# Challenges for gene therapies: delivery, durability and complications

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[redacted]

## Disclosures

Founder, equity holder, member of the Board and scientific advisor of Forcefield Therapeutics Inc, London

Co-founder, equity holder, member of the Board and scientific advisor of Purespring Therapeutics Inc, London

Founder, equity holder, member of the Board and scientific advisor of Heqet Therapeutics, Italy

Member of the Scientific Advisory Boards of Trizell Holding SA, Lausanne and DINAQR AG, Zurich-London

# Prevention and therapy of **heart failure**

REMARKABLE !!!!!!

**Protection from cardiomyocyte loss**

No drug or treatment

**Improvement of residual cardiac function**

Digitalis
Diuretics
Aldosterone antagonists
ACE inhibitors
Beta blockers
Angiotensin receptor neprilysin inhibitors (ARNIs)
Angiotensin receptor blockers (ARBs)
$I_f$ channel blockers (ivabradine)
SGLT2 inhibitors
*Myosin activators (omecamtiv mecarbil)*

**Generation of new cardiomyocytes**

No drug or treatment

# Next generation cardiac therapies will be biologics

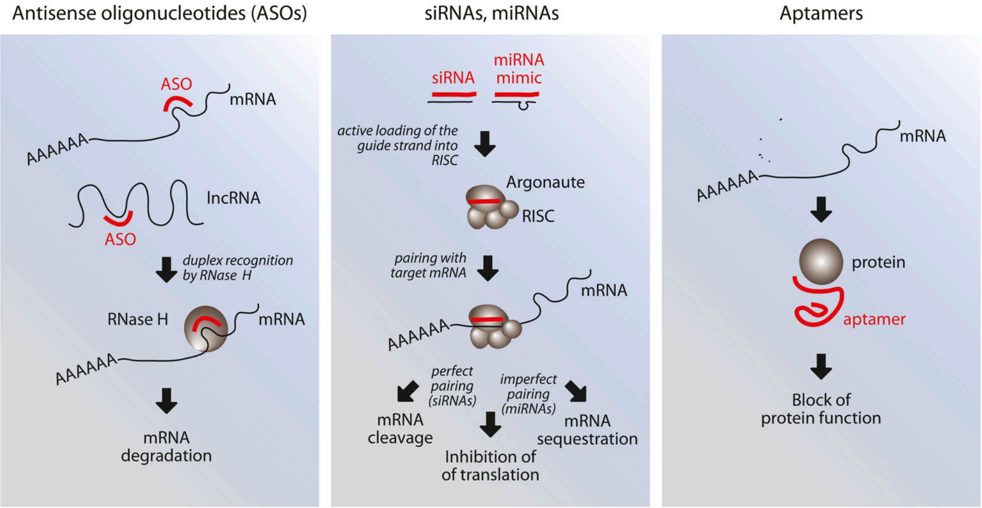
Biologics

- Recombinant proteins (e.g. cytokines, hormones, enzymes)
- Peptides
- Vaccines
- Monoclonal antibodies
- Nucleic acids (antisense, ncRNAs, mRNAs)

Advanced Therapies

- Viral vectors for gene therapy and gene editing
- Cells and 3D tissue

# Small, non coding RNA (ncRNA) therapeutics




M. Giacca & A.M. Shah, 2022, Eur. Heart J.

# FDA/EMA approved small ncRNAs (2022)

9 ASOs
4 siRNAs

| Product (Commercial name; Developer/Manufacturer) | Length | Modifications | Vehicle | Route of administration | Indication | Target organ | Target gene and mechanism | Year of approval |
|---|---|---|---|---|---|---|---|---|
| **Antisense oligonucleotides (ASOs)** | | | | | | | | |
| **Fomivirsen** (Vitravene; Isis Pharmaceuticals, Novartis) | 21-mer | PS | None | Intravitreal | CMV retinitis | Eye | CMV IE-2 mRNA | 1998 (FDA), 1999 (EMA); 2002 withdrawn |
| **Mipomersen** (Kynamro; Ionis Pharmaceuticals, Kastle Therapeutics) | 20-mer | PS, 2'-MOE, GapmeR | None | Subcutaneous | Familiar hypercholesterolaemia (FH) | Liver | Apolipoprotein B (ApoB) mRNA | 2013 (FDA); 2019 withdrawn |
| **Nusinersen** (Spinraza; Ionis Pharmaceuticals, Biogen) | 18-mer | PS, 2'-MOE | None | Intrathecal | Spinal muscular atrophy (SMA) | | SMN2 pre-mRNA splicing (exon 7 inclusion) | 2017 (EMA), 2016 (FDA) |
| **Eteplirsen** (Exondys 51, Sarepta Therapeutics) | 30-mer | PMO | None | Intravenous | Duchenne muscular dystrophy (DMD) | Skeletal muscle | Dystrophin pre-mRNA splicing (exon 51 skipping) | 2016 (FDA) |
| **Inotersen** (Tesgedi; Ionis Pharmaceuticals, Akcea Therapeutics) | 20-mer | PS, 2'-MOE, GapmeR | None | Subcutaneous | Hereditary transthyretin amyloidosis | Liver | Transthyretin (TTR) mRNA | 2018 (EMA), 2018 (FDA) |
| **Golodirsen** (Vyondys 53; Sarepta Therapeutics) | 25-mer | PMO | None | Intravenous | Duchenne muscular dystrophy (DMD) | Muscle | Dystrophin pre-mRNA splicing (exon 53 skipping) | 2019 (FDA) |
| **Viltolarsen** (Viltepso, NS Pharma) | 21-mer | PMO | None | Intravenous | Duchenne muscular dystrophy (DMD) | Muscle | Dystrophin pre-mRNA splicing (exon 53 skipping) | 2020 (FDA) 2020 (EMA) |
| **Volanesorsen** (Waylivra; Ionis Pharmaceuticals, Akcea Therapeutics) | 20-mer | PS, 2'-MOE, GapmeR | None | Subcutaneous | Familiar chylomicronaemia syndrome (FCS) | Liver | Apolipoprotein C3 (ApoC3I) mRNA | 2019 (EMA) |
| **Casimersen** (Amondys 45; Sarepta Therapeutics) | 22-mer | PMO | None | Intravenous | Duchenne muscular dystrophy (DMD) | Muscle | Dystrophin pre-mRNA splicing (exon 45 skipping) | 2021 (FDA) |
| **Small interfering RNAs (siRNAs)** | | | | | | | | |
| **Patisiran** (Onpattro; Anylam Pharmaceuticals) | 21-nt ds | 2'-*O*-Me | SNALP LNP | Intravenous | Hereditary transthyretin amyloidosis | Liver | Transthyretin mRNA | 2018 (EMA), 2019 (FDA) |
| **Givosiran** (Givlaari; Anylam Pharmaceuticals) | 21-nt ds | PS, 2'-*O*-Me, 2'-F, GalNAc-conjugated | None | Subcutaneous | Acute hepatic porphyria (AHP) | Liver | Delta aminolevulinic acid synthase 1 (ALAS1) mRNA | 2020 (EMA), 2019 (FDA) |
| **Inclisiran** (Leqvio; Novartis Pharmaceuticals) | 22-nt ds | PS, 2'-*O*-Me, 2'-F, GalNAc-conjugated | None | Subcutaneous | Primary hypercholesterolaemia or mixed dyslipidaemia | Liver | Proprotein convertase subtilisin/kexi)n type 9 (PCSK9) mRNA | 2020 (EMA) 2021 (FDA) |
| **Lumasiran** (Oxlumo; Anylam Pharmaceuticals) | 21-nt ds | PS, 2'-*O*-Me, 2'-F, GalNAc-conjugated | None | Subcutaneous | Primary hyperoxaluria type 1 (PH1) | Liver | Hydroxyacid oxidase-1 (HAO1) mRNA | 2020 (EMA), 2020 (FDA) |

# The search for new therapeutic leads

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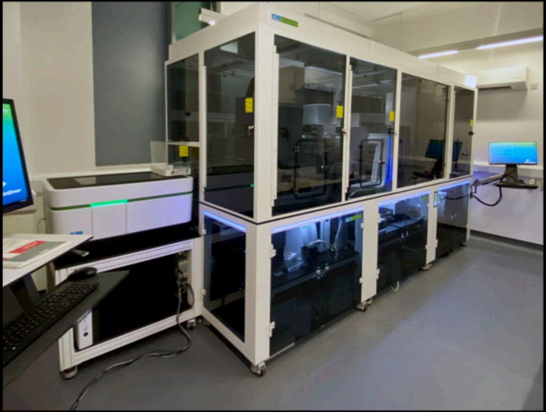
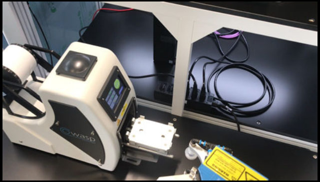
# The approach we like:

- Based on **selection** or **screening** (not on educated guesses)
- **Systematic** (exhaustive)
- **Functional** (not phenotypic)
- **In vivo** (if possible)

# High Throughput Screening Facility at the School of Cardiovascular Medicine & Sciences

Human/Mouse whole Genome siRNAs (*~18,000 siRNAs*)
Human/Mouse synthetic microRNA mimics (*2042 mature sequences, miRBase v. 19.0*)
Human miRCURY LNA inhibitors (*1972 molecules*)
2 FDA/EMA-approved small molecule libraries (*>3000 molecules*)
Mouse secreted factors (*1198 cDNAs*)

Miguel Mano

# ncRNAs for cardiac regeneration

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# The holy grail of **cardiac regeneration**

Cardiac regeneration by stem cells

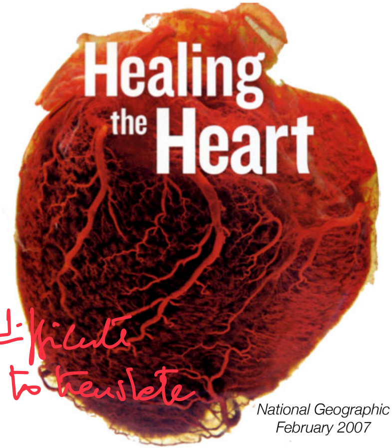
~~Bone marrow stem cells~~
~~Mesenchymal stromal cells~~
~~Cardiospheres~~
~~Adult cardiac stem cells~~
Cardiac Progenitor Cells (CPCs)

Cardiac regeneration by cardiomyocytes

ES-derived cardiomyocytes
iPS-derived cardiomyocytes

Engineered Heart Tissue

difficile to translate



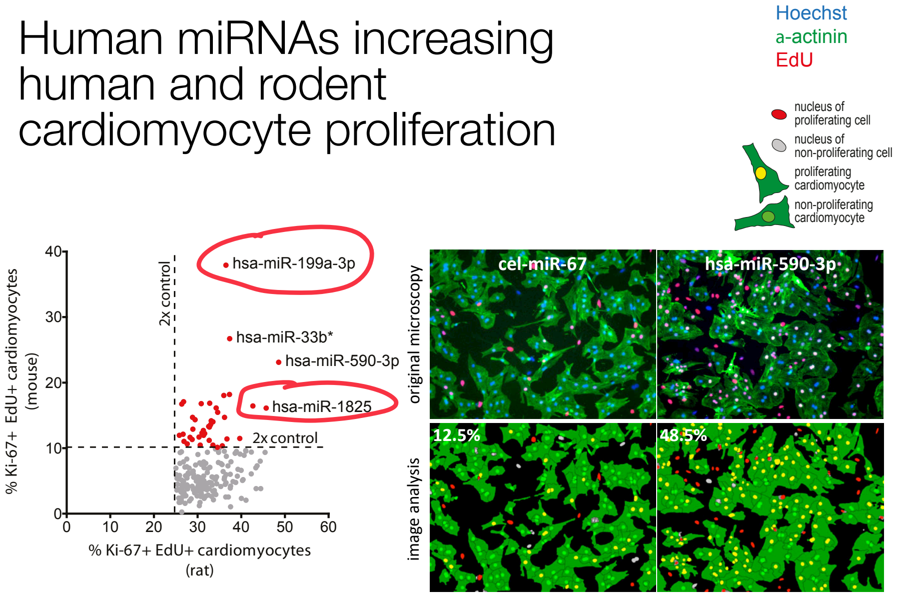

*National Geographic*
*February 2007*

Cardiac regeneration by stimulation of the endogenous capacity of cardiomyocytes to proliferate

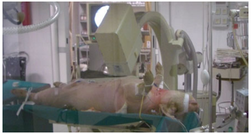
Cardiac regeneration by transdifferentiation of fibroblasts to cardiomyocytes

→ interesting for research

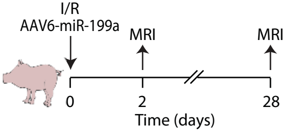
# Human miRNAs increasing human and rodent cardiomyocyte proliferation

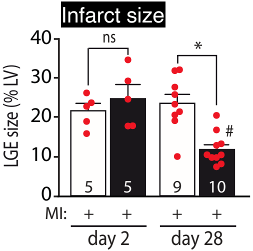


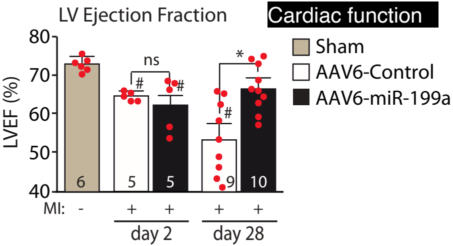

Eulalio et al. 2012. Nature 492, 376



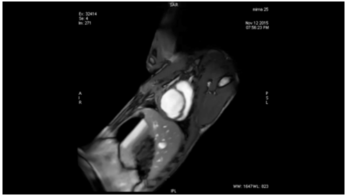
# AAV6-miR-199a reduces infarct size and fibrosis and improves cardiac function in pigs



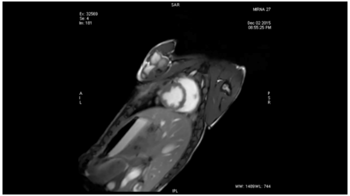




MI + AAV6-Control

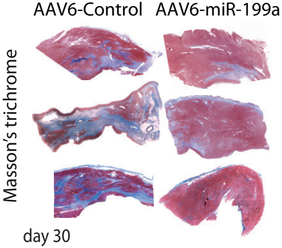


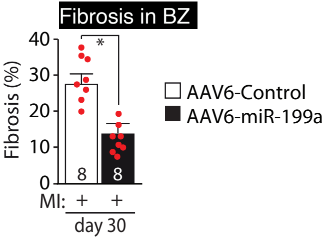
MI + AAV6-miR-199a



*30 days after MI and treatment*

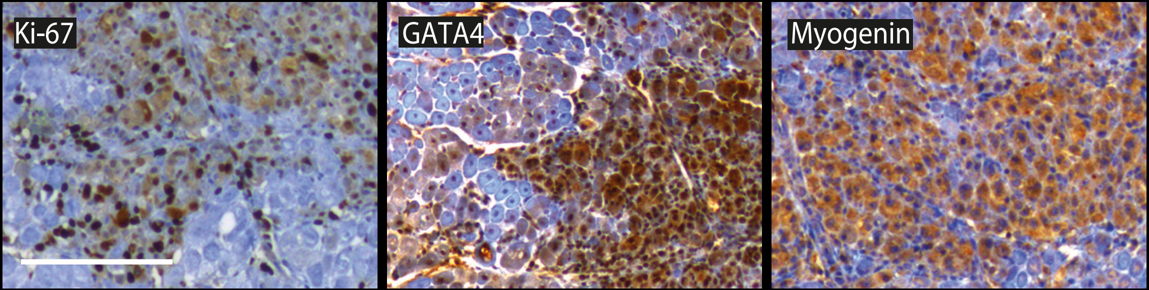
REVASCULARIZATION →




Gabisonia, K, et al. 2019. Nature 569, 418

# Small cell clusters in hearts injected with AAV6-miR-199a




Highly proliferating, GATA4+, myogenin+

*Early myoblast progenitors?*

Gabisonia, K, et al. 2019. Nature 569, 418

# ncRNAs that increase AAV permissivity

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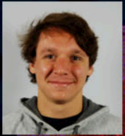
# FDA/EMA approved gene therapy products

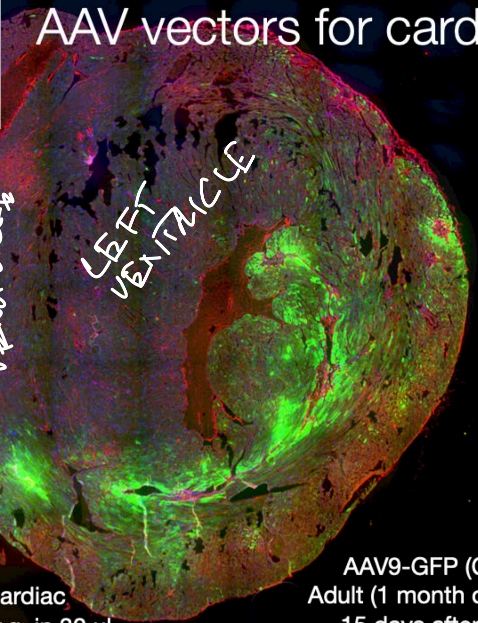
| Product | Company | Year | Therapeutic gene | Disease | Vector | Prevalence / Incidence | Price (USD) |
|---|---|---|---|---|---|---|---|
| Glybera | UniQure | 2012 | Lipoprotein lipase | Lipoprotein lipase deficiency (LPLD) | AAV1 | 1:1,000,000 | 1M |
| Strimvelis | GlaxoSmithKline | 2016 | Adenosine deaminase | ADA-SCID | RV | 1:100,000 | 665K |
| **Luxturna** | Spark Therapeutics | 2017 | RPE65 | Leber's congenital amaurosis (LCA) | AAV2 | <1:100,000 | 435K/eye |
| **Zynteglo** | Bluebird Bio | 2022 | Beta globin | Beta thalassaemia | LV | 1:100,000 | 2.8M |
| **Zolgensma** | Novartis/Avexis | 2019 | SMN1 | Spinal muscular atrophy (SMA) | AAV9 | 1-2:100,000 | 2.1M |
| **Hemgenix** | Behring | 2022 | Factor IX | Haemophilia B | AAV5 | 3.7:100,000 | 3.5M |
| **Skysona** | Bluebird Bio | 2022 | ABCD1 | X-linked adrenoleukodystrophy | LV | 5:100,000 | 3M |
| **Libmeldy** | Orchard Therapeutics | 2020E | Arylsulfatase A (ARSA) | Metachromatic leukodystrophy (MLD) | LV | 2.5-10:100,000 | 3.9M |
| **Upstaza** | PTC Therapeutics | 2022E | AADC | Aromatic L-amino acid decarboxylase deficiency | AAV2 | 1.1:100,000 | |
| **Roctavian** | BioMarin | 2022E | Factor VIII-SQ | Haemophilia A | AAV5 | 12:100,000 | 2.5M |
| **Yescarta** | Kite Pharma/Gilead | 2017 | CAR-T (CD-19) | Diffuse Large B-cell NHL | LV | 4:100,000 per year | 373K |
| **Kymriah** | Novartis | 2017 | CAR-T (CD-19) | B-cell lymphoma | LV | 1.7:100,000 | 475K |
| **Breyanzi** | Bristol Myers Squibb | 2022 | CAR-T (CD19) | B-cell lymphoma | LV | 1,7:100,000/year | 471K |
| **Abecma** | Bristol Myers Squibb | 2021 | CAR-T (BCMA) | Multiple myeloma | LV | 1-2:100,000/year | 480K |
| **Carvykti** | Janssen | 2023 | CAR-T (BCMA) | Multiple myeloma | LV | 1-2:100,000/year | 490K |
| **Tecartus** | Kite Pharma | 2020 | CAR-T (CD-19) | Mantle cell lymphoma | LV | 4-8/1M/year | 373K |
| **Adstiladrin** | Ferring | 2023 | Interferon alfa-2b | Bladder cancer | Ad | 32:100,000/year | ~200K |
| **Imlygic** | Amgen/BioVex | 2023 | Oncolytic herpesvirus | Melanoma | Herpesvirus | 25:100,000/year | 65K |

https://www.fda.gov/vaccines-blood-biologics/cellular-gene-therapy-products/approved-cellular-and-gene-therapy-products
https://www.genetherapynet.com/gene-therapy-products-on-the-market.html
https://www.pei.de/EN/medicinal-products/atmp/gene-therapy-medicinal-products/gene-therapy-node.html

# AAV vectors for cardiomyocyte gene transfer



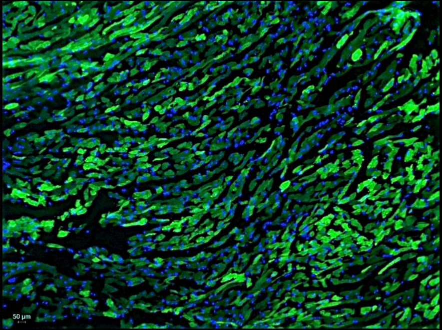
Edoardo Schneider

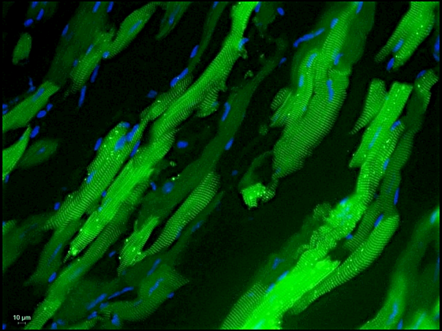



Intracardiac
6.6x10$^{10}$ v.g. in 30 µl

Intravenous (retro-orbital)
2.2x10$^{11}$ v.g. in 100 µl

AAV9-GFP (CMV promoter)
Adult (1 month old) C57BL/6 mice
15 days after administration




# Mouse heart transduction using AAV9 vectors

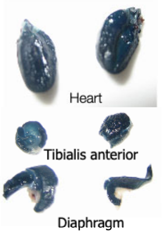
i.p., i.v. or intramyocardial injection

AAV9-ZsgGreen
(Zoanthus green fluorescent protein)

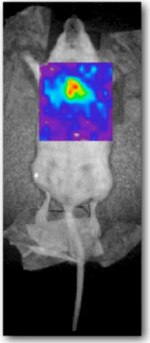
IP injection @day 7
$5 \times 10^{11}$ vg/mouse

3 months post-injection
$1 \times 10^{8}$ - $1 \times 10^{9}$ vg/heart

AAV9-LacZ




AAV9-Luc



# Addressing high dose AAV toxicity – ‘one and done’ or ‘slower and lower’?

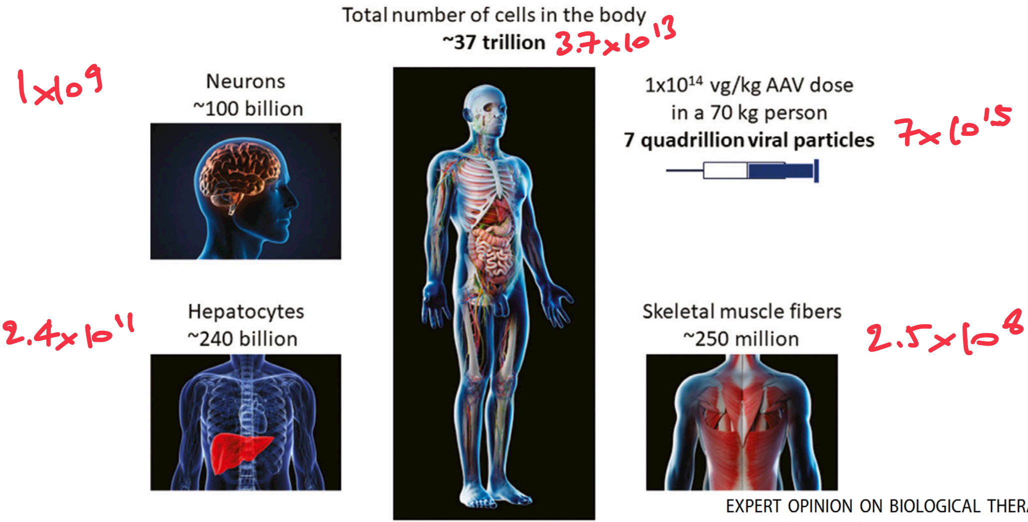
Takashi Kei Kishimoto[a] and Richard Jude Samulski[b]

EXPERT OPINION ON BIOLOGICAL THERAPY
2022, VOL. 22, NO. 9, 1067–1071

[a]Selecta Biosciences, Watertown, MA, USA; [b]Gene Therapy Center and Department of Pharmacology, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA

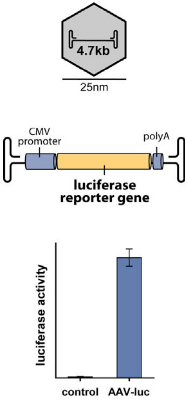
- **Vector doses of 1E14 vg/kg or higher required for efficacy in neuromuscular diseases**
- One third of 1400 patients administered systemically with Zolgensma (AAV9 for SMA) have experienced at least one adverse event of **hepatotoxicity**
- Four patients with X-linked my-tubular myopathy (XLMTM) **died** after receiving a vector dose of 3.5E14 vg/kg with signs of severe hepatotoxicity (Audientes)
- High vector doses associated with **thrombotic microangiopathy** (TMA) in clinical trials for Danon disease (Rocket Pharma) and SMA (9 cases, one death), and with atypical **haemolytic ureic syndrome** (aHUS) associated with complement activation in two clinical trials for DMD.
- Two cases of **myocarditis** in DMD Phase 3 clinical trial (Pfizer)
- Risk of **hepatocellular carcinoma** (HCC) and **dorsal root ganglia** (DRG) **toxicity** in animal models of AAV gene therapy

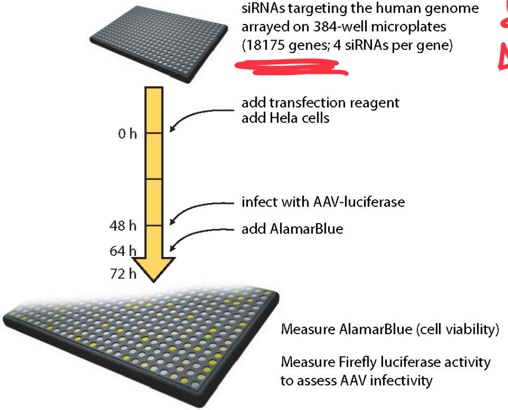
# Can we lower AAV doses by rendering cells more permissive?



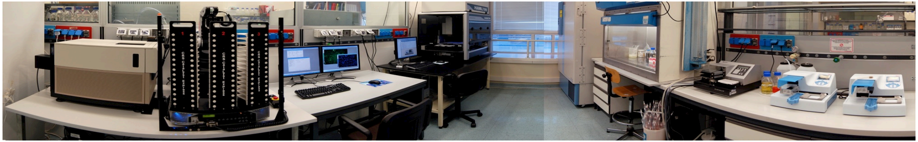

EXPERT OPINION ON BIOLOGICAL THERAPY
2022, VOL. 22, NO. 9, 1067–1071

# High-throughput screening of a whole genome siRNA and miRNA libraries for AAV transduction

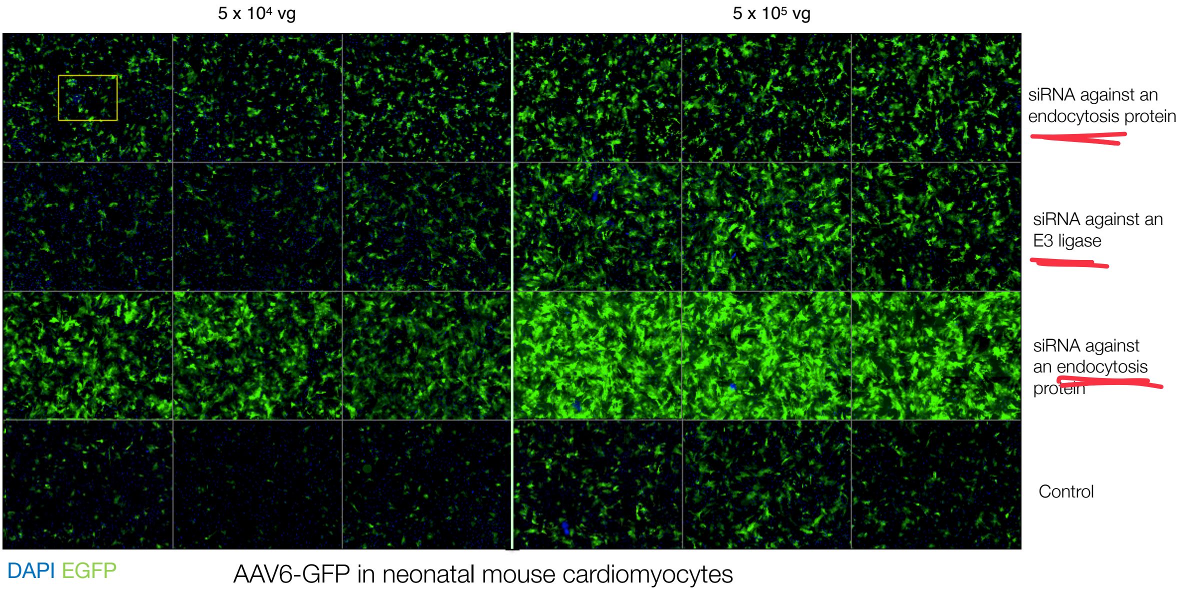



siRNAs against all human genes



# Screenings for AAV transduction




AAV6-GFP in neonatal mouse cardiomyocytes

# ncRNAs that increase precise gene editing

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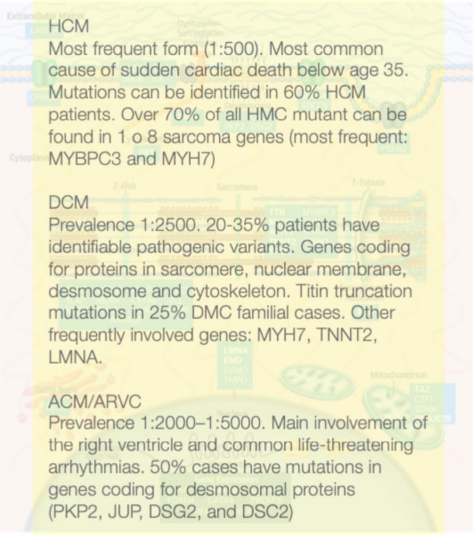
# Cardiomyopathies with Mendelian inheritance

- Most common cause of heart failure in young individuals
- Current medical approaches do not differ significantly from generic treatment for heart failure
- Most frequent mode of inheritance is autosomal dominant, because of either loss-of-function (e.g. truncating variants) or gain-of-function (missense variants)
- Autosomal dominance and large size of the cDNA coding for the affected genes are severe obstacles to cDNA gene therapy

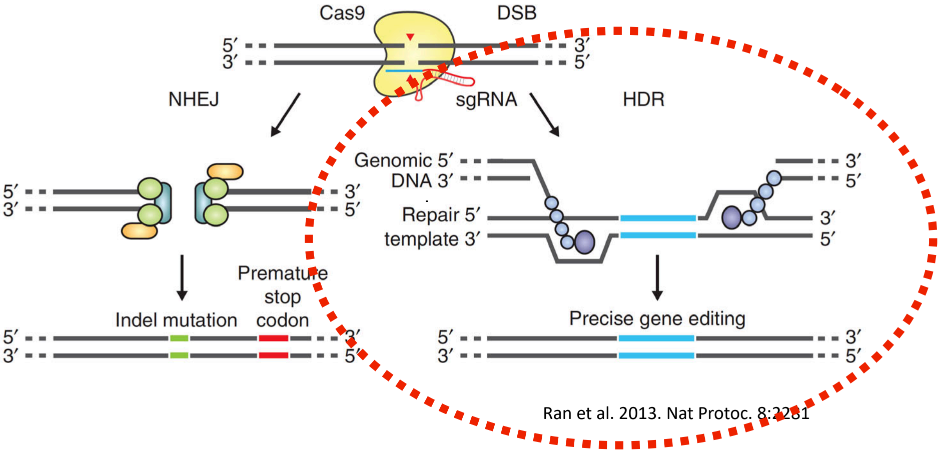


HCM
Most frequent form (1:500). Most common cause of sudden cardiac death below age 35. Mutations can be identified in 60% HCM patients. Over 70% of all HMC mutant can be found in 1 o 8 sarcoma genes (most frequent: MYBPC3 and MYH7)

DCM
Prevalence 1:2500. 20-35% patients have identifiable pathogenic variants. Genes coding for proteins in sarcomere, nuclear membrane, desmosome and cytoskeleton. Titin truncation mutations in 25% DMC familial cases. Other frequently involved genes: MYH7, TNNT2, LMNA.

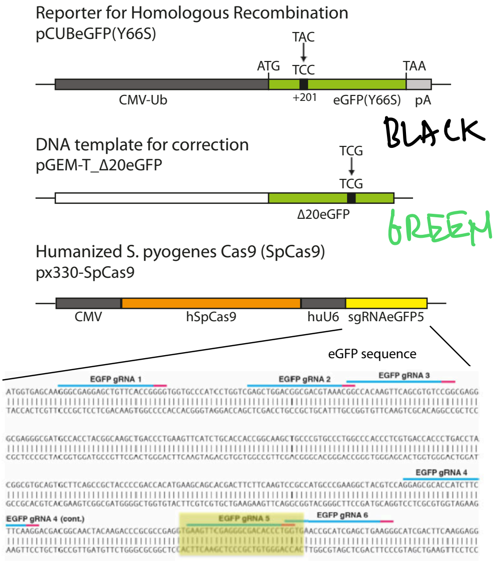
ACM/ARVC
Prevalence 1:2000–1:5000. Main involvement of the right ventricle and common life-threatening arrhythmias. 50% cases have mutations in genes coding for desmosomal proteins (PKP2, JUP, DSG2, and DSC2)

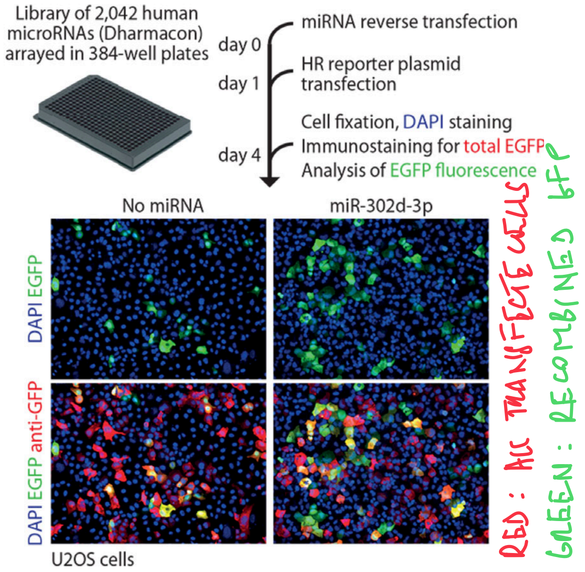
# Genome Editing with CRISPR/Cas9



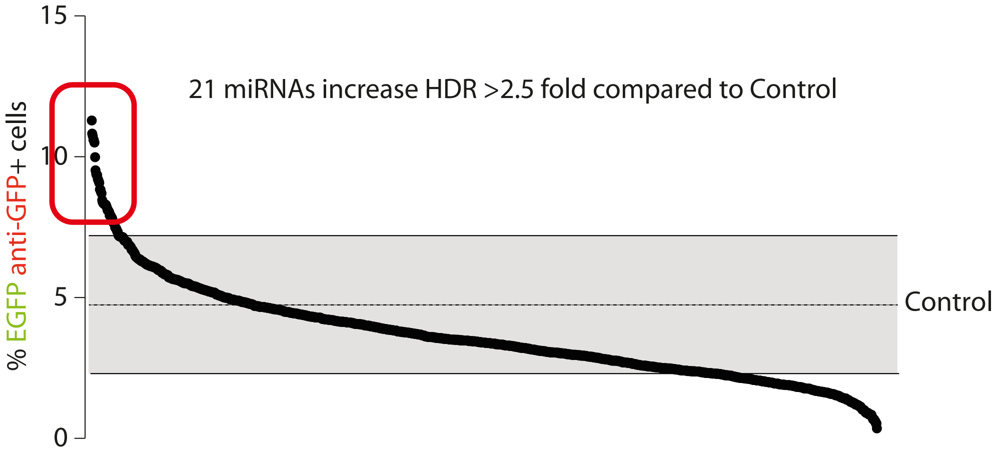

Ran et al. 2013. Nat Protoc. 8:2281

# An assay to detect homology-directed repair

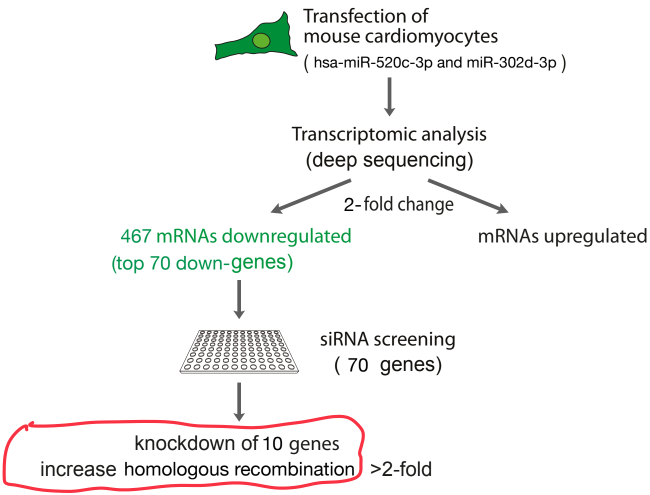


# HTS for miRNAs enhancing HDR

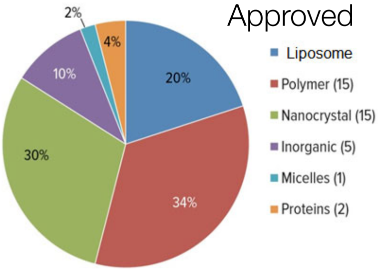


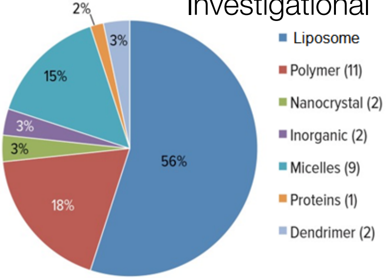
# Effect of the top miR-302d-3p and miR-520c-3p common downregulated genes on HDR in cardiomyocytes

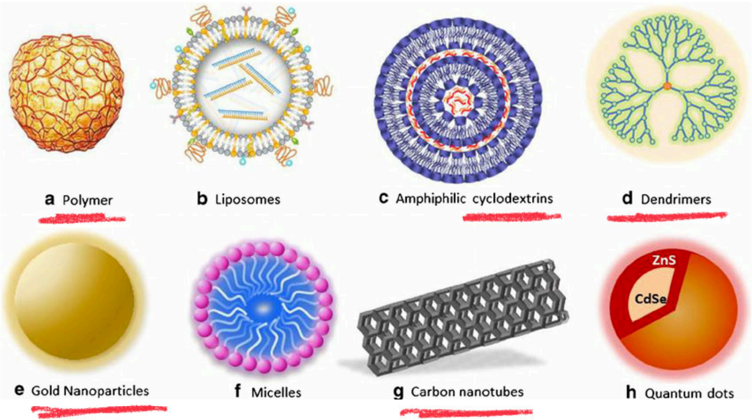


# Cardiac delivery of RNA therapeutics

# **Nanocarriers** for therapeutic ncRNA delivery



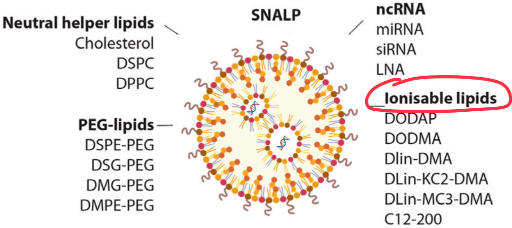




From: Ventola, 2017

# Stable Nucleic Acid-Lipid nanoParticles (SNALPs)

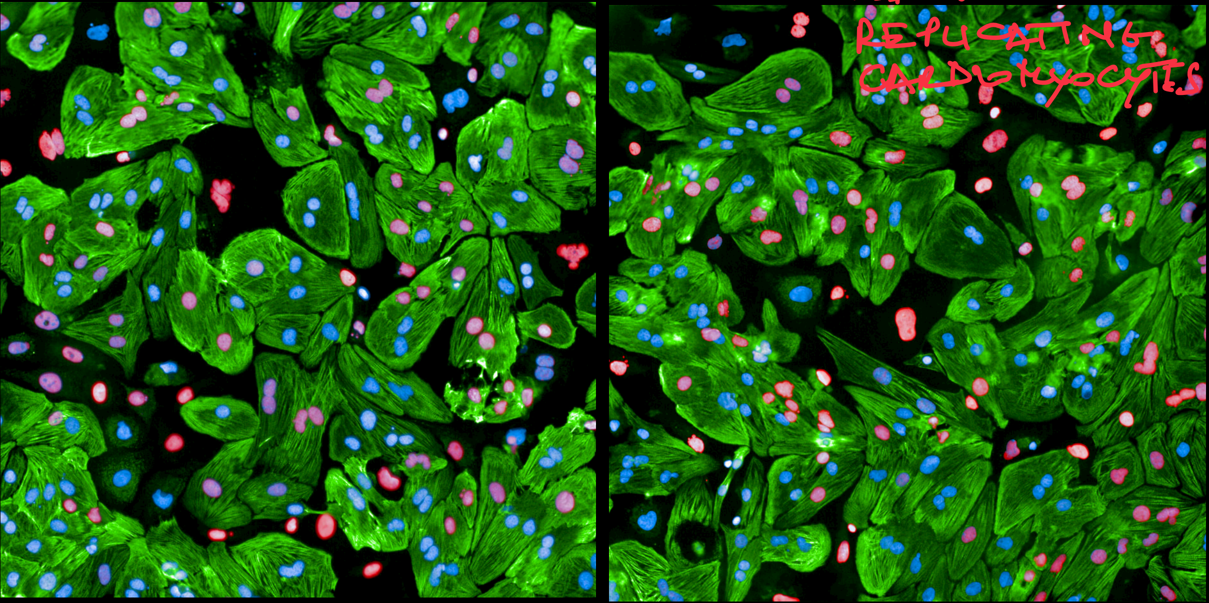
| ***Product*** | **Patisiran** | **BNT162b2** (Pfizer-BioNTech COVID-19 vaccine) | **mRNA-1273** (Moderna COVID-19 vaccine) |
|---|---|---|---|
| ***LNP technology*** | SNALP | SNALP | SNALP |
| ***Therapeutic RNA*** | Anti-TTR siRNA | SARS-CoV-2 Spike modified mRNA | SARS-CoV-2 Spike modified mRNA |
| ***Ionizable lipids*** | DLin-MC3-DMA | ALC-0315 | SM-102 |
| ***Neutral lipids*** | DSPC | DSPC | DSPC |
| | Cholesterol | Cholesterol | Cholesterol |
| ***PEG lipids*** | $PEG_{2000}$-C-DMG | $PEG_{2000}$ | $PEG_{2000}$-C-DMG |
| ***Reference*** | [46] | [35] | [34] |




Huntington J, Pachauri M, Ali H and Giacca M. 2021. Curr Opin Genet Dev 70, 48

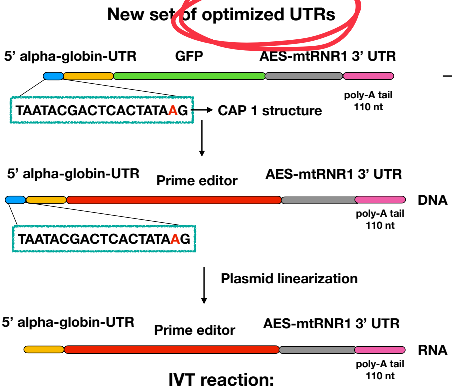
MC3-SNALP1 2.5:1 JSNALP2 2.5:1

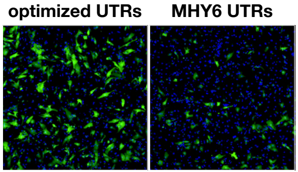
RED NUCLEI = REPLICATING CARDIOMYOCYTES



cTnT EdU DAPI

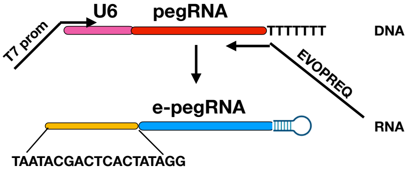
# Optimisation of in vitro transcription (IVT) for optimal mRNA translation in cardiomyocytes




**IVT reaction:**

- T7 polymerase
- Cap 1 analog (tri-link)
- pseudouridine
- PolyA-tail is already in the backbone



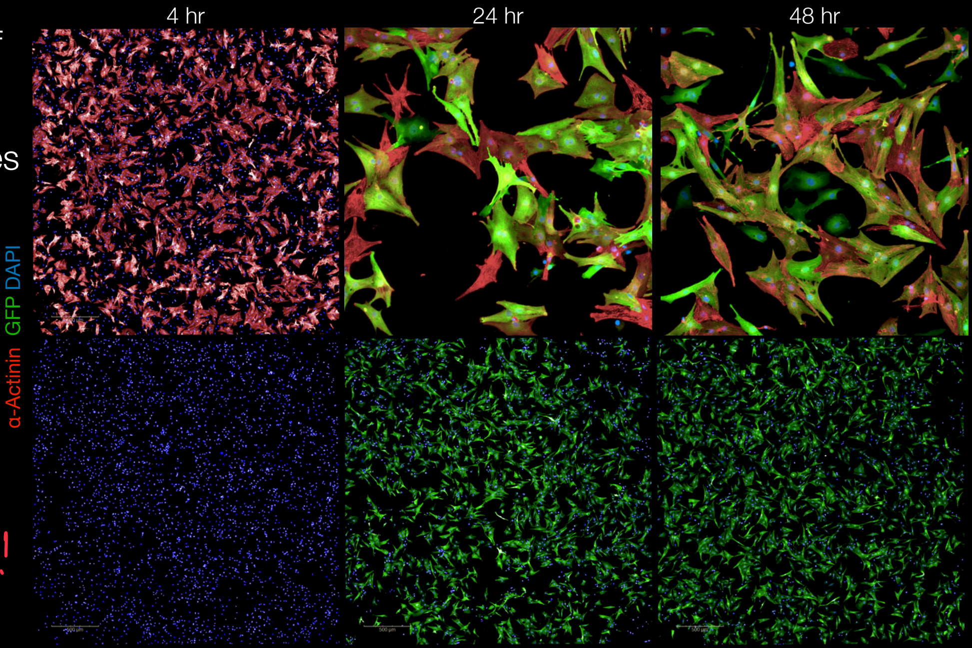
# Transfection of GFP mRNA in mouse cardiomyocytes by SNALPs

*SNALP3*

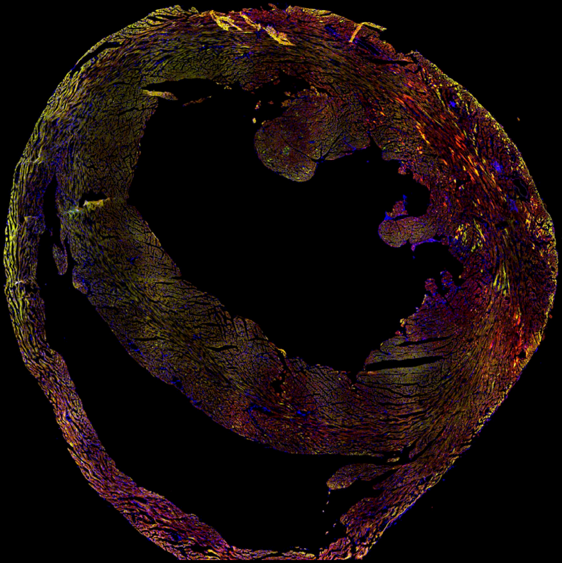
| | |
|---|---|
| MC3 | 40% |
| DOPE | 22% |
| Cholesterol | 35% |
| C16 Ceramide PEG | 3% |

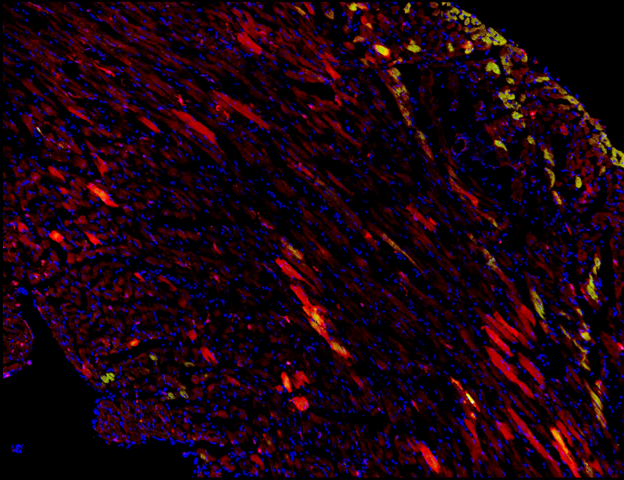
>95% efficiency!!

Josef Huntington



# JSNALP9-GFP mRNA



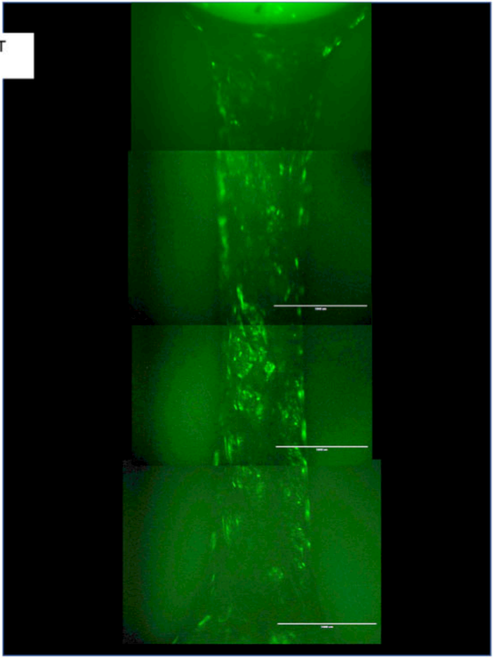


Native GFP GFP DAPI

# Pre-clinical human models

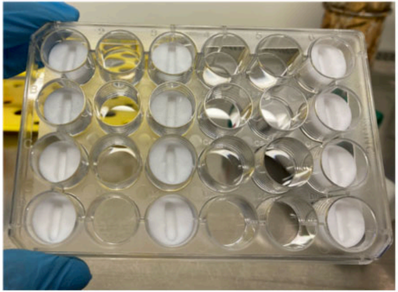
## 2nd experiment

Outer layer of the EHT
Live microscopy



# LNP - mGFP

- Lipid nanoparticles on EHT before macroscopic contraction (5days)
- Transduction chamber overnight
- EHT fixed after 24h
- 8ug/mL in 200uL Volume
- White posts



Transduction chamber

Giorgia Rizzari
Florian Weinberger
Thomas Eschenhagen

# Preparation of viable adult ventricular myocardial slices from large and small mammals

Samuel A Watson[1], Martina Scigliano[1], Ifigeneia Bardi[1], Raimondo Ascione[2], Cesare M Terracciano[1] & Filippo Perbellini[1]

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# Biomimetic electromechanical stimulation to maintain adult myocardial slices in vitro

Samuel A. Watson[1], James Duff[1], Ifigeneia Bardi[1], Magdalena Zabielska[2], Santosh S. Atanur[3], Richard J. Jabbc
André Simon[4], Alejandra Tomas[5], Ryszard T. Smolenski[2], Sian E. Harding[1], Filippo Perbellini[1] &
Cesare M. Terracciano[1]

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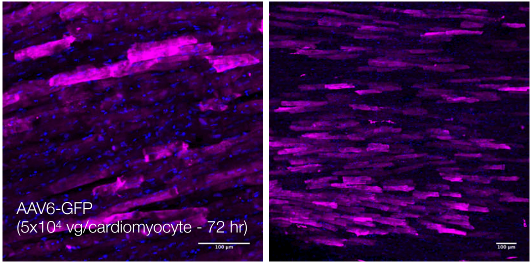
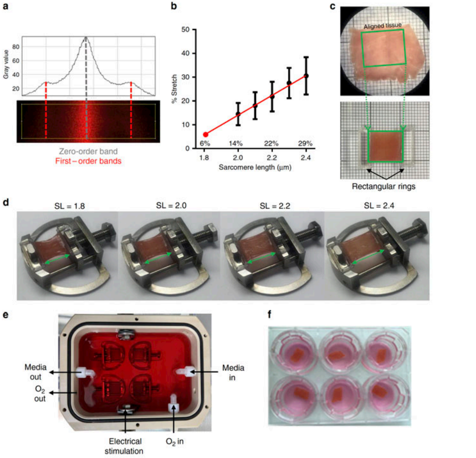


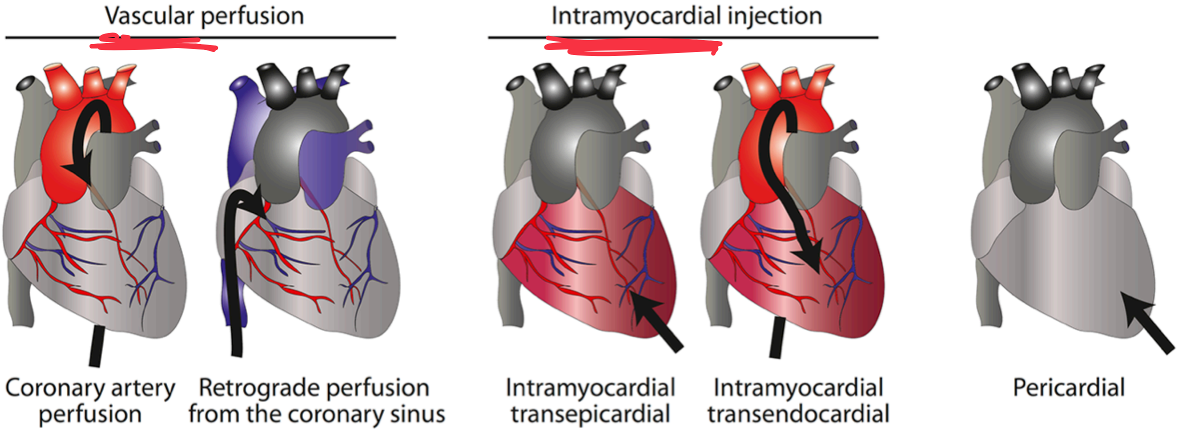

DAPI GFP




ig. 1 Application of electromechanical stimulation to rat myocardial slices. **a** Assessment of laser diffraction pattern. Peaks correspond to diffraction bands –the bright, central band corresponds to a zero-order band (grey), while the smaller bands on the left and right correspond to the less intense first-order ands (red). The distance between the zero-order and first-order band can be measured and used to calculate sarcomere length. **b** Percentage stretch equired to set the average diastolic rat myocardial slice sarcomere length. Rat myocardial slices were progressively stretched until a diffraction pattern quivalent to SL = 2.0 µm was achieved. The % stretch was then measured using calipers. This was repeated at 0.1-µm intervals until SL = 2.4 µm. A linear egression was used to estimate SL ($r^2 = 0.4776$, $y = 41.67 \times -69.26$) (SL = 2.0 $N = 11$, SL = 2.1 $N = 16$, SL = 2.2 $N = 14$, SL = 2.3 $N = 15$ and SL = 2.4 $N =$ 2). **c** Top—rat myocardial slice visualised using a macroscope. The slice is placed on a mm grid and the green rectangle highlights the aligned portion of he myocardial slice. Bottom—custom-made 3D-printed plastic rectangular rings are attached to opposite ends of the aligned portion of the myocardial lice using surgical glue. Rings are attached perpendicular to myofibril orientation. **d** Myocardial slice attached to the posts of a custom-made stretcher ising rings. Images show the different stretches required to achieve SL = 1.8–2.4 µm in rat myocardial slices. **e** Custom-made culture chamber. Myocardial lices are superfused with culture media. Media was oxygenated directly in the culture chamber. Field stimulation was provided via carbon electrodes. **f** Six-vell plate with Transwell inserts. Unloaded myocardial slices placed on a porous membrane and each well filled with 1 mL of culture media. N = number of nyocardial slices. Mean ± standard error is shown on graphs. Source data are provided as a Source Data file

Sam Watson

# Routes for myocardial delivery




M Giacca & AM Shah, 2022, Eur Heart J

# Take home messages

- Advanced therapies based on mRNAs and small ncRNAs
- Lead nucleic acid search through in vivo and in vitro systematic screenings
- Transient modification of cell properties using ncRNAs for proliferation, vector permissivity and precise gene editing
- Cardiac RNA delivery using lipid nanoparticles

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