MB: Single treatment 24 hrs post surgery with co-loaded SonoVue TM and Ang1 plasmid (0.25mg/ml) cocktail resulting in 1.5x10 ⁸ MB/ml; A 2 ml volume was infused at 12 ml/hr.	Direct readout: At 30 days post surgery, they showed a 3x increase in Ang1 compared to a negative control plasmid delivery. Other details: Compared to untreated controls, they observed an increase in microvascular density, decrease in sympathetic nerve density, and increase in LVEF and LVFS.
Sun et al. 2020 ²⁹¹	SDF-1/Rat/#6/ caudal vein (IV)	US system: Single-element Probe/Frequency: 1 MHz Pressure/Power: 2W/cm ² Pulse length: DC of 50% Interval: PRF of 1 kHz Total duration: 2 min. MB: One to three treatments (in 2 day intervals) of in-house lipid agent (4x10 ⁹ MB/ml) in a 0.5ml volume with 250 ug of plasmid	Direct readout: Compared to controls, authors also observed a ~2x increase in SDF-1 after a single US+MB treatment. Other details: Main results are increased BMSC survival, reduced myocardial apoptosis, reduced infarct size, increased vascular density, and improved cardiac function (LVEF and LVFS at 4 weeks post) compared

		DNA infused at 15 ml/hr. Post-treatment, infusion of shRNA-PHD2 modified BMSCs (1ml).	to the controls following a three treatment regime.
Zhang et al. 2017 ²⁹²	shRNA for PHD2/Rat/#1/ tail vein (IV)	US system: Sonitron 2000V (Nepa Gene) Probe/Frequency: 1 MHz Pressure/Power: 2W/cm ² Pulse length: Not specified. Interval: DC of 50% Total duration: 2 min. MB: Three treatments with in-house gene carrying lipid bubble (~4x10 ⁹ MB/ml) containing 600 µg/kg of shRNA 0.5ml sample was infused at 15 ml/hr.	Direct readout: At 4 days post treatment, they observed a 57% decrease in PHD2 mRNA levels and a 39% decrease in PHD2 protein levels compared to untreated controls. Other details: Treatments on day 0 (10 minutes after ligation), day 2 and day 4 with cationic microbubbles. LVFS and LVEF significantly increased at 4 weeks.
Deng et al. 2015 ²⁹³	Ang1/Rabbit/#1/ ear vein (IV)	US system: iE33 (Philips) Probe/Frequency: M3S/1.7 MHz Pressure/Power: MI of 1.3 Pulse length: Single flash frame Interval: Triggered every 4-8 cardiac cycles Total duration: 5 min. MB: Single treatment. Co-injection of either SonoVueTM or ICAM-1 decorated SonoVueTM and 100 µg of plasmid in a 1ml volume. Details on infusion speed/time were not provided.	Direct readout: Compared to single treatment with standard SonoVueTM, the targeted MB treatment resulted in approximately 1.7x increase in Ang-1 mRNA and 1.2x increase in Ang-1 protein expression. Pure negative controls expressed no human Ang-1. Other details: Compared to controls, a 1.6x increase in microvessel density, and LVEF, LVIDd, LVAWd all increased at 2 weeks.
Liu et al. 2015 ¹⁶¹	miR-21/Porcine/#5/ ear vein and LAD	US system: Not specified. Probe/Frequency: 1 MHz Pressure/Power: 1-3 W/cm ² Pulse length: Not specified. Interval: DC of 50% Total duration: 20 min. MB: Single treatment with in-house lipid bubble; co-injection with miR-21 (4 mg) in a volume of 6 ml (~1.9 x 10 ⁹ MB/ml). Details on infusion speed/time were not provided.	Direct readout: Approximately 12-15x higher levels of miR-21 using after 4 days in the heart, with the higher delivery efficiency observed via intracoronary injection versus IV. Other details: The authors assayed for PDCD4 protein expression, a downstream target of miR-21, and showed a significant decrease of PDCD4 (~40% decrease) compared to control.
Li et al. 2015 ²⁹⁴	MSCs/Rat/#6/ caudal vein (IV)	US system: Acuson S2000 (Siemens) Probe/Frequency: 2 MHz Pressure/Power: MI=1.3. Pulse length: Not specified.	Direct readout: Results show increase in the number of homing MSCs (1.3x increase) in the US treated group compared to non-treated controls.

		Interval: Not specified. Total duration: 10 min. MB: Single treatment. In-house lipid bubble (0.1ml/kg @ 7.5x10 ⁹ MB/ml) co-injection with MSCs. Details on infusion speed/time were not provided.	Other details: Higher protein expression of SDF-1 (1.9x) and CXCR4 (~1.5x) were observed in the ischemic myocardium treated vs control groups.
Sun et al. 2013 ²⁶⁷	AKT/Rat/#1/ tail vein (IV)	US system: Vivid 7 (GE) Probe/Frequency: M3S/1.6 MHz Pressure/Power: MI=1.3 Pulse length: Single flash frame Interval: ECG triggered every fourth end-systole Total duration: 20 min. MB: Single treatment with in-house gene carrying lipid bubble or DefinityTM containing 0.2 mg/kg of plasmid DNA. 0.4ml sample was infused at 1.2 ml/hr.	Direct readout: Three days post treatment, protein levels of AKT were increased using either DefinityTM (1.3x) or the in-house agent (1.7x) compared to untreated control. Other details: Other protein targets included p-AKT (~1.7x for DefinityTM; 2.5x for in-house), survivin (1.1x for DefinityTM; 1.3x for in-house) and p-BAD (2x for DefinityTM; 3x for in-house). Treatment with the in-house agent lowered infarct size, increased infarct thickness, reduced apoptosis, increased vascular density (after 3 days), and improved cardiac function/perfusion (21 days) compared to using DefinityTM and to non-treated controls.
Zhou et al. 2013 ²⁹⁵	HGF/Rat/#1/ femoral vein (IV)	US system: CGZZ ultrasonic gene transfection instrument Probe/Frequency: 0.3 MHz Pressure/Power: 2 Pulse length: 10s CW Interval: PRI of 10s Total duration: 5 min. MB: Single treatment with in-house cationic gene carrying bubbles with 1 mg of plasmid DNA and/or TAT peptide. 1ml sample was infused at 12 ml/hr.	Direct readout: Single treatment on day 3 post-ligation. RT-qPCR data indicates an approximately 4-fold increase in HGF mRNA compared to untreated controls. Other details: A synergistic effect was found when using MBs contained both TAT peptide and HGF, resulting in a 6.5-fold increase in HGF expression compared to untreated controls (~1.5x more than US+MB without TAT). All analysis was performed on day 10 post-ligation (7 days post treatment). MVD was found to modestly increase with gene-carrying MBs compared to US+MBs alone, but represents an approximate factor of 2-2.5-fold increase over negative controls.

Ling et al. 2013 ²⁹⁶	MSCs/Canine/#6/femoral vein (IV)	US system: UTG 1025 Probe/Frequency: 1 MHz Pressure/Power: 0.5-2 W/cm ² Pulse length: 5s (PRF of 1 kHz) Interval: DC of 50% Total duration: 10 min. MB: Three treatments with 2ml of in-house lipid MB (2x10 ⁸ MB/ml) on day 1, 4 and 7 post-MI with or without MSCs. Details on infusion speed/time were not provided.	Direct readout: None reported. Other details: In study 1, results indicate increased levels of SDF-1, VCAM-1 and VEGF expression at highest power. In study 2, canine MSCs were injected post US treatment and reveals improved myocardial perfusion (assessed via SPECT) and heart function (LVEF, LVIDs and LVFS) at 4 weeks post-treatment.
Yuan et al. 2012 ²⁹⁷	HGF/Canine/#1/direct LV injection	US system: UTG 1025 Probe/Frequency: 1 MHz Pressure/Power: 1 W/cm ² Pulse length: 30s (CW) Interval: DC of 75% Total duration: 1 min. MB: Single treatment with in-house lipid bubble (0.1 ml) co-injected with 100 µg of plasmid DNA intramyocardially delivered in 5 locations (total of 0.5 ml of MB and 500 µg of DNA).	Direct readout: None reported. Other details: Single treatment 5 min. post ligation. After 28 days, authors observed decreased LV mass, decreased infarct size, and increased MVD. They also observed a 1.8x-fold increase in VEGF levels compared to HGF+US group (no MBs).
Zhong et al. 2012 ²⁹⁸	MSCs/Canine/#6/femoral vein (IV)	US system: UTG 1025 Probe/Frequency: 1 MHz Pressure/Power: 0.5-2 W/cm ² Pulse length: not specified. Interval: DC of 50% Total duration: 10 min. MB: Three treatments with in-house lipid bubbles (2ml volume at 2x10 ⁸ MBs/ml) on day 7, 9 and 11 post-MI. On day 14, MSCs were injected (5ml at 1.35x10 ⁶ cells/ml). Details on infusion speed/time were not provided.	Direct readout: The number of MSCs increased by 4.57-fold compared to untreated controls, as measured via immunohistochemistry. Other details: Following a single treatment of only US+MBs, mRNA levels of VEGF, SDF-1, VCAM-1 and IL-1b were increased in the infarcted myocardium by a factor of 4x, 5x, 4x and 5x respectively versus controls.
Fujii et al. 2011 ²⁷⁰	SCF, SDF-1α/Rat/#6/tail vein (IV)	US system: Sequoia (Siemens) Probe/Frequency: 15L8 Pressure/Power: MI=1.6 Pulse length: single frame Interval: every 1.8-2 s in contrast mode Total duration: 20 min. MB: Starting 7 days post-ligation, up to 6 treatments with DefinityTM co-incubated with 0.6	Direct readout: Levels of SCF protein at 4 weeks post-treatment increased statistically by an approximate factor of 1.7 after 6 treatments compared to untreated control, a steady improvement over a single treatment (~1.1x) and three treatments (~1.4x). Similar results were found for SDF-1α.

		mg/kg of plasmid DNA infused at 1.5 ml/hr. Heart was scanned from base to apex (3 min. cycle)	Other details: On day 24, stem cell homing (as measured via c-kit and CXCR4), and myofibroblast recruitment increased while infarct size decreased with more treatment repeats.
Xu et al. 2010 ²⁹⁹	MSCs/Rabbit/#6/auricular vein (IV)	US system: Vivid 7 (GE) Probe/Frequency: 1.7 MHz Pressure/Power: MI=1.3 Pulse length: not specified. Interval: every 2 s Total duration: 10 min. MB: At 7 days post-MI, single treatment with in-house lipid bubble (0.1ml/kg) with or without MSCs. Details on infusion speed/time were not provided.	Direct readout: The number of MSCs increased by 1.45-fold compared to untreated controls, as measured via immunohistochemistry. Other details: Treatment increased MVD (via contrast echocardiography and staining), increased level of VEGF in ischemic myocardium, V-CAM1 expression and improved heart function (LVEF, LVFS) at 4 weeks post-treatment.
Fujii et al. 2009 ³⁰⁰	SCF, VEGF/Mouse/#6, #1/tail vein (IV)	US system: Sequoia (Siemens) Probe/Frequency: 15L8; 8 MHz Pressure/Power: MI=1.6 Pulse length: single frame Interval: every 0.5 s in contrast mode Total duration: 20 min. MB: Single treatment 7 days post-MI of DefinityTM incubated with 0.6 mg/kg of plasmid DNA and infusion over 1 minute. Heart was scanned from base to apex (3 min. cycle)	Direct readout: Measured 14 days post treatment, VEGF and SCF protein within the whole heart were approximately 1.2 and 1.3-fold greater than untreated control. Other details: Five days after treatment, progenitor recruitment was found to be higher in both treatment groups as compared to control, and MVD was also increased compared to controls on day 14.
Kondo et al. 2004 ²⁶⁰	HGF Plasmid/Rat/#1/femoral vein (bubbles), direct LV injection (plasmid)	US system: Sonos 5500 (Philips) Probe/Frequency: S3 / 1.3 MHz Pressure/Power: MI=1.8 Pulse length: 3 frames Interval: Triggered every 8th end-systole Total duration: 4 min. MB: Single injection of OptisonTM at 12ml/hr over 5 min combined with LV-injection of naked plasmid (1.5 mg)	Direct readout: HGF levels were approximately 3-fold higher in the US+MB group versus the HGF only group as measured via immunohistology. Other details: At three weeks, both LV mass infarct size decreased, while capillary and arterial density increased in treated groups compared to HGF injections alone.
Ischemic Heart Disease (I/R injury)			
Erikson et al. 2017 ³⁰¹	TRAF3IP2, p65, JNK1 Mouse/#3/	US system: Sonicator 730 (Mettler Electronics) Probe/Frequency: 1 MHz Pressure/Power: 0.6 W/cm2	Direct readout: Protein levels in the heart of treated mice were significantly lower for all three tested MBs; resulting in approximately a

	jugular vein (IV)	Pulse length: Not specified Interval: Not specified Total duration: 15 min. MB: Albumin-based MBs bound to AS-ODN (100 µg) were arterially infused 'slowly' over 10 min. Three agents were tested, each binding to either TRAF3IP2, p65, or JNK1 oligo.	1.75x, 1.5x and 1.5x decrease in TRAF3IP2, p65, and JNK1 using their respectively targeted bubbles. Other details: AS-ODN targeting TRAF3IP2, p65, or JNK1 all attenuated I/R-induced myocardial injury (infarct size) at 24 hrs post delivery.
Mofid et al. 2017 ¹⁷¹	S100A6/Rat/#2/ jugular vein (IV)	US system: Sonos 5500 Probe/Frequency: S12/ 5 MHz Pressure/Power: 120V Pulse length: single frame Interval: every 10 cardiac cycles Total duration: 30 min. MB: Single pre-treatment with in-house cationic MBs (1x10⁹ MBs) coupled to S100A6 plasmid (500 µg) 2 days prior to I/R infused over 5 min. Infusion speed not provided. Heart was scanned from base to apex.	Direct readout: The authors demonstrate an approximate 8-fold increase in S100A6 within the anterior LV compared to normal myocardium on day 1 post I/R (3 days post treatment). Other details: Increased expression of S100A6 up to 28 days post cardiac I/R, reduced infarct size and improved left ventricular systolic function, with less myocyte apoptosis, attenuated cardiac hypertrophy, and less cardiac fibrosis compared to untreated controls.
Du et al. 2017 ³⁰²	GDF11/Mouse/#6/ tail vein (IV)	US system: Vivid 7 (GE Healthcare) Probe/Frequency: M3S/1.6 MHz Pressure/Power: MI=1.3 Pulse length: single frame Interval: ECG triggered every 4th cardiac cycle Total duration: 20 min. MB: Two treatments (day 3 and 6 post I/R) of in-house gene-carrying cationic lipid bubbles infused at a rate of 1.2ml/hr	Direct readout: The mRNA levels of GDF11 were about 1.3-fold higher in treated versus untreated hearts, as measured 9 days post I/R (three days post treatment). Other details: Treatment effective in old mice (not young mice), whereby it improved cardiac function (LVFS), reduced infarct size, decreased senescence markers (p16, p53), and increased proliferation of cardiac stem cell antigen (Sca-1+) cells and homing of endothelial progenitor cells and angiogenesis.
Kwekkeboom et al. 2016 ¹⁶⁰	Antagomir /Mouse/ tail vein (IV)	US system: Sonos 5500 (Philips) Probe/Frequency: S3 and S12, 1.5, 6 and 7 MHz Pressure/Power: MI=1.4-1.8, B-mode and Power Doppler mode Pulse length: 1 s Interval: every 5 s Total duration: 15 min.	Direct readout: The authors found an increase in antagomir ranging from 2 to 2.5-fold in US treated diseased heart as compared to untreated controls, as measured via fluorescence microscopy. Other details: While they show interesting improvement in healthy rodent hearts, no significant

		MB: Single treatment with cationic in-house lipid bubbles (2 x 10 ⁹ MB/ml) conjugated to 0.75mg/kg of antagomir in 0.1 ml volume. Details on infusion speed/time were not provided.	differences in functional delivery for I/R hearts. Delivery of antagomir was maximal when using the treatment paradigm with the highest MI and more frequent pulses (Doppler mode with S12 probe).
Yan et al. 2014 ³⁰³	Timp3/Rat/#5/ tail vein (IV)	US system: Vivid 7 (GE) Probe/Frequency: M3S/1.6 MHz Pressure/Power: MI=1.3 Pulse length: single frame Interval: ECG trigger interval of four cardiac cycles Total duration: 20 min. MB: Single treatment of an in-house, targeted lipid agent decorated with MMP2 antibody co-injected (0.4 ml) with naked Timp3 plasmid (0.2 mg/kg) and infused at 1.2ml/hr for 20 min.	Direct readout: Anti-MMP2 microbubble treated groups resulted in an approximate 2.1x increase in TIMP3 protein expression within the scar/border zone at 3 days post treatment as compared to untreated controls. Other details: Non-targeted US treatment still exhibited an increase in TIMP3 protein at 3 days post-treatment (~1.6x) All increases in protein levels were no longer present by 3 weeks post treatment. MMP2 and MMP9 activity were reduced, resulting in smaller and thicker infarcts and improved cardiac function (LVEF, LVIDs, LVIDd).
Erikson et al. 2003 ²⁶³	Antisense TNF- α oligo /Rat/#2/ jugular vein (IV)	US system: Sonicator 730 (Mettler Electronics) Probe/Frequency: 1 MHz Pressure/Power: 0.6 W/cm ² Pulse length: Not specified. Interval: Not specified. Total duration: 15 min. MB: Either single or triple treatment through IV or direct LV injection of in-house albumin-based gene carrying bubbles (100 μ g) infused at 4ml/hr in a total volume of 1 ml.	Direct readout: There was no significant difference in TNF- α mRNA levels between positive controls and single or triple treated rats when performed via venous administration, but a decrease of ~39% (i.e. ~1.6-fold decrease) in protein level was observed. Other details: The authors also tested direct injection within the LV, with the US-treated groups showing no difference in mRNA but a slightly more attenuated level of TNF- α protein expression (~56.6% decrease). Timing experiments showed that treatment just before I/R was more efficient than just after I/R, as well as lower IL-1 β and ICAM-1 levels.
Cardiomyopathy (CM)			
Qin et. al 2023 ²⁶⁶	Sirt3/Porcine/#5/ ear vein (IV)	US system: EPIQ 5 (Philips) Probe/Frequency: S5-1 Pressure/Power: MI=1.2-1.35	Direct readout: Seven days after treatment, human Sirt3 protein levels

		Pulse length: single frame Interval: triggered every 4 cardiac cycles Total duration: 30 min. MB: Three treatments using an in-house gene-carrying lipid bubble (no MB concentration given,) containing 6-7 mg of plasmid diluted into a 50 ml sample and infused via ear vein at 100 ml/hr.	were 2.8x higher than in untreated controls. Other details: Short term assessment performed seven days post-treatment demonstrated decrease levels of ROS. Long term benefits were determined two months after treatment preventing LV thickness and mass increase, as well as decrease in pressure overload associated apoptosis and fibrosis. Protein and mRNA levels of ANP, BNP, and α-SMA were decreased compared to controls two months post-surgery.
Gao et al. 2021 ³⁰⁴	FGF21/Mouse/#5/ tail vein (IV)	US system: Single element Probe/Frequency: 0.5 MHz Pressure/Power: 2W Pulse length: Not specified. Interval: DC of 50% Total duration: 5 min. MB: With a regime of two times a week for 8-weeks, in-house gene-carrying polymer bubbles (no MB concentration given) were loaded with 100 μg/kg of FGF21. Details on infusion speed/time were not provided.	Direct readout: None reported. Other details: After the 8-week treatment period, the US-treated groups demonstrated a decrease in hypertrophic phenotypes, fibrosis, and apoptosis. Further, an increase in cardiac function (LVEF, LVFS and E/A ratio) was observed.
Zheng et al. 2020 ³⁰⁵	FGF1/Rat/#1/ tail vein (IV)	US system: Acuson Sequoia 512C (Siemens) Probe/Frequency: 15L8-w/12-14 MHz Pressure/Power: MI=1.9 Pulse length: single frame Interval: Not specified. Total duration: Not specified. MB: Twice a week for 12 weeks, injections consist of a co-administration of SonoVueTM (2x108 MB/ml) with nanoliposomes bound to FGF1 (15 μg/kg). Details on infusion speed/time were not provided.	Direct readout: None reported. Other details: US was applied until bubbles were no longer visible in the LV. After treatment, assessment of cardiac function was performed demonstrating an improved cardiac function (LVEF and LVFS) compared to non-US-treated controls. Further, increase in myocardial capillary density was observed and an improvement in the regional myocardial blood flow, the hemodynamics (LVESP and LVEDP) and the myocardial ultrastructure (using SEM).
Kopechek et al. 2019 ¹⁵⁹	AntimiR-23a/Mouse/#2/	US system: Sonos 7500 (Philips) Probe/Frequency: S3/1.3 MHz Pressure/Power: MI=1.0 Pulse length: 4 frames	Direct readout: After the two-week protocol, miR-23a levels were found to have decreased by 43% (i.e. a 1.73-

	jugular vein (IV)	Interval: 1s Total duration: 20 min. MB: Three treatments consisting of in-house, gene-carrying lipid bubble; 100 µl infusion (@2x10 ⁹ MB/ml) containing 35 pmol of anti-miR at 0.4ml/hr for 15 min. via the jugular vein.	fold decrease) compared to untreated, disease model controls. Other details: CM was induced with phenylephrine, which was delivered daily starting at day 0. Treatments were delivered on days 0, 3 and 7. Treated hearts demonstrated no disease related increase in cardiac mass on day 7. As well, LVFS was shown to be preserved on day 14. Isolated hearts on day 14 demonstrated a significant decrease in miR-23 compared to non treated diseased hearts.
Cardiomyopathy (DOX-induced, Adriamycin-induced)			
Sun et al. 2020 ¹⁶²	miR-21/Mouse/#5/ tail vein (IV)	US system: Not specified. Probe/Frequency: 0.66 MHz Pressure/Power: MI=1.6 Pulse length: Not specified. Interval: DC of 50% Total duration: 1 min. MB: Weekly treatments spanning 4 weeks of a co-injection of SonoVueTM (2x10 ⁸ MB/ml) with exosome-encapsulated miR-21 (100 µg; achieved through electroporation) in 0.2ml. Details on infusion speed/time were not provided.	Direct readout: The authors observed an approximate 4.8-fold increase in miR-21 within the heart tissue of treated mice (US+MB and miR-21 loaded exosomes) as compared to untreated controls. Other details: Assessment of cardiac function performed 28 days post-delivery, demonstrating improved cardiac function (LVEF and E/A ratio) compared to non-treated controls.
Tian et al. 2017 ³⁰⁶	MaFGF/R at/#5/ tail vein (IV)	US system: Acuson Sequoia 512C (Siemens) Probe/Frequency: 15L8-w/12-14 MHz Pressure/Power: Not specified. Pulse length: 10s Interval: 1s off-interval. Total duration: 33s MB: Co-injection of SonoVueTM (2x10 ⁸ MB/ml) with in-house gene-loaded nanoliposomes (3µg/kg of MaFGF) in 1ml via the caudal vein over 6 weeks with two treatments weekly. Details on infusion speed/time were not provided.	Direct readout: None reported. Other details: Hearts were treated until bubbles were no longer visible. Prevention of diseased related cardiac physiology (HW/BW) and dysfunction (LVEF, LVFS, LVIDd, LVIDs) was observed post-final treatment. Disease-associated oxidative stress, fibrosis and apoptosis were shown to be attenuated in treated samples. An increased expression of anti-apoptotic proteins (pAkt and Bcl-2), as well as the decreased expression in pro-apoptotic protein (Bax) were also observed.

Chen et al. 2015 ¹⁷⁷	GLP-1/Rat/#5/ not specified (IV)	US system: Sonos 5500 (Philips) Probe/Frequency: S3/1.3 MHz Pressure/Power: MI=1.4-1.5 Pulse length: 4 frames Interval: ECG triggered every 4 cardiac cycles Total duration: Not specified. MB: Treatment regime consisted of a 2-week window with three treatments per week using an in-house gene-carrying lipid bubble; each injection consisted of 50 nmol/kg of plasmid. Details on infusion speed/time were not provided.	Direct readout: Levels of GLP-1 mRNA exhibit an 84-fold increase in US treated hearts versus control rat hearts (these are healthy hearts that did not express any GLP-1). Other details: A secondary experiment delivered GLP-1 and treated 2 weeks after adriamycin-induced CM to determine recovery potential. A decrease of CM-associated phenotypes (LV mass and wall thickness) and improved cardiac function (LVFS) were observed in both treatments. As well, regeneration and proliferation of adult cardiomyocytes were evident in treated hearts.
Lee et al. 2014 ²⁶⁵	Survivin/Rat/#3/ not specified (IV)	US system: Sonos 5500 (Philips) Probe/Frequency: S12/ 5 MHz Pressure/Power: 120V Pulse length: single frame Interval: every 10 cardiac cycles Total duration: 5 min. MB: Single treatment with in-house, gene-carrying lipid bubbles via a 1 ml volume (109 MBs with 500 µg DNA) infused over 5 min. Heart was scanned from base to apex.	Direct readout: At 7 days post treatment, survivin expression increased by approximately 2.8-fold in treated versus untreated hearts. Other details: Increased expression of survivin lasted for up to 3 weeks post-delivery. Improved cardiac function (LVFS and LVEDV) and decrease in fibrosis was demonstrated 3 weeks post-delivery compared to non-treated hearts. As well, a decrease in disease-associated apoptosis was observed 1 week post-delivery in survivin treated hearts.
Heart Transplant Rejection			
Wang et al. 2020 ¹⁷⁴	galectin-7/Rat/#5/ tail vein (IV)	US system: Not specified Probe/Frequency: 1 MHz Pressure/Power: 0.8 MPa / 2 W/cm² Pulse length: Not specified. Interval: DC of 50% Total duration: 2 min. MB: Four treatments of in-house, gene-carrying lipid bubbles of 200 µl (0.39 nmol of siRNA per MB; no MB concentration given). Treatment performed 30s after injection. Details on infusion speed/time were not provided.	Direct readout: Three days post-treatment regime, galectin-7 levels were about a factor of 2 lower in treated hearts compared to controls, as measured both by immunofluorescence and rt-qPCR. Other details: No significant reduction in galectin-7 levels was found in non-heart issue. A decrease in apoptotic cells and immune cell penetration, indicating prevention of rejection, was also observed.

Yi et al. 2020 ¹⁷⁶	antimiR-155/Mouse /#5/not specified (IV)	US system: Sonitron 2000 (Nepa Gene) Probe/Frequency: 2 MHz Pressure/Power: 2W/cm2 Pulse length: Not specified. Interval: DC=50% Total duration: 2 min. MB: Three treatments (day 1, 3 and 5 after transplant) consisting of in-house, gene-carrying lipid bubbles. Volume administered was 150 µl (@ 2x10 ⁹ MB/ml) with 0.8nmol of antimiR. Details on infusion speed/time were not provided.	Direct readout: On the day 7 post transplant, mRNA levels of miR-155 in treated hearts dropped to 40% of native levels (i.e. a 2.5-fold decrease) as compared to untreated controls. Other details: anitmiR-155 delivered with US also ensured a longer survival of allograft heart compared to non-US controls. Treated hearts also demonstrated reduced expression of inflammatory cytokines (<i>e.g.</i> IL-6) up to 7 days after heart transplant.
Fibrosis			
Feroze et al. ¹⁵⁸	miR-29b mimic/Mouse/jugular vein (IV)	US system: Sonos 7500 (Philips) Probe/Frequency: S3/1.3 MHz Pressure/Power: MI=1.0 Pulse length: 4 frames Interval: 1s Total duration: 20 min. MB: Three treatments of in-house, gene-carrying lipid bubble (4 × 10 ⁸ MBs/mL) consisting of 3.5 × 10–8 µg/MB of miR at 1.2ml/h for 15 min.	Direct readout: One day after a single treatment, there was a 2.26-fold increase of miR-29b expression within the heart in the treatment cohort versus sham-treated controls. Other details: After three treatments, fibrotic transcripts and α-SMA levels were lower in the treated versus sham groups. Heart physiology was also improved in the treatment groups. Echo data confirming a decrease in LVED volume and an increase in fractional shortening and ejection fraction were confirmed on day 10.
Li et al. 2023 ³⁰⁷	Gal-3/Rat/#4/not specified	US system: Sonitron 2000 (Nepa Gene) Probe/Frequency: 1 MHz Pressure/Power: 2 W/cm2 Pulse length: Not specified. Interval: DC of 50% Total duration: 20 min. MB: Three treatments (day 1, 3 and 7 post-operation) using an in-house, gene-carrying lipid bubble of a volume of 500 µl consisting of 120 µg of Gal-3 shRNA and	Direct readout: The authors observed a 3x and 5x decrease in native Gal-3 mRNA levels at 7 and 21 days post MI, respectively. Other details: Gal-3 protein levels did not change by day 7, and were significantly lower by day 21. LVEF, LVFS, LVIDd and LVIDs were elevated by 21 days compared to untreated controls, as measured by echo.

		infused over 20 min. Details on infusion speed were not provided.	
Coronary Microembolization (CME)			
Su et al. 2015 ³⁰⁸	miR-21/Pig/#5/marginal ear vein (IV)	US system: Not specified. Probe/Frequency: 1 MHz Pressure/Power: 2 W/cm2 Pulse length: 10s Interval: DC of 50% Total duration: 20 min. MB: Single treatment with in-house, gene-carrying lipid bubble at a volume of 6 ml infusion consisting of 2mgml-1 of plasmid. Details on infusion speed/time were not provided.	Direct readout: There was a 4.48-fold increase in miR-21 within the heart of treated pigs versus control. Other details: Nine hours post-CME introduction improved cardiac function (LVEF, LVIDd, LVFS and CO) was shown compared to CME group. As well, a lower expression of gene associated with CME induced inflammation (PDCD4/NF-κB/TNF-α)

a: Targeted pathways as defined in Table 2. GOI, gene of interest; CX43, connexin-43; PRI, pulse repetition interval; PRF, pulse repetition frequency; DC, duty cycle; PHD2, prolyl hydroxylase-2; MB, microbubble; TAT, transactivating transcriptional activator; HGF, hepatocyte growth factor; MVD, microvascular density; MSC, mesenchymal stem cells; SCF, stem cell factor; TRAF3IP2, TRAF interaction protein 2; TIMP3, TIMP metalloproteinase inhibitor 3; TNF-α, tissue necrosis factor; Sirt3, sirtuin-3; MaFGF, Mutant acidic fibroblast growth factor; Gal-3, galectin-3; ROS, reactive oxygen species; E/A, mitral ratio of peak early to late diastolic filling velocity; LVEF, LV ejection fraction; LVFS, fractional shortening; LVESP, LV end-systolic pressure; LVEDP, LV end-diastolic pressure; SEM, scanning electron microscopy; HW, heart weight; BW, body weight; LVIDd, left ventricular internal dimension-diastole; LVIDs, left ventricular internal dimension-systole; LVEDV, LV end-diastolic volume; LVAWd, LV anterior wall thickness in diastole; CO, cardiac output;

Chapter 3: Image-guided local delivery of microRNA-1 via a single treatment of microbubble-mediated clinical ultrasound ameliorates hypertrophic heart disease in a sex-dependent manner

Preprint manuscript (submitted for publication): Singh, D., He, S., Hazel, K., McManus, M., Bergdahl, A., & Helfield, B. (2025). Image-guided local delivery of microRNA-1 via a single treatment of microbubble-mediated clinical ultrasound ameliorates hypertrophic heart disease in a sex-dependent manner.

3.1 Foreword:

Pathological left-ventricle hypertrophy remains a major global challenge in the field of cardiology. The application of miR-1 replacement therapy has emerged as a promising therapeutic strategy; however, the major challenge involves delivering the gene therapeutic in a non-invasive and efficient manner. In this chapter, we demonstrated the feasibility of ultrasound and microbubble-mediated as a targeted delivery platform miR-1 to hypertrophic cardiomyocyte *in-vitro* and *in-vivo*.

3.2 Abstract

3.2.1 Background and Aims:

Heart failure is typically preceded by maladaptive cardiac hypertrophy, an independent indicator of adverse cardiovascular outcome. MicroRNA replacement, particularly miR-1, is an exciting new molecular therapy approach directly targeting the hypertrophic phenotype, yet lacks an effective *in-vivo* delivery platform. This study investigates the use of clinical contrast ultrasound as an image-guided, non-viral, and highly localized miR-1 delivery technique in *in-vivo* models of hypertrophy.

3.2.2 Methods:

Both male and female pathological LV hypertrophy rat models were treated using clinical ultrasound, contrast agent microbubbles, and miR-1 mimic. Real-time quantitative PCR, immunoblotting, echocardiography, histology, flow cytometry, multiplex immunoassays, high-resolution respirometry, and treadmill assays were utilized to assess molecular, functional, metabolic and physiological changes.

3.2.3 Results:

Ultrasound-mediated miR-1 delivery to isolated rat cardiomyocytes completely reversed the hypertrophic phenotype, rescuing miR-1 levels, attenuating pro-hypertrophic genes, and restored cell size. Rats treated with a single dose confirmed the *in-vitro* results, with delivery localized entirely to the LV. Treatment in disease models showed complete miR-1 rescue, heart weight and cardiomyocyte size recovery, improvements in LV function, partial restoration of serum levels of over 20 analytes and mitochondrial respiratory function. Sex-stratified analysis highlights a stronger therapeutic efficiency in male rats, with more heterogeneity in the female group.

3.2.4 Conclusions:

This study demonstrated that localized and physiologically relevant amounts of miR-1 can be delivered to the diseased heart using only a single treatment based on clinical contrast echocardiography techniques. This platform mitigated many of the pathophysiological characteristics of cardiac hypertrophy, with a more pronounced effect in the male cohort.

3.2.5 Translational Perspective:

Maladaptive cardiac hypertrophy is independently associated with an increased risk of adverse cardiovascular outcome. Here, we demonstrate the utility of adapting clinical echocardiography as a platform for real-time image-guided targeted miR-1 replacement, localized entirely within the heart. This is the first study to demonstrate in a sex-dependent manner that with only a single treatment, such a technique restores aberrant protein expression, cardiomyocyte and heart size, while significantly mitigating many functional, metabolic and physiological changes brought forth by the disease. This study lays the foundation for a clinical contrast ultrasound dual imaging and cardiac therapy platform towards a first-in-human clinical trial.

3.3 Introduction

MicroRNAs (miRs) are short-noncoding nucleotide segments (22-nt) capable of regulating the expression of a multiple of genes simultaneously by binding to complementary target mRNAs, resulting in mRNA inhibition and/or degradation³⁰⁹. Since its initial discovery over three decades ago, for which the 2024 Nobel Prize in Physiology or Medicine was awarded to Dr. Victor Ambros and Dr. Gary Ruvkun, investigations into miR biology have expanded considerably. miRs play critical roles in regulation of multiple cellular activities and biological processes (*e.g.* cellular differentiation, metabolism, development) throughout different tissue types³¹⁰. It is also now well understood that miR dysfunction has strong links to numerous diseases³¹¹, including cancer³¹², autoimmune disorders³¹³, neurological conditions³¹⁴, and cardiovascular disease³¹⁵. In fact, their relevance within a broad range of diseases along with its ability to modulate the translation of multiple genes simultaneously has made miR replacement and/or inhibition therapy one of the most exciting emerging fields of molecular therapeutics³¹⁶.

Perhaps one of the first studies linking miRs to heart disease was published in 2006, revealing that distinct miR expression patterns were associated with heart failure and cardiac hypertrophy in both mice and humans³¹⁷. Since then, over 30 miRs have been comprehensively validated pre-clinically and/or clinically to play a role in cardiovascular disease³¹⁸. Of particular interest is the increasing unraveling of the roles of miR in the context of pathological left-ventricle hypertrophy (LVH)^{319–321}. LVH can be characterized by the abnormal growth of cardiomyocytes resulting in myocardial remodeling, and over time leading to contractile dysfunction and heart failure⁴. Studies have further demonstrated the sex-stratification of this disease, in terms of disease prevalence, progression, and severity³²². In this vein, miR-1 is the most abundantly expressed miR within cardiac tissue and has been shown to be a negative regulator of cardiac hypertrophy^{92,323}. miR-1 is even downregulated during the early asymptomatic stages of hypertrophy³²⁴, making it not only a biomarker but a potentially valuable therapeutic target⁸⁵. In fact, previous work has shown the feasibility of miR-1 replacement for treatment of hypertrophy, along with its impact on hypertrophic-dependent pathways (calcineurin-NFAT) to reverse pathological hypertrophic phenotypes^{83,91}. Along with the excitement of 19 ongoing clinical trials of miR-based therapeutics³¹⁸ including one for cardiovascular disease (a miR-132-3p inhibitor called CDR132L³²⁵), this presents an exciting path for targeted miR-1-based LVH therapy.

A major and recurring issue with miR therapy pertains to its selective and targeted delivery^{326,327}. Direct intramyocardial injection³²⁸, aside from its invasiveness, poses challenges in achieving homogeneous tissue distribution of the payload, while AAV techniques³²⁹ may elicit strong immunogenic responses and targeting issues, limiting the potential for serial treatments³³⁰. Recently, ultrasound-based techniques (US) have made tremendous progress as a non-viral, targeted, and image-guided drug/gene delivery platform²²⁹; currently in clinical trials for brain

disorders^{238,331,332}. This approach takes advantage of US contrast agent microbubbles, typically employed as a diagnostic agent for contrast echocardiography. Microbubbles vibrate as they circulate through the acoustic focus²⁰³, and under specific US conditions can be made to reversibly increase plasma membrane and vascular permeability^{231,333} for the delivery of otherwise impermeable macromolecules^{334,335}. Indeed, as echocardiography is already an essential tool for cardiac hypertrophy diagnosis³³⁶, an opportunity to extend routine contrast-enhanced echocardiography techniques towards targeted cardiac miR-1 therapy presents as a logical progressive step. In fact, pre-clinical work to date using plasmids has demonstrated that US-based cardiac gene therapy is viable^{169,270,337,338}.

Here, we first demonstrate the complete reversion of the hypertrophic phenotype using microbubble-mediated US delivery of miR-1 *in-vitro*, and that therapeutic efficiency is strongly correlated with salient microbubble disruption echoes. Next, using a clinical US scanner and clinical microbubble agent, we show that image-guided miR-1 delivery restores the attenuation of downstream pro-hypertrophic proteins in healthy rat models, and that the delivery is restricted to the heart only. Following this, we demonstrate that a single treatment rescues miR-1 levels in LVH model rats, and either completely or partially restores protein levels, cardiomyocyte size, LV mass and heart function, levels of serum cytokines, mitochondrial respiration, and functional aerobic physiology in a manner that is highly dependent on sex; with a more prominent effect observed in males. Collectively, this study demonstrates sex-dependent restoration of LVH phenotype using clinically available systems and agent with only a single US treatment.

3.4 Methods

For detailed methodologies, please see the supplementary material of this Chapter. All animal work was approved by Concordia University's Animal Research Ethics Committee in accordance with the Canadian Council on Animal Care.

3.4.1 Neonatal rat cardiomyocyte isolation and hypertrophy model

Neonatal rat cardiomyocytes were isolated from 1-3 day old Sprague Dawley pups (Charles Rivers, Senneville, QC, Canada) using the neonatal cardiomyocyte isolation kit by Worthington Biochemical (Lakewood, USA). Upon isolation, cells were plated with DMEM media and supplemented with 5% fetal bovine serum, 5% horse serum and 1.7% penicillin and streptomycin (Wisent Bioproducts, Saint-Bruno, QC) and validated using cardiac TnT staining; see Supplementary Fig. 3.7. Neonatal cardiomyocytes were used for at most 14 days post-isolation. Hypertrophy was induced in the cardiomyocytes by first incubating the cells in serum starved media (5-fold dilution of enriched media) for 24 hours. After this incubation period, 100uM of (R)-(-)-phenylephrine hydrochloride (Sigma-Aldrich) was diluted in serum starved media and incubated with the cells for 48 hours.

3.4.2 In-vivo hypertrophy model

A hypertrophic model was developed in both male and female Sprague Dawley rats (100-150g; Charles River, Senneville, QC, Canada). For this hypertrophic model, an osmotic minipump (model 2002; Alzet, Santa Clara, CA, USA) filled with (R)-(-)-phenylephrine hydrochloride (80mg/kg/day) was implanted subcutaneously in the dorsal area of the animals (Day 0). Throughout the implantation of the minipump, the animals were placed under general anesthesia, 4% isoflurane (Kent Scientific, Torrington, CT, USA), and were administered a local anesthetic of carprofen (5 mg/kg) at the site of incision. After surgery, the animals were returned to their cages

and monitored daily. Sham surgery consisted of a saline-filled minipump. These rodents were then treated with US 7-days post-implant (D7).

3.5 Results

3.5.1 Microbubble-mediated US viably delivered miR-1 to neonatal cardiomyocytes in an acoustic pressure-dependent manner, and is positively correlated to microbubble signal.

We started our examination of US-assisted delivery by first using fluorescent surrogate molecules. Here, two fluorescent molecules were chosen to span 0.6-10 kDa to provide insight into whether the variation in molecular weight alters delivery efficiency. Utilizing our customized *in-vitro* US tank (Supplementary Fig. 3.8), fluorescent molecules were delivered to freshly isolated healthy neonatal cardiomyocytes and quantified by microscopy and flow cytometry. Over a range of clinically achievable mechanical indices (MI from 0.0625-0.25), we observe a general trend of increasing molecular delivery with MI, regardless of cargo size (Fig. 3.1). Next, with a fundamental understanding of the competencies of this approach, we developed a model to induce hypertrophy in these cardiomyocytes using phenylephrine. We assessed miR-1 (~7 kDa) delivery using US to both healthy and hypertrophic neonatal cardiomyocytes, resulting in a similar MI-dependent trend (Fig. 3.1F). While there was a numerical but not a significant increase at the lowest MI (1.56-fold, $p=0.07$; 1.29-fold, $p=0.1$ for healthy and disease cardiomyocytes, respectively), statistical significance was achieved as the treatment intensity increased, which was maximal at MI=0.25 (2.88-fold, $p=0.001$; 12.90-fold, $p<0.001$) relative to non-treated sham controls. While post-treatment cell viability demonstrated an inverse relationship (at both 48 and 96 hours), significant and viable miR-1 delivery was attained with modest acoustic intensity (MI=0.0625 and 0.125). We additionally tested the concept of preventative miR replacement, with US-assisted miR-1 delivery pre-hypertrophy treatment completely preventing the characteristic loss of miR-1

while maintaining viability (Fig. 3.1J&K). Treatment in the absence of miR-1 results in no effect (Fig. 3.1L). Simultaneous monitoring of microbubble acoustic activity during these treatments (Fig. 3.9) confirms a positive correlation between miR-1 levels and inertial broadband cavitation signal ($r=0.77$, $p=0.003$; Fig. 3.1N) – itself a surrogate of microbubble disruption that informs our pre-clinical work.

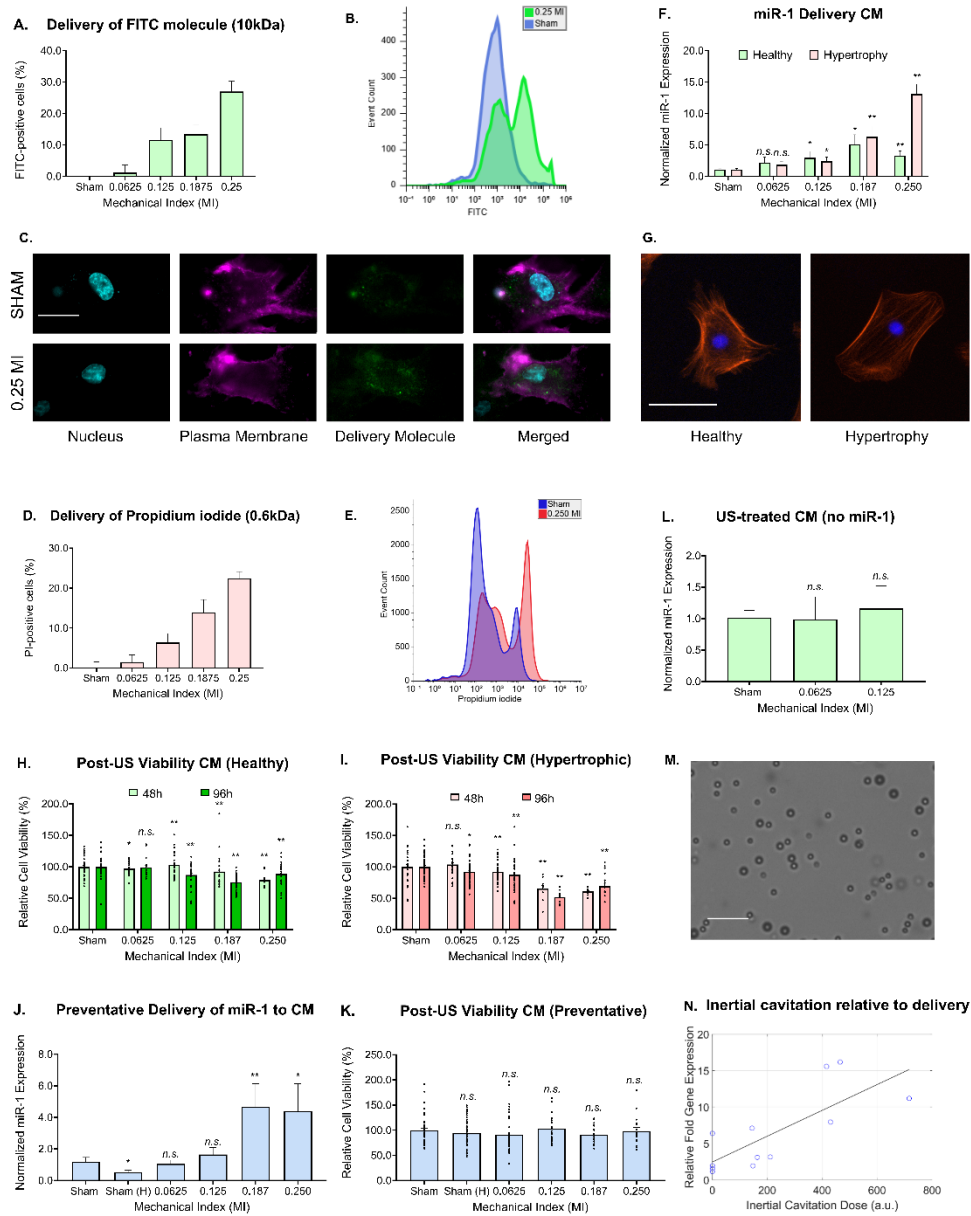


Figure 3.1: Ultrasound-mediated delivery of miR-1 to neonatal cardiomyocytes in-vitro is dependent on MI and is correlated with microbubble disruption. (A) Quantification of FITC-dextran (10kDa) uptake in in ultrasound-treated cardiomyocytes (0.0625 to 0.25 MI), quantified by flow cytometry. (B) Representative flow cytometry histogram of FITC-positive cardiomyocyte treated at 0.25 MI relative to sham treatment. (C) Fluorescence microscopy of FITC-positive ultrasound treated cardiomyocyte (0.25 MI) relative to sham treatment. Cells were stained for nucleus (DAPI; blue), plasma membrane (CellMask; magenta) and delivery molecules (FITC, green). Scale bar = 10µm. (D) Quantification of 0.6 kDa uptake in cardiomyocytes post-ultrasound treatment (0.0625 to 0.25 MI) quantified by flow cytometry. (E) Representative flow cytometry histogram of uptake in ultrasound treated cardiomyocyte (0.25 MI) relative to sham treatment. (F) miR-1 expression, determined by RT-qPCR, post-ultrasound treatment (0.0625 to 0.25 MI) in both healthy (green) and hypertrophic (pink) cardiomyocytes. (G) Representative microscopy images of healthy and hypertrophic cardiomyocyte demonstrating phenylephrine induced changes in cell size. Scale bar = 10µm. MTT viability assays were performed 48 hours and 96 hours post-ultrasound treatment in (H) healthy and (I) hypertrophic cardiomyocytes. (J) miR-1 expression in ultrasound mediated delivery of miR-1 to pre-hypertrophic cardiomyocyte (0.0625 to 0.25 MI). (K) Cell viability was determined by MTT assay 96 hours post-ultrasound treatment. (L) miR-1 expression shown ultrasound treated cells (0.0625-0.125 MI) in the absence of miR-1. (M) Representative brightfield image of microbubbles used for ultrasound-mediated delivery. Scale bar = 10 µm. (N) Relative gene expression as a function of inertial cavitation dose (a.u.). All samples are normalized to Sham controls. Statistical analysis was performed with a two-tailed student unpaired T-test. (*: $p < 0.05$, **: $p < 0.001$). Errors bars determined by SEM.

3.5.2 miR-1 replacement by US demonstrates complete reversion of the hypertrophy phenotype in-vitro

Upon confirmation that miR-1 could successfully be delivered to diseased cardiomyocytes, we followed up using an MI=0.125 to analyse the regression of hypertrophic phenotypes (Fig. 3.2). First, we confirm the expected drop in miR-1 48-hours post hypertrophy treatment (0.40-fold expression; $p=0.002$ relative to healthy myocytes) and demonstrate miR-1 rescue after ultrasound (0.76-fold, $p=n.s.$ from healthy control; $p=0.02$ hypertrophied cells; Fig. 3.2C). Three days post-delivery, immunoblotting was performed on three pro-hypertrophic proteins with known roles in LVH and well-established as downstream targets of miR-1^{25,83,92}. As previously documented, expression of these proteins in hypertrophic cardiomyocytes was significantly increased relative to healthy controls (TWF1, 1.27-fold, $p=0.002$; CX43, 1.45-fold, $p<0.001$; MEF2A, 1.25, $p=0.013$). US-assisted miR-1 delivery helped reverse this by decreasing the expression of each of

these proteins to almost healthy levels (TWF1, 0.8-fold, $p<0.0001$; CX43, 0.91-fold, $p<0.001$; MEF2A, 0.90, $p=0.003$ as compared to healthy cardiomyocytes; Fig. 3.2D), confirming functional miR-1 replacement. Further, our delivery resulted in a complete reversion of cardiomyocyte cell size (1.0-fold; $p<0.001$), originally enlarged due to hypertrophy (1.29-fold; $p<0.001$ compared to healthy – Fig. 3.2E). As a final proof-of-principle *in-vitro*, we assessed myokine secretion from the three groups (healthy; hypertrophic, and US-treated hypertrophic). Under hypertrophic conditions, six cytokines with well established roles in pathological hypertrophy and cardiovascular disease were shown to significantly increase relative to healthy samples (Fraktaline: 2.1-fold, $p=0.010$; MIP-1 α : 1.3-fold, $p=0.018$; IP-10: 1.8-fold, $p=0.024$; MCP-1: 1.5-fold, $p=0.023$; VEGF: 1.7-fold, $p=0.016$; IL-17A: 1.7-fold, $p=0.013$). Post-US treatment, each of these cytokines was demonstrated to completely recover to baseline or slightly lower levels (Fraktaline: 0.4-fold, $p=0.046$; ; MIP-1a: 0.6-fold, $p=0.004$; IP-10: 0.4-fold, $p=0.065$; MCP-1: 0.5-fold, $p=0.031$; VEGF: 0.4-fold, $p=0.033$; IL-17A: 0.4-fold, $p=0.02$; fold-change with respect to diseased state) - see Fig. 3.2F.

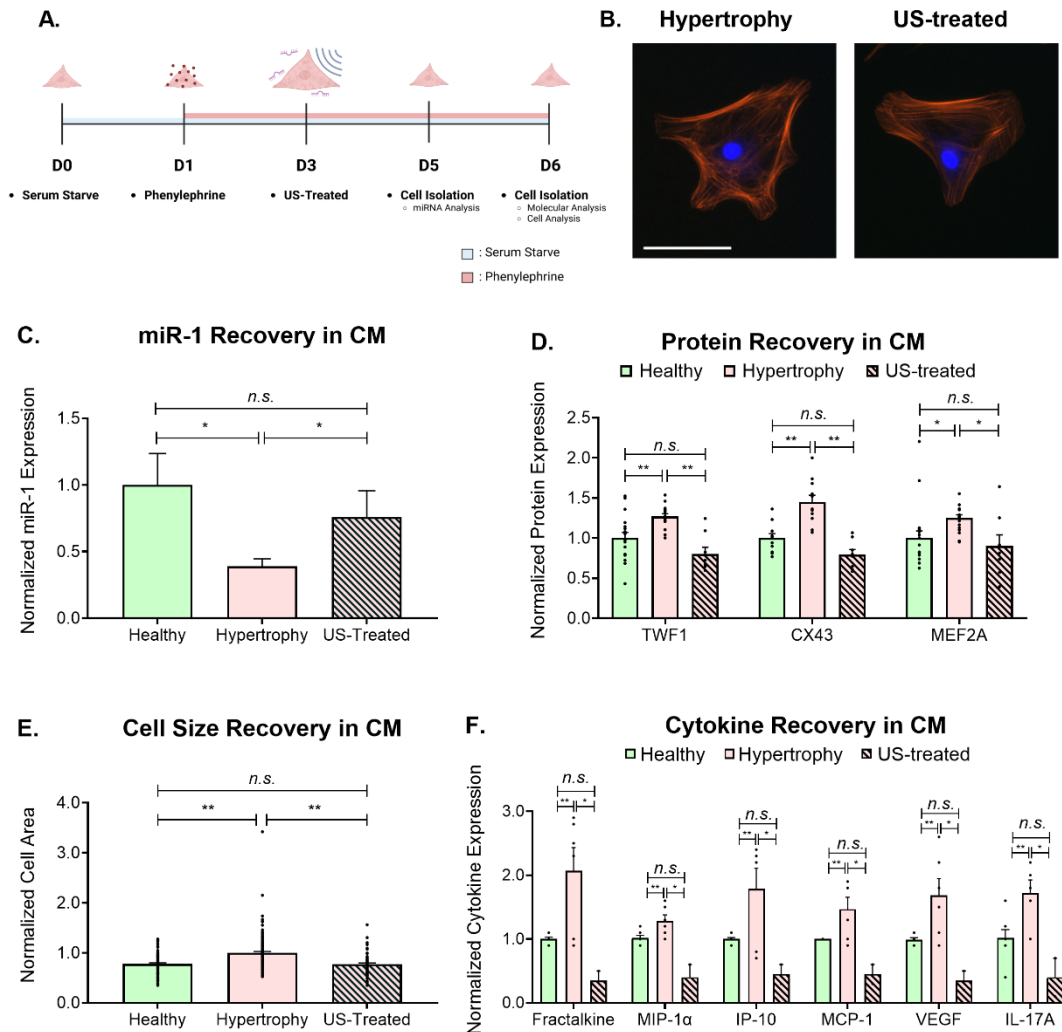


Figure 3.2: Recovery of hypertrophy phenotype via ultrasound-assisted miR-1 delivery in-vitro. (A) Experimental in-vitro timeline of neonatal myocyte treatment. Cardiomyocytes are serum starved on Day 0, followed by inducing hypertrophy with phenylephrine on Day 1. Ultrasound mediated delivery of miR-1 is performed on Day 3. miR-1 expression is measured on Day 5 and cell and protein analysis are performed on Day 6. (B) Cell area of hypertrophic and ultrasound treated cardiomyocytes relative to healthy cardiomyocytes. Representative fluorescence microscopy images of hypertrophic and ultrasound-treated cardiomyocytes stained with DAPI (nuclei; blue) and phalloidin (F-actin; orange) Scale bar = 100µm. (C) Levels of miR-1 expression are decreased in hypertrophic state and recover post US-treatment. (D) Expression of hypertrophic associated proteins (TWF1, CX43, MEF2A) in hypertrophic and ultrasound treated cardiomyocytes relative to healthy. (E) Cell size is rescued post-US. (F) Hypertrophic associated cytokine and chemokines (Fractalkine, IL-17A, MCP-1, MIP-1a, VEGF, and IP-10) increase in hypertrophic conditions and decrease post-ultrasound delivery of miR-1. *: $p < 0.05$, **: $p < 0.001$. Errors bars determined by SEM.

3.5.3 Targeted and site-specific functional miR-1 delivery to the heart can be achieved with clinical US and contrast agent microbubbles *in vivo*

We next sought to confirm US-mediated delivery of miR-1 specifically to the heart in healthy male and female rodent models (Fig. 3.3). First, to determine the overall stability of our intended therapeutic within the circulation, a degradation assay was performed in which we estimate a miR-1 half-life of approximately 12 min (Fig. 3.3B). Following this, we aimed to show US-mediated miR-1 deposition can be achieved in a single treatment with a highly accessible clinical US scanner, via co-injection with clinical microbubble agent (DefinityTM), and well within FDA safety limits ($MI < 2$) – Fig. 3.3A. Here, we placed the US probe in the parasternal short-axis view and interleaved our therapeutic US pulses with standard contrast-enhanced US (CEUS) sequences to confirm microbubble disruption within the LV (motivated by its correlation with miR-1 delivery; Fig. 3.1N), see Fig. 3.3C. Hearts were harvested within 30 minutes post-treatment and demonstrated an approximate 1.7-fold increase in miR-1 expression within the LV relative to non-treated shams, shown in both male ($p=0.04$) and female ($p=0.03$) models using RT-qPCR (Fig. 3.3E&F). Three-days post-treatment, a second subset of animals was sacrificed to assess cardiac protein levels. Indeed, we confirmed a decrease in expression of pro-hypertrophic associated proteins TWF1, CX43 and MEF2A (Fig. 3.3G&H) in both sexes, with a slightly larger magnitude of effect in female rats. In order to quantify the spatial specificity of our cardiac-targeted miR-1 delivery by US, the expression of miR-1 was measured in non-cardiac, off-target tissue, confirming no increase in miR-1 expression in the kidney ($p=0.24$) or liver ($p=0.34$) (Fig. 3.3D). This demonstrates rather robust spatial confinement, as this was achieved within the relatively small size of the rat heart using a US probe (and acoustic beam) designed for human patients. Taken together with the *in-vitro* proof-of-principle work, these datasets (Figs 3.1-3) convinced us of the feasibility of microbubble-assisted US for localized miR-1 replacement in the context of

LVH, including the real-time confirmation of microbubble disruption within the LV and our therapeutic pulsing sequence on a clinical US system.

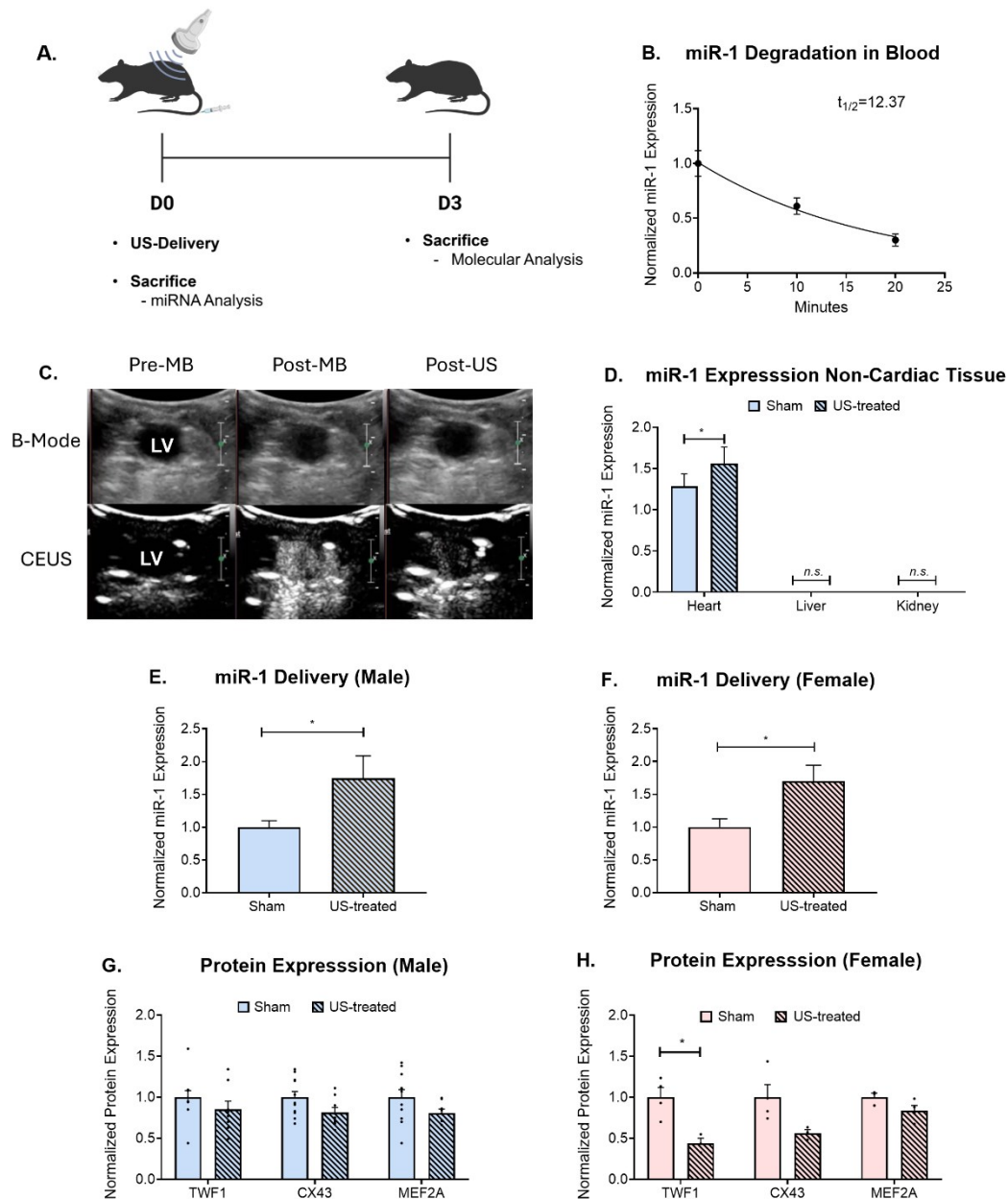


Figure 3.3: Single treatment using a clinical ultrasound system provides targeted and site-specific delivery of miR-1 to the heart in healthy male and female murine models in-vivo. (A) Experimental in-vivo timeline with single ultrasound treatment performed on Day 0. Post-treatment, a subset of the animals are sacrificed for miR measurement in the heart, while the other treated animals are sacrificed on Day 3 for molecular analysis. (B) Stability of miR-1 in blood, linear regression used

to determine the half-life of miR-1 in blood (12.37 min.). (C) Representative short axis echocardiographic images of the left-ventricle in both B-mode and CEUS before the microbubble injection (Pre-MB), post-injection, with microbubble filling the cavity (Post-MB) and the disappearance of the bubbles from the cavity after ultrasound treatment (Post-US). (D) miR-1 expression in non-cardiac tissue (liver and kidney) in ultrasound treated animals relative to native levels in hearts. miR-1 expression in the heart of (E) male and (F) female models post-ultrasound treatment, relative to non-treated sham. On Day 3, expression of hypertrophic associated proteins (TWF1, CX43, MEF2A) in ultrasound treated models, relative to non-treated sham for (G) male and (H) female animals. Statistical analysis was performed with a two-tailed student unpaired T-test. (*: $p < 0.05$, **: $p < 0.001$). Errors bars determined by SEM.

3.5.4 A single *in vivo* treatment of clinical US-mediated cardiac therapy rescues miR-1 and restores the attenuation of pro-hypertrophic proteins

Moving towards the assessment of the *in vivo* therapeutic potential of this platform, we next conducted our US-mediated treatment paradigm on a phenylephrine-induced cardiac hypertrophy rat model using surgically implanted osmotic mini-pumps (Fig. 3.4). Here, we demonstrate US-mediated miR-1 delivery using three experimental groups; *i*) sham-operated (PBS-filled mini-pump), *ii*) hypertrophic, and *iii*) hypertrophic and US treated. Seven days post-surgery, we confirm the characteristic drop in LV miR-1 compared to sham-operated rats in both sexes (0.69-fold, $p = 0.03$; 0.54-fold, $p = 0.003$ in males and females, respectively). Following this, we initiated our single treatment regime consisting of a continuously infused co-injection of clinical DefinityTM microbubbles and miR-1 mimic (0.6 ml combined volume). Microbubble disruption was confirmed during the interleaving contrast US imaging, and the total treatment duration (~15 min.) was determined upon echocardiographic evidence that there were no more microbubbles in circulation. In the first set of assays, our therapeutic platform achieved results consistent with our healthy datasets, which is to say the immediate restoration of miR-1 levels

within the heart (quantified on day 7), and subsequent (quantified on day 10; 3 days post-US) attenuation and restoration of salient downstream proteins.

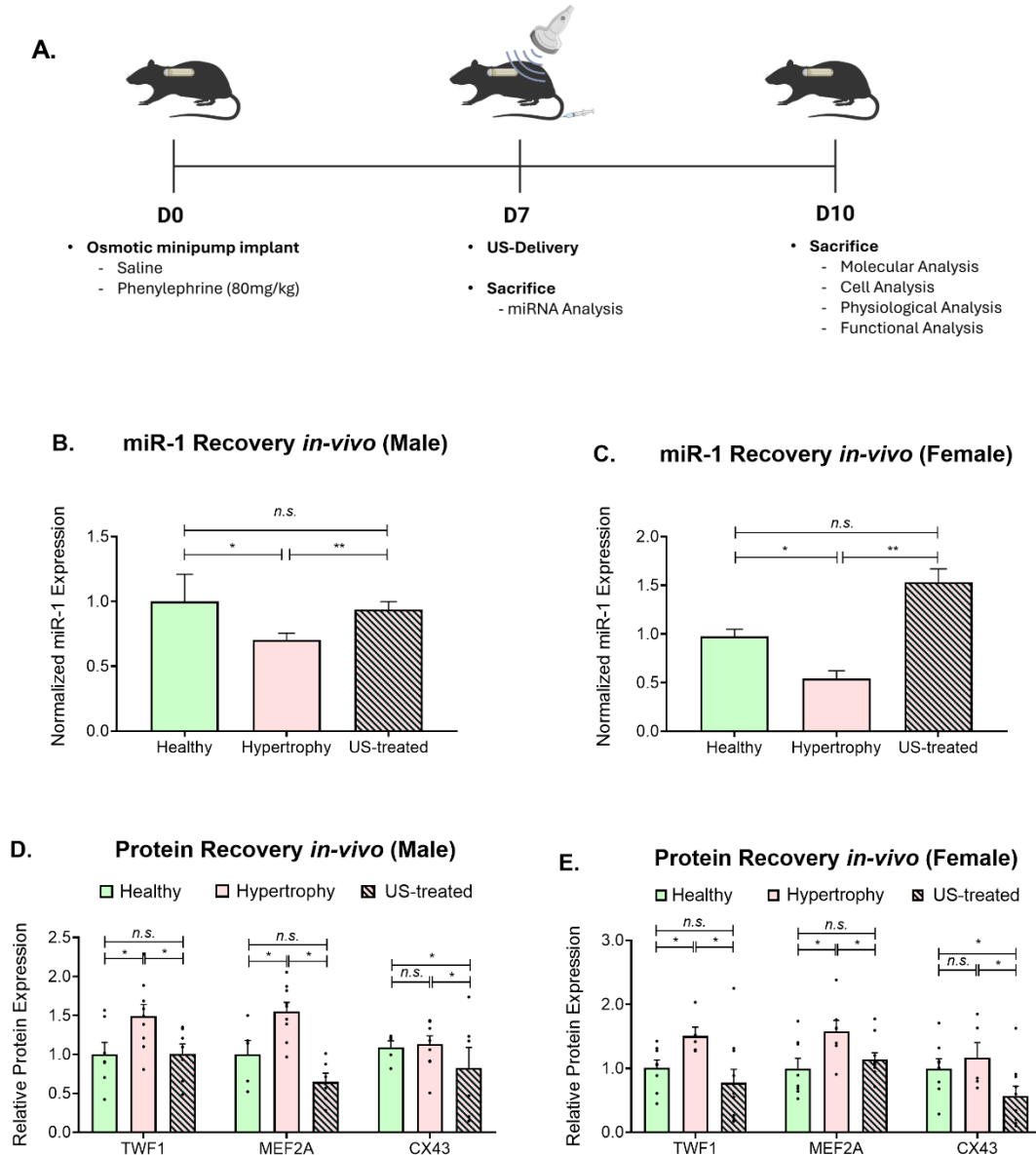


Figure 3.4: miR-1 recovery and the attenuation of hypertrophic proteins after a single ultrasound treatment in male and female *in-vivo* hypertrophic models. (A) Experimental timeline of *in-vivo* experiments. Osmotic pump (with saline or phenylephrine) are implanted on Day 0. Ultrasound delivery of miR-1 is performed on Day 7, with a subsection of treated animals undergoing miR quantification of the hearts post-treatment. On Day 10, another subsection of treated animals undergoes molecular, cellular, physiological and functional analysis. miR-1 expression of hypertrophic hearts, on Day 7, is significantly decrease relative to healthy hearts, and ultrasound

treatment shows miR-1 returning to approximate native levels, shown in (B) male and (C) female models. Three days post ultrasound treatment, on Day 10, expression of hypertrophic associated proteins (TWF1, MEF2A, CX43) are measured, demonstrating a significant increase in hypertrophic models relative to healthy hearts, followed by a decrease towards healthy levels in ultrasound treated hearts in (D) male and (E) female models. *: $p<0.05$, **: $p<0.001$. Errors bars determined by SEM.

3.5.5 The efficiency of miR-1 replacement in reversing the hypertrophic phenotype is sex-dependent, with a more pronounced result in males and a more heterogeneous response in females.

While we demonstrated the more direct success of miR-1 replacement, we further examined more downstream physiological, molecular and functional features. First, we demonstrate a return of cardiomyocyte size assessed through histology, and reversal of the enlarged heart weight to body weight ratio for both sexes (Fig. 3.5A-E). Next, we assessed left ventricular posterior wall thickness in diastole (LVPWd) and fractional shortening (FS) via high-frequency short-axis M-mode echocardiography (L17-5 Philips probe; Fig. 3.5H). Indeed, clinical studies have demonstrated marked increases in both LVPWd and FS in patients with LV hypertrophy (*e.g.* hypertrophic cardiomyopathy^{339,340}), which we confirm here in our model. After our single-treatment, the recovery on day 10 of these two physiological metrics was more pronounced in males, demonstrating strong reversals in both LVPWd (1.29-fold vs. 1.12-fold, $p<0.02$) and FS ($87\pm6\%$ vs $78\pm2\%$, $p<0.001$), compared to a partial response in female LV thickness (1.17-fold vs. 1.13-fold, $p=n.s$) and FS ($91\pm2\%$ vs. $83\pm4\%$, $p<0.02$), see Fig.3.5I-5L.

As accumulating evidence suggests correlations between local inflammation and cardiac hypertrophy, we hypothesized that local miR-1 delivery – in helping to return the cardiac phenotype - may additionally restore serum levels of pro-inflammatory cytokines that have been correlated with LVH and LV thickness^{42,341}. Our results, outlined in Fig. 3.5M-P, indicate a much more pronounced effect in male rat models, both in terms of cytokine dysregulation in disease and

its recovery post-treatment. miR-1 delivery with US restored the dysregulation of well-known pro-inflammatory cytokines IL-1, IL-6, IL-18, Fractalkine, IL17-A, as well as other immune-cell related markers – broadly consistent with our initial *in-vitro* myokine datasets. On the whole, out of the 27 solutes analysed, US-assisted miR-1 therapy numerically reversed the changes brought about by LVH in 25 cases, for a total reversal of ~93%. The response from the female model was much more heterogenous, with serum levels of pro-inflammatory cytokines lower than or unchanged from healthy controls; and a concomitant result post-miR-1 therapy.

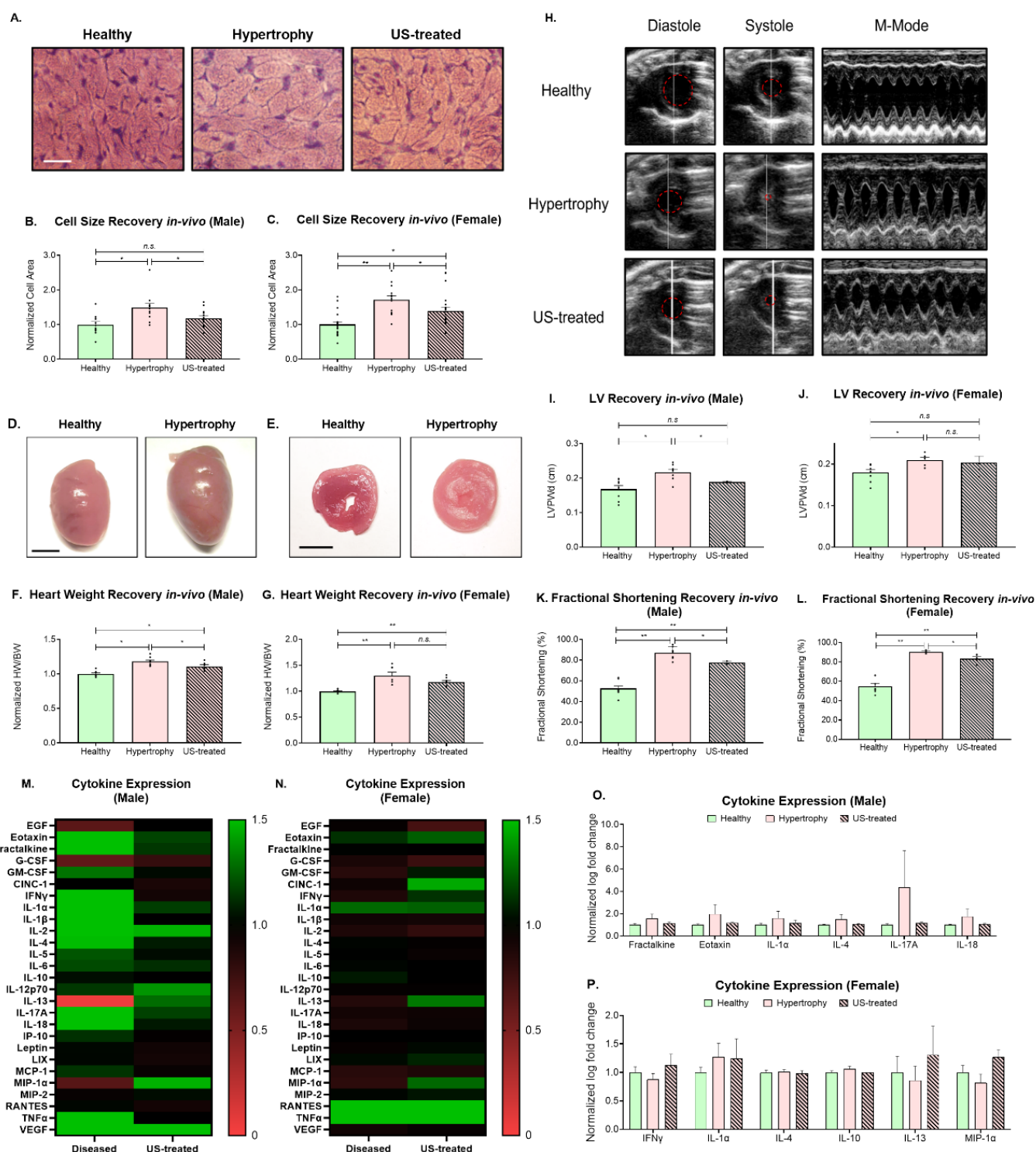


Figure 3.5: Sex dependent response to ultrasound-mediated miR-1 replacement therapy in reversing hypertrophic phenotypes. (A) High magnification of hematoxylin and eosin-stained cardiac cross sections, quantified by microscopy. Scale bar=25μm. Quantification of (B) male and (C) female cardiomyocyte size, determined by microscopy, from hypertrophic and ultrasound

treated models relative to non-treated healthy cardiomyocytes. (D) Representative healthy and hypertrophic heart images. Scale bar = 5mm. (E) Representative healthy and hypertrophic cardiac cross section. Scale bar = 5mm. Heart weight to body weight ratio measured in (F) male and (G) female rats depicting hypertrophic and ultrasound-treated models relative to healthy control. (H) Representative echocardiograph B-mode images of left-ventricle in healthy, hypertrophic and ultrasound treated hearts at diastole and systole, with corresponding M-mode images. Left-ventricle thickness measured in echocardiography, in (I) male models and (J) female hypertrophic and ultrasound-treated models relative to healthy control. Fractional shortening measured through echocardiography, in (K) male and (L) female hypertrophic and ultrasound-treated models relative to healthy control. Heatmap expression of 27 cytokines isolated from serum of hypertrophic and ultrasound-treated rats relative to healthy for (M) male and (N) female models. Of the array, 6 cytokines which had the greatest change were selected from both (O) male and (P) female models. *: $p < 0.05$, **: $p < 0.001$. Errors bars determined by SEM.

Finally, we evaluated heart bioenergetics and physiology; first by measuring the respiratory capacity of mitochondria isolated from fresh LV using high resolution respirometry. State 2 respiration was assessed by the sequential addition of malate, glutamate, and pyruvate to measure oxygen consumption by cardiac tissue in the absence of ADP or oxidative phosphorylation (OXPHOS), commonly referred to as LEAK respiration. High-resolution respirometry revealed no consistent or statistically significant differences in State 2 oxygen consumption among the experimental groups (Fig. 3.6A-C). State 3 respiration, reflecting complex I-driven oxygen consumption in the presence of saturating ADP (*i.e.* ADP-stimulated OXPHOS), also did not differ significantly between groups. However, upon subsequent addition of succinate to stimulate complex II, a significant increase in oxygen consumption (Complex I+II respiration) was observed in male rats following US treatment ($p < 0.05$) while hypertrophic female rats showed a significant reduction in maximal oxygen flux ($p < 0.05$) suggesting a sex-dependent response. Addition of oligomycin, an ATP synthase inhibitor, revealed a reduction in LEAK respiration within the hypertrophic groups and a recovery post-US treatment ($p < 0.05$). Finally, maximal uncoupled respiration assessed via FCCP titration showed no significant group differences in electron transport system (ETS) capacity.

While not all statistically significant, the main finding from the high-resolution respirometry data is that mitochondrial respiration is dysfunctional in hypertrophic cohorts (*i.e.* lower oxygen consumption), and focused cardiac miR-1 delivery via US trends towards its recovery, altering the cardiac tissue's basal or ADP-stimulated oxygen consumption. The significant increase in Complex I+II respiration in US-treated males compared to hypertrophic animals implies that miR-1 may enhance mitochondrial respiratory function and potentially restore ATP production capacity. This observation aligns with previous findings indicating that mitochondrial respiratory capacity is largely dependent on mitochondrial content³⁴². Indeed, we demonstrate a significant decrease in VDAC expression in hypertrophic hearts for both sexes, followed by a restoration post-US treatment in males (Supplementary Fig. 3.10). The lack of broader statistically significant group differences may reflect the relatively short intervention period and the inherently slow turnover rate of mitochondrial proteins³⁴³, potentially limiting the detection of adaptive bioenergetic changes within the timeframe of this study.

Following this, we examined aerobic capacity on a functional level by employing a maximal endurance treadmill stress test. After an initial acclimatization period, rats followed a graded exercise protocol up to 2 hours. The hypertrophy groups demonstrated a significant decrease in activity – quantified here at time to exhaustion - relative to sham-operated control (0.22-fold, $p=0.02$; 0.25-fold, $p=0.01$ for males and females respectively). After a single US treatment, we observed a numerical, trending increase in exercise capacity (~65% increase from LVH group), representing partial disease mitigation in both sexes (Fig. 3.6D-E).

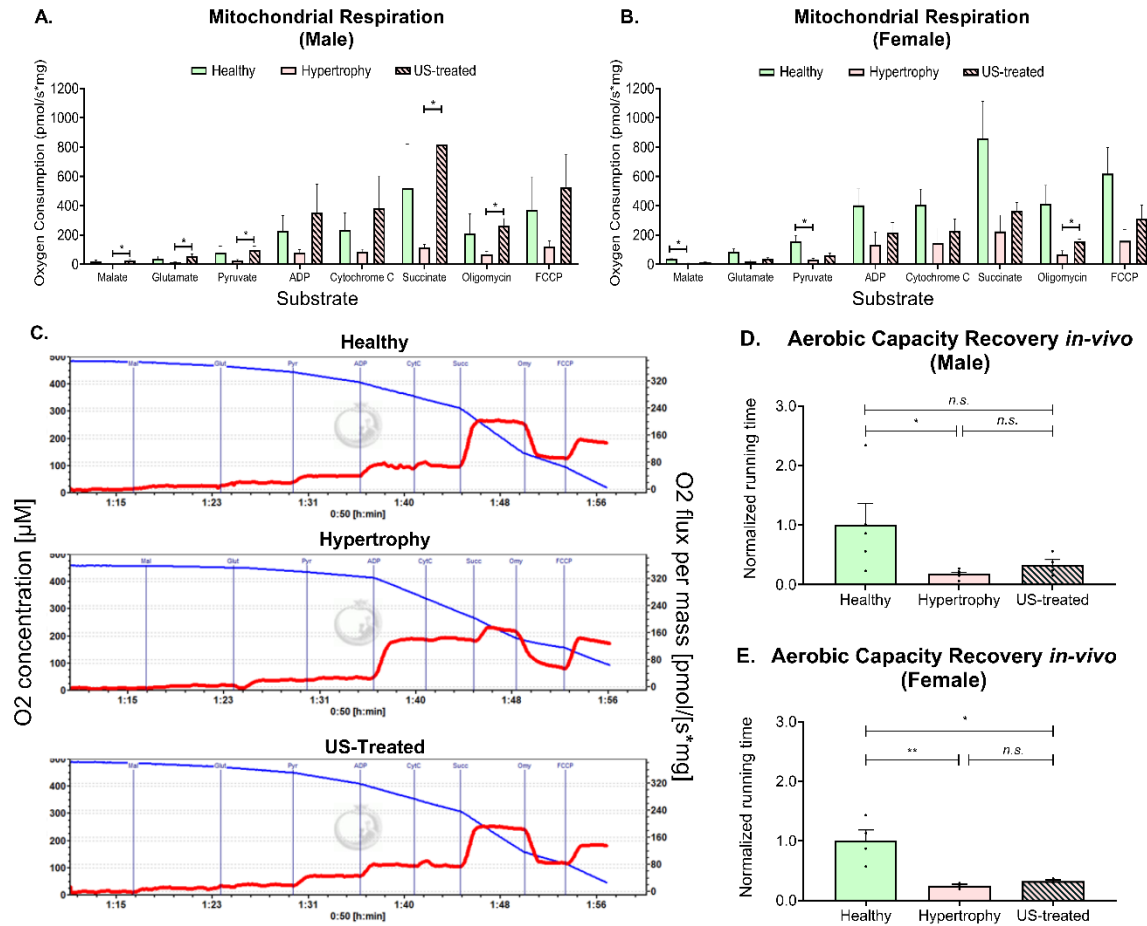


Figure 3.6: Mitochondrial and aerobic response to miR-1 replacement therapy in male and female hypertrophic murine model in-vivo. Mitochondrial oxygen flux of cardiac tissue in hypertrophic and ultrasound treated models in (A) male and (B) female rats relative to healthy control. Green bars represent healthy cohorts, pink represent hypertrophic cohort, and pink hash-marked bars represent the US-treated groups. (C) Representative respirometry trace in male healthy, hypertrophic and ultrasound treated cardiac tissues. Mitochondrial dysfunction is clearly observed in both sexes within the hypertrophic group, with partial recovery post-US. Aerobic capacity measured by treadmill endurance testing demonstrate significant decrease in hypertrophic models relative to healthy controls, and numerical improvement in ultrasound treated groups for (D) male and (E) female models. *: $p < 0.05$, **: $p < 0.001$. Errors bars determined by SEM.

3.6 Discussion

Heart failure is typically preceded by maladaptive cardiac hypertrophy, a compensatory enlargement in response to increased LV wall stress³⁴⁴, which often develops in pathological settings that include hypertension, obesity, mitral regurgitation, and various genetic cardiomyopathies. Here, we are specifically targeting LVH itself via local miR-1 replacement, as opposed to focussing on its upstream drivers. Indeed, LVH is an intrinsically valuable therapeutic target: there is strong clinical evidence that LVH on its own is an independent predictor of adverse cardiovascular outcomes, including myocardial infarction, arrhythmias, and heart failure^{345,346}. Over the last several decades, many studies have confirmed that LVH reduction is positively associated with improved health outcomes^{347,348}. Current pharmacological treatments for heart failure, including ACE inhibitors (*e.g.* Enalapril³⁴⁹), angiotensin receptor blockers (*e.g.* Valsartan³⁵⁰), angiotensin receptor–neprilysin inhibitors (ARNIs; *e.g.* sacubitril/valsartan³⁵¹), β -blockers (*e.g.* Metoprolol³⁵²), and calcium channel blockers (*e.g.* amlodipine besylate³⁵³) vary in their ability to exhibit LVH regression, dependent on a myriad of factors (age, sex, BMI, type and severity of LVH, disease etiology, among others). All told, the overall result is that only ~50% of patients receiving heart failure therapies – whether surgical or pharmacological – do indeed exhibit LVH regression to some extent³⁴⁴. There is therefore an urgent need to develop new therapies informed by molecular studies to target LVH specifically, thereby helping to restore cardiovascular health in non-responding patients, as well as to potentially provide a longer-lasting therapy in those that do.

To this end, the landscape of miR-based therapies for cardiovascular disease is rapidly expanding, in both preclinical and clinical contexts. Several key studies have demonstrated the feasibility of miR-directed therapy in reversal of the maladaptive processes involved in heart failure, such as cardiac fibrosis (*e.g.* miR-21³⁵⁴, miR-24³⁵⁵, miR-25³⁵⁶, miR-29¹⁵⁸) and hypertrophy

(*e.g.* miR-1⁹¹, miR-133³⁵⁷, miR-208³⁵⁸, miR-223³²¹). In fact, robust preclinical research^{359,360} recently led to the first clinical trial for miR therapeutics in heart failure in 2019⁹⁰, in which the Phase 1b trial demonstrated the sustained inhibition of miR-132 using a small molecular inhibitor CDR132L. The small study (28 patients) showed that CDR132L was safe and well tolerated, exhibited linear plasma pharmacokinetics with no signs of accumulation, and suggested cardiac improvement. The ongoing larger, follow-up study (HF-REVERT)³²⁵ is a Phase 2 trial aimed at assessment of CDR132L at 5mg/kg and 10mg/kg given as three single IV doses as an add on to standard of care therapy in patients with heart failure.

Despite this exciting progress, one of the major obstacles towards clinical use is the local and specific establishment of effective concentrations of miR product within the target tissues and cells – essentially a delivery challenge^{318,361,362}. Indeed, due to their hydrophilic nature, oligonucleotides do not effectively penetrate tissue, and their distribution within cardiac tissue is thus limited. Aside from viral techniques, which carry a strong immunogenicity risk, various non-viral strategies to improve this are currently under investigation, including functional manipulation (*e.g.* locked nucleic acid; PEGylation, conjugation to cell-penetrating peptides, and coupling to receptor ligands, antibody fragments, or aptamers) and/or embedding them within nanoparticles (*e.g.* lipid nanoparticles, extracellular vesicles).

Here, we demonstrated the image-guided and targeted delivery of miR-1 mimic in which we employed commercially available oligonucleotide, and a clinical US scanner and FDA approved US contrast agent. One of the major advantages of this technique is the inherent reproducibility of this platform, with established systems and reagents used in conjunction with one another. Indeed, the use of a clinical US scanner obviates the need for new technological development, and by using DefinityTM we remove the acoustic variability brought forth by non-

commercial or in-house drug-loaded bubble formulations; which can result from heterogeneities in bubble size distributions, physical properties, and agent manipulation^{163,203}. The image-guided nature of this platform - a distinct feature among alternative non-viral approaches that is borne out of extending the use of a clinical imaging agent – enables quantifiable reproducibility in real-time via contrast echo. In fact, clinical echocardiography with or without contrast agents is one of the main diagnostic tools in identifying LVH^{363,364}, which integrates nicely with an extended use towards miR therapeutics presented here. Further, it is well established that microbubble-mediated US can elicit localized and reversible cellular and vascular permeability and thereby circumvents the requirement of payload endosomal escape – a current issue with other leading non-viral strategies. This has been shown in multiple contexts (*e.g.* endothelial cells²²²), including during the opening of the blood-brain-barrier³⁶⁵. Indeed, under MRI guidance, the application of microbubble-mediated blood-brain-barrier opening is rapidly emerging as a clinical tool in the treatment of neurological disorders, including Alzheimer’s disease³⁶⁶, brain metastases²³⁸, cerebral cavernous malformations³³³, among others. Additionally, it is important to note that we were able to deliver large amounts of miR-1 localized to the heart and completely or partially reverse many LVH phenotypes with only a single treatment (~15 min.). The non-invasive and non-immunogenic nature of our echocardiography-based platform presented here allows us to easily extend this approach longitudinally – without the usual challenges that accompany viral methods.

Our rationale for targeting miR-1 is to address multiple dimensions of LVH; from a structural, transcriptomic, and an electrical function viewpoint. First, we demonstrate rescue of TWF1, which is a cytoskeleton regulatory that binds to actin monomers to prevent their assembly into filaments. Known to be downregulated in LVH⁸³, its restoration contributes to the re-establishing of cytoskeletal stability and mechanical resilience of cardiomyocytes. MEF2A has

been identified as a salient transcription factor with a robust role in determining the cardiac transcriptome³⁶⁷. Indeed, it is activated via the dysregulation of several upstream signaling pathways, including the calcineurin-NFAT axis³⁶⁸, MAPK pathway³⁶⁹ and calcium-calcineurin dependent signaling³⁷⁰. The restoration of MEF2A demonstrated here offers a way to broadly suppress the hypertrophic response. Finally, Cx43 is the dominant gap junction protein in LV cardiomyocytes, forming channels that allow the passage of ions and small signaling molecules between cells, well known to be impaired in the diseased heart³⁷¹. By mitigating this downregulation, our targeted miR-1 approach helps re-establish the rapid, coordinated spread of electrical impulses that trigger synchronized contraction – and indeed, Cx43 itself is an emerging target currently under-investigation for cardiovascular disease³⁷².

While sex-based differences in cardiovascular medicine are generally under-appreciated³⁷³, it is well established that there are major differences between the sexes when it comes to LVH and its progression to heart failure³⁷⁴. Furthermore, there is mounting evidence highlighting the role of biological sex on the efficiency of miR-based therapies³⁷⁵, including those targeting LVH^{376,377}. As a physical delivery method, it is not surprising that our technique elicited similar levels of cardiac miR-1 accumulation independent of sex (Fig. 3.3). However, the heterogeneous response of downstream indicators of disease (and successful treatment) between male and female models does suggest sex-dependent regulation of functional miR. Previous works have highlighted the cardioprotective role conferred by estrogen in the context of LVH by modulation of specific miRs and mitochondrial proteins^{377,378}, in which disease phenotypes and miR therapies were generally attenuated in female models – broadly consistent with our results (Fig. 3.4-6). This highlights the need for careful consideration of sex-dependent miR-based treatment paradigms.

It is important here to acknowledge some of our study limitations. Firstly, while we showed the protective effect of excess miR-1 delivery in resisting hypertrophy *in-vitro*, we did not test relative dose timings (between miR-1 and contrast microbubble injections) in our *in-vivo* models. Exploring the dose-response relationship for this delivery method remains an important aspect to consider. Related to this, the pharmacologically induced hypertrophy model employed here does not fully recapitulate the hemodynamic burden that characterizes pressure-overload hypertrophy. The use of the surgically implanted mini pumps of a finite volume were thus unable to capture protracted longitudinal treatment plans. This at least partly explains the trending data we observe in the physiological readouts, as these improvements generally occur on longer timescales than those explored here. Despite this, we were able to deliver a clinically meaningful dose of functional miR-1 directly to diseased heart in a single treatment. Finally, we only examined our image-guided miR replacement technique in murine models; further translational testing in large animal LVH models is necessary.

In summary, our findings – which are summarized in Table 1 - indicate that functional miR-1 delivery can be performed using a clinical US system in combination with clinical US contrast agents, providing an image-guided, targeted and safe approach for the treatment of pathological LVH. In isolated cardiomyocytes, as well as in healthy and disease rat models, we showed that a single treatment of approximately 15 min. in duration restores native cardiac miR-1 levels, rescues downstream salient protein expression, and either completely or partially restores multiple serum factors, LV function, and physiology in a sex-dependent manner. Taken together, we believe that future developments of such a clinically ready image-guided targeted US delivery platform may be able to propel non-viral miR therapy into clinical success.

	<i>In-vitro</i> Neonatal CM		<i>In-vivo</i> Murine Model			
			Male		Female	
Assay	Healthy vs Hypertrophy	Hypertrophy vs US	Healthy vs Hypertrophy	Hypertrophy vs US	Healthy vs Hypertrophy	Hypertrophy vs US
miRNA expression						
Protein expression						
Cytokine level						
Cell Size						
HW:BW						
LV thickness						
Fractional Shortening						
Mito. Respiration						
Aerobic Capacity						

Table 4: Overview of experimental outcomes of ultrasound treatment of hypertrophic in-vitro neonatal cardiomyocyte model and in-vivo male and female murine models. Summary of significant changes across 9 different experiments in diseased hypertrophic models relative to healthy controls and in ultrasound treated models relative to hypertrophic diseased models. Outcomes are reported as complete recovery (dark green; US-assisted recovery, $p < 0.05$), partial recovery (light green; US-assisted recovery, no statistical significance but trending) or no differences (grey, no obvious US-assisted recovery, no statistical significance).

3.7 Supplementary Methods

3.7.1 Microbubble contrast agent

For the *in-vitro* work, we synthesized an in-house lipid agent similar to commercial DefinityTM; see Memari et al.²¹⁹ for details. All *in-vivo* studies were performed with DefinityTM (Lantheus Medical Imaging, Billerica, MA, USA).

3.7.2 Ultrasound mediated delivery and cavitation detection *in-vitro*

A custom designed ultrasound tank was used to treat the cardiomyocytes samples, see Fig. 3.8. The samples consisted of a suspension of cardiomyocytes (healthy or hypertrophic – see Fig. 3.7), microbubbles (1:250 cell to bubble ratio), and a payload (either miR-1 mimic, 84nM; Thermofisher Scientific, Waltham, MA, USA; FITC-Dextran (5mg/ml; Sigma-Aldrich, St. Louis, MO, USA), or propidium iodide (10µg/ml P1304MP; Thermofisher Scientific). The samples were placed within a mylar sealed acrylic chamber, above a magnetic stir plate, which ensured the samples were homogeneous throughout treatment. The tank was filled with degassed water at 37°C, submerging two transducers that were co-axial positioned to face the sample chamber³⁷⁹. A primary transducer (1 MHz, $f=25.4$ mm, $f\#=1.33$; Olympus) was used to treat the samples (20 cycles, 200 µs pulse repetition interval) for 2 minutes with a variation in peak-negative pressures (0.062 MI, 0.125 MI, 0.1875 MI and 0.250 MI). The pulse was generated using an arbitrary function generator (AFG31000, Tektronix, Beaverton, OR, USA) and amplified using an RF power amplifier (model 105A100B, Amplifier Research, Souderton, PA, USA). The secondary transducer (3.5MHz; Olympus) was used to measure the passive cavitation of the bubbles within our sample chamber. Received bubble echoes were amplified (AU-1579, 0.7–200 MHz, MITEQ, Hauppauge, NY, USA), bandpass-filtered, and then digitized (Gage Razor Express CompuScope, Lockport, IL,

USA) for analysis using custom MATLAB software (MathWorks, Natick, MA, USA). Joint time–frequency analysis was performed with a window size of 50 ms and a 90% overlap. A Hamming window was applied to the RF data prior to obtaining the fast Fourier transform (FFT) to assess both stable (harmonic) bubble echoes and inertial (wideband emissions) behaviour, indicative of bubble collapse. Post-ultrasound treatment, the samples were removed from the chamber and left at room temperature for 5 minutes to recover, before undergoing three washes by centrifuging at 100xg at 4°C for 5 minutes. After washes, the cells were seeded and incubated at 37°C in 5% CO₂. miR-1 analysis was performed 48-hours post-ultrasound treatment, and all other assays (protein, size and cytokine measurements) were performed 72-hours post-treatment. For all *in-vitro* work, datasets consisted of at least $n=3$ independent samples per condition per experiment.

3.7.3 Ultrasound mediated delivery *in-vivo*

Before beginning the *in-vivo* ultrasound treatment, animals were placed under 4% isoflurane anesthesia and a C5-2 probe (iU22; Philips Healthcare, Bothell, WA, USA) was positioned on the chest in order to provide a short-axis view of the left-ventricle. A solution of DefinityTM (216 µl) and miR-1 mimic (0.6mg/kg) was then continuously injected through the tail vein (25G infusion set) at a constant speed of 4.8ml/hour using an injection pump. Once the microbubbles were present in the heart, as confirmed by ultrasound contrast imaging, the animals were treated using the same C5-2 transducer with a high-MI flash pulse at 1.34 MI and 40 frames over a 10 second pulse repetition, for approximately 7 minutes. After treatment, a subset of animals were euthanized for miR quantification in the heart. The other subsets were euthanized 3 days later (D10, for animals that have received osmotic pumps), in which changes in protein content, cellular phenotype, physiological and functional activity of the heart were measured. Euthanasia was

performed by CO₂ asphyxiation, followed cervical dislocation, as approved in our animal ethics protocol. For all *in-vivo* studies, animal cohorts consisted of at least $n=4$ rats per group per sex.

3.7.4 Tissue extraction and processing

For the *in-vivo* samples, the animal tissues (such as the heart, liver and kidneys) were isolated post-euthanasia. For the cardiac tissue, the atria were removed before moving forward. The isolated tissue was rinsed with ice cold saline before being placed in a solution of isopentane, within a dewar filled with liquid nitrogen. The tissues were left in the isopentane solution for approximately 1 minute or until frozen solid. Once frozen, the tissue samples were either prepared for histological analysis or further processed for miR and protein isolation. This was performed by first homogenizing the frozen tissue into a fine powder using a prechilled mortar and pestle. The miR was then isolated from the homogenized tissue using the miRvana™ miR isolation kit (Invitrogen, Waltham, MA, USA). For protein isolation, the homogenized tissue was added to a solution of RIPA buffer with PMSF, followed by a 1-hour incubation at 4°C, with occasional vortexing. The samples were then centrifuged at 10,000xg for 20 minutes at 4°C, after which the supernatant was collected.

3.7.5 Flow cytometry and fluorescence microscopy

To measure the delivery efficiency of ultrasound-mediated delivery *in-vitro*, fluorescent particles were first delivered to *in-vitro* cardiomyocytes following the ultrasound treatment regime described above. Propidium iodide or 10kDa FITC-dextran were delivered to the cardiomyocytes, and after the washing steps, these samples were resuspended in PBS and filtered through a 35µm cell strainer before being placed on ice. The samples were then read using a FACSMelody™ flow cytometer (Beckton Dickson, Franklin Lakes, NJ, USA). Analysis of the results and formation of flow plots were performed using Floreada.io software (<https://floreada.io/>). A subset of sample was

seeded on a glass bottom dish and stained with Hoechst (1:1000, Thermofisher Scientific) to visualize the nucleus and Cell MASK™ orange (Thermofisher Scientific) to highlight the plasma membrane. These samples were then imaged using the Nikon Eclipse TiE inverted C2 confocal microscope (100x objective; Nikon, Melville, NY, USA).

3.7.6 Real time quantitative PCR

For both *in-vitro* and *in-vivo*, experiment miR was isolated following the miRvana™ miR isolation kit (Invitrogen). Once isolated, miR concentrations and purity were determined using the Varioskan™ LUX microplate reader (Thermofisher Scientific). Complementary DNA was of isolated miR was produced using reverse transcription with the TaqMan microR reverse transcription kit (Applied Biosystems, Waltham, USA). The product number (PN) of the reverse transcriptase PCR primers are hsa-miR-1 (PN:4464062 RT) and U6 (PN:4427975 RT 001973). The reaction was incubated in a thermal cycler (Applied Biosystems) for 30 min at 16°C, 30 min at 42°C, 5 min at 85°C and then held at 4°C. Following this, real-time quantitative PCR (RT-qPCR) analysis was performed on the collected cDNA using a QuantStudio™ 3 system (Applied Biosystems) incubating for 20s at 95°C, and then 40 cycles for 1 second at 95°C and 20s at 60°C. For this procedure, TaqMan™ Fast advance master mix (Applied Biosystems) was used along with qPCR primers for miR-1 (PN:4464062 TM) and U6 (PN:4427975 TM 001973) (Applied Biosystems). The relative level of miR-1 was calculated by normalizing to the housekeeping gene U6 using the $\Delta\Delta CT$ method.

3.7.7 miR blood degradation assay

To evaluate the stability of miR-1 in whole blood, samples were collected from rats into an EDTA-coated tube (Becton Dickson, Franklin Lanes, NJ, USA). Equal volumes of the blood were aliquoted into separate Eppendorf tubes and placed in a 37°C water bath for 5 minutes to allow the

samples to reach physiological temperatures. Once the appropriate temperature was reached, miR-1 was added to the blood sample and incubated at 37°C for different periods of time (0, 10, 20 minutes). Once the incubation period was complete, the samples were removed, and miR isolation was performed using the miRvana™ miR isolation kit. miR-1 was then quantified by qPCR as described above. The half-life of miR-1 was calculated using a one-phase decay non-linear regression model on GraphPad Prism (GraphPad Software, Boston, MA, USA).

3.7.8 Immunoblotting

The proteins from the *in-vitro* cardiomyocytes samples were isolated using a scraping buffer. The samples were then centrifuged for 10 minutes at 5,000 rpm in 4°C to remove the supernatant, followed by resuspending in RIPA buffer with PMSF. These samples were then incubated on ice for 5 minutes before being centrifuged for 15 minutes at 12,000 rpm in 4°C. The supernatant was then collected. Following this, for both *in-vitro* and *in-vivo* isolated proteins, the concentrations were determined with Coomassie blue which was measured using a Varioskan™ LUX microplate reader at 595nm. The proteins were loaded at equal concentration and separated using a 1.5mm 7.5% acrylamide SDS gel (Biorad), with a PageRuler™ Plus prestained protein ladder (ThermoFisher Scientific). From the gel, the proteins were transferred onto an activated PVDF membrane overnight. This membrane was then blocked for 1 hour in 5% bovine serum albumin (Wisent) dissolved in tris-buffer saline (TBS). Primary antibodies 1:1000 TWF1, 1:1000 CX43 (Abcam, Cambridge, UK), 1:1000 GAPDH and 1:1000 MEF2A (ThermoFisher Scientific) were diluted in TBST-2% BSA and incubated for 1 hour at room temperature. Following this, the membrane was rinsed with TBST (5 times, for 3 minutes). After washing, HRP secondary antibodies (anti rabbit, anti mouse) were incubated with the gel for 1 hour. Another TBST wash was performed, and enhanced chemiluminescence reagent was incubated with the gel for 1 minute

while rinsing. The proteins bands were developed using an Amersham Imager 600 system (GE healthcare, Chicago, IL, USA). Relative protein band density was subsequently analysed using the ImageJ software, normalized to standard housekeeping protein GAPDH.

3.7.9 Cell viability analysis

Post-*in-vitro* ultrasound treatment, cardiomyocyte cell viability was determined by an MTT assay (Sigma Aldrich), in which subset of each sample was seeded in a 96 well plate in triplicates. The assay was performed either 48 hours or 96 hours post-treatment, where seeded cells were rinsed with PBS, before being incubated with an MTT solution for 4 hours at 37°C in 5% CO₂ as per the manufacturer protocol. DMSO was then added for 10 minutes to solubilize the formazan. After this final incubation, cells were measured at absorbance of 540 nm using the Varioskan™ LUX microplate reader.

3.7.10 Cell size analysis

Cardiomyocyte cell area was analysed 96-hours after treatment. Seeded cells were washed with PBS and fixed with 3.75% paraformaldehyde. After fixing, the cells were then washed once more with PBS and then incubated with cell staining dyes Phalloidin (Invitrogen) for cell area coverage and DAPI (Sigma Aldrich) for cell count. Images were captures using the EVOS M700 imaging system (20x objective; Thermofisher Scientific) and the cell area was calculated using ImageJ software.

3.7.11 Cytokine/Chemokine profiling

Profiling of cytokine and chemokine from *in-vitro* supernatant and *in-vivo* serum samples was performed by Eve Technologies (Alberta, Canada). The supernatant of the *in-vitro* cardiomyocyte samples was collected 48 hours and 72 hours post-treatment. The samples were

then centrifuged at 3000xg at 4° C, as instructed by the service provider. The serum was collected from *in-vivo* rat blood samples on Day 10 of the *in-vivo* treatment timeline. Samples were centrifuged at 1000xg for 10 min at 4°C. The collected *in-vitro* and *in-vivo* samples were sent to Eve Technologies, in which profiling was performed using a multiplex platform (Luminex™ 200 system; Luminex, Austin, TX, USA). Specifically, the Rat Cytokine/Chemokine 27-Plex Discovery Assay® Array (RD27) panel was chosen. This panel assays for the expression of the following cytokine/chemokines: Eotaxin | EGF | Fractalkine | IFN γ | IL-1 α | IL-1 β | IL-2 | IL-4 | IL-5 | IL-6 | IL-10 | IL-12p70 | IL-13 | IL-17A | IL-18 | IP-10 | GRO/KC | TNF α | G-CSF | GM-CSF | MCP-1 | Leptin | LIX | MIP-1 α | MIP-2 | RANTES | VEGF-A.

3.7.12 Histological assessment of cell size

Frozen tissue samples were covered in the OCT media and placed on a bed of dry ice until solidified. The samples were then sliced in the short-axis orientation into sections of 8 μ m thickness using a Leica CM3050 S cryostat. Hematoxylin and eosin staining was then performed on the sliced tissue, followed by imaging. Cell size analysis of images was performed using the Amscope image analysis software in a single-blinded manner.

3.7.13 LV remodeling and function

Echocardiography was performed on animals under isoflurane anesthesia, on Day 10, using the Philips L17-5 transducer. Short axis M-mode images were acquired to quantify the LV wall thickness and fractional shortening. Post-euthanasia, on Day 10, the heart was isolated and rinsed. The heart weight was then measured and divided by the animal body weight, providing a heart/body weight index.

3.7.14 Mitochondrial respiration measurements

Assessment of cardiac mitochondrial oxygen consumption was performed using high-resolution respirometry (Oxygraph2k, Oroboros Instruments, Innsbruck, Austria). To avoid potential oxygen limitation, all experiments were carried out under hyper-oxygenated conditions and followed previously described protocols³⁸⁰. For each experiment, approximately 2 mg of freshly isolated left ventricular (LV) tissue was placed into 2 mL of mitochondrial respiration medium (MiR05) within each respirometry chamber, which were maintained at a physiological temperature of 37°C. To initiate the respiratory assay, malate (2 mM), glutamate (10 mM), and pyruvate (6 mM) were sequentially introduced to stimulate LEAK respiration supported by complex I activity and to facilitate the establishment of a proton gradient across the inner mitochondrial membrane. Subsequently, ADP (5 mM) was added to activate oxidative phosphorylation, enabling the assessment of complex I-dependent, phosphorylating respiration. Cytochrome c (10 µM) was then added to test the integrity of the outer mitochondrial membrane. Following this, succinate (10 mM) was introduced to stimulate complex II and to assess maximal coupled respiration through both complexes I and II. To inhibit ATP synthase and measure non-phosphorylating, LEAK respiration under these conditions, oligomycin (2 µg/mL) was then added. Finally, FCCP (1 µM) was added to test for uncoupling. For this assay, we assessed $n=4$ rats per sex per treatment group

3.7.15 Functional aerobic capacity

The aerobic capacity of each animal group was determined by performing a maximal endurance test performed on Day 10, using a custom rodent treadmill and an established protocol³⁸¹. One day prior to the assay, the animals were placed on the treadmill at least 10 minutes with the motor turned on at 0 m/min to acclimate to the sounds and vibrations of the treadmill. On

the day of the experiment, the animals were placed on the treadmill at a low speed of 8 m/min for a period of 5 minutes in order to give the animals an opportunity to warm-up. Following this warm-up period, the speed was then increased to 12 m/min until exhaustion. The time from the warm-up to exhaustion was then recorded by participants blinded to the groups in which the rats belonged (single-blinded). The exhaustion was determined when the animal is at the base of the treadmill for more than 2 consecutive minutes.

3.7.16 Statistics

All statistical analysis was performed in GraphPad Prism version 10 software (GraphPad Software Inc.). As written in the appropriate figure captions within the manuscript, data are presented as mean $\pm$ standard error of the mean (SEM). Sample replicates are given within each subsection of the corresponding supplementary methods. Two independent groups were compared by unpaired Student's t-test and correlations were performed using Pearson coefficient. All tests were performed two-sided and statistical significance was considered for $p < 0.05$.

3.8 Supplementary Figures

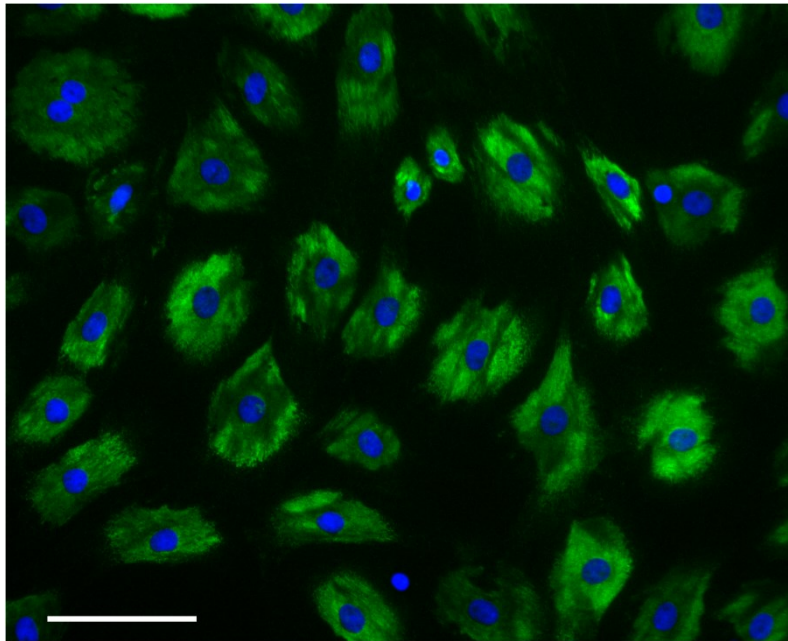


Figure 3.7: Neonatal cardiomyocyte isolation. Representative immunofluorescence microscopy image of isolated neonatal cardiomyocytes. Cells were stained for nucleus (blue), and cardiac troponin-T (green), a known marker for cardiomyocytes. Scale bar = 100 μ m.

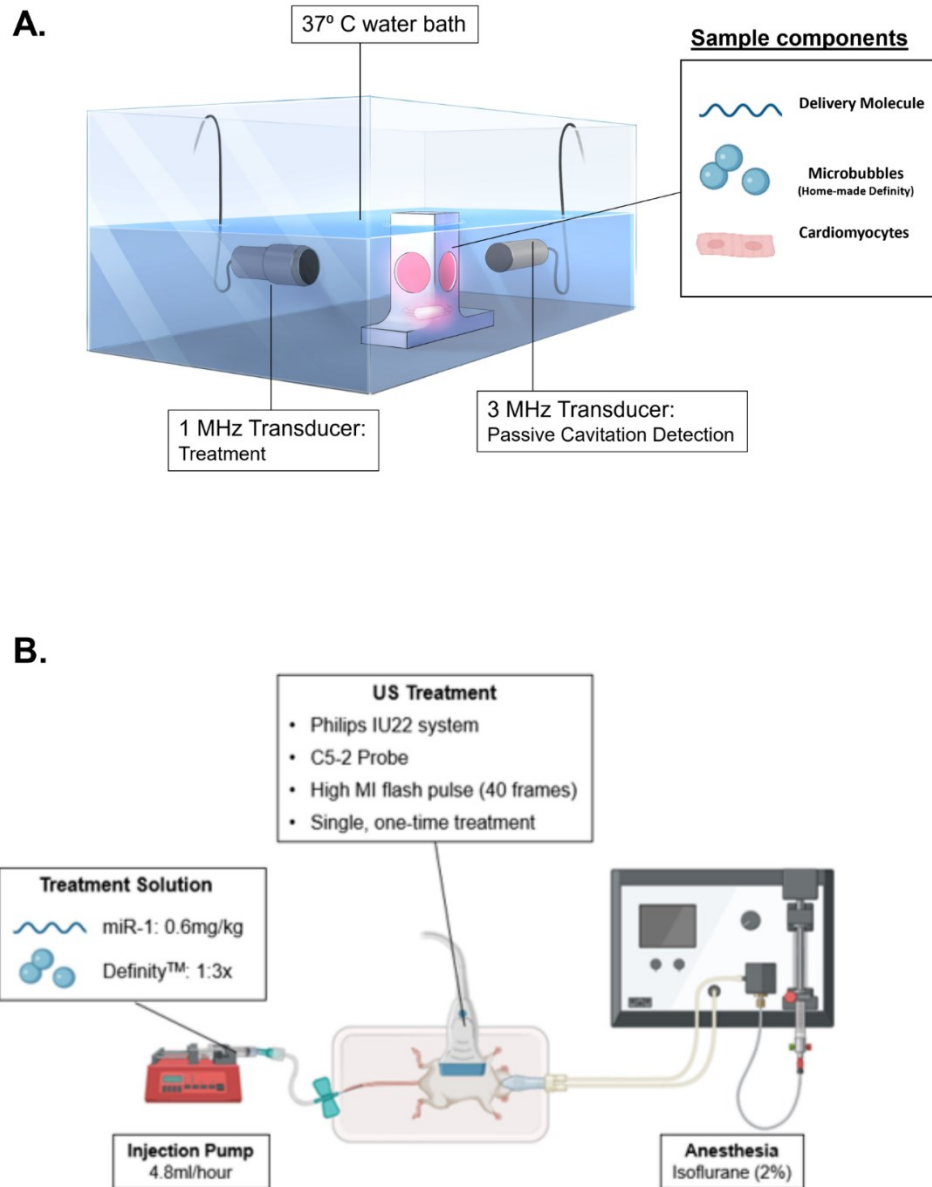


Figure 3.8: The experimental configurations for ultrasound-assisted miR-1 delivery. (A) Custom ultrasound tank for *in-vitro* cardiomyocyte treatment. This consists of two co-aligned single-element transducers for therapy (1 MHz) and passive cavitation detection. (B) *In-vivo* treatment set-up using a clinical ultrasound system. Here, we obtain a parasternal short-axis view of the LV using a C5-2 probe, which serves both as an imaging and therapeutic probe. See text for treatment parameter details.

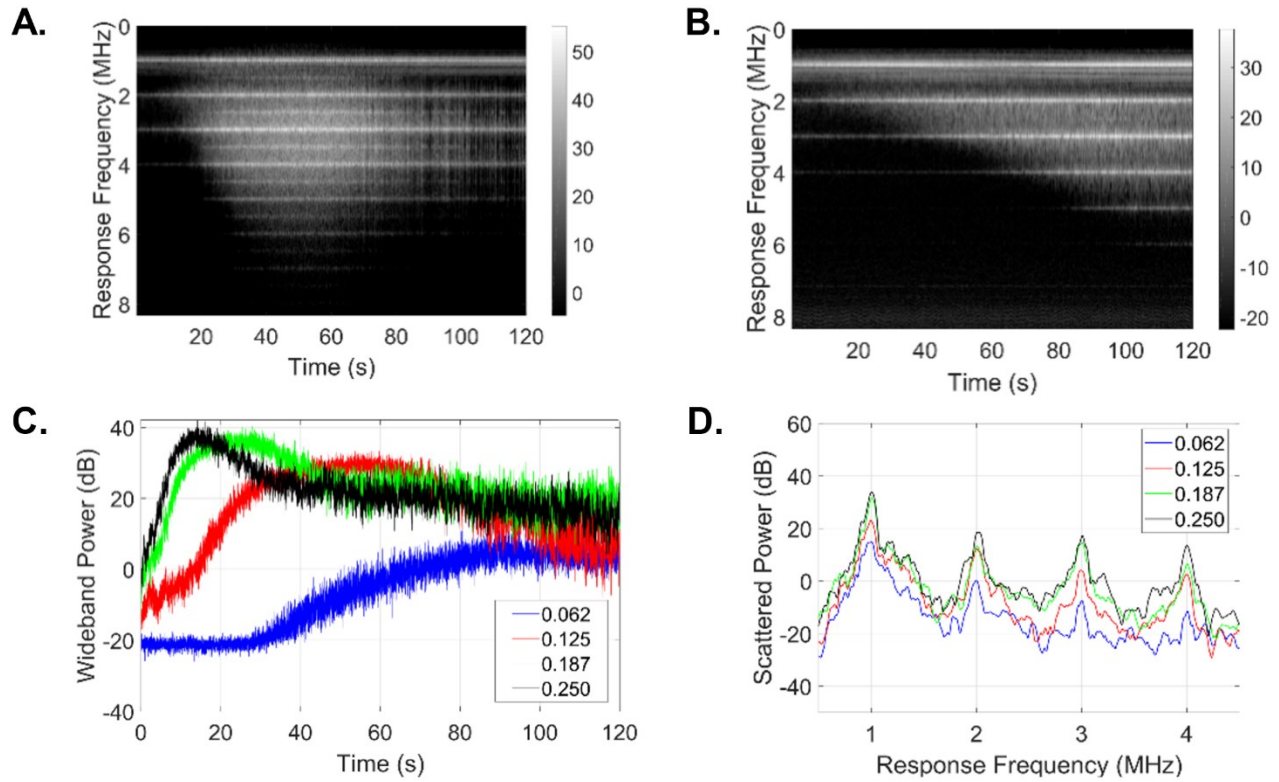


Figure 3.9: Real-time passive cavitation detection during in-vitro delivery of miR-1 to cardiomyocytes. Joint time-frequency analysis at an MI of (A) 0.062 and (B) 0.125, in which the response frequency of the microbubbles (y-axis) is plotted against the two-minute treatment time. Here, the grayscale encodes the power of the bubble signal in decibels. This time-frequency depiction clearly shows prolonged harmonic content (stable cavitation) along with the initiation of broadband spectral emissions (inertial cavitation). (C) Quantification of the cumulative spectral power from the inertial cavitation activity. (D) Representative example of the power spectra a few seconds into treatment (*i.e.* a single slice at a given time-point from a time-frequency plot).

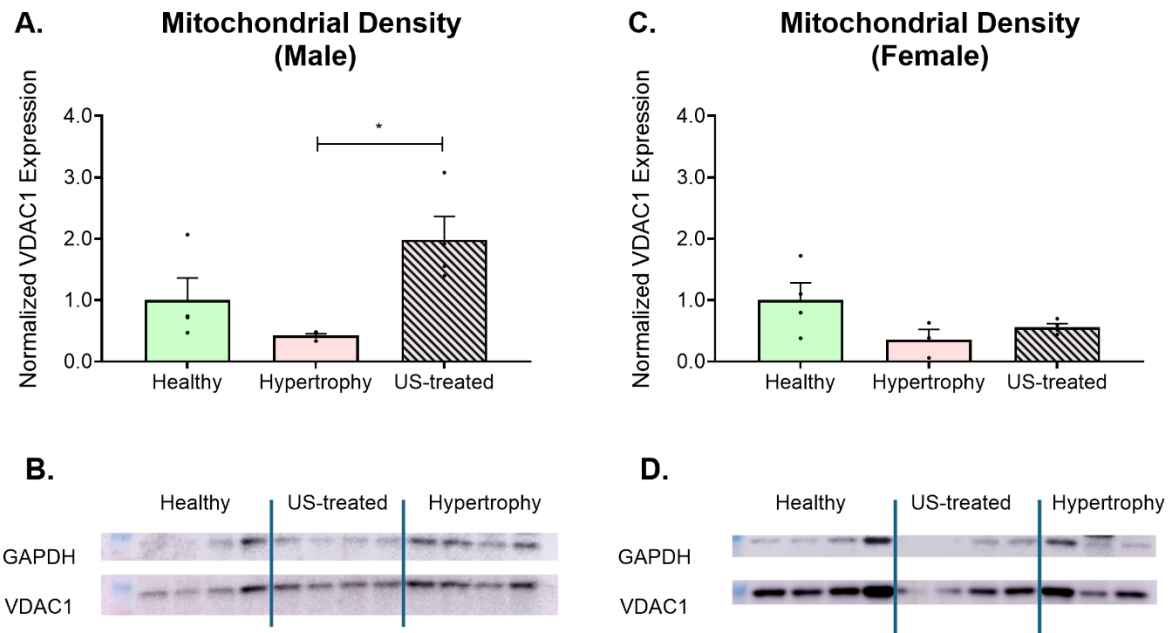


Figure 3.10: Sex-dependent expression changes in mitochondrial VDAC protein levels. Both (A,B) male and (C,D) female hypertrophy models demonstrate a significant decrease in mitochondrial content (pink bars) compared to control animals; US-assisted miR-1 delivery results in increased levels and rescue of VDAC in males, with no effect in females.

Chapter 4: Conclusion and future directions

4.1 Foreword:

In this thesis, I was able to demonstrate the feasibility of ultrasound and microbubbles as a method of delivering miR to cardiomyocytes *in-vitro* and to cardiac tissues *in-vivo*. Furthermore, I established the therapeutic potential of miR-1 in reversing hypertrophic phenotypes using a clinical ultrasound system. In the following section, I will summarize the broader implications of this work and provide a discussion on the limitations and future directions of this thesis.

4.2 Overview of thesis

Although current clinical treatment strategies for cardiovascular disease have improved patient outcomes, the mortality rate remains high. Gene therapy has emerged as a potential approach to overcome the limitations of the existing treatment. However, the success of these gene therapeutics is dependent on a delivery platform that could provide a targeted, non-invasive delivery to the cardiac tissue. Ultrasound and microbubble-mediated delivery address many of these challenges, offering an inherently image-guided and non-invasive strategy capable of performing repeated treatments without any risk to the patient. Though the progress in ultrasound delivery for cardiovascular diseases is not as clinically advanced as neurological applications, there is still a vast body of preclinical studies that have shown the feasibility of this technique. In Chapter 2, I provided a systematic overview of the *in-vivo* cardiac ultrasound-delivery studies performed to date, providing the opportunity to not only assess the state of the field but also quantify the effectiveness of the different techniques. From this analysis, I was able to plot general trends in

cardiac-mediated ultrasound delivery, gaining an understanding of parameters that may result in higher delivery efficiencies, such as animal model, microbubble dosing, and transmit frequencies.

In Chapter 3, I investigated the feasibility of miR-1 delivery to cardiomyocytes using ultrasound and microbubbles. Beginning with our *in-vitro* study, I utilized a custom ultrasound tank to treat the cardiomyocytes while simultaneously monitoring microbubble cavitation during delivery. With this experimental setup, I determined the optimal acoustic conditions required for viable delivery of miR-1 to both healthy and hypertrophic cardiomyocytes. The cavitation data obtained provided insight into the bubble physics during delivery, demonstrating a correlation between delivery efficiency and microbubble inertial cavitation (i.e., disruption). This finding highlights how contrast echocardiography can be used during treatments to provide real-time confirmation of delivery by using high-MI flash pulses that result in bubble disruption. Following this, I quantified treatment of hypertrophy through the decrease in hypertrophy-associated proteins (TWF1, MEF2A, CX43), recovery of cell size, and downregulation of disease-associated cytokines. This study not only demonstrates the delivery feasibility of miR-1 *in-vitro* but also establishes an experimental framework that can be applied in an *in-vivo* setting. Notably, the treatment of these hypertrophic phenotypes underscores the therapeutic potential of a miR-1 therapeutic strategy on a cellular level, supporting the translation to a pre-clinical model.

To build upon these findings, I transitioned to an *in-vivo* model, in which the complexities of a physiological system allowed us to investigate variables that cannot be tested in our *in-vitro* model. As a proof of concept, I began by optimizing ultrasound-mediated delivery in healthy rats. This resulted in a significant delivery of miR-1 to the heart, with minimal off-target expression in non-cardiac tissue. I then applied this approach to a hypertrophic rat model and observed recovery across multiple assays. These include molecular (decrease in hypertrophic cytokine and protein

expression, recovery in mitochondrial oxidation), cellular (decrease in cell size), physiological (decrease in LV thickness and heart-weight), and functional assays (recovery in fractional shortening and functional aerobic capacity). Importantly, these experiments were performed in male and female models to account for sex-dependent variation in the pathology of hypertrophy and the efficiencies in miR-1 therapy. Though our miR delivery efficiency is comparable between sexes, the reversion of the disease phenotype is more apparent in the male model, suggesting a sex-dependent regulation of functional miR.

Together, these findings demonstrate the feasibility of ultrasound and microbubble as a non-invasive, image-guided cardiac-specific delivery strategy and provide a translation proof-of-concept for miR-1 as a therapeutic strategy for pathological hypertrophy.

4.3 Limitations and future directions

Some of the limitations of our *in-vitro* study primarily revolve around the experimental setup. While I was able to treat the cardiomyocytes directly, the model does not account for several *in-vivo* variables, such as tissue attenuation of the ultrasound beam, the influence of blood flow, multicellular environment, and the efficiencies in extravasation. By transitioning to an *in-vivo* model, I was able to evaluate the changes in delivery efficiencies under physiological conditions to assess the impact of these additional variables.

Within our *in-vivo* hypertrophic model, the duration of phenylephrine administration was restricted by the infusion pump, meaning I could only perform an acute study of hypertrophy. Although the molecular and physiological phenotypes of hypertrophy are present within our experimental timeline, the acute nature of the model does limit our understanding of the long-term therapeutic potential of our treatment strategy. For instance, an acute hypertrophy model is unlikely

to present late-stage phenotypes of pathological hypertrophy, such as cardiomyocyte death and replacement fibrosis.

Therefore, this model does not allow us to evaluate the downstream consequences of the disease, such as the development of arrhythmia and the transition to eccentric hypertrophy, with the eventual development of HFrEF^{37,43,46,382}. To address these limitations, future works would incorporate a chronic pressure-overload model, potentially through the use of an extended duration infusion system. Such a model would enable the assessment of the capability of miR-1 therapy in the context of chronic hypertrophy remodeling, including the impact on myocyte viability, capillary density, regression of fibrosis, prevention of arrhythmias, and preserved systolic function.

Moreover, although the pharmacological induction of hypertrophy (through PE) initiates the same molecular pathways involved in pathological hypertrophy³⁸³, it does lack the hemodynamic burden associated with clinical presentations of pressure-overload hypertrophy. To replicate this, an aortic banding model could be utilized and would better replicate pressure overload conditions such as aortic stenosis, while also producing a chronic model of hypertrophy^{384,385}.

However, if the hypertrophic model were prolonged, it would likely require additional delivery of miR-1 in a multi-treatment regimen. This would elucidate some areas lacking in this study, such as the duration of miR-1 recovery and whether sustained recovery of hypertrophy is feasible after multiple miR-1 deliveries. Furthermore, perhaps I would also have the opportunity to exhibit a stronger recovery in some of our assays, such as the stress test, mitochondrial recovery, and cytokine regulation. Overall, this would provide a more thorough clinical perspective of this therapeutic strategy.

Although this thesis focuses on the therapeutic role of miR-1 on cardiac hypertrophy, it is well established that multiple miRs contribute to the development of this disease^{67,386}. There have been studies that have demonstrated the therapeutic applications of different miRs in response to cardiac hypertrophy (e.g. miR-133³⁸⁷, miR-10³⁸⁸, miR-672³⁸⁹). Therefore, a limitation of this work is that the contribution or therapeutic potential of these other miRs are not explored. Since pathological hypertrophy likely arises from a coordinated dysregulation of multiple miRs, a future study could investigate the delivery of a combination of miR and anti-miR, alongside miR-1. This strategy may be more efficient at restoring native miRNA expression in the diseased cardiomyocytes and thereby improve the overall therapeutic response to hypertrophy.

Alternatively, in familial hypertrophy, a single point mutation in the sarcomeric genes (e.g. MYH7, MYBPC3)³⁹⁰ can induce HCM. These mutations alter the sarcomeric function, disrupting contractility and triggering maladaptive remodeling. Although there have been studies that have utilized miR therapy to alleviate the phenotypes associated with HCM³⁹¹, they do not address genetic mutation responsible for the onset of the disorder. Emerging studies have demonstrated the applications of CRISPR-Cas9 gene editing as an alternative treatment strategy. By using this technique, single-point mutations are either directly repaired or inactivated, thereby providing a more permanent repair of these mutations. Recent preclinical work has demonstrated the AAV9-mediated delivery of Cas9 to correct for the MYH7 mutation, preventing pathological cardiac remodeling and fibrosis³⁹². Although there are still technical challenges remaining, these studies indicate an alternative to RNA-based therapeutics for genetic cardiomyopathies. Our future work could explore the feasibility of using ultrasound and microbubbles as a targeted method of delivering CRISPR-Cas9 to the heart for the treatment of HCM.

The use of a clinical ultrasound system for this study not only eliminates the need to develop a custom transducer system for treatment but also highlights the relative ease in translating this technique to a clinical setting. However, in this work, we are applying a human ultrasound probe to a small animal model, which has limitations. Some of these include suboptimal targeting and acoustic energy dispersion. Therefore, to address this, the next logical step would be to utilize an animal model with larger hearts, such as swine models. The size of the heart would ensure that the ultrasound beam targets solely the cardiac tissue, limiting exposure to nearby organs, and would enable more precise delivery to specific areas in the myocardium. The use of a larger animal model may also demonstrate a higher delivery efficiency, as seen from our analysis performed in Chapter 2 (Figure 2.3). This would address any underestimation of delivery efficiency from the murine model experiments relative to what might be achievable in a heart more closely matched in size to the intended clinical application. Overall, the use of a clinical ultrasound system may operate more efficiently on a heart that is more physiologically comparable to that of a human anatomy.

In our work, I have begun to explore the difference in therapeutic response on the basis of sex. Even with similar delivery efficiencies, the decrease in recovery indicates a difference in mechanism in response to miR-1. Given the substantial role of estrogen in the heart, our future work could further explore this by utilizing cycle-synchronized cohorts in order to determine whether the impact of this variable on treatment effectiveness³⁷⁴.

Finally, for a clinical perspective, I envision a multi-treatment regimen in which therapeutic delivery of miR-1 would be administered multiple times over a set period, as opposed to a single intervention. I do not anticipate miR therapy completely replacing existing clinical treatments for cardiac hypertrophy (pharmacological, exercise, dietary change)³⁴⁴. Instead, I propose a

complementary strategy, aimed at enhancing current therapeutic approaches. Moreover, depending on the underlying source of the hypertrophy (e.g., hypertension, aortic stenosis), I envision this as a combination approach using conventional clinical methods to halt the source of the hypertrophy, while miR therapy could be used in parallel to reverse the maladaptive remodeling in the heart.

4.4 Conclusion

In this thesis, I demonstrated the feasibility of ultrasound and microbubbles-mediated delivery of miR-1 to hypertrophic cardiomyocytes *in-vitro* and *in-vivo* models. I further showed the therapeutic potential of miR-1 for reversing hypertrophic phenotypes. Collectively, these findings establish ultrasound-mediated delivery as a promising non-invasive, real-time image-guided platform for cardiac gene therapy. The data collected provides translation insight for the clinical application of ultrasound-mediated delivery in treating cardiovascular disorders.

Appendix

Manuscript published as: He, S., **Singh, D.**, & Helfield, B. (2022). An Overview of Cell Membrane Perforation and Resealing Mechanisms for Localized Drug Delivery. *Pharmaceutics*, 14(4), 886. <https://doi.org/10.3390/pharmaceutics14040886>

5.1 Foreword

The permeabilization and subsequent resealing of the plasma membrane are critical events in ultrasound-mediated delivery, ensuring both high delivery efficiency and viability of the target cells. To provide a better insight into the biological processes involved, the following literary review was prepared alongside Dr. Stephanie He and Dr. Brandon Helfield. This review summarizes the physical techniques utilized to permeate the plasma membrane to enable intracellular delivery, and provides an overview of the cellular repair mechanisms involved in restoring membrane integrity, with an emphasis on the role of cytoskeletal remodeling.

5.2 Abstract

Localized and reversible plasma membrane disruption is a promising technique employed for the targeted deposition of exogenous therapeutic compounds for the treatment of disease. Indeed, the plasma membrane represents a significant barrier to successful delivery, and various physical methods using light, sound, and electrical energy have been developed to generate cell membrane perforations to circumvent this issue. To restore homeostasis and preserve viability, localized cellular repair mechanisms are subsequently triggered to initiate a rapid restoration of plasma membrane integrity. Here, we summarize the known emergency membrane repair responses, detailing the salient membrane sealing proteins as well as the underlying cytoskeletal remodeling that follows the physical induction of a localized plasma membrane pore, and we

present an overview of potential modulation strategies that may improve targeted drug delivery approaches

5.3 Introduction

Drug delivery techniques have revolutionized the field of precision medicine, helping to convert promising therapeutics into successful therapies³⁹³. The overall concept is to locally deliver high concentrations of therapeutics, either actively or passively, to the disease site and minimize off-target deposition. In so doing, the major limitations of systemic drug administration can be curtailed, including low solubility, poor biodistribution, unfavorable pharmacokinetics, and lack of selectivity³⁹⁴. Indeed, the major classes of therapeutic compounds, including small molecules, proteins and peptides, monoclonal antibodies, nucleic acids, and live cells, have all been incorporated into drug delivery systems and made significant contributions towards the treatment of disease⁴⁹. Despite this exciting progress, there remain significant challenges in designing drug delivery tools, most notably in maintaining therapeutic stability, target specificity, and penetration of biological barriers (e.g., cell membranes)³⁹⁵.

The current techniques for intracellular delivery can be broadly characterized into two subtypes: carrier-mediated delivery and membrane-permeating delivery^{49,394,396,397}. Carrier-mediated approaches rely on biochemical constructs, including drug-loaded nanoparticles, viral vectors, and extracellular vesicles, to overcome some of the limitations of naked drug delivery³⁹⁵. Systems based on nanoparticle or microparticle constructs have allowed the deposition of otherwise low-solubility drugs, enabled the trafficking of small molecules to their site of action, and increased drug retention in tumour sites³⁹⁸. Environmental modifications, including the addition of cell-penetrating peptides, can aid in plasma membrane penetration and endosomal escape⁴⁹.

Membrane-permeating strategies are physical methods that use an external force to puncture the cell membrane and allow direct access to the intracellular space, thereby bypassing the need to overcome the plasma membrane barrier and escape from early endosomes. However, unlike carrier-based methods, the target cells must respond in a timely manner to repair the temporary damage sustained to the plasma membrane^{399–401}. Individual eukaryotic cells can quickly repair their plasma membranes after injury through a sequential, highly localized process that restores internal homeostasis and prevents cell death^{400–402}. The physical perforation methods currently used for drug delivery use external forces of different origins, including electric fields, ultrasound, and light, and thus are expected to result in characteristically different pore dynamics, including the spatiotemporal coordination of the key components involved in wound repair.

While aspects of cellular membrane pore repair mechanisms have been previously reviewed, the current manuscript aims to link the physical methods of membrane perforation with membrane repair biomechanics and to identify techniques that may be implemented for the development of improved drug delivery systems. We first provide a brief description of the main physical methods employed for local or targeted drug/gene delivery into cells and discuss what is known about the spatial–temporal behaviour of plasma membrane perforations using these modalities. Following this, we provide a description of the subcellular and molecular events that restore bilayer integrity, highlighting the protein families implicated in membrane repair and the cytoskeletal-based mechanisms involved in pore resealing. Finally, we offer a discussion on the interplay between the fundamental study of wound repair on an individual cell level with targeted drug/gene delivery paradigms.

5.4 Overview of Physical Plasma Membrane Permeation Techniques

The physical disruption of the plasma membrane results from the spatially and temporally regulated deposition of energy. The following section provides a brief overview of the more common approaches to achieving this (Table 5).

Table 5. A summary of the salient features of the main physical methods used to generate plasma membrane perforations.

Method	Pore Features	Relative Advantages	Relative Disadvantages
Microinjection	Single pore of similar size to the fine-tipped glass micropipette (~200–1000 nm).	- Extremely efficient (~100%) - Precise control over payload concentration	- Very low throughput - Highly technical - Not applicable for in vivo drug delivery
Sonoporation	Pore radii ranging from sub-micron to ~10 μm . Single pore per bubble, with the possibility of multiple pores per cell.	- In vivo translatability - Drug/gene loaded constructs for added spatial targeting - Image-guided - Non-invasive	Highly dependent on ultrasound transmit and physical acoustic parameters
Electroporation	Pore radii generally < 1nm, with up to 10^9 pores per cm^2 .	- Very good efficiency - Efficient for ex vivo applications	- Semi-invasive procedure - Limited in vivo applications - Requires therapeutic co-injection
Photoporation	Pore radii ranging from ~10–2000 nm.	Very good efficiency, depending on the laser mode of operation	- Low throughput - Limited in vivo applications - Requires therapeutic co-injection

5.4.1 Microinjection

Perhaps the most direct and established technique for membrane permeabilization is microinjection using a fine-tipped micropipette, typically characterized by an outer diameter on the order of 200–1000 nm. Used to create a single membrane pore on a single cell for therapeutic delivery, this approach requires a precision translational stage and a micro-injector performed under a high-gain objective microscope ⁴⁰³. Since its original application over forty years ago, microinjection has been a reliable technique for delivering nucleic acid to the cell cytoplasm or directly into the nucleus, which bypasses cytoplasmic degradation. Microinjection is an extremely efficient method for a variety of payloads, irrespective of particle size and charge, including peptides, proteins, and oligonucleotides, and the exact number of DNA molecules can be precisely controlled. It is, however, a low-throughput technique that is best suited for specialty applications. Indeed, it is currently widely used to generate transgenic animals ⁴⁰⁴ through microinjection of a transgenic construct into the pronucleus of a fertilized egg (oocyte or zygote), including mice, pigs, goats, and cattle (e.g., ^{405,406}), and it is also used in forms of in vitro fertilization ⁴⁰⁷.

5.4.2 Sonoporation

One of the more recent techniques to increase plasma membrane permeability uses ultrasound energy. Biomedical ultrasound is widely employed as an imaging modality for anatomical assessment, as well as to provide information on blood flow characteristics. As an acoustic wave is transmitted into the body, reflections are generated at tissue interfaces and recorded to generate an image ⁴⁰⁸. Ultrasound contrast agent, which consists of a solution of encapsulated bubbles typically between 1 and 10 μm in size, gives rise to strong scattered echoes from the vasculature in which they are confined—much stronger than red blood cells ⁴⁰⁹. Contrast-enhanced ultrasound imaging is currently employed clinically in cardiology and radiology applications to improve the

delineation of vessel lumen and to enable the visualization of the microcirculation¹²⁷. Microbubbles vibrate within an ultrasound field, expanding and contracting about their resting size, and exhibit a rich variety of dynamic behaviours that are functions of the transmit conditions (acoustic frequency, peak-negative pressure, pulse duration, and duty cycle), the intrinsic bubble properties (size, shell characteristics, and constituents), and the local boundary conditions (vessel constraints)²⁰³. These behaviours range from stable and spherically symmetric vibrations to shape distortions, bubble fragmentation, and violent bubble collapse. It has been shown through numerical simulations and careful experimental investigations that microbubble oscillations create complex local fluid dynamic patterns; when situated adjacent to vessels, they can create local shear stresses that may ultimately modulate vasoactivity²¹⁴, vascular permeability²²⁷, and local cell membrane perforation⁴¹⁰. Indeed, a microbubble acts as a force actuator, focusing ultrasonic energy on the millimeter scale (typical wavelengths $0.75 \leq \lambda \leq 3$ mm) to micro-manipulate neighboring plasma membranes¹³¹ or generate an individual sub-micron- to micron-sized membrane perforation^{210,223}—a process termed sonoporation. Under the assumption that blood is a Newtonian fluid, the shear stress τ due to a vibrating microbubble of size R_0 can be estimated as

$$\tau \approx 2(\mu\rho)^{1/2}(\pi f)^{3/2}(\varepsilon R_0), \quad (1)$$

where μ is the fluid viscosity, ρ is the fluid density, f is the transit frequency, and ε is the maximum radial excursion of the bubble.

Among other design factors, recent work has demonstrated that microbubble proximity to the target cell is a key parameter in sonoporation efficiency, requiring distances on the order of a microbubble diameter or less between them⁴¹¹. Efforts to minimize microbubble–cell distances are currently being investigated, including the coupling of targeting ligands within the bubble encapsulation to promote site-specific microbubble accumulation (e.g., $\alpha_V\beta_3$ ⁴¹²), and novel

ultrasound pulse sequences to initiate microbubble translation towards neighboring cells using acoustic radiation force. Investigations employing static techniques post-treatment, including scanning electron microscopy and atomic force microscopy, reveal sonoporation-induced pore diameters ranging from 10 to 1200 nm in diameter^{133,413–415} and, depending on the acoustic conditions, broadly consistent with microscopy studies that infer these spatial scales from intracellular fluorescence tracer uptake dynamics interpreted via diffusion models⁴¹⁶. Real-time microscopy approaches that directly observe and quantify membrane perforation during sonoporation events^{223,417} have shown that these pores exhibit rapid opening timescales (< 1 min) and longer resealing timescales (> 1 – 10 min) and can resemble transmembrane apertures. Given the fundamental nature of sonoporation, that is, the generation of very-high-magnitude shear stress ($\sim kPa$) acting on very short timescales ($\sim \mu s$), the spatial–temporal characteristics of microbubble-assisted membrane perforation and their relationship to different cell types are not well understood.

Since the discovery of the potential for microbubble-mediated therapeutic delivery in the 1990s⁴¹⁸, there have been many investigations into sonoporation efficiency within the fields of cardiovascular disease, brain disorders⁴¹⁹, cancer⁴²⁰, and immunotherapy⁴²¹ that highlight the successful delivery of therapeutic macromolecules, plasmid DNA, mRNA, oligonucleotides, and associated viral vectors. Perhaps the simplest approach towards microbubble-mediated drug delivery is via a co-injection, whereby local therapeutic macromolecules migrate to the extravascular or intracellular space through sonoporation-derived perforations due to passive diffusion. Current pre-clinical and clinical trials using MR-guided microbubble-mediated blood–brain barrier disruption employ this technique for localized drug delivery²³⁸. Through advances in microbubble synthesis techniques, other platforms are being developed that incorporate therapeutic payloads into the bubble itself, including drug loading within the encapsulation

material and strategies that attach payloads to the surface of the microbubble shell (e.g., electrostatic interactions ⁴²² or nanoparticle linkage ²²⁸). For gene delivery applications, these constructs have shown an increased resistance to nucleic acid degradation within blood serum ¹⁴⁴ and thus exhibit a significantly longer half-life than otherwise unshielded gene approaches.

Given that microbubbles are natively designed and currently clinically approved for ultrasound contrast imaging, sonoporation- and ultrasound-microbubble-assisted therapies present an inherently image-guided in vivo approach to targeted drug delivery. These therapies fit many requirements of an ideal gene delivery platform, such as minimal procedural invasiveness, limited off-target deposition due to tight acoustic focusing and biochemical ligands, ease of repeated treatments, and a good safety profile in preclinical studies (see Table 5). Additionally, current advances in device development have introduced techniques for passively detecting and quantifying regions of microbubble-treatment in real-time for the purposes of treatment monitoring and quality control ²²⁴.

5.4.3 Electroporation

Electroporation is a technique whereby cellular membranes exhibit increased permeability to macromolecules when exposed to an external electric field. While the mechanisms are not yet fully elucidated, it is generally accepted that nanopores are generated within the plasma membrane upon exposure to high-magnitude electric fields of a given duration. Under physiological conditions, a cell maintains a potential difference across its plasma membrane of approximately -50 to -80 mV, in which its intracellular contents maintain a slightly negative charge compared to the extracellular environment ⁴²³. Under an applied external E-field E_{ext} , the induced transmembrane potential $\Delta\Psi_m$ across a cell membrane of effective radius R_c is generally given by ⁴²⁴:

$$\Delta\Psi_m = f_s E_{ext} R_c \cos \theta, \quad (2)$$

where θ is the polar angle between the normal vector of the electric field and the site on the membrane at which $\Delta\Psi_m$ is evaluated and f_s is a dimensionless term related to the electrical properties of the cytosol, plasma membrane, and the extracellular compartment, typically taken as $f_s \approx 1.5$ for most mechanistic studies⁴²⁵. To achieve enhanced cell membrane permeability, a transmembrane potential threshold on the order of $\Delta\Psi_m \approx 1\text{ V}$ is required, slightly dependent on cell type^{425,426}. The generation and characterization of these nanopores are dependent on the pulse parameters, including pulse height, width, and duration. Electroporation typically generates many pores within the plasma membrane, with theoretical estimates of pore density on the order of 10^9 pores/cm²⁴²⁷, the majority of which are $< 1\text{ nm}$ in radius⁴²⁸. The kinetics of the transmembrane transport that is achieved with this approach can be approximated in five stages: i) pore initiation ($\sim 0.1\text{--}1\text{ }\mu\text{s}$); ii) expansion ($\sim\text{ms}$), lasting as long as the pulse remains above the threshold value; iii) stabilization ($\sim\text{ms}$), a stable decrease in permeability while the pulse is turned off; iv) resealing (seconds to hours), the return to baseline permeability; and v) the gradual cessation of residual memory effects (hours), which refers to the observation that cells, even after full membrane resealing, still exhibit alterations in their physiological processes before returning to their equilibrium state⁴²⁹.

Similar to other types of physical permeation strategies, electroporation can be divided into two distinct types: reversible electroporation (RE), in which the nanopores are transient and the plasma membrane integrity is restored, or irreversible electroporation (IRE), in which the perforations do not reseal, resulting in cell death. Indeed, electroporation has been used successfully to introduce a variety of molecules into cells (e.g.,^{430,431}), including ions, drugs, RNA, micro-RNA, and DNA. While many studies have investigated the optimal parameters, a starting

point for conditions that achieve cell permeation with high cell viability (i.e., RE) is applying eight square waves of 100 μ s in duration at a frequency of 1 Hz and an amplitude of 1.2 kVcm⁻¹—with the recognition that other factors, including cell size (Equation (1)), temperature, and the desired therapeutic agent, may play a role in these applied parameters ⁴²⁵. Ex vivo applications typically involve blood cells treated outside the body and then reintroduced to provide therapeutic benefits. Electroporation has been employed on stem cells ⁴³², to introduce chimeric-antigen receptor genes in T cells ⁴³³, and to modify red blood cells ⁴³⁴. For in vivo applications of this technique, naked injection of the therapeutic into the target tissue is required prior to the application of the external E-field. The E-field is generated via electrodes placed in direct contact with the tissue, and therefore target regions are limited to those that both the therapeutic and the electrodes can access safely. This being said, in vivo electroporation has been demonstrated in the liver, bladder, brain, muscle, and skin (e.g., ^{435,436}).

It is important to note that recent works have investigated IRE as a primary, desired endpoint ⁴²³. As a non-thermal tissue ablation modality capable of treating clinically sized volumes of tissue, under certain conditions, this approach allows for the preservation of collagenous and other protein/lipid-based structures, including the vasculature ⁴³⁷. This relative advantage over other ablative approaches, as well as its relatively short treatment time requirement, has motivated investigations into many soft-tissue cancer types (e.g., ^{438,439}), including pancreas, prostate, liver, lung, and brain—resulting in more than 50 clinical trials since its inception over a decade ago ⁴²³.

5.4.4 Photoporation

Photoporation, otherwise referred to as optoporation, is a technique in which highly focused light is the source of membrane perforation. In this technique, a laser beam is typically focused on a spot with a size on the order of 0.5–1 μ m by a high numerical aperture microscope objective lens

to the plasma membrane of a cultured monolayer. Photoporation has been demonstrated using continuous-wave light exposure, as well as pulsed laser modes, including pulse durations in the millisecond, nanosecond, and femtosecond timescales (e.g., ⁴⁴⁰). Modifying the operating mode of the laser and its physical characteristics, such as wavelength and energy density, alter the physical and chemical mechanisms for induced cell membrane perforation ⁴⁴¹. Continuous-wave operation likely relies on heat deposition to induce membrane perforation and is often performed in the presence of an absorbing dye in the culture media. Although it causes perforation on a single cell level with high resulting cell viability, the pores generated by continuous-wave approaches are not as efficient as other modes ⁴⁴⁰. Pulse laser sources with very high irradiances (e.g., $10^{10} - 10^{12}$ Wcm⁻²) locally generate large E-fields ($10^6 - 10^7$ Vcm⁻¹) compared to the average intramolecular Coulomb fields, resulting in the breakdown of target molecules in the focal region ⁴⁴². With slightly longer pulses in the nanosecond range, this can be accompanied by heating, bubble formation, and thermoelastic stress ⁴⁴³ that can expand the spatial scale of perforation to tens of microns ⁴⁴⁴. Pore sizes generated by this technique range from ~10 to 1000 nm, depending on the parameters of the laser source ⁴⁴⁵. Currently, the most widely adopted approach to optoporation is the use of femtosecond lasers (~<200 fs pulse durations), typically at 800nm ⁴⁴⁰. Since the original demonstration over thirty years ago to deliver DNA into rat kidney cells ⁴⁴⁶, a wide array of membrane-impermeable substances have been delivered in this way with high cell viability, including dyes, nanoparticles, DNA, and mRNA (e.g., ^{447,448}). Additionally, photoporation can be achieved in combination with gold nanoparticles to increase the likelihood of perforation for a given set of input conditions (e.g., ⁴⁴⁹), presumably due to the local amplification of the E-field ⁴⁴¹. Optoporation provides a means to robustly perforate cell

membranes; however, it is mostly restricted to *in vitro* applications due to the low penetration depth *in-vivo* and the requirement for complex optical setups.

5.5 Plasma Membrane Repair Mechanisms

Pore generation within the plasma membrane launches an immediate cellular response to restore homeostasis and preserve cell viability. In this section, the known mechanisms of cell membrane repair are described (Figure 5.1) and summarized in Table 6.

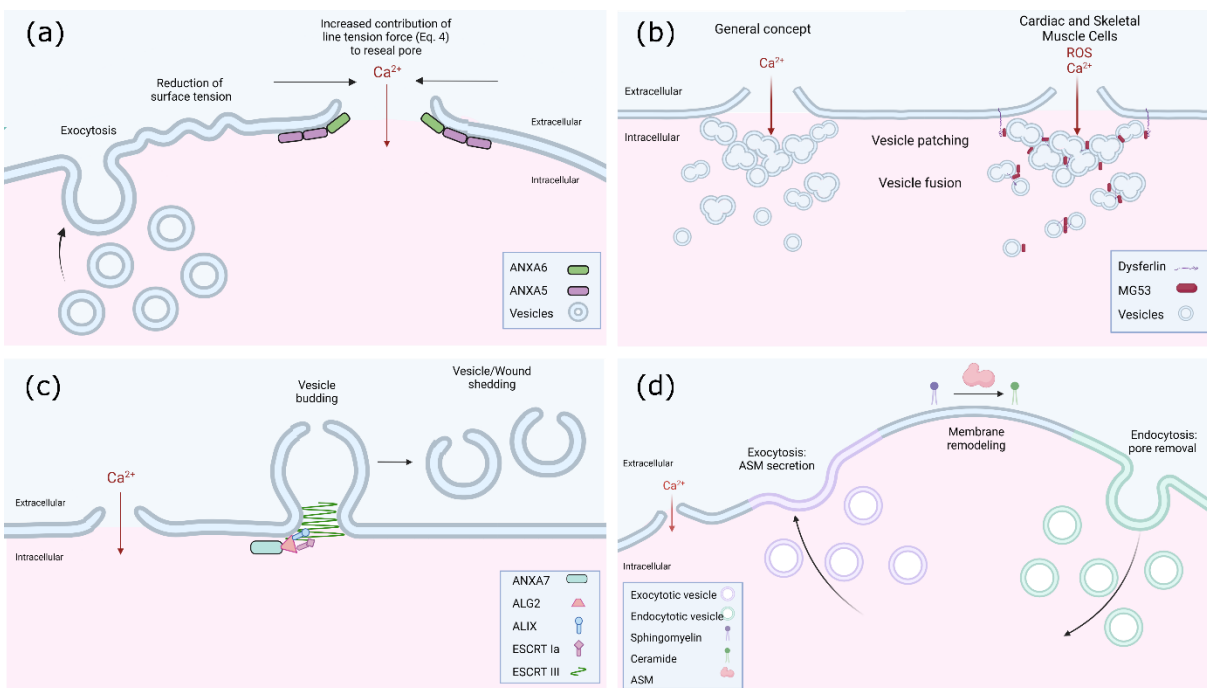


Figure 5.1. Summary of known plasma membrane resealing mechanisms. (a) The tension–reduction hypothesis; (b) the patch hypothesis; (c) the ESCRT mechanism; and (d) exocytosis/endocytosis. See text for details.

Table 6. A summary of the key proteins involved in plasma membrane repair. See text for details.

Protein Family	Role	Ca ²⁺ Binding	Estimated Pore Sizes in Which Proteins Have Been Observed	Suggested Plasma Membrane Repair Mechanism(s)
Annexins	Play a role in membrane patching, fusion, reshaping, reducing membrane tension, removing damaged membrane, limiting pore expansion	Yes	~nm- μ m ⁴⁵⁰⁻⁴⁵²	Patch, Tension Reduction, Exocytosis/Endocytosis, Membrane Budding (A7 ⁴⁵³)
SNARE proteins	Mediates fusion of membranes	No		Patch, Tension Reduction,
SYT7	Helps activate SNAREs	Yes	0.5–3 μ m ^{454,455}	Exocytosis/Endocytosis
S100A11	Implicated in membrane and cytoskeletal dynamics, interacts with A2	Yes	0.5–1.2 μ m ^{450,456}	Tension Reduction ⁴⁵⁷
Dysferlin	In muscle cells, accumulates at the site of membrane damage, interacts with some annexins, MG53, BIN1, EHD1/2	Yes	nm scale (Cai, Weisleder, Ko, Komazaki, et al., 2009a), μ m scale ⁴⁵¹	Patch ⁴⁵⁹ , Exocytosis/Endocytosis
MG53	In muscle cells, it is tethered to plasma membrane and intracellular vesicles and, upon ROS stimulus, oligomerizes and accumulates at wound sites	No	nm scale ⁴⁵⁹	Patch ⁴⁵⁹ , Tension Reduction ⁴⁶⁰
ESCRT-III	Involved in membrane budding	No		
ESCRT-I	Recruits ESCRT-III	No	<100 nm ⁴⁶¹ , >1	
ALIX	Recruits ESCRT machinery	Yes	μ m ⁴⁶²	Membrane Budding
ALG-2	Recruits ALIX	Yes		
ASM	Outer plasma membrane remodeling to initiate inward vesicle budding. Converts sphingomyelin into ceramide	No	nm scale ⁴⁶³	Exocytosis/Endocytosis

5.5.1 Physical Intuition

As a first approach to understanding the dynamics of membrane pore resealing, it is perhaps instructive to consider the flux through a circular pore in the absence of any biological wound

response. Assuming the cytoplasm is a simple fluid leaking out of a single pore of radius r_p , the flow per second normalized to initial cell volume $\tilde{Q}$ is given by the following relation ⁴⁶⁴:

$$\tilde{Q} = \left(\frac{\Delta P}{4\pi\eta_0} \right) \left(\frac{r_p}{R} \right)^3, \quad (3)$$

where ΔP is the pressure difference across the membrane, R is the effective cell radius, and η_0 is the cytosolic viscosity. Assuming $\Delta P \approx 10$ Pa, an intracellular viscosity range from $\eta_0 \approx 1$ to 200 times that of pure water, and a pore size of 10% of the cell size, the leakage rate is on the order of 80%-84% of cell volume per second. From such a purely physical analysis, the necessity of viable cell membrane resealing occurring within seconds to minutes is clear. Membrane pore resealing times, quantified as a cessation of intracellular influx, have been reported over an array of input sources (e.g., ultrasound and microinjection) and across different cell types to range on this timescale^{222,223,465–467}.

5.5.2 Repair Triggers

It is well established that calcium ions are involved in a plethora of signaling pathways and cellular processes and, as such, intracellular Ca^{2+} concentration is well regulated. Due to the 10,000-fold gradient maintained across the plasma membrane ⁴⁶⁸, a localized breach of the plasma membrane results in an immediate calcium ion influx and is considered the universal trigger that launches the mechanisms of perforation repair ⁴⁰¹. Indeed, it presumably dictates the magnitude of plasma membrane repair as entry levels are approximately correlated to pore size, duration, and/or density, while an excessive level of intracellular Ca^{2+} is cytotoxic ⁴⁰⁰. Previous works have demonstrated that membrane resealing timescales increase in low Ca^{2+} environments and fail to reseal in the complete absence of extracellular Ca^{2+} ^{400,469,470}. Calcium entry through membrane perforations selectively activates Ca^{2+} -dependent cellular responses depending on both the local

Ca²⁺ concentration and the relative affinity of Ca²⁺ binding proteins ⁴⁷¹. This enables cells to mount a spatially and temporally regulated response to steep Ca²⁺ influx ⁴⁵⁷. The extracellular Ca²⁺ concentrations required for successful resealing have been reported in the μ M to mM range ⁴⁷², depending on the cell type and the wound generating mechanism, and tolerable increases in intracellular Ca²⁺ also vary between cell types. This highlights that tight control over Ca²⁺ dynamics is required to maintain cell viability following membrane perforation.

Oxidation at the plasma membrane is another trigger that initiates repair mechanisms in skeletal and cardiac cells ⁴⁷³. Previous studies conducted on myotubes have elucidated the role of the protein Mitsugumin 53 (MG53) in muscle cell repair⁴⁵⁹. In the presence of a reducing agent in the extracellular environment, the accumulation of MG53 is hindered compared to the addition of an oxidizing agent, which increases the rate of MG53 accumulation at the site of cellular injury. MG53 also interacts with dysferlin to be translocated to the region where the concentration of free radicals is highest to seal the pore in a “patching” manner, typically within a minute after membrane injury⁴⁵⁹. The role of MG53 in membrane fusion, budding, and exocytosis is modulated by muscle-specific caveolin-3 (Cav3) for proper sarcolemma repair during muscle contractions and differentiation ⁴⁵⁹.

5.5.3 Plasma Membrane Repair Hypotheses

A breach of the plasma membrane disrupts the tension sustained by the lipid bilayer. Nanosized pores are frequently and transiently formed on cellular membranes as the cell naturally synthesizes organelles, moves, or contracts ⁴⁷⁴. In these cases, the lipid bilayer can reseal these pores without requiring a cascade of proteins. Given this, there have been many experimental and theoretical investigations of wound healing on pure lipid vesicles, whereby the passive pore-

opening and resealing dynamics are due to the force balance per unit length between two opposing forces:

$$F = 2\sigma(r_p) - \frac{\lambda}{r_p}, \quad (4)$$

where $\sigma(r_p)$ is the plasma membrane surface tension that acts to pull the pore of radius r_p open, and λ is the line tension that is associated with the energy penalty of having exposed hydrophobic lipids along the pore perimeter and acts to close the pore. The relatively simplistic model given above predicts that pore size, opening, and resealing times are a function of lipid composition and cell viscosity ^{464,475}.

Early experiments in sea urchin cells and mammalian cells revealed the localized fusion of intracellular vesicles with the plasma membrane, leading to two fundamental hypotheses for wound resealing ⁴⁰¹: (i) the ‘tension reduction’ hypothesis stipulates that the excessive membrane surface area delivered via the exocytosis of pre-existing intracellular vesicles serves to rapidly decrease the membrane surface tension to promote wound closure (see Equation (4)); and (ii) the fusion of pre-existing vesicles in the vicinity of the pore, including lysosomes, create a ‘patch’ that merges with the plasma membrane to seal the wound. Although the mechanisms underlying plasma membrane repair in mammalian cells are not fully elucidated and may differ between cell types and the source of the perforation, increasing evidence suggests that annexins are one of the first to be recruited to the wound site ⁴⁵³. The annexin family, consisting of twelve proteins (A1–A11, A13), is made up of phospholipid-binding proteins that are triggered to migrate from the cytosol to the plasma membrane under local increases of Ca²⁺ concentration as early as 10–45 s post-perforation ⁴⁷⁶. As each of these proteins exhibits its own Ca²⁺ sensitivity threshold, this family presents a broad Ca²⁺ sensing mechanism that responds dynamically during a wound repair event. There is evidence that annexins play a role in the immediate ‘patching’ of the perforation in

an attempt to minimize cytosolic loss and intracellular Ca^{2+} increase. Annexin protein A5, when recruited to the perforation site, has been shown to form a 2D protein array to temporarily delay diffusion and limit wound expansion ⁴⁷⁷. Annexin protein A4 binds to the plasma membrane adjacent to the opening and changes conformation to reshape the lipid bilayer, while annexin A6 constricts the wound to prevent it from expanding and promote closure ⁴⁵². Synaptotagmin 7 (SYT7), a member of the synaptotagmin protein family, is a Ca^{2+} -sensing protein present on the membrane of lysosomes that plays a critical role in lysosomal fusion and membrane patching, as its inhibition leads to impaired membrane repair ⁴⁷⁸. Further, SYT7, among other members of its protein family, regulates the formation of soluble N-ethylmaleimide sensitive factor attachment protein receptor complexes (SNARE), which are a large group of small proteins that are key mediators of all intracellular membrane fusion events⁶⁵. As opposed to SYT7 acting as a Ca^{2+} -dependent modulator of membrane fusion, another Ca^{2+} sensor, ALG-2, modulates membrane fission machinery to mediate pore repair. ALG-2 recruits important membrane-trafficking scaffold proteins, including ALG-2 interacting protein X (ALIX), which then recruits components of the endosomal sorting complex required for transportation (ESCRT) machinery ⁴⁰⁰. ESCRT-III, normally associated with its role in multivesicular body biogenesis, has been shown to translocate to the wound site through the recruitment of ALIX, ESCRT-1a, and ESCRT-II ^{453,461,479}. Indeed, under specific perforation conditions, a lack of ALG-2 or ALIX results in failed membrane repair ⁴⁶². The ESCRT machinery has, therefore, recently been suggested as a third alternative mechanism in addition to the patching and membrane tension reduction hypotheses, in which membrane lesions are actively removed through the formation of vesicles outwards from the damaged membrane—the shedding of membrane buds ^{461,480}.

While lysosome fusion was originally only thought to contribute to membrane patching, recent observations of massive Ca^{2+} -dependent endocytosis following lysosomal exocytosis⁴⁸¹ have revealed a fourth hypothesis for membrane pore repair: the ‘exocytosis/endocytosis’ pathway. The fusion of lysosomes to the site of injury promotes the release of lysosomal enzyme acid sphingomyelinase (ASM), which remodels the outer leaflet of the plasma membrane. This novel form of endocytosis, which takes place within seconds of membrane injury, has been shown to be induced by the modification of plasma membrane lipid sphingomyelin into ceramide⁴⁸², resulting in the formation of large domains capable of inward budding⁴⁸³.

It should be stated here that, although presented as separate mechanisms, it is possible that multiple approaches to plasma membrane repair occur simultaneously and synergistically. For example, ESCRT-III recruitment to the cell membrane has been observed when membrane tension is low⁴⁷⁹, and lysosomal (and other intracellular vesicles) fusion can contribute to all pathways. The threshold perforation size for these mechanisms is not yet fully elucidated but likely plays a role in the extent to which one mechanism is favoured over others.

5.6 Cytoskeletal Remodeling during Perforation

In the context of physical permeation strategies, cell membrane perforation often results in a local disruption of the actin cortex. As a consequence, pore recovery depends on the spatial–temporal coordination between key protein families involved in membrane recovery, cytoskeletal architecture, and vesicle fusion. The following section outlines the salient proteins involved in these processes and is summarized in Table 7.

Table 7. A summary of the involvement of cytoskeletal remodeling post-pore formation. See text for details.

Cytoskeletal Remodeling	Main Proteins Involved	Estimated Pore Sizes in Which Proteins Have Been Observed
Contractile Ring 580	Actin, myosin II, GTPases (Cdc42, Rho), Arp2/3,	~ μm scale 524,527
S100A11-A2 535	S100A11, annexins A1, A2	0.5–1.2 μm 535,537
Repair Cap 574,581	Annexins A1, A2, A5 and A6, dysferlin, EHD1/2, MG53, BIN1	~ μm scale 574
Exocytosis/Endocytosis (e.g., 540)	Myosin family, kinesin, actin, microtubules, GTPases, formins, SNARE complexes	

5.6.1 Initial Reaction: Deconstruction of Actin Network

Local Ca^{2+} influx due to membrane perforation activates cytoskeletal-remodeling proteins, such as INF2 and calpain protease, that allow for the disassembly of the local cortical actin⁴⁸⁹. This disassembly helps reduce membrane surface tension and improves access for intracellular vesicles to fuse with the plasma membrane⁴⁰². Indeed, studies have shown that the presence of actin depolymerization agents, such as DNase 1, acts to enhance the reparation of damaged cell membranes, while actin stabilizing agent results in a decrease in the resealing rate⁴⁹⁰. Further, this disassembly has been shown to occur for small and large membrane lesions⁴⁹¹ and is likely a requirement irrespective of perforation size. Similar to the actin network, the microtubule network also undergoes a brief period of local disassembly at the damaged site⁴⁹². The perforation pathway triggers the recruitment of the microtubule-associated protein 1 (EB1), which both promotes the re-assembly of microtubules and facilitates the transport of lipids to the wound area⁴⁹³. Indeed, given that directional vesicle transport to the site of perforation is controlled by myosin and kinesin

activity through the reorganized elongated microtubules⁴⁹⁴, a rapid repair and remodeling of the breached cytoskeletal architecture is required for viable perforation repair.

5.6.2 Resealing and Remodeling: Actomyosin Contractile Ring

One such remodeling mechanism is the formation of an actomyosin ring that has been shown in multiple models, including *Xenopus* oocytes and *Drosophila* embryos⁴⁹⁵. For this process, two elements of the cytoskeleton are required: actin and myosin II. These are both recruited to the wound edge, assemble as contractile arrays surrounding the perforation, and continuously contract throughout the repair process. The spatial–temporal regulation of the actomyosin ring is regulated by Ca²⁺-dependent Rho GTPases, specifically Cdc42 and RhoA⁴⁵⁷. Indeed, the Rho family of GTPases⁴⁹⁶ plays a major role in cytoskeletal regulation and, consequently, is involved in cell migration, adhesion, cytokinesis, and perforation repair. GTPases act as molecular switches that can modulate signal transduction pathways in response to a specific stimulus. Activated RhoA accumulates in a ring around the perforation area (i.e., a RhoA activity zone) and spatially overlaps with myosin II, while the concentric Cdc42 activity zone overlaps with the actin ring⁴⁹⁷. Myosin II is recruited by the RhoA activation of Rho-associated kinase (ROK)⁴⁸⁶, and the Cdc42 activity zone is responsible for the recruitment and polymerization of branched actin filaments through downstream effectors, such as N-WASP and p21-activated kinases, that control the Arp2/3-dependent actin assembly⁴⁹⁷. Overall, the formation of these zones has been shown to occur within 30–45 s in *Xenopus* and *Drosophila*⁴⁹⁷ and has been shown to be active for the repair of wounds within the micrometer range^{489,498}. Microtubules are also shown to be essential in the recruitment of Arp2/3 and myosin II and help focus the zone of actin and myosin II assembly at the wound edge⁴⁸⁵. Together, these proteins create an actomyosin cable around the wounded area that is coupled to the plasma membrane by junction proteins such as E-cadherin and B-catenin. In E-

cadherin-deficient cell models, wound overexpansion and improperly formed actomyosin rings are observed, yet complete wound repair remains possible, suggesting that other proteins are involved in the tethering of the actomyosin ring to the plasma membrane ⁴⁹⁹. The circular constriction shortens the actomyosin cable that pulls the membrane closer together to close the wound and is often referred to as the ‘purse-string’ mechanism. This mechanism is also seen in multicellular epithelial models, in which the Rho GTPase and ROCK are essential for the assembly actomyosin ring intracellularly between the nearby cells ⁵⁰⁰. This actomyosin ring is then anchored to the membrane through adherence junction and tight junction and ZO-1 ⁵⁰¹, while constriction is initiated by the phosphorylation of myosin regulatory light chain (MLC) ⁵⁰².

Though this repair model has been fully explored and described in embryonic mammalian models and multicellular epithelial models, the formation of the contractile ring is still yet to be seen in single somatic cells⁵⁰³. However, even though the ring itself is not seen, individual components are still shown to play a major role in cytoskeletal restructuring for membrane repair. Cdc42 and Rho, for example, are shown to be translocated from the cytosol to the membrane for cytoskeletal reorganization in shear-stress-damaged bovine aortic endothelial cells⁵⁰⁴. In laser-wounded skeletal muscle cells, RhoA activity is induced by Ca²⁺ influx⁵⁰⁵, and F-actin accumulation at the wound site has been shown post-perforation in human endothelial cells²²² cancer cells⁴⁵⁰, and muscle cells⁵⁰⁶.

5.6.3 Resealing and Remodeling: S100A11-A2

In somatic cell models, another cytoskeleton remodeling mechanism has been demonstrated that utilizes the Ca²⁺ binding protein S100A11 and annexin A2. These proteins have been shown to be active in wounded mammalian cells, such as in cancer cells and vascular endothelial cells ^{450,456}. With the influx of extracellular calcium, the EF hand-type protein, S100A11, is activated

and binds to F-actin along with annexin A2, which is also capable of Ca^{2+} -dependent membrane binding⁴⁵⁰. The binding of these proteins to the cortical actin of the wounded membrane restricts the depolymerization of the F-actin while also promoting the buildup and increase in the polymerization of actin at the wounded zone⁴⁵⁰. The increase of cortical F-actin results in wound closure, and it is suggested to be related to the purse-string closure mechanism due to the buildup of cortical actin being analogous to the actin activity in wounded *Xenopus* oocytes⁴⁵⁰. Concurrently, annexin A1 accumulates on the damaged region of the membrane and labels it for excision⁴⁵⁰. In combination, the S100A11-A2 complex is able to bind to the membrane and pull it closer together, while annexin A1 removes damaged membranes. The necessity of this complex was shown in S100A11- and A2-depleted endothelial cells, in which pore resealing was either delayed or failed completely in both laser- and glass-beads-induced membrane damage models⁵⁰⁷. Overall, these components provide an alternate cytoskeletal remodeling mechanism for wounded membranes.

5.6.4 Resealing and Remodeling: Repair Cap

In muscle cells, another mechanism referred to as a “repair cap” is shown to occur post-membrane-wounding^{506,508}. In this instance, an influx of Ca^{2+} results in the translocation of annexins towards the wounded membrane, particularly annexin A1, A2, A5, and A6; these accumulate and form a “cap” along the damaged region of the plasma membrane. This annexin-rich cap is supported both by a “shoulder” structure, shown to be essential for the repair cap formation, and consisting of proteins including dysferlin, EHD1/2, MG53, and BIN1^{400,506}, and by F-actin recruitment to the non-annexin region below the cap. The formation of the repair cap is shown to be both Ca^{2+} -and actin-dependent, demonstrating the necessity of cytoskeleton remodeling for this membrane repair mechanism⁵⁰⁹.

5.6.5 Resealing and Remodeling: Exo/Endocytosis Events

All of the aforementioned plasma membrane repair pathways include steps that involve membrane fusion, exocytosis, or endocytosis, and in this manner the cytoskeleton plays a vital part in perforation repair (Table 7). All of the highly regulated steps of exocytosis, including the shuttling of vesicles to the peripheral membrane, vesicular fusion with the plasma membrane, and the release of vesicular content, are regulated by the actin cytoskeleton⁴⁸⁸. As exocytic vesicle size varies as a function of secretory cell type ($\sim 0.01\text{--}1\text{ }\mu\text{m}$), so too does the timescale over which exocytosis occurs ($\sim 0.1\text{--}100\text{ s}$), exhibiting an inverse relationship⁴⁸⁸. Vesicle transport along actin filaments requires actin-based motor proteins such as kinesin or members of the myosin V family⁴⁹⁴. While the molecular details differ between different vesicles, myosin Va is associated with several vesicles and has been shown to play a major role in this regard (e.g., endothelial cells⁵¹⁰ and neurons⁵¹¹). After membrane fusion by SNARE complexes⁵¹², it has been suggested that the final expulsion of the vesicles utilizes an actin coat/ring at the base of the vesicle⁵¹³. This mechanism is shown in laser-ablated HUVECs, in which Ca^{2+} influx results in the exocytosis of Weibel–Palade bodies, secretory organelles involved in the initiation of inflammation. In this model, Rho GTPases result in the recruitment of an actin ring at the base of the vesicle, while the contraction is enabled by myosin II-mediated constriction⁵¹⁴.

As stated above, the exocytosis-mediated release of ASM has been shown to result in a ceramide-coated membrane, ultimately triggering membrane invagination⁵¹⁵. Indeed, studies have demonstrated that the transcriptional silencing of ASM inhibits membrane repair, while adding exogenous ASM can cause it to recover⁴⁸². Recent work has suggested that the form of endocytosis that is stimulated by this mechanism is caveolin-dependent⁵¹⁶, implying that the usual role of the cytoskeleton in caveolin-mediated endocytosis is at play during perforation resealing under this pathway.

5.7 Future Perspectives

The investigation of individual cell wound repair mechanisms in the context of targeted drug and gene delivery can offer tremendous insight into the development of strategies designed to improve treatment outcomes through the modulation of perforation kinetics. This is especially of interest in optimizing in vivo techniques (e.g., sonoporation), whereby elucidating the mechanisms of perforation repair in human cells can aid in treatment design and planning. These strategies, whether physical or pharmacological, have applications both in targeted genetic delivery techniques (e.g., ischemia and cardiovascular disease) where preservation of cell viability is paramount and in cancer-related treatments where perhaps immediate and selective cancer cell death is the primary objective. Perhaps the most universal approach to altering the repair kinetics of individual cells is the administration of a Ca^{2+} chelator (e.g., BAPTA-AM) either during or immediately post-perforation⁵¹⁷. As the key trigger and regulator of membrane repair, the local modulation of Ca^{2+} influx affects the spatial and temporal evolution of wound repair, potentially altering perforation size, repair timescale, and long-term cell viability via the activation/inhibition of Ca^{2+} -dependent cellular responses⁵¹⁸. The depletion of extracellular Ca^{2+} also induces the dissociation of intracellular junctions⁵¹⁹, including adherens and tight junctions, promoting paracellular drug transport. This may have a particular impact in aiding the targeted delivery of therapeutics to the brain via the reversible opening of the blood–brain barrier⁵²⁰, an area in which microbubble-mediated focused ultrasound therapy, in particular, is rapidly progressing⁴¹⁹. Further, there are novel advancements in the field of nanoparticle synthesis designed to modify the Ca^{2+} environment and affect local Ca^{2+} homeostasis. These techniques incorporate materials that are either Ca^{2+} -coated⁵²¹ or Ca^{2+} -binding⁵²² to influence the repair dynamics in wound healing applications.

The properties of plasma membranes, including lipid fluidity and phospholipid packing, play a role in their repair dynamics (e.g., the ‘tension-reduction’ hypothesis for perforation resealing). Indeed, lipid composition is regulated in response to pathological as well as pharmacological triggers. Membrane lipid alterations have been shown to be involved in many diseases, including cancer, atherosclerosis, and neurodegenerative diseases (e.g., ⁵²³). One such example is the regulation of plasma-membrane-incorporated cholesterol, which may be dysfunctional in cancer cells ⁵²⁴ and result in variations in membrane fluidity and surface tension. Membrane cholesterol content is a key factor in modulating perforation repair dynamics, as has been demonstrated and physically modeled using giant unilamellar vesicles, resulting in shorter pore lifetimes with increasing cholesterol content ⁴⁷⁵. Localized co-treatment with cholesterol depletion drugs, for example Filipin, ultimately decreases lipid raft number and membrane rigidity and may provide a means to ensure rapid resealing post-drug-delivery. Alternative lipid-altering strategies, such as incorporating pluronic polymers as part of the drug payload (e.g., Tween and polyethylene-glycol) ⁵²⁵, have the potential to preserve and amplify cell membrane recovery ⁵²⁶ and to facilitate intracellular drug transport ⁵²⁷. Indeed, as polyethylene-glycol is a typical constituent of ultrasound-stimulated microbubbles used for sonoporation, microbubbles present a novel intrinsic vehicle for the local and targeted modulation of cell membrane viscoelasticity, as recently demonstrated ⁵²⁸. It is well known that the composition of the outlet leaflet of the plasma membrane modifies its local surface tension and can trigger specific membrane repair mechanisms (e.g., the ‘exocytosis/endocytosis’ pathway), and this presents a unique strategy for tailoring drug/gene delivery treatment efficiency and timescales. Short-chain ceramides (with chain lengths four to eight carbons long) have been shown to readily incorporate into the outlet leaflet of the plasma membrane ⁵²⁹. In contrast to their long-chained counterparts, short-chain ceramides are not able to

form lipid rafts nor increase membrane rigidity ⁵³⁰. Therefore, short-chain ceramides can be employed externally to increase membrane fluidity and have been used in conjunction with cancer therapeutics to improve intracellular drug permeability ⁵³¹. Indeed, the field of membrane lipid therapy, whereby the modulation of cell membrane composition can be achieved pharmacologically through membrane structure reorganization, regulation of enzymatic activity, and modulation of lipid-based gene expression ⁵³², can be incorporated into targeted membrane-permeation strategies to tailor pore kinetics. In fact, cancer treatments based on membrane lipid therapy have been investigated in clinical trials of patients with advanced solid tumors (e.g., NCT02201823 and NCT01792310).

Aside from direct plasma membrane alteration, phospholipid-binding and cytoskeletal proteins offer promising targets for modulating perforation recovery dynamics, one of which is the annexin family of proteins. Previous studies have shown that treatment with annexin-1-derived peptides (e.g., Anx-12–26) elicits cyto-protective actions and shortens cardiomyocyte injury recovery time ⁵³³, facilitates wound healing in vivo ⁵³⁴, promotes epithelial repair ⁵³⁵, and can aid in the restoration of adheren junctions and the normalization of barrier integrity ⁵³⁶. Recent studies have also demonstrated that treatment post-injury with recombinant annexin 6 enhances membrane repair capacity ⁵³⁷. Further, annexin 5 has been investigated both as a diagnostic and as a therapeutic tool due to its strong binding affinity to phosphatidylserine, which results in an imaging surrogate for apoptosis in vivo and a potential anticoagulant, as it binds to activated platelets to prevent thrombin formation ⁵³⁸. Additionally, annexin 5 has anti-inflammatory properties due to its potential to modulate nitric oxide signalling cascades, which has launched recent clinical trials investigating the delivery of recombinant human annexin 5 in patients with sepsis and COVID-19 (NCT04850339, NCT04748757). Tension development through the modulation of GTPases and

cytoskeletal organization is also a promising approach to altering perforation kinetics ⁵³⁹. The selective pharmacological inhibition of tension development, including the blocking of myosin II (blebbistatin), Rho-associated protein kinases (Y27632), and myosin light chain (ML-7), has been shown to result in a decrease in pore retraction time and prolongation of total perforation recovery, demonstrated in human dermal and lung microvascular endothelial cells ⁵⁴⁰, as well as neuroendocrine cells ⁵⁴¹.

5.8 Conclusions

Cellular recovery from plasma membrane perforation is critical to ensure successful drug delivery using physical techniques. Indeed, fundamental molecular biology studies on individual cell membrane pore repair have brought tremendous insight into the arrangement of the key molecular actors at play. In coordination with salient Ca^{2+} -dependent proteins, including the annexin family, various plasma resealing mechanisms have been put forth, including the ‘tension-reduction’, ‘patch’, ‘shedding’, and ‘exocytosis/endocytosis’ pathways. In conjunction with cytoskeletal reorganization involving actin and/or myosin and their roles in vesicular trafficking, these mechanisms likely work in concert to achieve rapid localized repair and control perforation dynamics. As such, physical membrane perforation techniques employed for targeted in vivo drug/gene delivery, including the use of microbubble-mediated ultrasound perforation, may benefit from co-administration with pharmacological agents to selectively modulate the spatio-temporal dynamics of membrane perforation to enhance therapeutic effectiveness.

References

1. Cuspidi C, Sala C, Negri F, Mancia G, Morganti A. Prevalence of left-ventricular hypertrophy in hypertension : an updated review of echocardiographic studies. *J Hum Hypertens* 2012;**26**:343–349.
2. Tsuda T. Clinical Assessment of Ventricular Wall Stress in Understanding Compensatory Hypertrophic Response and Maladaptive Ventricular Remodeling. *J Cardiovasc Dev Dis* 2021;**8**:122.
3. Soonpaa MH, Kim KK, Pajak L, Franklin M, Field LJ. Cardiomyocyte DNA synthesis and binucleation during murine development. *Am J Physiol Heart Circ Physiol* 1996;**271**:2183–2189.
4. Nakamura M, Sadoshima J. Mechanisms of physiological and pathological cardiac hypertrophy. *Nat Rev Cardiol* 2018;**15**:387–407.
5. Nakamura M, Verboon JM, Parkhurst SM. Prepatterning by RhoGEFs governs Rho GTPase spatiotemporal dynamics during wound repair. *Journal of Cell Biology* 2017;**216**:3959–3969.
6. Bernardo BC, Weeks KL, Pretorius L, McMullen JR. Molecular distinction between physiological and pathological cardiac hypertrophy: Experimental findings and therapeutic strategies. *Pharmacol Ther* 2010;**128**:191–227.
7. Sala V, Gallo S, Leo C, Gatti S, Gelb BD, Crepaldi T. Signaling to Cardiac Hypertrophy : Insights from Human and Mouse RASopathies. *Molecular medicine* 2012;**18**:938–947.
8. Force T, Hajjar R, Monte F Del, Rosenzweig A, Choukroun G. Signaling pathways mediating the response to hypertrophic stress in the heart. *Gene Expr* 1999;**7**:337–348.
9. Rapacciuolo A, Esposito G, Caron K, Mao L, Thomas SA, Rockman HA. Important Role of Endogenous Norepinephrine and Epinephrine in the Development of In Vivo Pressure-Overload Cardiac Hypertrophy. *J Am Coll Cardiol* 2001;**38**:876–882.
10. Nishida M, Kurose H. Roles of TRP channels in the development of cardiac hypertrophy. *Naunyn Schmiedebergs Arch Pharmacol* 2008;**378**:395–406.
11. Hulot J-S, Fauconnier J, Ramanujam D, Chaanine A, Aubart F, Sassi Y, Merkle S, Cazorla O, Ouillé A, Dupuis M, Hadri L, Jeong D, Mühlstedt S, Schmitt J, Braun A, Bénard L, Saliba Y, Lagerbauer B, Nieswandt B, Lacampagne A, Hajjar RJ, Lompré A-M, Engelhard S. A critical role for STIM1 in cardiac hypertrophy. *Circulation* 2011;**23**:1–7.
12. Terracio L, Rubin K, Gullberg D, Balog E, Carver W, Jyring R, Borg TK. Expression of collagen binding integrins during cardiac development and hypertrophy. *Circ Res* 1991;**68**:734–744.
13. Bazgir F, Nau J, Nakhaei-Rad S, Amin E, Wolf MJ, Saucerman JJ, Lorenz K, Ahmadian MR. The Microenvironment of the Pathogenesis of Cardiac Hypertrophy. *Cells*.

14. Tham YK, Bernardo BC, Ooi JYY, Weeks KL, McMullen JR. Pathophysiology of cardiac hypertrophy and heart failure : signaling pathways and novel therapeutic targets. *Arch Toxicol* 2015;**89**:1401–1438.
15. Takano APC, Senger N, Barreto-chaves MLM. The endocrinological component and signaling pathways associated to cardiac hypertrophy. *Mol Cell Endocrinol* 2020;**518**:110972.
16. Heineke J, Molkentin JD. Regulation of cardiac hypertrophy by intracellular signalling pathways. *Nat Rev Mol Cell Biol* 2006;**7**:589–600.
17. Ye J, Cardona M, Llovera M, Comella JX, Sanchis D. Translation of Myocyte Enhancer Factor-2 is induced by hypertrophic stimuli in cardiomyocytes through a Calcineurin-dependent pathway. *J Mol Cell Cardiol* 2012;**53**:578–587.
18. Lehman JJ, Kelly DP. Gene Regulatory Mechanisms Governing Energy Metabolism during Cardiac Hypertrophic Growth. *Heart Fail Rev* 2002;**7**:175–185.
19. Reiser PJ, Portman MA, Ning X, Moravec CS. Human cardiac myosin heavy chain isoforms in fetal and failing adult atria and ventricles. *Am J Physiol Heart Circ Physiol* 2001;**280**:1814–1820.
20. Herron TJ, McDonald KS. Small amounts of α -myosin heavy chain isoform expression significantly increase power output of rat cardiac myocyte fragments. *Circ Res* 2002;**90**:1150–1152.
21. Mahesh P. Gupta. Factors controlling cardiac myosin-isoform shift during hypertrophy and heart failure. *J Mol Cell Cardiol* 2007;**23**:1–7.
22. Lips DJ, DeWindt LJ, Kraaij DJW Van, Doevendans PA. Molecular determinants of myocardial hypertrophy and failure: Alternative pathways for beneficial and maladaptive hypertrophy. *Eur Heart J* 2003;**24**:883–896.
23. Andersson KB, Birkeland JAK, Finsen AV, Louch WE, Sjaastad I, Wang Y, Chen J, Molkentin JD, Chien KR, Sejersted OM, Christensen G. Moderate heart dysfunction in mice with inducible cardiomyocyte-specific excision of the *Serca2* gene. *J Mol Cell Cardiol* 2009;**47**:180–187.
24. Curcio A, Torella D, Iaconetti C, Pasceri E, Sabatino J, Sorrentino S, Giampà S, Micieli M, Polimeni A, Henning BJ, Leone A, Catalucci D, Ellison GM, Condorelli G, Indolfi C. MicroRNA-1 Downregulation Increases Connexin 43 Displacement and Induces Ventricular Tachyarrhythmias in Rodent Hypertrophic Hearts. *PLoS One* 2013;**8**:1–17.
25. Bacova BS, Andelova K, Sykora M, Benova TE, Kurahara LH, Slezak J, Tribulova N. Distinct Cardiac Connexin-43 Expression in Hypertrophied and Atrophied Myocardium May Impact the Vulnerability of the Heart to Malignant Arrhythmias . A Pilot Study. *Physiol Res* 2023;**72**:37–45.

26. Tagawa H, Koide M, Sato H, Zile MR, Carabello BA, Cooper IV G. Cytoskeletal role in the transition from compensated to decompensated hypertrophy during adult canine left ventricular pressure overloading. *Circ Res* 1998;**82**:751–761.
27. Zile MR, Green GR, Schuyler GT, Aurigemma GP, Miller DC, Cooper G. Cardiocyte Cytoskeleton in Patients With Left Ventricular Pressure Overload Hypertrophy. *J Am Coll Cardiol* 2001;**37**:1080–1084.
28. Pan C, Wang S, Liu C, Ren Z. Actin-Binding Proteins in Cardiac Hypertrophy. *Cells*.
29. Rosca MG, Tandler B, Hoppel CL. Mitochondria in cardiac hypertrophy and heart failure. *J Mol Cell Cardiol* 2014;**55**:31–41.
30. Moris D, Spartalis M, Spartalis E, Karachaliou GS, Karaolanis GI, Tsourouflis G, Tsilimigras DI, Tzatzaki E, Theocharis S. The role of reactive oxygen species in the pathophysiology of cardiovascular diseases and the clinical significance of myocardial redox. *Ann Transl Med* 2017;**5**:1–11.
31. Li Z, Wang J, Yang X. Functions of Autophagy in Pathological Cardiac Hypertrophy. *Int J Biol Sci* 2015;**11**:672–678.
32. Teiger E, Dam TV, Richard L, Wisnewsky C, Tea BS, Gaboury L, Tremblay J, Schwartz K, Hamet P. Apoptosis in pressure overload-induced heart hypertrophy in the rat. *Journal of Clinical Investigation* 1996;**97**:2891–2897.
33. Li X, Lan Y, Wang Y, Nie M, Lu Y, Zhao E. Telmisartan suppresses cardiac hypertrophy by inhibiting cardiomyocyte apoptosis via the NFAT/ANP/BNP signaling pathway. *Mol Med Rep* 2017;**15**:2574–2582.
34. Heinzl FR, Hohendanner F, Jin G, Sedej S, Edelmann F. Myocardial Hypertrophy and Its Role in Heart Failure with Preserved Ejection Fraction Left Ventricular Hypertrophy – Clinical Presentation. *J Appl Physiol (1985)* 2015;**119**:1233–1242.
35. Maillet M, Berlo JH Van, Molkentin JD. Molecular basis of physiological heart growth: fundamental concepts and new players. *Nat Rev Mol Cell Biol* 2015;**14**:38–48.
36. Caturano A, Vetrano E, Galiero R, Salvatore T, Docimo G, Epifani R, Alfano M, Sardu C, Marfella R, Rinaldi L, Sasso FC. Cardiac Hypertrophy: From Pathophysiological Mechanisms to Heart Failure Development. *Reviews in Cardiovascular Medicine*.
37. Oka T, Akazawa H, Naito AT, Komuro I. Angiogenesis and Cardiac Hypertrophy: Maintenance of Cardiac Function and Causative Roles in Heart Failure. *Circ Res* 2014;**114**:565–571.
38. Breisch EA, Houser SR, Carey RA, Spann JF, Bove AA. Myocardial blood flow and capillary density in chronic pressure overload of the feline left ventricle. *Cardiovasc Res* 1980;**14**:469–475.

39. Frieler RA, Mortensen RM. Immune Cell and Other Non-Cardiomyocyte Regulation of Cardiac Hypertrophy and Remodelin. *Ciruculation* 2015;**131**:1019–1030.
40. Zhang W, Lavine KJ, Epelman S, Evans SA, Weinheimer CJ, Barger PM, Mann DL. Necrotic myocardial cells release Damage-Associated Molecular Patterns that provoke fibroblast activation in vitro and trigger myocardial inflammation and fibrosis in vivo. *J Am Heart Assoc* 2015;**4**:1–19.
41. Fang L, Ellims AH, Beale AL, Taylor AJ, Murphy A, Dart AM. Systemic inflammation is associated with myocardial fibrosis, diastolic dysfunction, and cardiac hypertrophy in patients with hypertrophic cardiomyopathy. *Am J Transl Res* 2017;**9**:5063–5073.
42. Kong P, Christia P, Frangogiannis NG. The pathogenesis of cardiac fibrosis. *Cellular and molecular life sciences* 2014;**71**:549–574.
43. Verheule S, Schotten U. Electrophysiological consequences of cardiac fibrosis. *Cells* 2021;**10**:3220.
44. Diwan A, Dorn GW. Decompensation of Cardiac Hypertrophy : Cellular Mechanisms and Novel Therapeutic Targets. *Physiology* 2007;**22**:56–64.
45. Messerli FH, Rimoldi SF, Bangalore S. The Transition From Hypertension to Heart Failure. *JACC Heart Fail* 2017;**5**:543–551.
46. Bytyçi I, Bajraktari G. Mortality in heart failure patients. *Anatolian J Cardio* 2015;**15**:63–68.
47. Sayin BY, Oto A. Left Ventricular Hypertrophy: Etiology-Based Therapeutic Options. *Cardiol Ther* 2022;**11**:203–230.
48. Stewart MP, Langer R, Jensen KF. Intracellular delivery by membrane disruption: Mechanisms, strategies, and concepts. *Chem Rev* 2018;**118**:7409–7531.
49. Mathew J, Sleight P, Lonn E, Johnstone D, Pogue J, Yi Q, Bosch J, Sussex B, Probstfield J, Yusuf S. Reduction of cardiovascular risk by regression of electrocardiographic markers of left ventricular hypertrophy by the angiotensin-converting enzyme inhibitor ramipril. *Circulation* 2001;**104**:1615–1621.
50. Ruggenenti P, Iliev I, Costa GM, Parvanova A, Perna A, Giuliano GA, Motterlini N, Ene-Iordache B, Remuzzi G. Preventing left ventricular hypertrophy by ACE inhibition in hypertensive patients with type 2 diabetes A prespecified analysis of the Bergamo Nephrologic Diabetes Complications Trial (BENEDICT). *Diabetes Care* 2008;**31**:1629–1634.
51. Bodi I, Mikala G, Koch SE, Akhter SA, Schwartz A. The L-type calcium channel in the heart : the beat goes on. *J Clin Invest* 2005;**115**:3306–3317.
52. John J V McMurray, Pfeffer MA. Heart Failure. *Lancet* 2005;**365**:1877–1889.

53. Yancy CW, Jessup M, Bozkurt B, Butler J, Casey DE, Drazner MH, Fonarow GC, Geraci SA, Horwich T, Januzzi JL, Johnson MR, Kasper EK, Levy WC, Masoudi FA, McBride PE, McMurray JJV, Mitchell JE, Peterson PN, Riegel B, Sam F, Stevenson LW, Tang WHW, Tsai EJ, Wilkoff BL. 2013 ACCF/AHA guideline for the management of heart failure: A report of the American college of cardiology foundation/american heart association task force on practice guidelines. *J Am Coll Cardiol* 2013;**62**:147–239.
54. Florian Rader, Oreziak A, Choudhury L, Saberi S, Fermin D, Wheeler MT, Abraham TP, Garcia-Pavia P, Zwas DR, Masri A, Owens A, Hegde SM, Seidler T, Fox S, Balaratnam G, Sehnert AJ, Olivotto I. Mavacamten Treatment for Symptomatic Obstructive Hypertrophic Cardiomyopathy. *JACC Heart Fail* 2024;**12**:164–177.
55. Ha M, Kim VN. Regulation of microRNA biogenesis. *Nat Rev Mol Cell Biol* 2014;**15**:509–524.
56. Yi R, Qin Y, Macara IG, Cullen BR. Exportin-5 mediates the nuclear export of pre-microRNAs and short hairpin RNAs. *Genes Dev* 2003;**17**:3011–3016.
57. Ando Y, Maida Y, Morinaga A, Burroughs AM, Kimura R, Chiba J, Suzuki H, Masutomi K, Hayashizaki Y. Two-step cleavage of hairpin RNA with 5' overhangs by human DICER. *BMC Mol Biol* 2011;**12**:6.
58. Iwasaki S, Kobayashi M, Yoda M, Sakaguchi Y, Katsuma S, Suzuki T, Tomari Y. Hsc70/Hsp90 chaperone machinery mediates ATP-dependent RISC loading of small RNA duplexes. *Mol Cell* 2010;**39**:292–299.
59. Kawamata T, Seitz H, Tomari Y. Structural determinants of miRNAs for RISC loading and slicer-independent unwinding. *Nat Struct Mol Biol* 2009;**16**:953–960.
60. Bartel DP. MicroRNA Target Recognition and Regulatory Functions. *Cell* 2010;**44**:141–157.
61. Hammond SM, Bernstein E, Beach D, Hannon GJ. An RNA-directed nuclease mediates post-transcriptional gene silencing in Drosophila cells. *Nature* 2000;**404**:293–296.
62. Behm-Ansmant I, Rehwinkel J, Doerks T, Stark A, Bork P, Izaurralde E. mRNA degradation by miRNAs and GW182 requires both CCR4:NOT deadenylase and DCP1:DCP2 decapping complexes. *Genes Dev* 2006;**20**:1885–1898.
63. Djuranovic S, Nahvi A, Green R. miRNA-mediated gene silencing by translational repression followed by mRNA deadenylation and decay. *Science (1979)* 2011;**23**:1–7.
64. Rao SK, Huynh C, Proux-Gillardeaux V, Galli T, Andrews NW. Identification of SNAREs Involved in Synaptotagmin VII-regulated Lysosomal Exocytosis. *Journal of Biological Chemistry* 2004;**279**:20471–20479.
65. Chistiakov DA, Orekhov AN, Bobryshev Y V. Cardiac-specific miRNA in cardiogenesis, heart function, and cardiac pathology (with focus on myocardial infarction). *J Mol Cell Cardiol* 2016;**94**:107–121.

66. Wehbe N, Nasser SA, Pintus G, Badran A, Eid AH, Baydoun E. MicroRNAs in Cardiac Hypertrophy. *Int J Mol Sci* 2019;**20**.
67. Zhao Y, Ransom JF, Li A, Vedantham V, Drehle M Von, Muth AN, Tsuchihashi T, Mcmanus MT, Schwartz RJ. Dysregulation of Cardiogenesis , Cardiac Conduction , and Cell Cycle in Mice Lacking miRNA-1-2. *Cell* 2007;**129**:303–317.
68. Rao PK, Toyama Y, Chiang HR, Gupta S, Bauer M, Medvid R, Reinhardt F, Liao R, Krieger M, Jaenisch R, Lodish HF, Blecloch R. Loss of cardiac microRNA-mediated regulation leads to dilated cardiomyopathy and heart failure. *Circ Res* 2009AD;**105**:585–594.
69. Zhao Y, Samal E, Srivastava D. Serum response factor regulates a muscle-specific microRNA that targets Hand2 during cardiogenesis. *Nature* 2005;**436**:214–220.
70. Ivey KN, Muth A, Arnold J, King FW, Yeh R, Jason E, Hsiao EC, Schwartz RJ, Conklin BR, Harold S, Srivastava D. MicroRNA Regulation of Cell Lineages in Mouse and Human Embryonic Stem Cells. *Cell Stem Cell* 2008;**2**:219–229.
71. Sluijter JPG, Mil A Van, Vliet P Van, Metz CHG, Liu J, Doevendans PA. MicroRNA-1 and -499 Regulate Differentiation and Proliferation in Human-Derived Cardiomyocyte Progenitor Cells. *Arterioscler Thromb Vasc Biol* 2010;**30**:859–868.
72. Heidrichs A, Saxby C, Carver-moore K, Huang Y. microRNA-1 regulates sarcomere formation and suppresses smooth muscle gene expression in the mammalian heart. *Elife* 2013;**2**:1–22.
73. Gan J, Mei F, Tang K, Su X, Lu G, Xu J, Siu H, Lee S, Ka K, Lee H. microRNA-1 inhibits cardiomyocyte proliferation in mouse neonatal hearts by repressing CCND1 expression. *Ann Transl Med* 2019;**1**:1–16.
74. Kim GH. MicroRNA Regulation of Cardiac Conduction and Arrhythmias. *Transl Res* 2013;**161**:381–392.
75. Rau F, Freyermuth F, Fugier C, Villemin J, Fischer M, Jost B, Dembele D, Gourdon G, Nicole A, Duboc D, Wahbi K. Misregulation of miR-1 processing is associated with heart defects in myotonic dystrophy. *Nat Struct Mol Biol* 2011;**18**:840–845.
76. Girmatsion Z, Biliczki P, Bonauer A, Wimmer-greinecker G, Scherer M, Moritz A, Bukowska A, Goette A, Nattel S, Hohnloser SH, Ehrlich JR. Changes in microRNA-1 expression and I K1 up-regulation in human atrial fibrillation. *Heart Rhythm* 2009;**6**:1802–1809.
77. Yang D, Wan X, Schwieterman N, Cavus O, Kacira E, Xu X, Laurita KR, Wold LE, Hund TJ, Mohler PJ, Deschênes I, Fu JD. MicroRNA-1 Deficiency Is a Primary Etiological Factor Disrupting Cardiac Contractility and Electrophysiological Homeostasis. *Circ Arrhythm Electrophysiol* 2024;**17**:34–48.

78. Michela P, Velia V, Aldo P, Ada P. Role of connexin 43 in cardiovascular diseases. *Eur J Pharmacol* 2015;**768**:71–76.
79. Bers DM. Cardiac excitation–contraction coupling. *Nature* 2002;**415**:198–205.
80. Terentyev D, Belevych AE, Terentyeva R, Martin MM, Malana GE, Kuhn DE, Abdellatif M, Feldman DS, Terry S, Gyorke S. MiR-1 Overexpression Enhances Ca²⁺ release and Promotes Cardiac Arrhythmogenesis by Targeting PP2A Regulatory Subunit B56 α and Causing CaMKII-Dependent Hyperphosphorylation of RyR2. *Circ Res* 2015;**104**:514–521.
81. Chan JY, Takeda M, Briggs LE, Graham ML, Lu JT, Horikoshi N, Weinberg EO, Aoki H, Sato N, Chien KR. Identification of Cardiac-Specific Myosin Light Chain Kinase. *Circ Res* 2008;**102**:571–580.
82. Li Q, Song XW, Zou J, Wang GK, Kremneva E, Li XQ, Zhu N, Lappalainen P, Yuan WJ, Qin YW, Jing Q. Attenuation of microRNA-1 derepresses the cytoskeleton regulatory protein twinfilin-1 to provoke cardiac hypertrophy. *J Cell Sci* 2010;**123**:2444–2452.
83. Li J, Dong X, Wang Z, Wu J. MicroRNA-1 in cardiac diseases and cancers. *Korean Journal of Physiology and Pharmacology* 2014;**18**:359–363.
84. Bagnall RD, Tsoutsman T, Shephard RE, Ritchie W, Semsarian C. Global MicroRNA Profiling of the Mouse Ventricles during Development of Severe Hypertrophic Cardiomyopathy and Heart Failure. *PLoS One* 2012;**7**:e44744.
85. Abdellatif M. Differential Expression of MicroRNAs in Different Disease States. *Circ Res* 2012;**110**:638–650.
86. Divakaran V, Mann DL. The Emerging Role of MicroRNAs in Cardiac Remodeling and Heart Failure. *Circ Res* 2008;**103**:1072–1083.
87. Wei Y, Peng S, Wu M, Sachidanandam R, Tu Z, Zhang S. Multifaceted roles of miR-1 s in repressing the fetal gene program in the heart. *Nature Publishing Group* 2014;**24**:278–292.
88. Dirkx E, Costa Martins PA da, Windt LJ De. Regulation of fetal gene expression in heart failure. *Biochim Biophys Acta Mol Basis Dis* 2013;**1832**:2414–2424.
89. Taubel J, Hauke W, Rump S, Viereck J, Batkai S, Poetzsch J, Rode L, Henning Weigt, Genschel C, Lorch U, Theek C, Levin AA, Bauersachs J, Solomon SD, Thum T. Novel antisense therapy targeting microRNA-132 in patients with heart failure : results of a first-in-human Phase 1b study. *Eur Heart J* 2021;**42**:178–188.
90. Karakikes I, Chaanine AH, Kang S, Mukete BN, Jeong D, Zhang S, Hajjar RJ, Lebeche D. Therapeutic cardiac-targeted delivery of miR-1 reverses pressure overload-induced cardiac hypertrophy and attenuates pathological remodeling. *J Am Heart Assoc* 2013;**2**:17–19.

91. Ikeda S, He A, Kong SW, Lu J, Bejar R, Bodyak N, Lee K, Ma Q, Kang PM, Golub TR, Pu WT. MicroRNA-1 Negatively Regulates Expression of the Hypertrophy-Associated Calmodulin and Mef2a Genes □ †. *Mol Cell Biol* 2009;**29**:2193–2204.
92. Dib N, Khawaja H, Varner S, McCarthy M, Campbell A. Cell therapy for cardiovascular disease: A comparison of methods of delivery. *Journal of Cardiovascular Translational Research*.
93. Vekstein AM, Wendell DC, DeLuca S, Yan R, Chen Y, Bishawi M, Devlin GW, Asokan A, Poss KD, Bowles DE, Williams AR, Bursac N. Targeted Delivery for Cardiac Regeneration: Comparison of Intra-coronary Infusion and Intra-myocardial Injection in Porcine Hearts. *Front Cardiovasc Med* 2022;**9**:1–10.
94. Shazly T, Smith A, Uline MJ, Spinale FG. Therapeutic payload delivery to the myocardium: Evolving strategies and obstacles. *JTCVS Open* 2022;**5**:185–194.
95. Anttila V, Saraste A, Knuuti J, Jaakkola P, Hedman M, Svedlund S, Lagerström-Fermér M, Kjaer M, Jeppsson A, Gan LM. Synthetic mRNA Encoding VEGF-A in Patients Undergoing Coronary Artery Bypass Grafting: Design of a Phase 2a Clinical Trial. *Mol Ther Methods Clin Dev* 2020;**18**:464–472.
96. Gwon C, Jeong J-O, Kim H-J, Woo Park S, Lee SH, Park S-J, Huh J-E, Lee Y, Kim S, Kim D-K. The feasibility and safety of fluoroscopy-guided percutaneous intramyocardial gene injection in porcine heart. *International Journal of Cardiology*.
97. Fukushima S, Varela-Carver A, Coppen SR, Yamahara K, Felkin LE, Lee J, Barton PJR, Terracciano CMN, Yacoub MH, Suzuki K. Direct intramyocardial but not intracoronary injection of bone marrow cells induces ventricular arrhythmias in a rat chronic ischemic heart failure model. *Circulation* 2007;**115**:2254–2261.
98. Zhao ZA, Han X, Lei W, Li J, Yang Z, Wu J, Yao M, Lu XA, He L, Chen Y, Zhou B, Hu S. Lack of cardiac improvement after cardiosphere-derived cell transplantation in aging mouse hearts. *Circ Res* 2018;**123**:E21–E31.
99. French BA, Mazur W, Geske RS, Bolli R. Direct in vivo gene transfer into porcine myocardium using replication-deficient adenoviral vectors. *Circulation* 1994;**90**:2414–2424.
100. Laakmann S, Fortmüller L, Piccini I, Grote-Wessels S, Schmitz W, Breves G, Kirchhof P, Fabritz L. Minimally invasive closed-chest ultrasound-guided substance delivery into the pericardial space in mice. *Naunyn Schmiedeberg's Arch Pharmacol* 2013;**386**:227–238.
101. Lamping KG, Rios CD, Chun JA, Ooboshi H, Davidson BL, Heistad DD. Intrapericardial administration of adenovirus for gene transfer. *Am J Physiol Heart Circ Physiol* 1997;**272**:310–317.
102. Traber GM, Yu AM. Special Section on Non-Coding RNAs in Clinical Practice: From Biomarkers to Therapeutic Tools-Minireview RNAi-Based Therapeutics and Novel RNA

- Bioengineering Technologies. *Journal of Pharmacology and Experimental Therapeutics* 2023;**384**:133–154.
103. Segal M, Slack FJ. Challenges identifying efficacious miRNA therapeutics for cancer. *Expert Opinion Drug Discovery* 2020;**176**:139–148.
 104. Akbarzadeh A, Rezaei-Sadabady R, Davaran S, Joo SW, Zarghami N, Hanifehpour Y, Samiei M, Kouhi M, Nejati-Koshki K. Liposome: Classification, preparation, and applications. *Nanoscale Res Lett* 2013;**8**:102.
 105. Allen TM, Cullis PR. Liposomal drug delivery systems: From concept to clinical applications. *Adv Drug Deliv Rev* 2013;**65**:36–48.
 106. Li M, Tang X, Liu X, Cui X, Lian M, Zhao M, Peng H, Han X. Targeted miR-21 loaded liposomes for acute myocardial infarction. *J Mater Chem B* 2020;**8**:10384–10391.
 107. Evers MJW, Du W, Yang Q, Kooijmans SAA, Vink A, Steenberg M van, Vader P, Jager SCA de, Fuchs SA, Mastrobattista E, Sluijter JPG, Lei Z, Schiffelers R. Delivery of modified mRNA to damaged myocardium by systemic administration of lipid nanoparticles. *Journal of Controlled Release* 2022;**343**:207–216.
 108. Chen P, Wang L, Fan X, Ning X, Yu B, Ou C, Chen M. Targeted delivery of extracellular vesicles in heart injury. *Theranostics* 2021;**11**:2263–2277.
 109. Cheng M, Yang J, Zhao X, Zhang E, Zeng Q, Yu Y, Yang L, Wu B, Yi G, Mao X, Huang K, Dong N, Xie M, Limdi NA, Prabhu SD, Zhang J, Qin G. Circulating myocardial microRNAs from infarcted hearts are carried in exosomes and mobilise bone marrow progenitor cells. *Nat Commun* 2019;**10**:959.
 110. Johnsen KB, Gudbergsson JM, Duroux M, Moos T, Andresen TL, Simonsen JB. On the use of liposome controls in studies investigating the clinical potential of extracellular vesicle-based drug delivery systems – A commentary. *Journal of Controlled Release* 2018;**269**:10–14.
 111. Gu J, You J, Liang H, Zhan J, Gu X, Zhu Y. Engineered bone marrow mesenchymal stem cell-derived exosomes loaded with miR302 through the cardiomyocyte specific peptide can reduce myocardial ischemia and reperfusion (I/R) injury. *J Transl Med* 2024;**22**:168.
 112. Romeo FJ, Mavropoulos SA, Ishikawa K. Progress in Clinical Gene Therapy for Cardiac Disorders. *Molecular Diagnosis and Therapy*.
 113. Zhang X, McLendon JM, Peck BD, Chen B, Song LS, Boudreau RL. Modulation of miR-29 influences myocardial compliance likely through coordinated regulation of calcium handling and extracellular matrix. *Mol Ther Nucleic Acids* 2023;**34**:102081.
 114. Yan H, Wang H, Zhu X, Huang J, Li Y, Zhou K, Hua Y, Yan F, Wang DZ, Luo Y. Adeno-associated virus-mediated delivery of anti-miR-199a tough decoys attenuates cardiac hypertrophy by targeting PGC-1alpha. *Mol Ther Nucleic Acids* 2021;**23**:406–417.

115. Fu Y, Chen J, Huang Z. Recent progress in microrna-based delivery systems for the treatment of human disease. *ExRNA* 2019;**1**:1–14.
116. Abu-Zidan FM, Hefny AF, Corr P. Clinical ultrasound physics. *J Emerg Trauma Shock* 2011;**4**:501–503.
117. Quarato CMI, Lacedonia D, Salvemini M, Tuccari G, Mastrodonato G, Villani R, Fiore LA, Scioscia G, Mirijello A, Saponara A, Sperandeo M. A Review on Biological Effects of Ultrasounds: Key Messages for Clinicians. *Diagnostics* 2023;**13**:1–29.
118. Şen T, Tüfekçioğlu O, Koza Y. Mechanical index. *Anatol J Cardiol* 2015;**15**:334–336.
119. Lang RM, Badano LP, Mor-avi V, Afilalo J, Armstrong A, Ernande L, Flachskampf FA, Foster E, Goldstein SA, Kuznetsova T, Lancellotti P, Muraru D. Recommendations for Cardiac Chamber Quantification by Echocardiography in Adults : An Update from the American Society of Echocardiography and the European Association of Cardiovascular Imaging. *Journal of the American Society of Echocardiography* 2015;**28**:1-39.e14.
120. Sirsi SR, Borden MA. Microbubble compositions, properties and biomedical applications. *Bubble Sci Eng Technol* 2009;**1**:3–17.
121. Hernot S, Klivanov AL. Microbubbles in Ultrasound-Triggered Drug and Gene Delivery. *Adv Drug Delivery Rev* 2008;**60**:1153–1166.
122. Ferrara K, Pollard R, Borden M. Ultrasound microbubble contrast agents: Fundamentals and application to gene and drug delivery. *Annu Rev Biomed Eng* 2007;**9**:415–447.
123. Ajmal S. Contrast-Enhanced Ultrasonography: Review and Applications. *Cureus* 2024;**13**:10–14.
124. Forsberg F, Shi WT, Goldberg BB. Subharmonic imaging of contrast agents. *Ultrasonics* 2000;**38**:93–98.
125. Lindner JR, Wei K. Contrast Echocardiography. *Curr Probl Cardiol* 2002;**27**:449–520.
126. Averkiou MA, Bruce MF, Powers JE, Sheeran PS, Burns PN. Imaging Methods for Ultrasound Contrast Agents. *Ultrasound Med Biol* 2020;**46**:498–517.
127. Calliada F, Campani R, Bottinelli O, Bozzini A, Sommaruga MG, SerTizio. Ultrasound contrast agents. *Eur J Radiol* 1998;**27**:S157–S160.
128. Phenix CP, Togtema M, Pichardo S, Zehbe I, Curiel L. High Intensity Focused Ultrasound Technology ,Its Scope and Applications in Therapy and Drug Delivery. *J Pharm Pharm Sci* 2014;**17**:136–153.
129. Lowerison MR, Sekaran NVC, Dong Z, Chen X, You Q, Llano DA, Song P. Super-Resolution Ultrasound Reveals Cerebrovascular Impairment in a Mouse Model of Alzheimer’s Disease. *The Journal of Neuroscience* 2024;**44**:1–14.

130. Wamel A van, Bouakaz A, Versluis M, Jong N de. Micromanipulation of endothelial cells: ultrasound-microbubble-cell interaction. *Ultrasound Med Biol* 2004;**30**:1255–1258.
131. Qiu Y, Zhang C, Tu J, Zhang D. Microbubble-induced sonoporation involved in ultrasound-mediated DNA transfection in vitro at low acoustic pressures. *J Biomech* 2012;**45**:1339–1345.
132. Schlicher RK, Radhakrishna H, Tolentino TP, Apkarian RP, Zarnitsyn V, Prausnitz MR. Mechanism of intracellular delivery by acoustic cavitation. *Ultrasound Med Biol* 2006;**32**:915–924.
133. Wamel A van, Kooiman K, Harteveld M, Emmer M, Cate FJ ten, Versluis M, Jong N de. Vibrating microbubbles poking individual cells: Drug transfer into cells via sonoporation. *Journal of Controlled Release* 2006;**112**:149–155.
134. Collis J, Manasseh R, Liovic P, Tho P, Ooi A, Petkovic-Duran K, Zhu Y. Cavitation microstreaming and stress fields created by microbubbles. *Ultrasonics* 2010;**50**:273–279.
135. Kodama T, Tomita Y, Koshiyama K, Blomley MJK. Transfection effect of microbubbles on cells in superposed ultrasound waves and behaviour of cavitation bubble. *Ultrasound Med Biol* 2006;**32**:905–914.
136. Ohl C, Wolfrum B. Detachment and sonoporation of adherent HeLa-cells by shock wave-induced cavitation. *Biochim Biophys Acta* 2003;**1624**:131–138.
137. Postema M, Wamel A van, Cate FJ, Jong N De. High-speed photography during ultrasound illustrates potential therapeutic applications of microbubbles. *Med Phys* 2005;**32**:3707–3711.
138. Juffermans LJM, Dijk A van, Jongenelen CAM, Drukarch B, Reijerkerk A, Vries HE de, Kamp O, Musters RJP. Ultrasound and Microbubble-Induced Intra- and Intercellular Bioeffects in Primary Endothelial Cells. *Ultrasound Med Biol* 2009;**35**:1917–1927.
139. Meijering BDM, Juffermans LJM, Wamel A Van, Henning RH, Zuhorn IS, Emmer M, Versteilen AMG, Paulus WJ, Gilst WH Van, Kooiman K, Jong N De, Musters RJP, Deelman LE, Kamp O. Ultrasound and microbubble-targeted delivery of macromolecules is regulated by induction of endocytosis and pore formation. *Circ Res* 2009;**104**:679–687.
140. ParK J, Fan Z, Kumon RE, El-Sayed MEH, Deng CX. Modulation of intracellular Ca²⁺ concentration in brain microvascular endothelial cells in-vitro by acoustic cavitation. *Ultrasound Med Biol* 2011;**36**:1176–1187.
141. Belcik JT, Davidson BP, Xie A, Wu MD, Yadava M, Qi Y, Liang S, Chon CR, Ammi AY, Field J, Harmann L, Chilian WM, Linden J, Lindner JR. Augmentation of muscle blood flow by ultrasound cavitation by ATP and purinergic signaling. *Circulation* 2018;**135**:1240–1252.

142. Heath CH, Sorace A, Knowles J, Rosenthal E, Hoyt K. Microbubble Therapy Enhances Anti-tumor Properties of Cisplatin and Cetuximab In Vitro and In Vivo. *Otolaryngol Head Neck Surg* 2012;**146**:938–945.
143. Carson AR, McTiernan CF, Lavery L, Grata M, Leng X, Wang J, Chen X, Villanueva FS. Ultrasound-targeted microbubble destruction to deliver siRNA cancer therapy. *Cancer Res* 2012;**72**:6191–6199.
144. Kwekkeboom RFJ, Lei Z, Bogaards SJP, Aiazian E, Kamp O, Paulus WJ, Sluijter JPG, Musters RJP. Ultrasound and Microbubble-Induced Local Delivery of MicroRNA-Based Therapeutics. *Ultrasound Med Biol* 2015;**41**:163–176.
145. Temmerman ML De, Dewitte H, Vandenbroucke RE, Lucas B, Libert C, Demeester J, Smedt SC De, Lentacker I, Rejman J. mRNA-Lipoplex loaded microbubble contrast agents for ultrasound-assisted transfection of dendritic cells. *Biomaterials* 2011;**32**:9128–9135.
146. Xie A, Belcik T, Qi Y, Morgan TK, Champaneri SA, Taylor S, Davidson BP, Zhao Y, Klibanov AL, Kuliszewski MA, Leong-Poi H, Ammi A, Lindner JR. Ultrasound-Mediated Vascular Gene Transfection by Cavitation of Endothelial-Targeted Cationic Microbubbles. *JACC Cardiovasc Imaging* 2013;**5**:1253–1262.
147. Yu FTH, Chen X, Wang J, Qin B, Villanueva FS. Low Intensity Ultrasound Mediated Liposomal Doxorubicin Delivery Using Polymer Microbubbles. *Mol Pharm* 2015;**13**:55–64.
148. Christiansen JP, French BA, Klibanov AL, Kaul S, Lindner JR. Targeted tissue transfection with ultrasound destruction of plasmid-bearing cationic microbubbles. *Ultrasound Med Biol* 2003;**29**:1759–1767.
149. Wang DS, Panje C, Pysz MA. Cationic versus Neutral Microbubbles for Gene Delivery in Cancer. *Radiology* 2012;**264**:721–732.
150. Vannan M, McCreery T, Li P, Han Z. Ultrasound-Mediated Transfection of Canine Myocardium by Intravenous Administration of Cationic Microbubble-Linked Plasmid DNA. *Journal of the American Society of Echocardiography* 2002;**15**:214–218.
151. Sundaram KM, Arora S, Chang SS, Penson DF. Therapeutic Ultrasound and Prostate Cancer. *Semin Intervent Radiol* 2017;**264**:721–732.
152. Yeo SY, Bratke G, Grüll H. High Intensity Focused Ultrasound for Treatment of Bone. *Cancers (Basel)* 2022;**15**.
153. Chang WS, Jung HH, Kweon EJ, Zadicario E, Rachmilevitch I, Chang JW. Unilateral magnetic resonance guided focused ultrasound thalamotomy for essential tremor : practices and clinicoradiological outcomes. *J Neurol Neurosurg Psychiatry* 2015;**86**:257–264.

154. Wintrich J, Pavlicek V, Millenaar D, Ukena C. Mapping of an atrial tachycardia after Epicor™ high-intensity focused ultrasound ablation: A case report. *J Electrocardiol* 2021;**67**:19–22.
155. Ichijo S, Shindo T, Eguchi K, Monma Y, Nakata T. Low - intensity pulsed ultrasound therapy promotes recovery from stroke by enhancing angio - neurogenesis in mice in vivo. *Sci Rep* 2021;**11**:4958.
156. Khokhlova V, Fowlkes J, Roberts W, Schade G, Xu Z, Khokhlova T, Hall T, Maxwell A, Wang Y, Cain C. *Histotripsy Methods in Mechanical Disintegration of Tissue: Toward Clinical Applications*. 2016.
157. Feroze RA, Kopechek J, Zhu J, Chen X, Villanueva FS. Ultrasound-Induced Microbubble Cavitation for Targeted Delivery of MiR-29b Mimic to Treat Cardiac Fibrosis. *Ultrasound Med Biol* 2023;**49**:2573–2580.
158. Kopechek JA, McTiernan CF, Chen X, Zhu J, Mburu M, Feroze R, Whitehurst DA, Lavery L, Cyriac J, Villanueva FS. Ultrasound and microbubble-targeted delivery of a microRNA inhibitor to the heart suppresses cardiac hypertrophy and preserves cardiac function. *Theranostics* 2019;**9**:7088–7098.
159. Kwekkeboom RFJ, Sluijter JPG, Middelaar BJ Van, Metz CH, Brans MA, Kamp O, Paulus WJ, Musters RJP. Increased local delivery of antagomir therapeutics to the rodent myocardium using ultrasound and microbubbles. *Journal of Controlled Release* 2016;**222**:18–31.
160. Liu Y, Li L, Su Q, Liu T, Ma Z, Yang H. Ultrasound-targeted microbubble destruction enhances gene expression of microRNA-21 in swine heart via intracoronary delivery. *Echocardiography* 2015;**32**:1407–1416.
161. Sun W, Zhao P, Zhou Y, Xing C, Zhao L, Li Z, Yuan L. Ultrasound targeted microbubble destruction assisted exosomal delivery of miR-21 protects the heart from chemotherapy associated cardiotoxicity. *Biochem Biophys Res Commun* 2020;**532**:60–67.
162. Yusefi H, Helfield B. Ultrasound Contrast Imaging: Fundamentals and Emerging Technology. *Front Phys* 2022;**10**:1–16.
163. Kooiman K, Roovers S, Langeveld SAG, Kleven RT, Dewitte H, O'Reilly MA, Escoffre JM, Bouakaz A, Verweij MD, Hynynen K, Lentacker I, Stride E, Holland CK. Ultrasound-Responsive Cavitation Nuclei for Therapy and Drug Delivery. *Ultrasound Med Biol* 2020;**46**:1296–1325.
164. Lipsman N, Meng Y, Bethune AJ, Huang Y, Lam B, Masellis M, Herrmann N, Heyn C, Aubert I, Boutet A, Smith GS, Hynynen K, Black SE. Blood–brain barrier opening in Alzheimer’s disease using MR-guided focused ultrasound. *Nat Commun* 2018;**9**:2336.
165. Tsao CW, Aday AW, Almarzooq ZI, Alonso A, Beaton AZ, Bittencourt MS, Boehme AK, Buxton AE, Carson AP, Commodore-Mensah Y, Elkind MSV, Evenson KR, Eze-Nliam C,

- Ferguson JF, Generoso G, Ho JE, Kalani R, Khan SS, Kissela BM, Knutson KL, Levine DA, Lewis TT, Liu J, Loop MS, Ma J, Mussolino ME, Navaneethan SD, Perak AM, Poudel R, Rezk-Hanna M, Roth GA, Schroeder EB, Shah SH, Thacker EL, Vanwagner LB, Virani SS, Voecks JH, Wang NY, Yaffe K, Martin SS. *Heart Disease and Stroke Statistics-2022 Update: A Report from the American Heart Association. Circulation.* 2022.
166. Matkar PN, Singh KK, Leong-Poi H, Singh KK. Cardiac gene therapy: Are we there yet? *Gene Ther* 2016;**23**:635–648.
 167. Cannatà A, Ali H, Sinagra G, Giacca M. Gene Therapy for the Heart Lessons Learned and Future Perspectives. *Circ Res* 2020:1394–1414.
 168. Chen HH, Matkar PN, Afrasiabi K, Kuliszewski MA, Leong-Poi H. Prospect of ultrasound-mediated gene delivery in cardiovascular applications. *Expert Opin Biol Ther* 2016;**16**:815–826.
 169. Leong-poi H, Kuliszewski MA, Lekas M, Sibbald M, Teichert-kuliszewska K, Klibanov AL, Stewart DJ, Lindner JR. Therapeutic Arteriogenesis by Ultrasound-Mediated VEGF 165 Plasmid Gene Delivery to Chronically Ischemic Skeletal Muscle. *Circ Res* 2007;**101**:295–303.
 170. Mofid A, Newman NS, Lee PJH, Abbasi C, Matkar PN, Rudenko D, Kuliszewski MA, Chen HH, Afrasiabi K, Tsoporis JN, Gramolini AO, Connelly KA, Parker TG, Leong-Poi H. Cardiac overexpression of S100A6 attenuates cardiomyocyte apoptosis and reduces infarct size after myocardial ischemia-reperfusion. *J Am Heart Assoc* 2017;**6**:1–16.
 171. Cao S, Deng Q, Wang Y, Zhou Y, Zhou Q. Ultrasound-targeted microbubble destruction-mediated Ang1 gene transfection improves left ventricular structural and sympathetic nerve remodeling in canines with myocardial infarction. *Ann Transl Med* 2021;**9**:221–221.
 172. Suzuki J ichi, Ogawa M, Takayama K, Taniyama Y, Morishita R, Hirata Y, Nagai R, Isobe M. Ultrasound-Microbubble-Mediated Intercellular Adhesion Molecule-1 Small Interfering Ribonucleic Acid Transfection Attenuates Neointimal Formation After Arterial Injury in Mice. *J Am Coll Cardiol* 2010;**55**:904–913.
 173. Wang Z, Jiang S, Li S, Yu W, Chen J, Yu D, Zhao C, Li Y, Kang K, Wang R, Liang M, Xu M, Ou Y, Li P, Leng X, Tian J, R-Porter T. Targeted galectin-7 inhibition with ultrasound microbubble targeted gene therapy as a sole therapy to prevent acute rejection following heart transplantation in a Rodent model. *Biomaterials* 2020;**263**:120366.
 174. Cao WJ, Rosenblat JD, Roth NC, Kuliszewski MA, Matkar PN, Rudenko D, Liao C, Lee PJH, Leong-Poi H. Therapeutic Angiogenesis by Ultrasound-Mediated MicroRNA-126-3p Delivery. *Arterioscler Thromb Vasc Biol* 2015;**35**:2401–2411.
 175. Yi L, Chen Y, Jin Q, Deng C, Wu Y, Li H, Liu T, Li Y, Yang Y, Wang J, Lv Q, Zhang L, Xie M. Antagomir-155 Attenuates Acute Cardiac Rejection Using Ultrasound Targeted Microbubbles Destruction. *Adv Healthc Mater* 2020;**9**:1–10.

176. Chen S, Chen J, Huang P, Meng XL, Clayton S, Shen JS, Grayburn PA. Myocardial regeneration in adriamycin cardiomyopathy by nuclear expression of GLP1 using ultrasound targeted microbubble destruction. *Biochem Biophys Res Commun* 2015;**458**:823–829.
177. Chen S, Chen J, Meng XL, Shen JS, Huang J, Huang P, Pu Z, McNeill NH, Grayburn PA. ANGPTL8 reverses established adriamycin cardiomyopathy by stimulating adult cardiac progenitor cells. *Oncotarget* 2016;**7**:80391–80403.
178. Chen H, Kuliszeski MA, Rudenko D, Leong-Poi H. Preclinical Evaluation of Proangiogenic Gene Therapy by Ultrasound Targeted Microbubble Destruction of Vascular Endothelial Growth Factor Minicircle DNA in a Model of Severe Peripheral Arterial Disease in Watanabe Heritable Hyperlipidemic Rabbits (abstrac. *Circulation* 2017;**136**:A19897.
179. McMahon D, O'Reilly MA, Hynynen K. Therapeutic Agent Delivery across the Blood-Brain Barrier Using Focused Ultrasound. *Annu Rev Biomed Eng* 2021;**23**:89–113.
180. Isner JM, Pieczek A, Schainfeld R, Blair R, Haley L, Asahara T, Rosenfield K, Razvi S, Walsh K, Symes JF. Clinical evidence of angiogenesis after arterial gene transfer of phVEGF165 in patient with ischaemic limb. *Lancet* 1996;**348**:370–374.
181. Losordo DW, Vale PR, Symes JF, Dunnington CH, Esakof DD, Maysky M, Ashare AB, Lathi K, Isner JM. Gene Therapy for Myocardial Angiogenesis. Initial clinical results with direct myocarial injection of phVEGF165 as sole therapt for myocardial ischemia. *Circulation* 1998;**98**:2800–2805.
182. Anttila V, Saraste A, Knuuti J, Hedman M, Jaakkola P, Laugwitz KL, Krane M, Jeppsson A, Sillanmäki S, Rosenmeier J, Zingmark P, Rudvik A, Garkaviy P, Watson C, Pangalos MN, Chien KR, Fritsche-Danielson R, Collén A, Gan LM. Direct intramyocardial injection of VEGF mRNA in patients undergoing coronary artery bypass grafting. *Molecular Therapy* 2023;**31**:866–874.
183. Ylä-herttuala S, Martin JF. Cardiovascular gene therapy. *The Lancet* 2000;**355**:213–222.
184. Yla-Herttuala S, Bridges C, Katz MG, Korpisalo P. Angiogenic gene therapy in cardiovascular diseases : dream or vision ? *Eur Heart J* 2017;**38**:1365–1371.
185. Cannatà A, Ali H, Sinagra G, Giacca M. Gene Therapy for the Heart Lessons Learned and Future Perspectives. *Circulation Research*.
186. Hammond HK, Penny WF, Traverse JH, Henry TD, Watkins MW, Yancy CW, Sweis RN, Adler ED, Patel AN, Murray DR, Ross RS, Bhargava V, Maisel A, Barnard DD, Lai NC, Dalton ND, Lee ML, Narayan SM, Blanchard DG, Gao MH. Intracoronary gene transfer of adenylyl cyclase 6 in patients with heart failure: A randomized clinical trial. *JAMA Cardiol* 2016;**1**:163–171.

187. Zacchigna S, Zentilin L, Giacca M. Adeno-associated virus vectors as therapeutic and investigational tools in the cardiovascular system. *Circulation Research*.
188. Rincon MY, VandenDriessche T, Chuah MK. Gene therapy for cardiovascular disease: Advances in vector development, targeting, and delivery for clinical translation. *Cardiovascular Research*.
189. St George JA. Gene therapy progress and prospects: Adenoviral vectors. *Gene Therapy*.
190. Karvinen H, Pasanen E, Rissanen TT, Korpisalo P, Vähäkangas E, Jazwa A, Giacca M, Ylä-Herttuala S. Long-term VEGF-A expression promotes aberrant angiogenesis and fibrosis in skeletal muscle. *Gene Ther* 2011;**18**:1180.
191. Kulkarni JA, Cullis PR, Meel R Van Der. Lipid Nanoparticles Enabling Gene Therapies: From Concepts to Clinical Utility. *Nucleic Acid Ther* 2018;**28**:146–157.
192. Zimmermann TS, Lee ACH, Akinc A, Bramlage B, Bumcrot D, Fedoruk MN, Harborth J, Heyes JA, Jeffs LB, John M, Judge AD, Lam K, McClintock K, Nechev L V., Palmer LR, Racie T, Röhl I, Seiffert S, Shanmugam S, Sood V, Soutschek J, Toudjarska I, Wheat AJ, Yaworski E, Zedalis W, Koteliansky V, Manoharan M, Vornlocher HP, MacLachlan I. RNAi-mediated gene silencing in non-human primates. *Nature* 2006;**441**:111–114.
193. Cullis PR, Hope MJ. Lipid Nanoparticle Systems for Enabling Gene Therapies. *Molecular Therapy*.
194. Ilahibaks NF, Lei Z, Sluijter JPG. Extracellular vesicles as vehicles for drug delivery to the heart. *Eur Heart J* 2024.
195. Sahoo S, Adamiak M, Mathiyalagan P, Kenneweg F, Kafert-Kasting S, Thum T. Therapeutic and Diagnostic Translation of Extracellular Vesicles in Cardiovascular Diseases: Roadmap to the Clinic. *Circulation*.
196. Li X, Salvia S La, Liang Y, Adamiak M, Kohlbrenner E, Jeong D, Chepurko E, Ceholski D, Lopez-Gordo E, Yoon S, Mathiyalagan P, Agarwal N, Jha D, Lodha S, Daaboul G, Phan A, Raisinghani N, Zhang S, Zangi L, Gonzalez-Kozlova E, Dubois N, Dogra N, Hajjar RJ, Sahoo S. Extracellular Vesicle-Encapsulated Adeno-Associated Viruses for Therapeutic Gene Delivery to the Heart. *Circulation* 2023;**148**:405–425.
197. Song Y, Zhang C, Zhang J, Jiao Z, Dong N, Wang G, Wang Z, Wang L. Localized injection of miRNA-21-enriched extracellular vesicles effectively restores cardiac function after myocardial infarction. *Theranostics* 2019;**9**:2346–2360.
198. Nawaz M, Heydarkhan-Hagvall S, Tangruksa B, González-King Garibotti H, Jing Y, Maugeri M, Kohl F, Hultin L, Reyahi A, Camponeschi A, Kull B, Christoffersson J, Grimsholm O, Jennbacken K, Sundqvist M, Wiseman J, Bidar AW, Lindfors L, Synnergren J, Valadi H. Lipid Nanoparticles Deliver the Therapeutic VEGFA mRNA In Vitro and In Vivo and Transform Extracellular Vesicles for Their Functional Extensions. *Advanced Science* 2023;**10**.

199. Lai RC, Arslan F, Lee MM, Sze NSK, Choo A, Chen TS, Salto-Tellez M, Timmers L, Lee CN, Oakley RM El, Pasterkamp G, Kleijn DPV de, Lim SK. Exosome secreted by MSC reduces myocardial ischemia/reperfusion injury. *Stem Cell Res* 2010;**4**:214–222.
200. Abreu RC de, Fernandes H, Costa Martins PA da, Sahoo S, Emanuelli C, Ferreira L. Native and bioengineered extracellular vesicles for cardiovascular therapeutics. *Nature Reviews Cardiology*.
201. Mentkowski KI, Lang JK. Exosomes Engineered to Express a Cardiomyocyte Binding Peptide Demonstrate Improved Cardiac Retention in Vivo. *Sci Rep* 2019;**9**.
202. Helfield B. A Review of Phospholipid Encapsulated Ultrasound Contrast Agent Microbubble Physics. *Ultrasound Med Biol* 2019;**45**:282–300.
203. Leighton TG. *The acoustic bubble*. London: Academic Press; 1994.
204. Wu J, Nyborg WL. Ultrasound, cavitation bubbles and their interaction with cells. *Adv Drug Deliv Rev* 2008;**60**:1103–1116.
205. Postema M, Wamel A van, Lancée CT, Jong N de. Ultrasound-induced encapsulated microbubble phenomena. *Ultrasound Med Biol* 2004;**30**:827–840.
206. Apfel RE, Holland CK. Gauging the likelihood of cavitation from short-pulse, low-duty cycle diagnostic ultrasound. *Ultrasound Med Biol* 1991;**17**:179–185.
207. Price RJ, Skyba DM, Kaul S, Skalak TC. Delivery of colloidal particles and red blood cells to tissue through microvessel ruptures created by targeted microbubble destruction with ultrasound. *Circulation* 1998;**98**:1264–1267.
208. Skyba DM, Price RJ, Linka a Z, Skalak TC, Kaul S. Direct in vivo visualization of intravascular destruction of microbubbles by ultrasound and its local effects on tissue. *Circulation* 1998;**98**:290–293.
209. Helfield B, Chen X, Watkins SC, Villanueva FS. Biophysical insight into mechanisms of sonoporation. *Proceedings of the National Academy of Sciences* 2016;**113**:9983–9988.
210. Park J, Fan Z, Kumon RE, El-Sayed MEH, Deng CX. Modulation of intracellular Ca²⁺ concentration in brain microvascular endothelial cells in vitro by acoustic cavitation. *Ultrasound Med Biol* 2010;**36**:1176–1187.
211. Beekers I, Mastik F, Beurskens R, Tang PY, Vegter M, Steen AFW van der, Jong N de, Verweij MD, Kooiman K. High-Resolution Imaging of Intracellular Calcium Fluctuations Caused by Oscillating Microbubbles. *Ultrasound Med Biol* 2020;**46**:2017–2029.
212. Belcik JT, Mott BH, Xie A, Zhao Y, Kim S, Lindner NJ, Ammi A, Linden JM, Lindner JR. Augmentation of Limb Perfusion and Reversal of Tissue Ischemia Produced by Ultrasound-Mediated Microbubble Cavitation. *Circ Cardiovasc Imaging* 2015;**8**:1–9.
213. Chen H, Kreider W, Brayman AA, Bailey MR, Matula TJ. Blood vessel deformations on microsecond time scales by ultrasonic cavitation. *Phys Rev Lett* 2011;**106**:1–4.

214. Sheikov N, McDannold N, Vykhodtseva N, Jolesz F, Hynynen K. Cellular mechanisms of the blood-brain barrier opening induced by ultrasound in presence of microbubbles. *Ultrasound Med Biol* 2004;**30**:979–989.
215. Sheikov N, McDannold N, Sharma S, Hynynen K. Effect of focused ultrasound applied with an ultrasound contrast agent on the tight junctional integrity of the brain microvascular endothelium. *Ultrasound Med Biol* 2008;**34**:1093–1104.
216. Fekri F, Christian R, Santos D, Karshafian R. Ultrasound Microbubble Treatment Enhances Clathrin-Mediated Endocytosis and Fluid- Phase Uptake through Distinct Mechanisms. 2016:1–22.
217. Goertz DE. An overview of the influence of therapeutic ultrasound exposures on the vasculature: High intensity ultrasound and microbubble-mediated bioeffects. *International Journal of Hyperthermia* 2015;**31**:134–144.
218. Memari E, Hui F, Yusefi H, Helfield B. Fluid flow influences ultrasound-assisted endothelial membrane permeabilization and calcium flux. *Journal of Controlled Release* 2023;**358**:333–344.
219. O'Reilly MA, Hough O, Hynynen K. Blood-Brain Barrier closure time after controlled ultrasound-induced opening is independent of opening volume. *J Ultrasound Med* 2017;**36**:475–483.
220. Rooij T van, Skachkov I, Beekers I, Lattwein KR, Voorneveld JD, Kokhuis TJA, Bera D, Luan Y, Steen AFW van der, Jong N de, Kooiman K. Viability of endothelial cells after ultrasound-mediated sonoporation: Influence of targeting, oscillation, and displacement of microbubbles. *Journal of Controlled Release* 2016;**238**:197–211.
221. Helfield B, Chen X, Watkins SC, Villanueva FS. Transendothelial Perforations and the Sphere of Influence of Single-Site Sonoporation. *Ultrasound Med Biol* 2020;**46**:1686–1697.
222. Hu Y, Wan JMF, Yu ACH. Membrane perforation and recovery dynamics in microbubble-mediated sonoporation. *Ultrasound Med Biol* 2013;**39**:2393–2405.
223. Jones RM, McMahon D, Hynynen K. Ultrafast three-dimensional microbubble imaging in vivo predicts tissue damage volume distributions during nonthermal brain ablation. *Theranostics* 2020;**10**:7211–7230.
224. Chen ZY, Lin Y, Yang F, Jiang L, Ge SP. Gene therapy for cardiovascular disease mediated by ultrasound and microbubbles. *Cardiovasc Ultrasound* 2013;**11**:1–14.
225. Yemane PT, Åslund AKO, Snipstad S, Bjørkøy A, Grendstad K, Berg S, Mørch Y, Torp SH, Hansen R, Davies C de L. Effect of Ultrasound on the Vasculature and Extravasation of Nanoscale Particles Imaged in Real Time. *Ultrasound Med Biol* 2019;**45**:3028–3041.

226. Cho EE, Drazic J, Ganguly M, Stefanovic B, Hynynen K. Two-photon fluorescence microscopy study of cerebrovascular dynamics in ultrasound-induced blood – brain barrier opening. *Journal of Cerebral Blood Flow Metabolism* 2011;**31**:1852–1862.
227. Lentacker I, Smedt SC De, Sanders NN. Drug loaded microbubble design for ultrasound triggered delivery. *Soft Matter* 2009;**5**:2161–2170.
228. Meng Y, Hynynen K, Lipsman N. Applications of focused ultrasound in the brain: from thermoablation to drug delivery. *Nat Rev Neurol* 2021;**17**:7–22.
229. Kofoed RH, Aubert I. Focused ultrasound gene delivery for the treatment of neurological disorders. *Trends in Molecular Medicine*.
230. Hynynen K, McDannold N, Vykhodtseva N, Jolesz FA. Noninvasive MR imaging-guided focal opening of the blood-brain barrier in rabbits. *Radiology* 2001;**220**:640–646.
231. McDannold N, Vykhodtseva N, Hynynen K. Blood-Brain Barrier Disruption Induced by Focused Ultrasound and Circulating Preformed Microbubbles Appears to Be Characterized by the Mechanical Index. *Ultrasound Med Biol* 2008;**34**:834–840.
232. Wu SY, Sanchez CS, Samiotaki G, Buch A, Ferrera VP, Konofagou EE. Characterizing Focused-Ultrasound Mediated Drug Delivery to the Heterogeneous Primate Brain in Vivo with Acoustic Monitoring. *Sci Rep* 2016;**6**.
233. Arvanitis CD, Askoxylakis V, Guo Y, Datta M, Kloepper J, Ferraro GB, Bernabeu MO, Fukumura D, McDannold N, Jain RK. Mechanisms of enhanced drug delivery in brain metastases with focused ultrasound-induced blood–tumor barrier disruption. *Proceedings of the National Academy of Sciences* 2018;**115**:E8717–E8726.
234. O'Reilly MA, Hynynen K. Real-time feedback-controlled focused ultrasound disruption by using an acoustic emissions – based controller. *Radiology* 2012;**263**:96–106.
235. Mainprize T, Lipsman N, Huang Y, Meng Y, Bethune A, Ironside S, Heyn C, Alkins R, Trudeau M, Sahgal A, Perry J, Hynynen K. Blood-Brain Barrier Opening in Primary Brain Tumors with Non-invasive MR-Guided Focused Ultrasound: A Clinical Safety and Feasibility Study. *Sci Rep* 2019;**9**.
236. Carpentier A, Stupp R, Sonabend AM, Dufour H, Chinot O, Mathon B, Ducray F, Guyotat J, Baize N, Menei P, Groot J de, Weinberg JS, Liu BP, Guemas E, Desseaux C, Schmitt C, Bouchoux G, Canney M, Idbaih A. Repeated blood–brain barrier opening with a nine-emitter implantable ultrasound device in combination with carboplatin in recurrent glioblastoma: a phase I/II clinical trial. *Nat Commun* 2024;**15**.
237. Meng Y, Reilly RM, Pezo RC, Trudeau M, Sahgal A, Singnurkar A, Perry J, Myrehaug S, Pople CB, Davidson B, Llinas M, Hyen C, Huang Y, Hamani C, Suppiah S, Hynynen K, Lipsman N. MR-guided focused ultrasound enhances delivery of trastuzumab to Her2-positive brain metastases. *Sci Transl Med* 2021;**13**.

238. Jones RM, Hynynen KR. Advances in acoustic monitoring and control of focused ultrasound-mediated increases in blood-brain barrier permeability. *British Journal of Radiology* 2019;**92**:1–13.
239. Lee H, Guo Y, Ross JL, Schoen S, Degertekin FL, Arvanitis C. Spatially targeted brain cancer immunotherapy with closed-loop controlled focused ultrasound and immune checkpoint blockade. *Sci Adv* 2022;**8**.
240. Anastasiadis P, Gandhi D, Guo Y, Ahmed AK, Bentzen SM, Arvanitis C, Woodworth GF. Localized blood-brain barrier opening in infiltrating gliomas with MRI-guided acoustic emissions-controlled focused ultrasound. *Proc Natl Acad Sci U S A* 2021;**118**.
241. Hughes A, Khan DS, Alkins R. Current and Emerging Systems for Focused Ultrasound-Mediated Blood–Brain Barrier Opening. *Ultrasound in Medicine and Biology*.
242. Sonoda S, Tachibana K, Uchino E, Okubo A, Yamamoto M, Sakoda K, Hisatomi T, Sonoda KH, Negishi Y, Izumi Y, Takao S, Sakamoto T. Gene transfer to corneal epithelium and keratocytes mediated by ultrasound with microbubbles. *Invest Ophthalmol Vis Sci* 2006;**47**:558–564.
243. Sakakima Y, Hayashi S, Yagi Y, Hayakawa A, Tachibana K, Nakao A. Gene therapy for hepatocellular carcinoma using sonoporation enhanced by contrast agents. *Cancer Gene Ther* 2005;**12**:884–889.
244. Chen S, Ding J-H, Bekeredjian R, Yang B-Z, Shohet R V, Johnston SA, Hohmeier HE, Newgard CB, Grayburn PA. Efficient gene delivery to pancreatic islets with ultrasonic microbubble destruction technology
245. Wolfram JA, Donahue K. Gene Therapy to Treat Cardiovascular Disease. *J Am Heart Assoc* 2013;**2**:1–12.
246. Giacca M, Zacchigna S. VEGF gene therapy: Therapeutic angiogenesis in the clinic and beyond. *Gene Ther* 2012;**19**:622–629.
247. Isner JM. Myocardial gene therapy. *Nature* 2002;**415**:234–239.
248. Monte F Del, Williams E, Lebeche D, Schmidt U, Rosenzweig A, Gwathmey JK, Lewandowski ED, Hajjar RJ. Improvement in survival and cardiac metabolism after gene transfer of sarcoplasmic reticulum Ca²⁺-ATPase in a rat model of heart failure. *Circulation* 2001;**104**:1424–1429.
249. Lai NC, Roth DM, Gao MH, Tang T, Dalton N, Lai YY, Spellman M, Clopton P, Hammond KK. Intracoronary adenovirus encoding adenylyl cyclase VI increases left ventricular function in heart failure. *Circulation* 2004;**110**:330–336.
250. Kato K, Yin H, Agata J, Yoshida H, Chao L, Chao J. Adrenomedullin gene delivery attenuates myocardial infarction and apoptosis after ischemia and reperfusion. *Am J Physiol Heart Circ Physiol* 2003;**285**:1506–1514.

251. Chatterjee S, Stewart AS, Bish LT, Jayasankar V, Kim EM, Pirolli T, Burdick J, Woo YJ, Gardner TJ, Sweeney HL. Viral gene transfer of the antiapoptotic factor Bcl-2 protects against chronic postischemic heart failure. *Circulation* 2002;**106**:212–217.
252. He Z, Li H, Zuo S, Pasha Z, Wang Y, Yang Y, Jiang W, Ashraf M, Xu M. Transduction of Wnt11 promotes mesenchymal stem cell transdifferentiation into cardiac phenotypes. *Stem Cells Dev* 2011;**20**:1771–1778.
253. Pepe M, Mamdani M, Zentilin L, Csiszar A, Qanud K, Zacchigna S, Ungvari Z, Puligadda U, Moimas S, Xu X, Edwards JG, Hintze TH, Giacca M, Recchia FA. Intramyocardial VEGF-B167 gene delivery delays the progression towards congestive failure in dogs with pacing-induced dilated cardiomyopathy. *Circ Res* 2010;**106**:1893–1903.
254. Pachori AS, Melo LG, Zhang L, Solomon SD, Dzau VJ. Chronic recurrent myocardial ischemic injury is significantly attenuated by pre-emptive adeno-associated virus heme oxygenase-1 gene delivery. *J Am Coll Cardiol* 2006;**47**:635–643.
255. Sundararaman S, Miller TJ, Pastore JM, Kiedrowski M, Aras R, Penn MS. Plasmid-based transient human stromal cell-derived factor-1 gene transfer improves cardiac function in chronic heart failure. *Gene Ther* 2011;**18**:867–873.
256. Askari AT, Unzek S, Popovic ZB, Goldman CK, Forudi F, Kiedrowski M, Rovner A, Ellis SG, Thomas JD, Dicorleto PE, Topol EJ, Penn MS. Effect of stromal-cell-derived factor 1 on stem-cell homing and tissue regeneration in ischaemic cardiomyopathy Arman. *Lancet* 2003;**362**:697–703.
257. Shohet R V., Chen S, Zhou Y-T, Wang Z, Meidell RS, Unger RH, Grayburn P a. Echocardiographic Destruction of Albumin Microbubbles Directs Gene Delivery to the Myocardium. *Circulation* 2000;**101**:2554–2556.
258. Bekeredjian R, Chen SY, Frenkel PA, Grayburn PA, Shohet R V. Ultrasound-targeted microbubble destruction can repeatedly direct highly specific plasmid expression to the heart. *Circulation* 2003;**108**:1022–1026.
259. Kondo I, Ohmori K, Oshita A, Takeuchi H, Fuke S, Shinomiya K, Noma T, Namba T, Kohno M. Treatment of acute myocardial infarction by hepatocyte growth factor gene transfer: The first demonstration of myocardial transfer of a ‘functional’ gene using ultrasonic microbubble destruction. *J Am Coll Cardiol* 2004;**44**:644–653.
260. Ueda H, Sawa Y, Matsumoto K, Kitagawa-Sakakida S, Kawahira Y, Nakamura T, Kaneda Y, Matsuda H. Gene transfection of hepatocyte growth factor attenuates reperfusion injury in the heart. *Annals of Thoracic Surgery* 1999;**67**:1726–1731.
261. Wang W, Tayier B, Guan L, Yan F, Mu Y. Pre-transplantation of bone marrow mesenchymal stem cells amplifies the therapeutic effect of ultrasound-targeted microbubble destruction-mediated localized combined gene therapy in post-myocardial infarction heart failure rats. *Ultrasound Med Biol* 2022;**48**:830–845.

262. Erikson JM, Freeman GL, Chandrasekar B. Ultrasound-targeted antisense oligonucleotide attenuates ischemia/reperfusion-induced myocardial tumor necrosis factor- α . *J Mol Cell Cardiol* 2003;**35**:119–130.
263. Ciarambino T, Menna G, Sansone G, Giordano M. Cardiomyopathies : An Overview. *International Journal of Molecular Sciences Review* 2021;**22**:1–25.
264. Lee PJH, Rudenko D, Kuliszewski MA, Liao C, Kabir MG, Connelly KA, Leong-poi H. Survivin gene therapy attenuates left ventricular systolic dysfunction in doxorubicin cardiomyopathy by reducing apoptosis and fibrosis. 2014:423–433.
265. Qin X, Cai P, Liu C, Chen K, Jiang X, Chen W, Li J, Jiao X, Guo E, Yu Y, Sun L, Tian H. Cardioprotective effect of ultrasound-targeted destruction of Sirt3-loaded cationic microbubbles in a large animal model of pathological cardiac hypertrophy. *Acta Biomater* 2023.
266. Sun L, Huang CW, Wu J, Chen KJ, Li SH, Weisel RD, Rakowski H, Sung HW, Li RK. The use of cationic microbubbles to improve ultrasound-targeted gene delivery to the ischemic myocardium. *Biomaterials* 2013;**34**:2107–2116.
267. Kooiman K, Vos HJ, Versluis M, Jong N de. Acoustic behavior of microbubbles and implications for drug delivery. *Adv Drug Deliv Rev* 2014:1–21.
268. Kooiman K, Foppen-Harteveld M, Steen AFW van der, Jong N de. Sonoporation of endothelial cells by vibrating targeted microbubbles. *J Control Release* 2011;**154**:35–41.
269. Fujii H, Li SH, Wu J, Miyagi Y, Yau TM, Rakowski H, Egashira K, Guo J, Weisel RD, Li RK. Repeated and targeted transfer of angiogenic plasmids into the infarcted rat heart via ultrasound targeted microbubble destruction enhances cardiac repair. *Eur Heart J* 2011;**32**:2075–2084.
270. Kaufmann B a, Sanders JM, Davis C, Xie A, Aldred P, Sarembock IJ, Lindner JR. Molecular imaging of inflammation in atherosclerosis with targeted ultrasound detection of vascular cell adhesion molecule-1. *Circulation* 2007;**116**:276–284.
271. Hamilton AJ, Huang SL, Warnick D, Rabbat M, Kane B, Nagaraj A, Klegerman M, McPherson DD. Intravascular Ultrasound Molecular Imaging of Atheroma Components In Vivo. *J Am Coll Cardiol* 2004;**43**:453–460.
272. Villanueva FS, Jankowski RJ, Klibanov S, Pina ML, Alber SM, Watkins SC, Brandenburger GH, Wagner WR. Microbubbles targeted to intercellular adhesion molecule-1 bind to activated coronary artery endothelial cells. *Circulation* 1998;**98**:1–5.
273. Lindner JR, Song J, Christiansen J, Klibanov a L, Xu F, Ley K. Ultrasound assessment of inflammation and renal tissue injury with microbubbles targeted to P-selectin. *Circulation* 2001;**104**:2107–2112.

274. Davidson BP, Kaufmann BA, Belcik JT, Xie A, Qi Y, Lindner JR. Detection of antecedent myocardial ischemia with multiselectin molecular imaging. *J Am Coll Cardiol* 2012;**60**:1690–1697.
275. Pochon S, Tardy I, Bussat P, Bettinger T, Brochot J, Wronski M von, Passantino L, Schneider M. BR55: a lipopeptide-based VEGFR2-targeted ultrasound contrast agent for molecular imaging of angiogenesis. *Invest Radiol* 2010;**45**:89–95.
276. Mott B, Packwood W, Xie A, Belcik JT, Taylor RP, Zhao Y, Davidson BP, Lindner JR. Echocardiographic Ischemic Memory Imaging Through Complement-Mediated Vascular Adhesion of Phosphatidylserine-Containing Microbubbles. *JACC Cardiovasc Imaging* 2016;**9**:937–946.
277. Zhou Q, Deng Q, Hu BO, Wang YJ, Chen JL, Cui JJ, Cao S, Song HN. Ultrasound combined with targeted cationic microbubble-mediated angiogenesis gene transfection improves ischemic heart function. *Exp Ther Med* 2017;**13**:2293–2303.
278. Takeuchi H, Ohmori K, Kondo I, Oshita A, Shinomiya K, Yu Y, Takagi Y, Mizushige K, Kangawa K, Kohno M. Potentiation of C-type natriuretic peptide with ultrasound and microbubbles to prevent neointimal formation after vascular injury in rats. *Cardiovasc Res* 2003;**58**:231–238.
279. Kobulnik J, Kuliszewski MA, Stewart DJ, Lindner JR, Leong-Poi H. Comparison of Gene Delivery Techniques for Therapeutic Angiogenesis. *J Am Coll Cardiol* 2009;**54**:1735–1742.
280. Smith AH, Kuliszewski MA, Liao C, Rudenko D, Stewart DJ, Leong-Poi H. Sustained improvement in perfusion and flow reserve after temporally separated delivery of vascular endothelial growth factor and angiopoietin-1 plasmid deoxyribonucleic acid. *J Am Coll Cardiol* 2012;**59**:1320–1328.
281. Kuliszewski MA, Kobulnik J, Lindner JR, Stewart DJ, Leong-Poi H. Vascular gene transfer of SDF-1 promotes endothelial progenitor cell engraftment and enhances angiogenesis in ischemic muscle. *Molecular Therapy* 2011;**19**:895–902.
282. Song J, Qi M, Kaul S, Price RJ. Stimulation of arteriogenesis in skeletal muscle by microbubble destruction with ultrasound. *Circulation* 2002;**106**:1550–1555.
283. Song J, Cottler PS, Klibanov AL, Kaul S, Price RJ. Microvascular remodeling and accelerated hyperemia blood flow restoration in arterially occluded skeletal muscle exposed to ultrasonic microbubble destruction. *Am J Physiol Heart Circ Physiol* 2004;**287**:2754–2761.
284. Yoshida J, Ohmori K, Takeuchi H, Shinomiya K, Namba T, Kondo I, Kiyomoto H, Kohno M. Treatment of ischemic limbs based on local recruitment of vascular endothelial growth factor-producing inflammatory cells with ultrasonic microbubble destruction. *J Am Coll Cardiol* 2005;**46**:899–905.

285. Zhang X, Tian X, Li P, Zhu H, Zhou N, Fang Z, Yang Y, Jing Y, Yuan J. Ultrasound-targeted microbubble destruction promotes myocardial angiogenesis and functional improvements in rat model of diabetic cardiomyopathy. *BMC Cardiovasc Disord* 2021;**21**:1–9.
286. Zhou NQ, Song YT, Liu WZ, Yue RZ, Tian XQ, Yang SC, Yin YL, Li P. Diagnostic ultrasound-mediated microbubble cavitation dose-dependently improves diabetic cardiomyopathy through angiogenesis. *Cell Biol Int* 2023;**47**:178–187.
287. Anderson CD, Arthur JA, Zhang Y, Bharucha N, Karakikes I, Shohet R V. Non-viral in vivo cytidine base editing in hepatocytes using focused ultrasound targeted microbubbles. *Mol Ther Nucleic Acids* 2023;**33**:733–737.
288. Paranjape AN, D’Aiuto L, Zheng W, Chen X, Villanueva FS. A multicellular brain spheroid model for studying the mechanisms and bioeffects of ultrasound-enhanced drug penetration beyond the blood–brain barrier. *Sci Rep* 2024;**14**.
289. Wu J, Xie F, Kumar T, Liu J, Lof J, Shi W, Everbach EC, Porter TR. Improved sonothrombolysis from a modified diagnostic transducer delivering impulses containing a longer pulse duration. *Ultrasound Med Biol* 2014;**40**:1545–1553.
290. Sun Z, Xie Y, Lee RJ, Chen Y, Jin Q, Lv Q, Wang J, Yang Y, Li Y, Cai Y, Wang R, Han Z, Zhang L, Xie M. Myocardium-targeted transplantation of PHD2 shRNA-modified bone mesenchymal stem cells through ultrasound-targeted microbubble destruction protects the heart from acute myocardial infarction. *Theranostics* 2020;**10**:4967–4982.
291. Zhang L, Sun Z, Ren P, You M, Zhang J, Fang L, Wang J, Chen Y, Yan F, Zheng H, Xie M. Localized delivery of shRNA against PHD2 protects the heart from acute myocardial infarction through ultrasound-targeted cationic microbubble destruction. *Theranostics* 2017;**7**:51–66.
292. Deng Q, Hu B, Cao S, Song HN, Chen JL, Zhou Q. Improving the efficacy of therapeutic angiogenesis by UTMD-mediated Ang-1 gene delivery to the infarcted myocardium. *Int J Mol Med* 2015;**36**:335–344.
293. Li L, Wu S, Liu Z, Zhuo Z, Tan K, Xia H, Zhuo L, Deng X, Gao Y, Xu Y. Ultrasound-Targeted Microbubble Destruction Improves the Migration and Homing of Mesenchymal Stem Cells after Myocardial Infarction by Upregulating SDF-1/CXCR4: A Pilot Study. *Stem Cells Int* 2015;**2015**.
294. Zhou Z, Zhang P, Ren J, Ran H, Zheng Y, Li P, Zhang Q, Zhang M, Wang Z. Synergistic effects of ultrasound-targeted microbubble destruction and TAT peptide on gene transfection: An experimental study in vitro and in vivo. *Journal of Controlled Release* 2013;**170**:437–444.
295. Ling ZY, Shu S yu, Zhong SG, Luo J, Su L, Liu ZZ, Lan X Bin, Yuan GB, Zheng YY, Ran HT, Wang ZG, Yin YH. Ultrasound targeted microbubble destruction promotes

- angiogenesis and heart function by inducing myocardial microenvironment change. *Ultrasound Med Biol* 2013;**39**:2001–2010.
296. Yuan QY, Huang J, Chu BC, Li XJ, Li XS, Si LY. A targeted high-efficiency angiogenesis strategy as therapy for myocardial infarction. *Life Sci* 2012;**90**:695–702.
 297. Zhong S, Shu S, Wang Z, Luo J, Zhong W, Ran H, Zheng Y, Yin Y, Ling Z. Enhanced homing of mesenchymal stem cells to the ischemic myocardium by ultrasound-targeted microbubble destruction. *Ultrasonics* 2012;**52**:281–286.
 298. Xu YL, Gao YH, Liu Z, Tan K Bin, Hua X, Fang ZQ, Wang YL, Wang YJ, Xia HM, Zhuo ZX. Myocardium-targeted transplantation of mesenchymal stem cells by diagnostic ultrasound-mediated microbubble destruction improves cardiac function in myocardial infarction of New Zealand rabbits. *Int J Cardiol* 2010;**138**:182–195.
 299. Fujii H, Sun Z, Li S-H, Wu J, Fazel S, Weisel RD, Rakowski H, Lindner J, Li R-K. Ultrasound-Targeted Gene Delivery Induces Angiogenesis After a Myocardial Infarction in Mice. *JACC Cardiovasc Imaging* 2009;**2**:869–879.
 300. Erikson JM, Valente AJ, Mummidi S, Kandikattu HK, Demarco VG, Bender SB, Fay WP, Siebenlist U, Chandrasekar B. Targeting TRAF3IP2 by genetic and interventional approaches inhibits ischemia/reperfusion-induced myocardial injury and adverse remodeling. *Journal of Biological Chemistry* 2017;**292**:2345–2358.
 301. Du GQ, Shao ZB, Wu J, Yin WJ, Li SH, Wu J, Weisel RD, Tian JW, Li RK. Targeted myocardial delivery of GDF11 gene rejuvenates the aged mouse heart and enhances myocardial regeneration after ischemia–reperfusion injury. *Basic Res Cardiol* 2017;**112**:1–14.
 302. Yan P, Chen KJ, Wu J, Sun L, Sung HW, Weisel RD, Xie J, Li RK. The use of MMP2 antibody-conjugated cationic microbubble to target the ischemic myocardium, enhance Timp3 gene transfection and improve cardiac function. *Biomaterials* 2014;**35**:1063–1073.
 303. Gao J, Liu J, Meng Z, Li Y, Hong Y, Wang L, He L, Hu B, Zheng Y, Li T, Cui D, Shen E. Ultrasound-assisted C3F8-filled PLGA nanobubbles for enhanced FGF21 delivery and improved prophylactic treatment of diabetic cardiomyopathy. *Acta Biomater* 2021;**130**:395–408.
 304. Zheng L, Shen CL, Li JM, Ma YL, Yan N, Tian XQ, Zhao YZ. Assessment of the preventive effect against diabetic cardiomyopathy of FGF1-loaded nanoliposomes combined with microbubble cavitation by ultrasound. *Front Pharmacol* 2020;**10**:1–12.
 305. Tian XQ, Ni XW, Xu HL, Zheng L, ZhuGe DL, Chen B, Lu CT, Yuan JJ, Zhao YZ. Prevention of doxorubicin-induced cardiomyopathy using targeted MaFGF mediated by nanoparticles combined with ultrasound-targeted MB destruction. *Int J Nanomedicine* 2017;**12**:7103–7119.

306. Li W, Jin Q, Zhang L, He S, Song Y, Xu L. Ultrasonic Microbubble Cavitation Deliver Gal-3 shRNA to Inhibit Myocardial Fibrosis after Myocardial Infarction. *Pharmaceutics* 2023;1–13.
307. Su Q, Li L, Liu Y, Zhou Y, Wang J, Wen W. Ultrasound-targeted microbubble destruction-mediated microRNA-21 transfection regulated PDCD4/NF- κ B/TNF- α pathway to prevent coronary microembolization-induced cardiac dysfunction. *Gene Ther* 2015;22:1000–1006.
308. Mohr AM, Mott JL. Overview of microRNA biology. *Seminars in Liver Disease*.
309. Saliminejad K, Khorram Khorshid HR, Soleymani Fard S, Ghaffari SH. An overview of microRNAs: Biology, functions, therapeutics, and analysis methods. *Journal of Cellular Physiology*.
310. Esteller M. Non-coding RNAs in human disease. *Nature Reviews Genetics*.
311. Calin GA, Croce CM. MicroRNA signatures in human cancers. *Nature Reviews Cancer*.
312. Pauley KM, Cha S, Chan EKL. MicroRNA in autoimmunity and autoimmune diseases. *J Autoimmun* 2009;32:189–194.
313. Brennan GP, Henshall DC. MicroRNAs as regulators of brain function and targets for treatment of epilepsy. *Nature Reviews Neurology*.
314. Rooij E Van, Olson EN. MicroRNA therapeutics for cardiovascular disease: Opportunities and obstacles. *Nature Reviews Drug Discovery*.
315. Bader AG, Brown D, Winkler M. The promise of microRNA replacement therapy. *Cancer Research*.
316. Rooij E Van, Sutherland LB, Liu N, Williams AH, McAnally J, Gerard RD, Richardson JA, Olson EN. A signature pattern of stress-responsive microRNAs that can evoke cardiac hypertrophy and heart failure. *Proc Natl Acad Sci U S A* 2006;103:18255–18260.
317. Lagnerbauer B, Engelhardt S. MicroRNAs as therapeutic targets in cardiovascular disease. *J Clin Invest* 2022;132:e159179.
318. Callis TE, Pandya K, Hee YS, Tang RH, Tatsuguchi M, Huang ZP, Chen JF, Deng Z, Gunn B, Shumate J, Willis MS, Selzman CH, Wang DZ. MicroRNA-208a is a regulator of cardiac hypertrophy and conduction in mice. *Journal of Clinical Investigation* 2009;119:2772–2786.
319. Carè A, Catalucci D, Felicetti F, Bonci D, Addario A, Gallo P, Bang ML, Segnalini P, Gu Y, Dalton ND, Elia L, Latronico MVG, Høydal M, Autore C, Russo MA, Dorn GW, Ellingsen Ø, Ruiz-Lozano P, Peterson KL, Croce CM, Peschle C, Condorelli G. MicroRNA-133 controls cardiac hypertrophy. *Nat Med* 2007;13:613–618.
320. Wang K, Long B, Liu F, Wang JX, Liu CY, Zhao B, Zhou LY, Sun T, Wang M, Yu T, Gong Y, Liu J, Dong YH, Li N, Li PF. A circular RNA protects the heart from pathological hypertrophy and heart failure by targeting miR-223. *Eur Heart J* 2016;37:2602a–2611a.

321. Butters A, Lakdawala NK, Ingles J. Sex Differences in Hypertrophic Cardiomyopathy: Interaction With Genetics and Environment. *Current Heart Failure Reports*.
322. Seok H, Lee H, Lee S, Ahn SH, Lee HS, Kim GWD, Peak J, Park J, Cho YK, Jeong Y, Gu D, Jeong Y, Eom S, Jang ES, Chi SW. Position-specific oxidation of miR-1 encodes cardiac hypertrophy. *Nature* 2020;**584**:279–285.
323. Sayed D, Hong C, Chen IY, Lypowy J, Abdellatif M. MicroRNAs play an essential role in the development of cardiac hypertrophy. *Circ Res* 2007;**100**:416–424.
324. Bauersachs J, Solomon SD, Anker SD, Antorrena-Miranda I, Batkai S, Viereck J, Rump S, Filippatos G, Granzer U, Ponikowski P, Boer RA de, Vardeny O, Hauke W, Thum T. Efficacy and safety of CDR132L in patients with reduced left ventricular ejection fraction after myocardial infarction: Rationale and design of the HF-REVERT trial. *Eur J Heart Fail* 2024;**26**:674–682.
325. Rupaimoole R, Slack FJ. MicroRNA therapeutics: Towards a new era for the management of cancer and other diseases. *Nature Reviews Drug Discovery*.
326. Zhang Y, Wang Z, Gemeinhart RA. Progress in microRNA delivery. *Journal of Controlled Release*.
327. Lesizza P, Prosdocimo G, Martinelli V, Sinagra G, Zacchigna S, Giacca M. Single-Dose Intracardiac Injection of Pro-Regenerative MicroRNAs Improves Cardiac Function after Myocardial Infarction. *Circ Res* 2017;**120**:1298–1304.
328. Xie J, Ameres SL, Friedline R, Hung JH, Zhang Y, Xie Q, Zhong L, Su Q, He R, Li M, Li H, Mu X, Zhang H, Broderick JA, Kim JK, Weng Z, Flotte TR, Zamore PD, Gao G. Long-term, efficient inhibition of microRNA function in mice using rAAV vectors. *Nat Methods* 2012;**9**:403–409.
329. Mingozzi F, High KA. Therapeutic in vivo gene transfer for genetic disease using AAV: progress and challenges. *Nat Rev Genet* 2011;**12**:341–355.
330. Abrahao A, Meng Y, Llinas M, Huang Y, Hamani C, Mainprize T, Aubert I, Heyn C, Black SE, Hynynen K, Lipsman N, Zinman L. First-in-human trial of blood–brain barrier opening in amyotrophic lateral sclerosis using MR-guided focused ultrasound. *Nat Commun* 2019;**10**:4373.
331. Mainprize T, Lipsman N, Huang Y, Meng Y, Bethune A, Ironside S, Heyn C, Alkins R, Trudeau M, Sahgal A, Perry J, Hynynen K. Blood-Brain Barrier Opening in Primary Brain Tumors with Non-invasive MR-Guided Focused Ultrasound: A Clinical Safety and Feasibility Study. *Sci Rep* 2019;**9**:321.
332. Fisher DG, Cruz T, Hoch MR, Sharifi KA, Shah IM, Gorick CM, Breza VR, Debski AC, Samuels JD, Sheehan JP, Schlesinger D, Moore D, Mandell JW, Lukens JR, Miller GW, Tvrdik P, Price RJ. Focused ultrasound-microbubble treatment arrests the growth and formation of cerebral cavernous malformations. *Nat Biomed Eng* 2025.

333. Helfield B, Chen X, Watkins SC, Villanueva FS. Biophysical insight into mechanisms of sonoporation. *Proc Natl Acad Sci U S A* 2016;**113**:9983–9988.
334. Hazel K, Singh D, He S, Guertin Z, Husser MC, Helfield B. Focused ultrasound and microbubble-mediated delivery of CRISPR-Cas9 ribonucleoprotein to human induced pluripotent stem cells. *Molecular Therapy* 2025.
335. Moura B, Aimo A, Al-Mohammad A, Keramida K, Gal T Ben, Dorbala S, Todiere G, Cameli M, Barison A, Bayes-Genis A, Bardeleben RS von, Bucciarelli-Ducci C, Delgado V, Mordi IR, Seferovic P, Savarese G, Čelutkienė J, Rapezzi C, Emdin M, Coats A, Metra M, Rosano G. Diagnosis and management of patients with left ventricular hypertrophy: Role of multimodality cardiac imaging. A scientific statement of the Heart Failure Association of the European Society of Cardiology. *Eur J Heart Fail* 2023;**25**:1493–1506.
336. Singh D, Memari E, He S, Yusefi H, Helfield B. Cardiac gene delivery using ultrasound: State of the field. *Mol Ther Methods Clin Dev* 2024;**32**.
337. Matkar PN, Leong-Poi H, Singh KK. Cardiac gene therapy: Are we there yet? *Gene Therapy*.
338. Ganame J, Mertens L, Eidem BW, Claus P, D’hooge J, Havemann LM, McMahon CJ, Elayda MAA, Vaughn WK, Towbin JA, Ayres NA, Pignatelli RH. Regional myocardial deformation in children with hypertrophic cardiomyopathy: Morphological and clinical correlations. *Eur Heart J* 2007;**28**:2886–2894.
339. Maron BJ. Hypertrophic Cardiomyopathy A Systematic Review. *JAMA* 2002;**287**:1308–1320.
340. Li X, Guo X, Chang Y, Zhang Naijin, Sun Yingxian. Serum inflammatory factors and fibrosis indicators in hypertensive patients. *Am J Transl Res* 2022;**14**:4097–4103.
341. Meinild Lundby AK, Jacobs RA, Gehrig S, Leur J de, Hauser M, Bonne TC, Flück D, Dandanell S, Kirk N, Kaech A, Ziegler U, Larsen S, Lundby C. Exercise training increases skeletal muscle mitochondrial volume density by enlargement of existing mitochondria and not de novo biogenesis. *Acta Physiologica* 2018;**222**.
342. Menzies RA, Gold PH. The turnover of mitochondria in a variety of tissues of young adult and aged rats. *Journal of Biological Chemistry* 1971;**246**:2425–2429.
343. Martin TG, Juarros MA, Leinwand LA. Regression of cardiac hypertrophy in health and disease: mechanisms and therapeutic potential. *Nat Rev Cardiol* 2023;**20**:347–363.
344. Vakili BA, Okin PM, Devereux RB. Prognostic implications of left ventricular hypertrophy. *Am Heart J* 2001;**141**:334–341.
345. Levy D, Garrison RJ, Savage D, Kannel WB, Castelli WP. Prognostic Implications of Echocardiographically Determined Left Ventricular Mass in the Framingham Heart Study. *New England Journal of Medicine* 1990;**322**:1561–1566.

346. Levy D, Salomon M, D'agostino RB, Belanger AJ, Kannel WB. Prognostic Implications of Baseline Electrocardiographic Features and Their Serial Changes in Subjects With Left Ventricular Hypertrophy. *Circulation* 1994;**90**:1786–1793.
347. Pierdomenico SD, Lapenna D, Cuccurullo F. Regression of echocardiographic left ventricular hypertrophy after 2 years of therapy reduces cardiovascular risk in patients with essential hypertension. *Am J Hypertens* 2008;**21**:464–470.
348. Konstam MA, Rousseau MF, Kronenberg MW, Udelson JE, Melin J, Stewart D, Dolan N, Edens TR, Ahn S, Kinan D, Howe DM, Kilcoyne L, Metherall J, Benedict C, Yusuf S, Pouleur H. Effects of the Angiotensin Converting Enzyme Inhibitor Enalapril on the Long-term Progression of Left Ventricular Dysfunction in Patients With Heart Failure. *Circulation* 1992;**86**:431–438.
349. Wong M, Staszewsky L, Latini R, Barlera S, Volpi A, Chiang YT, Benza RL, Gottlieb SO, Kleemann TD, Rosconi F, Vandervoort PM, Cohn JN. Valsartan benefits left ventricular structure and function in heart failure: Val-HeFT echocardiographic study. *J Am Coll Cardiol* 2002;**40**:970–975.
350. Desai AS, Solomon SD, Shah AM, Claggett BL, Fang JC, Izzo J, McCague K, Abbas CA, Rocha R, Mitchell GF. Effect of sacubitril-valsartan vs enalapril on aortic stiffness in patients with heart failure and reduced ejection fraction: A randomized clinical trial. *JAMA* 2019;**322**:1077–1084.
351. Colucci WS, Kolia TJ, Adams KF, Armstrong WF, Ghali JK, Gottlieb SS, Greenberg B, Klibaner MI, Kukin ML, Sugg JE. Metoprolol reverses left ventricular remodeling in patients with asymptomatic systolic dysfunction: The REversal of VEntricular Remodeling with Toprol-XL (REVERT) Trial. *Circulation* 2007;**116**:49–56.
352. Rosendorff C, Dubiel R, Xu J, Chavanu KJ. Comparison of Olmesartan Medoxomil Versus Amlodipine Besylate on Regression of Ventricular and Vascular Hypertrophy. *American Journal of Cardiology* 2009;**104**:359–365.
353. Cavarretta E, Condorelli G. MiR-21 and cardiac fibrosis: Another brick in the wall? *European Heart Journal*.
354. Wang J, Huang W, Xu R, Nie Y, Cao X, Meng J, Xu X, Hu S, Zheng Z. MicroRNA-24 regulates cardiac fibrosis after myocardial infarction. *J Cell Mol Med* 2012;**16**:2150–2160.
355. Jeong D, Yoo J, Lee P, Kepreotis S V., Lee A, Wahlquist C, Brown BD, Kho C, Mercola M, Hajjar RJ. miR-25 Tough Decoy Enhances Cardiac Function in Heart Failure. *Molecular Therapy* 2018;**26**:718–729.
356. Matkovich SJ, Wang W, Tu Y, Eschenbacher WH, Dorn LE, Condorelli G, Diwan A, Nerbonne JM, Dorn GW. MicroRNA-133a protects against myocardial fibrosis and modulates electrical repolarization without affecting hypertrophy in pressure-overloaded adult hearts. *Circ Res* 2010;**106**:166–175.

357. Montgomery RL, Hullinger TG, Semus HM, Dickinson BA, Seto AG, Lynch JM, Stack C, Latimer PA, Olson EN, Eva Van Rooij ; Therapeutic Inhibition of miR-208a Improves Cardiac Function and Survival During Heart Failure. 2011.
358. Devaux Y, Badimon L. CDR132L: Another brick in the wall towards the use of miRNAs to treat cardiovascular disease. *European Heart Journal*.
359. Hinkel R, Batkai S, Bähr A, Bozoglu T, Straub S, Borchert T, Viereck J, Howe A, Hornaschewitz N, Oberberger L, Jurisch V, Kozlik-Feldmann R, Freudenthal F, Ziegler T, Weber C, Sperandio M, Engelhardt S, Laugwitz KL, Moretti A, Klymiuk N, Thum T, Kupatt C. AntimiR-132 Attenuates Myocardial Hypertrophy in an Animal Model of Percutaneous Aortic Constriction. *J Am Coll Cardiol* 2021;**77**:2923–2935.
360. Peng B, Chen Y, Leong KW. MicroRNA delivery for regenerative medicine. *Advanced Drug Delivery Reviews*.
361. Li Z, Rana TM. Therapeutic targeting of microRNAs: Current status and future challenges. *Nature Reviews Drug Discovery*.
362. Nagueh SF, Phelan D, Abraham T, Armour A, Desai MY, Dragulescu A, Gilliland Y, Lester SJ, Maldonado Y, Mohiddin S, Nieman K, Sperry BW, Woo A. Recommendations for Multimodality Cardiovascular Imaging of Patients with Hypertrophic Cardiomyopathy: An Update from the American Society of Echocardiography, in Collaboration with the American Society of Nuclear Cardiology, the Society for Cardiovascular Magnetic Resonance, and the Society of Cardiovascular Computed Tomography. *Journal of the American Society of Echocardiography* 2022;**35**:533–569.
363. Urbano-Moral JA, Gonzalez-Gonzalez AM, Maldonado G, Gutierrez-Garcia-Moreno L, Vivancos-Delgado R, Mora-Martin M De, Rodriguez-Palomares JF, Evangelista-Masip A. Contrast-Enhanced Echocardiographic Measurement of Left Ventricular Wall Thickness in Hypertrophic Cardiomyopathy: Comparison with Standard Echocardiography and Cardiac Magnetic Resonance. *Journal of the American Society of Echocardiography* 2020;**33**:1106–1115.
364. Sheikov N, McDannold N, Sharma S, Hynynen K. Effect of focused ultrasound applied with an ultrasound contrast agent on the tight junctional integrity of the brain microvascular endothelium. *Ultrasound Med Biol* 2008;**34**:1093–1104.
365. Rezai AR, D’Haese P-F, Finomore V, Carpenter J, Ranjan M, Wilhelmsen K, Mehta RI, Wang P, Najib U, Vieira Ligo Teixeira C, Arsiwala T, Tarabishy A, Tirumalai P, Claassen DO, Hodder S, Haut MW. Ultrasound Blood–Brain Barrier Opening and Aducanumab in Alzheimer’s Disease. *New England Journal of Medicine* 2024;**390**:55–62.
366. Schlesinger J, Schueler M, Grunert M, Fischer JJ, Zhang Q, Krueger T, Lange M, Tönjes M, Dunkel I, Sperling SR. The cardiac transcription network modulated by gata4, mef2a, nkx2.5, srf, histone modifications, and microRNAs. *PLoS Genet* 2011;**7**.

367. Oort RJ Van, Rooij E Van, Bourajjaj M, Schimmel J, Jansen MA, Nagel R Van Der, Doevendans PA, Schneider MD, Echteld CJA Van, Windt LJ De. MEF2 activates a genetic program promoting chamber dilation and contractile dysfunction in calcineurin-induced heart failure. *Circulation* 2006;**114**:298–308.
368. Han J, Molkentin JD. Regulation of MEF2 by p38 MAPK and its implication in cardiomyocyte biology. *Trends Cardiovasc Med* 2000;19–22.
369. Wilkins BJ, Molkentin JD. Calcium-calcineurin signaling in the regulation of cardiac hypertrophy. *Biochem Biophys Res Commun* 2004;**322**:1178–1191.
370. Fontes MSC, Veen TAB Van, Bakker JMT De, Rijen HVM Van. Functional consequences of abnormal Cx43 expression in the heart. *Biochim Biophys Acta Biomembr* 2012;**1818**:2020–2029.
371. Schulz R, Gorge PM, Gorge A, Ferdinandy P, Lampe PD, Leybaert L. Connexin 43 is an emerging therapeutic target in ischemia/reperfusion injury, cardioprotection and neuroprotection. *Pharmacology and Therapeutics*.
372. Burgess SN. Understudied, Under-Recognized, Underdiagnosed, and Undertreated: Sex-Based Disparities in Cardiovascular Medicine. *Circulation: Cardiovascular Interventions*.
373. Regitz-Zagrosek V, Kararigas G. Mechanistic Pathways of Sex Differences in Cardiovascular Disease. *Physiol Rev* 2017;**97**:1–37.
374. Sohrabji F, Selvamani A. Sex differences in miRNA as therapies for ischemic stroke. *Neurochemistry International*.
375. Bernardo BC, Ooi JYY, Matsumoto A, Tham YK, Singla S, Kiriazis H, Patterson NL, Sadoshima J, Obad S, Lin RCY, McMullen JR. Sex differences in response to miRNA-34a therapy in mouse models of cardiac disease: identification of sex-, disease- and treatment-regulated miRNAs. *Journal of Physiology* 2016;**594**:5959–5974.
376. Fliegner D, Schubert C, Penkalla A, Witt H, Kararigas G, Dworatzek E, Staub E, Martus P, Noppinger PR, Kintscher U, Gustafsson J-Å, Regitz-Zagrosek V. Female sex and estrogen receptor-attenuate cardiac remodeling and apoptosis in pressure overload. *Am J Physiol Regul Integr Comp Physiol* 2010;**298**:1597–1606.
377. Queirós AM, Eschen C, Fliegner D, Kararigas G, Dworatzek E, Westphal C, Sanchez Ruderisch H, Regitz-Zagrosek V. Sex- and estrogen-dependent regulation of a miRNA network in the healthy and hypertrophied heart. *Int J Cardiol* 2013;**169**:331–338.
378. He S, Singh D, Helfield B. Flow rate modulates focused ultrasound-mediated vascular delivery of microRNA. *Mol Ther Nucleic Acids* 2025;**36**.
379. Kuznetsov A V., Veksler V, Gellerich FN, Saks V, Margreiter R, Kunz WS. Analysis of mitochondrial function in situ in permeabilized muscle fibers, tissues and cells. *Nat Protoc* 2008;**3**:965–976.

380. Wisløff U, Helgerud J, Johan Kemi O, Ellingsen Ø. Intensity-controlled treadmill running in rats: VO₂max and cardiac hypertrophy. *Am J Physiol Heart Circ Physiol* 2001;**280**:1301–1310.
381. Noll NA, Lal H, Merryman WD. Mouse Models of Heart Failure with Preserved or Reduced Ejection Fraction. *American Journal of Pathology* 2020;**190**:1596–1608.
382. Lamortes VJ, Thorburns J, Abshers D, Spiegelss A, Brown JH, Chien KR, Feramiscosln JR, Knowltons KU. Gq- and Ras-dependent Pathways Mediate Hypertrophy of Neonatal Rat Ventricular Myocytes following cw , -Adrenergic Stimulation *. *J Biol Chem* 1994;**269**:13490–13496.
383. Cho JS, Cho EJ, Lee J, Yang K, Park M, Park G. Myocardial Mechanics in a Rat Model with Banding and Debanding of the Ascending Aorta. *J Cardiovasc Ultrasound* 2014;**22**:189–195.
384. Bjørnstad JL, Skrbic B, Sjaastad I, Bjørnstad S, Christensen G. A mouse model of reverse cardiac remodelling following banding-debanding of the ascending aorta. *Acta Physiol* 2012;**205**:92–102.
385. Roma-Rodrigues C, Raposo LR, Fernandes AR. MicroRNAs Based Therapy of Hypertrophic Cardiomyopathy: The Road Traveled so Far. *Biomed Res Int* 2015;**2015**.
386. Diniz GP, Lino CA, Guedes EC, Nascimento Moreira L do, Barreto-Chaves MLM. Cardiac microRNA-133 is down-regulated in thyroid hormone-mediated cardiac hypertrophy partially via Type 1 Angiotensin II receptor. *Basic Res Cardiol* 2015;**110**:1–11.
387. Wang D, Zhai G, Ji Y, Jing H. microRNA-10a targets T-box 5 to inhibit the development of cardiac hypertrophy. *Int Heart J* 2017;**58**:100–106.
388. Lu Y, Wu F. A new miRNA regulator, miR-672, reduces cardiac hypertrophy by inhibiting JUN expression. *Gene* 2018;**648**:21–30.
389. Marian AJ, Braunwald E. Hypertrophic cardiomyopathy: Genetics, pathogenesis, clinical manifestations, diagnosis, and therapy. *Circ Res* 2017;**121**:749–770.
390. Zalivina I, Barwari T, Yin X, Langley SR, Barallobre-barreiro J, Wakimoto H, Zampetaki A, Mayr M, Avkiran M, Eminaga S. Inhibition of miR-199a-3p in a murine hypertrophic cardiomyopathy (HCM) model attenuates fibrotic remodeling. *Journal of Molecular and Cellular Cardiology Plus* 2023;**6**:100056.
391. Reichart D, Newby GA, Wakimoto H, Lun M, Gorham JM, Curran JJ, Raguram A, DeLaughter DM, Conner DA, Marsiglia JDC, Kohli S, Chmatal L, Page DC, Zabaleta N, Vandenberghe L, Liu DR, Seidman JG, Seidman C. Efficient in vivo genome editing prevents hypertrophic cardiomyopathy in mice. *Nat Med* 2023;**29**:412–421.
392. Allen TM, Cullis PR. Drug delivery systems: Entering the mainstream. *Science (1979)* 2004;**303**:1818–1822.

393. Manzari MT, Shamay Y, Kiguchi H, Rosen N, Scaltriti M, Heller DA. Targeted drug delivery strategies for precision medicines. *Nat Rev Mater* 2021;**6**:351–370.
394. Peer D, Karp JM, Hong S, Farokhzad OC, Margalit R, Langer R. Nanocarriers as an emerging platform for cancer therapy. *Nat Nanotechnol* 2007;**2**:751–760.
395. Langer R. New Methods of Drug Delivery. *Science (1979)* 1990;**249**:1527–1533.
396. Peer D, Karp JM, Hong S, Farokhzad OC, Margalit R, Langer R. Nanocarriers as an emerging platform for cancer therapy. *Nat Nanotechnol* 2007;**2**:751–760.
397. Dong P, Rakesh KP, Manukumar HM, Mohammed YHE, Karthik CS, Sumathi S, Mallu P, Qin HL. Innovative nano-carriers in anticancer drug delivery-a comprehensive review. *Bioorg Chem* 2019;**85**:325–336.
398. Andrews NW, Corrotte M. Plasma membrane repair. *Current Biology* 2018;**28**:R392–R397.
399. Jimenez AJ, Perez F. Plasma membrane repair: the adaptable cell life-insurance. *Curr Opin Cell Biol* 2017;**47**:99–107.
400. McNeil PL, Kirchhausen T. An emergency response team for membrane repair. *Nat Rev Mol Cell Biol* 2005;**6**:499–505.
401. Horn A, Jaiswal JK. Cellular mechanisms and signals that coordinate plasma membrane repair. *Cellular and Molecular Life Sciences* 2018;**75**:3751–3770.
402. Zhang Y, Yu LC. Single-cell microinjection technology in cell biology. *BioEssays* 2008;**30**:606–610.
403. Gordon JW, Scangos GA, Plotkin DJ, Barbosa JA, Ruddle FH. Genetic transformation of mouse embryos by microinjection of purified DNA. *Proc Natl Acad Sci U S A* 1980;**77**:7380–7384.
404. Hofmann A, Zakhartchenko V, Weppert M, Sebald H, Wenigerkind H, Brem G, Wolf E, Pfeifer A. Generation of transgenic cattle by lentiviral gene transfer into oocytes. *Biol Reprod* 2004;**71**:405–409.
405. Auerbach AB. Production of functional transgenic mice by DNA pronuclear microinjection. *Acta Biochim Pol* 2004;**51**:9–31.
406. Bongso TA, Sathananthan AH, Wong PC, Ratnam SS, Ng SC, Anandakumar C, Ganatra S. Human fertilization by micro-injection of immotile spermatozoa. *Human Reproduction* 1989;**4**:175–179.
407. Szabo TL. *Diagnostic Ultrasound Imaging: Inside and Out*. 1st edition. London, UK: Elsevier; 2004.
408. Medwin H. Counting bubbles acoustically : a review. *Ultrasonics* 1977:7–13.

409. Helfield BL, Chen X, Qin B, Watkins SC, Villanueva FS. Mechanistic Insight into Sonoporation with Ultrasound-Stimulated Polymer Microbubbles. *Ultrasound Med Biol* 2017;**43**:2678–2689.
410. Qin P, Xu L, Han T, Du L, Yu ACH. Effect of non-acoustic parameters on heterogeneous sonoporation mediated by single-pulse ultrasound and microbubbles. *Ultrason Sonochem* 2016;**31**:107–115.
411. Kooiman K, Foppen-Harteveld M, Steen AFW van der, Jong N de. Sonoporation of endothelial cells by vibrating targeted microbubbles. *J Control Release* 2011;**154**:35–41.
412. Mehier-Humbert S, Bettinger T, Yan F, Guy RH. Plasma membrane poration induced by ultrasound exposure: Implication for drug delivery. *Journal of Controlled Release* 2005;**104**:213–222.
413. Prentice P, Cuschieri A, Dholakia K, Prausnitz M, Campbell P. Membrane disruption by optically controlled microbubble cavitation. *Nat Phys* 2005;**1**:107–110.
414. Zeghimi A, Escoffre JM, Bouakaz A. Role of endocytosis in sonoporation-mediated membrane permeabilization and uptake of small molecules: A electron microscopy study. *Phys Biol* 2015;**12**.
415. Fan Z, Liu H, Mayer M, Deng CX. Spatiotemporally controlled single cell sonoporation. *Proceedings of the National Academy of Sciences* 2012;**109**:16486–16491.
416. Helfield B, Black JJ, Qin B, Pacella J, Chen X, Villanueva FS. Fluid Viscosity Affects the Fragmentation and Inertial Cavitation Threshold of Lipid-Encapsulated Microbubbles. *Ultrasound Med Biol* 2016;**42**:782–794.
417. Bao S, Thrall BD, Miller DL. Transfection of a reporter plasmid into cultured cells by sonoporation in vitro. *Ultrasound Med Biol* 1997;**23**:953–959.
418. Hynynen K. Ultrasound for drug and gene delivery to the brain. *Adv Drug Deliv Rev* 2008;**60**:1209–1217.
419. Escoffre JM, Piron J, Novell A, Bouakaz A. Doxorubicin delivery into tumor cells with ultrasound and microbubbles. *Mol Pharm* 2011;**8**:799–806.
420. Bulner S, Prodeus A, Garipey J, Hynynen K, Goertz DE. Enhancing Checkpoint Inhibitor Therapy with Ultrasound Stimulated Microbubbles. *Ultrasound Med Biol* 2019;**45**:500–512.
421. Kopechek JA, Carson AR, McTiernan CF, Chen X, Hasjim B, Lavery L, Sen M, Grandis JR, Villanueva FS. Ultrasound targeted microbubble destruction-mediated delivery of a transcription factor decoy inhibits STAT3 signaling and tumor growth. *Theranostics* 2015;**5**:1378–1387.
422. Aycock KN, Davalos R V. Irreversible Electroporation: Background, Theory, and Review of Recent Developments in Clinical Oncology. *Bioelectricity* 2019;**1**:214–234.

423. Schwan HP. Electrical properties of tissue and cell suspensions. *Adv Biol Med Phys* 1957;**5**:147–209.
424. Gehl J. Electroporation: Theory and methods, perspectives for drug delivery, gene therapy and research. *Acta Physiol Scand* 2003;**177**:437–447.
425. Teissié J, Rols MP. An experimental evaluation of the critical potential difference inducing cell membrane electroporation. *Biophys J* 1993;**65**:409–413.
426. Shil P, Bidaye S, Vidyasagar PB. Analysing the effects of surface distribution of pores in cell electroporation for a cell membrane containing cholesterol. *J Phys D Appl Phys* 2008;**41**.
427. Krassowska W, Filev PD. Modeling electroporation in a single cell. *Biophys J* 2007;**92**:404–417.
428. Kotnik T, Rems L, Tarek M, Miklavcic D. Membrane Electroporation and Electroporation: Mechanisms and Models. *Annu Rev Biophys* 2019;**48**:63–91.
429. Frandsen SK, Vissing M, Gehl J. A comprehensive review of calcium electroporation —A novel cancer treatment modality. *Cancers (Basel)* 2020;**12**:1–21.
430. Teixeira M, Py BF, Bosc C, Laubret D, Moutin MJ, Marvel J, Flamant F, Markossian S. Electroporation of mice zygotes with dual guide RNA/Cas9 complexes for simple and efficient cloning-free genome editing. *Sci Rep* 2018;**8**:1–9.
431. Aslan H, Zilberman Y, Arbeli V, Sheyn D, Matan Y, Liebergall M, Li JZ, Helm GA, Gazit D, Gazit Z. Nucleofection-based Ex vivo nonviral gene delivery to human stem cells as a platform for tissue regeneration. *Tissue Eng* 2006;**12**:877–889.
432. Chicaybam L, Abdo L, Viegas M, Marques LVC, Sousa P de, Batista-Silva LR, Alves-Monteiro V, Bonecker S, Monte-Mór B, Bonamino MH. Transposon-mediated generation of CAR-T cells shows efficient anti B-cell leukemia response after ex vivo expansion. *Gene Ther* 2020;**27**:85–95.
433. Lizano C, Sanz S, Luque J, Pinilla M. In vitro study of alcohol dehydrogenase and acetaldehyde dehydrogenase encapsulated into human erythrocytes by an electroporation procedure. *Biochim Biophys Acta Gen Subj* 1998;**1425**:328–336.
434. Heller R, Jaroszeski M, Atkin A, Moradpour D, Gilbert R, Wands J, Nicolau C. In vivo gene electroinjection and expression in rat liver. *FEBS Lett* 1996;**389**:225–228.
435. Fang Z, Chen L, Moser MAJ, Zhang W, Qin Z, Zhang B. Electroporation-Based Therapy for Brain Tumors: A Review. *J Biomech Eng* 2021;**143**.
436. Maor E, Ivorra A, Leor J, Rubinsky B. The effect of irreversible electroporation on blood vessels. *Technol Cancer Res Treat* 2007;**6**:307–312.

437. Lee JM, Choi HS, Chun HJ, Kim ES, Keum B, Seo YS, Jeon YT, Lee HS, Um SH, Kim CD, Kim HB. EUS-guided irreversible electroporation using endoscopic needle-electrode in porcine pancreas. *Surg Endosc* 2019;**33**:658–662.
438. Rubinsky J, Onik G, Mikus P, Rubinsky B. Optimal Parameters for the Destruction of Prostate Cancer Using Irreversible Electroporation. *Journal of Urology* 2008;**180**:2668–2674.
439. Stevenson DJ, Gunn-Moore FJ, Campbell P, Dholakia K. Single cell optical transfection. *J R Soc Interface* 2010;**7**:863–871.
440. Schneckenburger H. Laser-assisted optoporation of cells and tissues – a mini-review. *Biomed Opt Express* 2019;**10**:2883.
441. Peng Q, Juzeniene A, Chen J, Svaasand LO, Warloe T, Giercksky KE, Moan J. Lasers in medicine. *Reports on Progress in Physics* 2008;**71**.
442. Vogel A, Noack J, Hüttman G, Paltauf G. Mechanisms of femtosecond laser nanosurgery of cells and tissues. *Appl Phys B* 2005;**81**:1015–1047.
443. Soughayer JS, Krasieva T, Jacobson SC, Ramsey JM, Tromberg BJ, Allbritton NL. Characterization of cellular optoporation with distance. *Anal Chem* 2000;**72**:1342–1347.
444. Fan Q, Hu W, Ohta AT. Efficient single-cell poration by microsecond laser pulses. *Lab Chip* 2015;**15**:581–588.
445. Tsukakoshi M, Kurata S, Nomiya Y, Ikawa Y, Kasuya T. A novel method of DNA transfection by laser microbeam cell surgery. *Applied Physics B Photophysics and Laser Chemistry* 1984;**35**:135–140.
446. Lei M, Xu H, Yang H, Yao B. Femtosecond laser-assisted microinjection into living neurons. *J Neurosci Methods* 2008;**174**:215–218.
447. Uchugonova A, König K, Bueckle R, Isemann A, Tempea G. Targeted transfection of stem cells with sub-20 femtosecond laser pulses. *Opt Express* 2008;**16**:9357.
448. Bergeron E, Boutopoulos C, Martel R, Torres A, Rodriguez C, Niskanen J, Lebrun JJ, Winnik FM, Sapieha P, Meunier M. Cell-specific optoporation with near-infrared ultrafast laser and functionalized gold nanoparticles. *Nanoscale* 2015;**7**:17836–17847.
449. Jaiswal JK, Lauritzen SP, Scheffer L, Sakaguchi M, Bunkenborg J, Simon SM, Kallunki T, Jäätelä M, Nylandsted J. S100A11 is required for efficient plasma membrane repair and survival of invasive cancer cells. *Nat Commun* 2014;**5**:1–13.
450. Croissant C, Gounou C, Bouvet F, Tan S, Bouter A. Annexin-A6 in Membrane Repair of Human Skeletal Muscle Cell: A Role in the Cap Subdomain. *Cells* 2020;**9**.
451. Boye TL, Maeda K, Pezeshkian W, Sønder SL, Haeger SC, Gerke V, Simonsen AC, Nylandsted J. Annexin A4 and A6 induce membrane curvature and constriction during cell membrane repair. *Nat Commun* 2017;**8**:1–10.

452. Häger SC, Nylandsted J. Annexins: players of single cell wound healing and regeneration. *Commun Integr Biol* 2019;**12**:162–165.
453. Bi GQ, Alderton JM, Steinhardt RA. Calcium-regulated exocytosis is required for cell membrane resealing. *Journal of Cell Biology* 1995;**131**:1747–1758.
454. Shen SS, Tucker WC, Chapman ER, Steinhardt RA. Molecular regulation of membrane resealing in 3T3 fibroblasts. *Journal of Biological Chemistry* 2005;**280**:1652–1660.
455. Koerdt SN, Gerke V. Annexin A2 is involved in Ca²⁺-dependent plasma membrane repair in primary human endothelial cells. *BBA - Molecular Cell Research* 2017;**1864**:1046–1053.
456. Boucher E, Mandato CA. Plasma membrane and cytoskeleton dynamics during single-cell wound healing. *Biochim Biophys Acta* 2015;**1853**:2649–2661.
457. Cai C, Weisleder N, Ko JK, Komazaki S, Sunada Y, Nishi M, Takeshima H, Ma J. Membrane repair defects in muscular dystrophy are linked to altered interaction between MG53, caveolin-3, and dysferlin. *Journal of Biological Chemistry* 2009;**284**:15894–15902.
458. Cai C, Weisleder N, Ko JK, Komazaki S, Sunada Y, Nishi M, Takeshima H, Ma J. Membrane repair defects in muscular dystrophy are linked to altered interaction between MG53, caveolin-3, and dysferlin. *Journal of Biological Chemistry* 2009;**284**:15894–15902.
459. Dias C, Nylandsted J. Plasma membrane integrity in health and disease: significance and therapeutic potential. *Cell Discov* 2021;**7**.
460. Jimenez AJ, Maiuri P, Lafaurie-Janvore J, Divoux S, Piel M, Perez F. ESCRT machinery is required for plasma membrane repair. *Science* 2014;**343**:1247136.
461. Scheffer LL, Sreetama SC handra, Sharma N, Medikayala S, Brown KJ, Defour A, Jaiswal JK. Mechanism of Ca²⁺-triggered ESCRT assembly and regulation of cell membrane repair. *Nat Commun* 2014;**5**:5646.
462. Castro-Gomes T, Corrotte M, Tam C, Andrews NW. Plasma membrane repair is regulated extracellularly by proteases released from lysosomes. *PLoS One* 2016;**11**:1–26.
463. Brochard-Wyart F, Gennes PG De, Sandre O. Transient pores in stretched vesicles: role of leak-out. *Physica A: Statistical Mechanics and its Applications* 2000;**278**:32–51.
464. Jimenez AJ, Perez F. Physico-chemical and biological considerations for membrane wound evolution and repair in animal cells. *Semin Cell Dev Biol* 2015;**45**:2–9.
465. Terasaki M, Miyake K, Mcneil PL. Large Plasma Membrane Disruptions Are Rapidly Resealed by Ca²⁺-dependent Vesicle–Vesicle Fusion Events. 1997;**139**:63–74.
466. Togo T. Disruption of the plasma membrane stimulates rearrangement of microtubules and lipid traffic toward the wound site. *J Cell Sci* 2006;**119**:2780–2786.

467. Bushinsky DA, Monk RD. Calcium. *Lancet* 1998;**352**:306–311.
468. Deng CX, Sieling F, Pan H, Cui J. Ultrasound-induced cell membrane porosity. *Ultrasound Med Biol* 2004;**30**:519–526.
469. McNeil PL, Vogel SS, Miyake K, Terasaki M. Patching plasma membrane disruptions with cytoplasmic membrane. *J Cell Sci* 2000;**113**:1891–1902.
470. Potez S, Luginbühl M, Monastyrskaya K, Hostettler A, Draeger A, Babiychuk EB. Tailored protection against plasmalemmal injury by annexins with different Ca²⁺ sensitivities. *Journal of Biological Chemistry* 2011;**286**:17982–17991.
471. Steinhardt RA, Bi G, Alderton JM. Cell Membrane Resealing by a Vesicular Mechanism Similar to Neurotransmitter Release. *Science (1979)* 1994;**263**:390–393.
472. Ottaviano FG, Handy DE, Loscalzo J. Redox regulation in the extracellular environment. *Circulation Journal* 2008;**72**:1–16.
473. Clarke MSF, Caldwell RW, Chiao H, Miyake K, McNeil PL. Contraction-induced cell wounding and release of fibroblast growth factor in heart. *Circ Res* 1995;**76**:927–934.
474. Karatekin E, Sandre O, Guitouni H, Borghi N, Puech P-H, Brochard-Wyart F. Cascades of transient pores in giant vesicles: line tension and transport. *Biophys J* 2003;**84**:1734–1749.
475. Draeger A, Monastyrskaya K, Babiychuk EB. Plasma membrane repair and cellular damage control: The annexin survival kit. *Biochem Pharmacol* 2011;**81**:703–712.
476. Bouter A, Gounou C, Bérat R, Tan S, Gallois B, Granier T, D’Estaintot BL, Pöschl E, Brachvogel B, Brisson AR. Annexin-A5 assembled into two-dimensional arrays promotes cell membrane repair. *Nat Commun* 2011;**2**.
477. Martinez I, Chakrabarti S, Hellevik T, Morehead J, Fowler K, Andrews NW. Synaptotagmin vii regulates Ca²⁺-dependent exocytosis of lysosomes in fibroblasts. *Journal of Cell Biology* 2000;**148**:1141–1149.
478. Mercier V, Larios J, Molinard G, Goujon A, Matile S, Gruenberg J, Roux A. Endosomal membrane tension regulates ESCRT-III-dependent intra-lumenal vesicle formation. *Nat Cell Biol* 2020;**22**:947–959.
479. Castro-Gomes T, Koushik AB, Andrews NW. ESCRT: nipping the wound in the bud? *Trends Biochem Sci* 2014;**39**:307–309.
480. Idone V, Tam C, Goss JW, Toomre D, Pypaert M, Andrews NW. Repair of injured plasma membrane by rapid Ca²⁺ dependent endocytosis. *Journal of Cell Biology* 2008;**180**:905–914.
481. Tam C, Idone V, Devlin C, Fernandes MC, Flannery A, He X, Schuchman E, Tabas I, Andrews NW. Exocytosis of acid sphingomyelinase by wounded cells promotes endocytosis and plasma membrane repair. *Journal of Cell Biology* 2010;**189**:1027–1038.

482. Grassmé H, Riethmüller J, Gulbins E. Biological aspects of ceramide-enriched membrane domains. *Prog Lipid Res* 2007;**46**:161–170.
483. Sonnemann KJ, Bement WM. Wound repair: toward understanding and integration of single-cell and multicellular wound responses. *Annu Rev Cell Dev Biol* 2011;**27**:237–263.
484. Mandato CA, Bement WM. Actomyosin Transports Microtubules and Microtubules Control Actomyosin Recruitment during *Xenopus* Oocyte Wound Healing. 2003;**13**:1096–1105.
485. Abreu-Blanco MT, Verboon JM, Parkhurst SM. Coordination of Rho family GTPase activities to orchestrate cytoskeleton responses during cell wound repair. *Current Biology* 2014;**24**:144–155.
486. Swaggart KA, Demonbreun AR, Vo AH, Swanson KE, Kim EY, Fahrenbach JP. Annexin A6 modifies muscular dystrophy by mediating sarcolemmal repair. *PNAS* 2014;**111**:6004–6009.
487. Porat-Shliom N, Milberg O, Masedunskas A, Weigert R. Multiple roles for the actin cytoskeleton during regulated exocytosis. *Cellular and Molecular Life Sciences* 2013;**70**:2099–2121.
488. Mellgren RL, Zhang W, Miyake K, Mcneil PL. Calpain Is Required for the Rapid , Calcium-dependent Repair of Wounded Plasma Membrane. *Journal of Biological Chemistry* 2007;**282**:2567–2575.
489. Togo T, Krasieva TB, Steinhardt RA. A Decrease in Membrane Tension Precedes Successful Cell-Membrane Repair. *Mol Biol Cell* 2000;**11**:4339–4346.
490. Godin LM, Vergen J, Prakash YS, Pagano RE, Hubmayr RD. Spatiotemporal dynamics of actin remodeling and endomembrane trafficking in alveolar epithelial type I cell wound healing. *Am J Physiol Lung Cell Mol Physiol* 2011;**300**:L615–L623.
491. O'Brien ET, Salmon ED, Erickson HP. How Calcium Causes Microtubule Depolymerization. 1997;**135**:125–135.
492. Togo T. Disruption of the plasma membrane stimulates rearrangement of microtubules and lipid traffic toward. *J Cell Sci* 2006;**1**:2780–2786.
493. Bi G, Morris RL, Liao G, Alderton JM, Scholey JM, Steinhardt RA. Kinesin- and Myosin-driven Steps of Vesicle Recruitment for Ca²⁺- regulated Exocytosis. *Journal of Cell Biology* 1997;**138**:999–1008.
494. Mandato CA, Bement WM. Contraction and polymerization cooperate to assemble and close actomyosin rings around *Xenopus* oocyte wounds. 2000:785–797.
495. Piekny A, Werner M, Glotzer M. Cytokinesis: Welcome to the Rho zone. *Trends Cell Biol* 2005;**15**:651–658.

496. Prehoda KE, Scott JA, Mullins RD, Lim WA. Integration of Multiple Signals Through Cooperative Regulation of the N-WASP – Arp2 / 3 Complex. 2000;**290**:801–807.
497. Mandato CA, Bement WM. Contraction and polymerization cooperate to assemble and close actomyosin rings around *Xenopus* oocyte wounds. *Journal of Cell Biology* 2001;**154**:785–797.
498. Abreu-Blanco MT, Watts JJ, Verboon JM, Parkhurst SM. Cytoskeleton responses in wound repair. *Cellular and Molecular Life Sciences* 2012;**69**:2469–2483.
499. Russo JM, Florian P, Shen LE, Graham WV, Maria S, Gitter AH, Mrsny RJ, Turner JR. Distinct Temporal-Spatial Roles for Rho Kinase and Myosin Light Chain Kinase in Epithelial Purse-String Wound Closure. *Gastroenterology* 2006;**128**:987–1001.
500. Danjo Y, Gipson IK. Actin ‘ purse string ’ filaments are anchored by E-cadherin-mediated adherens junctions at the leading edge of the epithelial wound , providing coordinated cell movement. *Journal of Cell Biology* 1998;**331**:3323–3331.
501. Tamada M, Perez TD, Nelson WJ, Sheetz MP. Two distinct modes of myosin assembly and dynamics during epithelial wound closure. 2007;**176**:27–33.
502. Dekraker C, Boucher E, Mandato CA. Regulation and Assembly of Actomyosin Contractile Rings in Cytokinesis and Cell Repair. 2018;**2066**:2051–2066.
503. Li S, Chen BPC, Azuma N, Hu Y, Wu SZ, Sumpio BE, Shyy JY, Chien S. Distinct roles for the small GTPases Cdc42 and Rho in endothelial responses to shear stress. *Journal of clinical investigation* 1999;**103**:1141–1150.
504. Horn A, Vandermeulen JH, Defour A, Hogarth M, Reed A, Scheffer L, Chandel NS, Jaiswal JK. Mitochondrial redox signaling enables repair of injured skeletal muscle cells. *Sci Signal* 2018;**10**:1–24.
505. Demonbreun AR, Quattrocelli M, Barefield DY, Allen M V., Swanson KE, McNally EM. An actin-dependent annexin complex mediates plasma membrane repair in muscle. *Journal of Cell Biology* 2016;**213**:705–718.
506. Ashraf APK, Gerke V. Plasma membrane wound repair is characterized by extensive membrane lipid and protein rearrangements in vascular endothelial cells. *Biochim Biophys Acta Mol Cell Res* 2021;**1868**:118991.
507. Ebstrup ML, Dias C, Sofie A, Heitmann B, Sønder SL, Nylandsted J. Actin Cytoskeletal Dynamics in Single-Cell Wound Repair. *Int J Mol Sci* 2021;**22**:1–16.
508. Li S, Chen BPC, Azuma N, Hu Y, Wu SZ, Sumpio BE, Shyy JY, Chien S. Distinct roles for the small GTPases Cdc42 and Rho in endothelial responses to shear stress. *Journal of clinical investigation* 1999;**103**:1141–1150.

509. Nightingale T, Cutler D. The secretion of von Willebrand factor from endothelial cells; an increasingly complicated story. *Journal of Thrombosis and Haemostasis* 2013;**11**:192–201.
510. Desnos C, Huet S, Fanget I, Chapuis C, Böttiger C, Racine V, Sibarita JB, Henry JP, Darchen F. Myosin Va mediates docking of secretory granules at the plasma membrane. *Journal of Neuroscience* 2007;**27**:10636–10645.
511. Zhu Q, Yamakuchi M, Lowenstein CJ. SNAP23 Regulates Endothelial Exocytosis of von Willebrand Factor. *PLoS One* 2015;**4**:14–22.
512. Nightingale TD, Cutler DF, Cramer LP. Actin coats and rings promote regulated exocytosis. *Trends Cell Biol* 2012;**22**:329–337.
513. Miklavc P, Hecht E, Hobi N, Wittekindt OH, Dietl P, Kranz C, Frick M. Actin coating and compression of fused secretory vesicles are essential for surfactant secretion – a role for Rho , formins and myosin II. *J Cell Sci* 2012;**125**.
514. April C, Page SEEL, Trajkovic K, Hsu C, Chiantia S, Rajendran L, Wenzel D, Wieland F, Schwille P, Brügger B, Simons M. Ceramide Triggers Budding of Exosome Vesicles into Multivesicular Endosomes. *Science (1979)* 2008;**319**.
515. Corrotte M, Almeida PE, Tam C, Castro-gomes T, Fernandes MC, Millis BA, Cortez M, Miller H, Song W, Mangel TK, Andrews NW. Caveolae internalization repairs wounded cells and muscle fibers. 2013:1–30.
516. Bullock A, Pickavance P, Haddow D, MacNeil S. Development of a calcium-chelating hydrogel for treatment of superficial burns and scalds. *Regenerative Med* 2010;**5**.
517. Hutcheson JD, Schlicher RK, Hicks HK, Prausnitz MR. Saving cells from ultrasound-induced apoptosis: Quantification of cell death and uptake following sonication and effects of targeted calcium chelation. *Ultrasound Med Biol* 2010;**36**:1008–1021.
518. Kondoh M, Yagi K. Tight Junction Modulators: Promising Candidates for Drug Delivery. *Curr Med Chem* 2007;**14**:2482–2488.
519. Brown RC, Davis TP. Calcium modulation of adherens and tight junction function: A potential mechanism for blood-brain barrier disruption after stroke. *Stroke* 2002;**33**:1706–1711.
520. Kawai K, Larson BJ, Ishise H, Carre AL, Nishimoto S, Longaker M, Lorenz HP. Calcium-based nanoparticles accelerate skin wound healing. *PLoS One* 2011;**6**.
521. Cabrera D, Walker K, Moise S, Telling ND, Harper AGS. Controlling human platelet activation with calcium-binding nanoparticles. *Nano Res* 2020;**13**:2697–2705.
522. Zalba S, Hagen TLM ten. Cell membrane modulation as adjuvant in cancer therapy. *Cancer Treat Rev* 2017;**52**:48–57.

523. Hendrich A, Michalak K. Lipids as a Target for Drugs Modulating Multidrug Resistance of Cancer Cells. *Curr Drug Targets* 2005;**4**:23–30.
524. Mehier-Humbert S, Bettinger T, Yan F, Guy R. Influence of polymer adjuvants on the ultrasound-mediated transfection of cells in culture. *European Journal of Pharmaceutics and Biopharmaceutics* 2009;**72**:567–573.
525. Shi R, Borgens RB. Anatomical repair of nerve membranes in crushed mammalian spinal cord with polyethylene glycol. *J Neurocytol* 2000;**29**:633–643.
526. Regev R, Katzir H, Yeheskely-Hayon D, Eytan GD. Modulation of P-glycoprotein-mediated multidrug resistance by acceleration of passive drug permeation across the plasma membrane. *FEBS Journal* 2007;**274**:6204–6214.
527. Carugo D, Aron M, Sezgin E, Bernardino de la Serna J, Kuimova MK, Eggeling C, Stride E. Modulation of the molecular arrangement in artificial and biological membranes by phospholipid-shelled microbubbles. *Biomaterials* 2017;**113**:105–117.
528. Quinn PJ. Lipid-lipid interactions in bilayer membranes: Married couples and casual liaisons. *Prog Lipid Res* 2012;**51**:179–198.
529. Goñi FM, Alonso A. Effects of ceramide and other simple sphingolipids on membrane lateral structure. *Biochim Biophys Acta Biomembr* 2009;**1788**:169–177.
530. Stover TC, Sharma A, Robertson GP, Kester M. Systemic delivery of liposomal short-chain ceramide limits solid tumor growth in murine models of breast adenocarcinoma. *Clinical Cancer Research* 2005;**11**:3465–3474.
531. Escribá P V., Busquets X, Inokuchi JI, Balogh G, Török Z, Horváth I, Harwood JL, Vigh L. Membrane lipid therapy: Modulation of the cell membrane composition and structure as a molecular base for drug discovery and new disease treatment. *Prog Lipid Res* 2015;**59**:38–53.
532. Ritchie RH, Gordon JM, Woodman OL, Cao AH, Dusting GJ. Annexin-1 peptide Anx-12-26 protects adult rat cardiac myocytes from cellular injury induced by simulated ischaemia. *Br J Pharmacol* 2005;**145**:495–502.
533. Huang JJ, Xia CJ, Wei Y, Yao Y, Dong MW, Lin KZ, Yu LS, Gao Y, Fan YY. Annexin A1-derived peptide Ac2-26 facilitates wound healing in diabetic mice. *Wound Repair and Regeneration* 2020;**28**:772–779.
534. Leoni G, Neumann PA, Kamaly N, Quiros M, Nishio H, Jones HR, Sumagin R, Hilgarth RS, Alam A, Fredman G, Argyris I, Rijcken E, Kusters D, Reutelingsperger C, Perretti M, Parkos CA, Farokhzad OC, Neish AS, Nusrat A. Annexin A1’containing extracellular vesicles and polymeric nanoparticles promote epithelial wound repair. *Journal of Clinical Investigation* 2015;**125**:1215–1227.
535. Purvis GSD, Solito E, Thiemermann C. Annexin-A1: Therapeutic potential in microvascular disease. *Front Immunol* 2019;**10**:1–8.

- 536. Demonbreun AR, Fallon KS, Oosterbaan CC, Bogdanovic E, Warner JL, Sell JJ, Page PG, Quattrocchi M, Barefield DY, McNally EM. Recombinant annexin A6 promotes membrane repair and protects against muscle injury. *Journal of Clinical Investigation* 2019;**129**:4657–4670.
- 537. Mui L, Martin CM, Tschirhart BJ, Feng Q. Therapeutic Potential of Annexins in Sepsis and COVID-19. *Front Pharmacol* 2021;**12**:1–13.
- 538. Burkel BM, Benink HA, Vaughan EM, Dassow G von, Bement WM. A Rho GTPase signal treadmill backs a contractile array. *Dev Cell* 2013;**23**:384–396.
- 539. Martinelli R, Kamei M, Sage PT, Massol R, Varghese L, Sciuto T, Toporsian M, Dvorak AM, Kirchhausen T, Springer TA, Carman C V. Release of cellular tension signals self-restorative ventral lamellipodia to heal barrier micro-wounds. *Journal of Cell Biology* 2013;**201**:449–465.
- 540. Neco P, Fernández-Peruchena C, Navas S, Gutiérrez LM, Álvarez De Toledo G, Alés E. Myosin II contributes to fusion pore expansion during exocytosis. *Journal of Biological Chemistry* 2008;**283**:10949–10957.