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RNA interference therapeutics for cardiac regeneration

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There is an impelling need to develop new therapeutics for myocardial infarction and heart failure. A novel and exciting therapeutic possibility is to achieve cardiac regeneration through the stimulation of the endogenous capacity of cardiomyocytes to proliferate. Proof-of-concept evidence of microRNA-induced cardiac regeneration is available in both small and large animals using viral vectors. However, a clinically more applicable strategy is the development of lipid-mediated nanotechnologies for the administration of RNA therapeutics as synthetic molecules. The recent success of the Stable Nucleic Acid Lipid Particle (SNALP) platform for the generation of nanosized, efficient and non-inflammatory lipid nanoparticles paves the way to the development of injectable nanoformulations of microRNAs through cardiac catheterisation.

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The clinical need for cardiac regeneration

There is an impelling clinical need to develop new therapeutic approaches for myocardial infarction (MI), in order to prevent maladaptive cardiac remodelling and fibrosis, two hallmarks of heart failure (HF) [1]. Pharmacological treatment for MI and HF has not evolved significantly since the mid-'90s (including the relatively recent angiotensin receptor-neprilysin inhibitor (ARNI) combination [2]), as most of the drugs are based on pathophysiological concepts developed over 30 years ago (reviewed in Ref. [3]). While there is hope in the cardiovascular effects of SGLT2 inhibitors to treat HF, the effect of these drugs was discovered serendipitously in the course of anti-diabetic clinical trials [4] and there still is no unanimous explanation for their mechanisms of action. Of note, for conditions that are as prevalent as MI and HF, no biological therapy (based on proteins, antibodies, nucleic acids or cells) is currently available.

Over the last decade, it has clearly emerged that a major reason underlying the prevalence of HF relates to the incapacity of the heart to regenerate after an insult. MI can kill as many as 25% CMs in the left ventricle in an acute manner [5]. In addition, CMs are killed during several other more chronic conditions, ranging from inherited cardiomyopathies to drug-induced cardiac toxicity. This contrasts with the rate of CM renewal in adult life, which is estimated to be less than 1% new CM generation per year [6,7], far too low to provide clinical benefit.

In contrast to other organs in which tissue formation and regeneration is sustained by stem cells that persist throughout life, cardiac progenitors generate CMs only during the first phases of cardiac development, while CM formation later depends on the proliferation of already committed cells [8]. As a consequence, cardiac repair after an insult depends on the capacity of CMs to enter the cell cycle and duplicate. Proliferation of CMs, however, remains robust throughout embryonic and foetal development, and suddenly stops after birth, for reasons that still escape our full understanding (*cf.* Ref. [9] and references cited therein). This contrasts with other species, such as amphibians and fish, in which CM replication, and thus cardiac regeneration after damage, can continue throughout life. Thus, the development of therapeutic strategies that stimulate cardiac proliferation in adulthood offers an unprecedented opportunity to achieve cardiac regeneration after MI and other conditions that determine CM loss.

Stimulation of cardiac regeneration by microRNAs

Over the last few years, there has been a flurry of new information on the possibility of achieving cardiac regeneration by stimulating the endogenous capacity of CMs to proliferate [10]. One of the most exciting strategies in this respect is to modulate the CM transcriptome through the RNA interference (RNAi) pathway. The microRNAs (miRNAs) that can stimulate CM proliferation can be classified into one of three groups. The first group is composed of miRNAs that are physiologically expressed in embryonic stem (ES) cells and are required to maintain

pluripotency. The prototype of these miRNAs is the miR-302-367 cluster [11]. A second group includes different molecules participating in the regulation of cancer cell proliferation. These comprise the miR-17∼92 cluster (also named OncomiR1) [12,13], and its paralogue clusters miR-106b∼25 and miR-106a∼363 [14,15]. Lastly, the third group includes a heterogenous series of miRNAs that were directly discovered through high throughput screenings for miRNAs that induce replication of neonatal rodent [16] or human [17] CMs. The most effective miRNAs of this group include miR-199a-3p, miR-590-3p and the primate-specific microRNA miR-1825. Despite its absence in the rodent genome, miR-1825 is among the most effective miRNA in driving proliferation of both mouse and rat cells when delivered exogenously [18•].

A more effective way to express microRNAs in CMs is through gene transfer of their pri-miRNA DNA using AAV vectors, which show specific tropism for CMs and other post-mitotic cells [19]. Expression of miR-199a from AAV serotype 9 (AAV) in mice [16] and AAV6 in pigs [20••] induces cardiac regeneration and marked improvement in cardiac function, as assessed over time by echocardiography and cardiac MRI respectively. The use of AAV vectors for cardiac regeneration, however, is fraught with two critical problems. First, these vectors persist and express their transgenes indefinitely. This is not desirable for a pro-regenerative molecule, especially as CM proliferation requires partial de-differentiation of these cells, with potential risk in terms of arrhythmogenicity [20••]. Second, generation of an effective, single-stranded, mature miRNA inside the cells depends on the activity of the RISC complex, which can generate both 5p and 3p active RNA molecules from the same double-stranded miRNA. In the case of miR-199a, while miR-199a-3p is pro-regenerative, miR-199a-5p can exert undesirable effects in the heart [21]. To overcome both these limitations of AAV vectors, a very appealing option is to directly deliver the desired miRNA strand transitorily as a synthetic molecule using lipid nanoformulations.

Lipid nanoformulations for non-coding RNA delivery

There has been recent excitement in the possibility of exploiting various nanotechnology tools for the delivery of RNAi therapeutics (reviewed in Ref. [22], also in reference to cardiovascular applications [23].

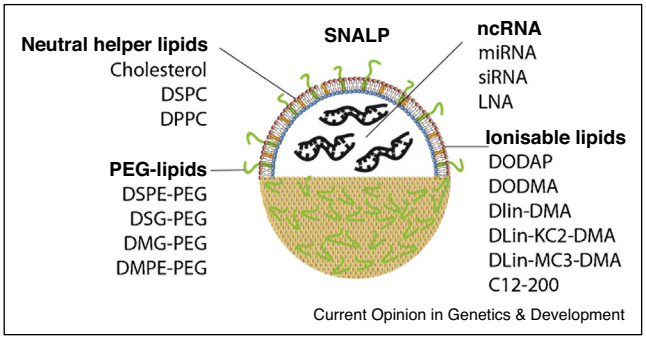
Traditionally, the transfection of small non-coding RNAs (ncRNAs) in cultured cells has taken advantage of cationic lipids (such as DOTMA, DOSPA or DOTAP, which bind the negatively charged RNA molecules), mixed with a neutral lipid with a helper function (such as DOPE) to form a lipoplex [24]. This kind of formulation forms the basis of the widely used reagents of the lipofectamine group (the original Lipofectamine is a 3:1 formulation of DOSPA and DOPE). In particular, DOPE has a cone shape, which is an optimal configuration to stabilise the transient hexagonal phase during endosomal membrane fusion and disruption, which is required for payload delivery to the cytosol [24]. Lipoplexes are usually formed by mixing preformed cationic liposomes with a ncRNA in an aqueous environment. For cardiac regeneration, a single intramyocardial injection miR-199a-3p or miR-590-3p or the intravenous administration of miR-19a/19b [25•] mimics using one of the commercial, lipofectamine-based formulations (RNAiMax) was shown to stimulate a regenerative response in mice [26].

Lipoplexes are efficient tools for cell transfection with miRNAs (or siRNAs). However, their large particle size (often >1 μm), dissociation of the nucleic acid molecules at the outer surface, aggregation by opsonization once in the circulation, toxicity and inflammation *in vivo* [27–30] significantly hamper their clinical utilization. Some of the shortcomings of positively charged lipoplexes can be overcome by the use of neutral lipids, which show improved biodistribution and reduced clearance from the circulation. An example of these neutral lipids is the commercial preparation MaxSuppressor *in vivo* RNA-LANCEr II, composed of DOPC, squalene oil, polysorbate 20 and an antioxidant. This formulation was used for the daily intravenous administration of the pro-regenerative miR302b/c [11], miR-19a/19b [25•] or miR-708 [31] in mice. Furthermore, miR-199a-3p has also been successfully used in lipid (DSPE-PEG) polymeric nanoparticles within a hydrogel to induce cardiac regeneration after intramyocardial injection [32•].

Significant progress in lipid nanoformulation amenable to clinical translation has been achieved by the use of ionisable cationic lipids. These lipids are positively charged at low pH and can thus complex with negatively charged nucleic acids, while become neutral when pH is raised to physiological values. This property, coupled with the possibility of loading the lipid-nucleic acid complexes into small particles, permits the generation of neutral lipid nanoparticles (LNPs) that are devoid of significant toxicity [24]. In particular, the SNALP (Stable Nucleic Acid Lipid Particle) family of LNPs has gained particular momentum in the last couple of years. The overall first market approval for an siRNA therapeutic was in 2018 for patisiran, an LNP carrying an anti-transthyretin siRNA for the treatment of transthyretin-induced amyloidosis [33••]. The use of SNALPs for RNA delivery then has achieved worldwide application starting from the late 2020 with the COVID-19 vaccines pioneered by Moderna (mRNA-1273 [34••]) and Pfizer/BioNTech (BNT162b2 [35••], for the administration of the SARS-CoV-2 Spike mRNA.

Typically, in SNALPs, the nucleic acid is surrounded by a lipid bilayer that forms a sphere with a mean diameter

Figure 1



Schematic representation of a Stable Nucleic Acid Lipid Particle (SNALP).
The figure lists the main lipids that can be used for the formulation of SNALPs, according to chemical characteristics.

~100 nm and a neutral charge. The lipid bilayer comprises three essential components: an ionizable cationic lipid (DODAP, DODMA, DLinDMA, DLin-KC2-DMA and DLin-MC3-DMA); a couple of neutral helper lipids (DSPC, cholesterol) and a PEG-derivatized lipid (Figure 1). The rationale for SNALP formation is that, at acidic pH, the positively charge lipid allows for RNA complexation, whereas, at physiological pH, the surface charge is near neutral, reducing the particle cytotoxicity; the other lipids help to stabilise the lipid bilayer [36]. The nucleic acid cargo entrapment efficiency within the LNP using ionisable lipids can reach 90% efficiency. In addition, DLin-MC3-DMA, which was used to formulate patisiran and other SNALPs, includes two unsaturated alyl chains, which give DLin-MC3-DMA the cone shape required to stabilise the hexagonal phase and improve endosomal escape [37]. Of the helper lipids, DSPC is cylinder-shaped and therefore forms a bilayer structure which provides increased stability to the LNPs, while cholesterol, similar to its role in biological membranes, provides stability in the presence of serum proteins and assist in membrane fusion to facilitate endosome escape [38]. Finally, the PEG-derivatized lipids provide an aqueous shield around the nanoparticle, which prolongs its persistence in blood. PEGylated lipids can come in various forms, the main two being permanent DSG-PEG, or diffusible DMG-PEG. Table 1 reports the formulations of some of the recently used LNPs and SNALPs.

Challenges in the formulation of SNALPs for cardiac regeneration

For a nanosized particle to be successful in delivering its ncRNA payload, it must remain stable in the circulation (or in the tissue in which it is directly administered), enter the cells efficiently, usually by endocytosis, avoid degradation by escaping the endosomes while *en route* to lysosomes, and localise in the cytoplasm where it can be loaded into the RNA-induced silencing complex (RISC).

Extracellular stability is usually ensured by the bilayer-forming properties of DSPC (all the approved SNALP therapies use this helper lipid [33••,34••,35••]) and by the inclusion of PEGylated lipids. In particular, the diffusible DMG-PEG allows for the protection from LNP fusion when these are in storage, while the lipid is shed from the surface upon injection, therefore allowing the LNP to transfect the target cells. On the other hand, increasing the PEG half-life by augmenting the acyl chain length increases the length of time PEG is retained on the LNP, and therefore the LNP circulation time [39]. PEG, however, is immunogenic, and therefore a balance needs to be struck in the PEG half-life that enables sufficient tissue accumulation of LNPs without evoking a strong immune response. PEG can also affect LNP size, with higher PEG content resulting in smaller LNPs [40]. As LNP size denotes the rate at which its components dissociate, smaller particles having a greater surface area to volume ratio exhibit the greatest dissociations and therefore reduced stability in circulation [41].

Table 1

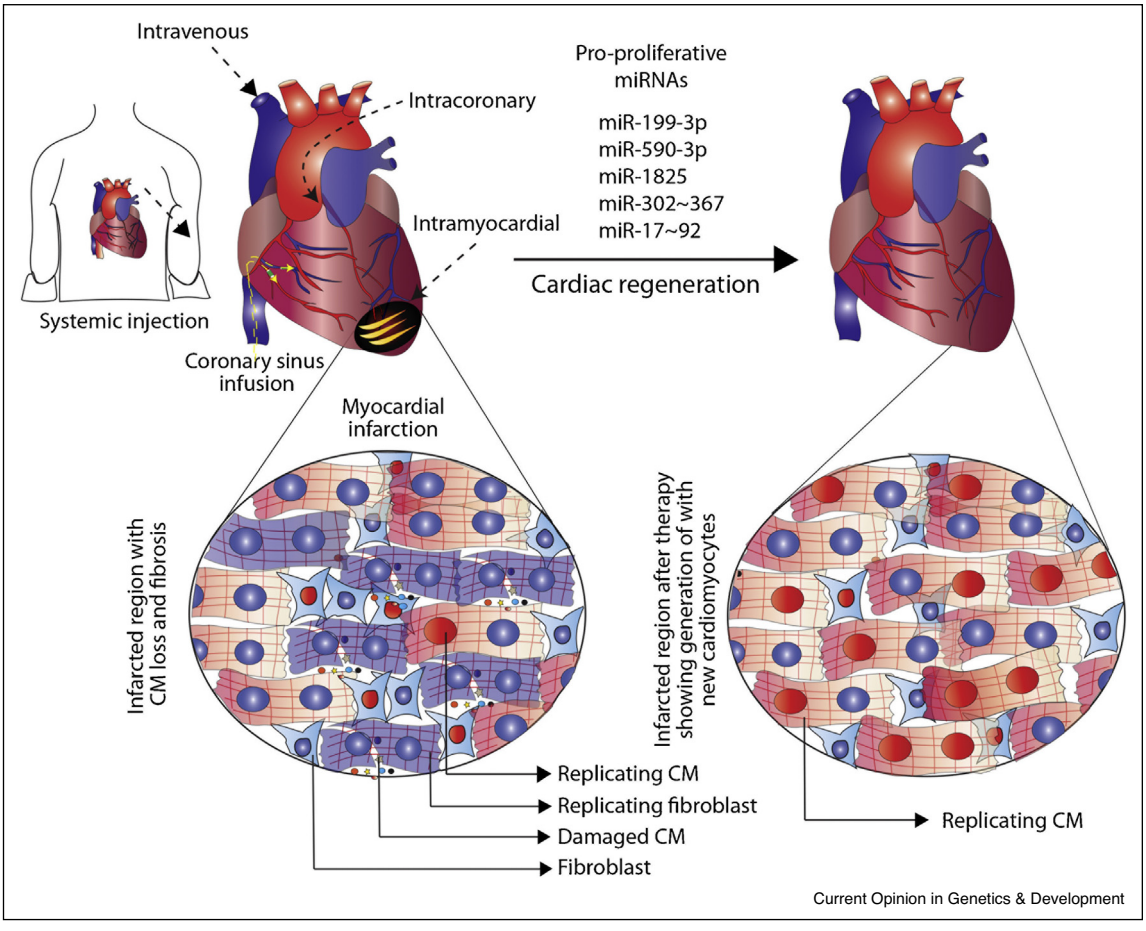
Lipid formulations in some of the RNA therapies based on LNP-mediated delivery that are currently approved or are in late-phase clinical trials

Product	Patisiran	BNT162b2 (Pfizer-BioNTech COVID-19 vaccine)	mRNA-1273 (Moderna COVID-19 vaccine)	EPHARNA	Atu027
LNP technology	SNALP	SNALP	SNALP	Neutral liposome	Cationic lipoplex
Therapeutic RNA	Anti-TTR siRNA	SARS-CoV-2 Spike modified mRNA	SARS-CoV-2 Spike modified mRNA	Anti-EphA2 siRNA	Anti-PKN3 siRNA
Ionisable lipids	DLin-MC3-DMA	ALC-0315	SM-102		AtuFECT01
Neutral lipids	DSPC Cholesterol	DSPC Cholesterol	DSPC Cholesterol	DOPC	DPhyPE
PEG lipids	PEG_{2000}-C-DMG	ALC-0159	PEG_{2000}-C-DMG		DSPE-PEG
Reference	[47]	[35••]	[34••]	[48]	[49]

Upon systemic administration, most LNPs accumulate in the liver, followed by spleen, kidneys, lungs and then the heart [42]. Particle size does not appear to significantly affect this distribution pattern [41]. This highlights a challenge in both LNP formulation and route of administration for their use in cardiac regeneration. However, most available biodistribution studies are carried out in healthy, non-inflamed tissues. Upon inflammation, there is increased vessel permeability, resulting in the so-called enhanced permeability and retention (EPR) effect. Whether the EPR effect after MI could be sufficient to promote preferential cardiac accumulation of LNPs upon their systemic administration remains to be determined.

One area that could dramatically improve biodistribution of an RNAi therapeutic after systemic administration is the direct modification of the RNA molecule, or of the LNP including it, with a cell or tissue targeting factor. This has determined the current success of RNAi therapies for the liver. The very recently approved givosiran (an siRNA against aminolevulinate synthase 1 for the treatment of acute hepatic porphyria [43•]), lumasiran (an siRNA against glycolate oxidase for primary hyperoxaluria type 1 [44]) and inclisiran (an siRNA targeting the PCSK9 mRNA for the treatment of hyperlipidaemias [45]), all developed by Alnylam Pharmaceuticals, are siRNAs conjugates with *N*-acetylgalactosamine (GalNAc) sugars, which bind with high affinity and specificity to asialoglycoprotein (ASGPR), a receptor abundantly expressed in hepatocytes. GalNAc conjugates have been so successful that up to 2/3 of all RNAi drugs currently in clinical trials are GalNAc conjugates [46]. Such an efficient system to target cardiomyocytes has yet to be identified.

Figure 2



Routes for RNAi therapeutics delivery.
These include injection into the coronary artery as during standard percutaneous coronary intervention or retrograde into the coronary sinus; or intramyocardial, through either direct injection after minithoracotomy or during bypass surgery; or after percutaneous catheterisation to reach the left ventricle, followed by trans-endocardial delivery. A targeted LNP could be injected systemically without the need for catheterisation. Several miRNAs (in particular, miR-199-3p, miR-590-3p, miR-1825, and the miR-302~367 and miR-17-92 clusters) can stimulate CM proliferation and can be formulated with lipid nanocarriers for *in vivo* administration.

Alternative (or additional) to organ targeting, the heart is amenable to direct, intra-tissue administration. This can be achieved through either surgical access, that is, mini-thoracotomy followed by trans-epicardial delivery, or intraventricular injection using a percutaneous catheter-based approach, to achieve trans-endocardial delivery (Figure 2). The trans-epicardial approach is straightforward in terms of myocardial access, but is fraught with the problems related to cardiac surgery. On the contrary, the percutaneous approach is a less invasive and more feasible delivery method. At least four catheters have been developed to deliver the treatment by a trans-endocardial approach through the ventricular cavity, a relatively common procedure performed in several cardiac catheterizations [3].

The most practicable approach for cardiac LNP delivery appears to be via intracoronary administration, as this procedure is routinely performed worldwide for left heart catheterization and percutaneous coronary intervention. LNP delivery can be performed by either infusion after complete or partial balloon occlusion of the coronary flow. As an alternative to anterograde coronary infusion, the LNP can be administered by retrograde infusion through the vein system. This approach, which is commonly used in cardiac surgery to deliver effective cardioplegia, can increase transfection efficacy, especially in a trans-epicardial fashion.

While all these interventional approaches have been extensively utilized in both large animals and patients for the administration of both viral vectors and cells in the past two decades [3], significant experience still need to be developed on the feasibility and efficacy of LNP delivery in these settings.

Conflict of interest statement

M.G. is listed as one of the inventors in a patent family on microRNAs that stimulate cardiomyocyte proliferation and their use for cardiac regeneration. The other authors declare no conflict of interest.

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References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. Metra M, Teerlink JR: **Heart failure**. *Lancet* 2017, **390**:1981-1995.

2. Califf RM: **LCZ696: too good to be true?** *Eur Heart J* 2015, **36**:410-412.

3. Cannata A, Ali H, Sinagra G, Giacca M: **Gene therapy for the heart lessons learned and future perspectives**. *Circ Res* 2020, **126**:1394-1414.

4. McMurray JJV, Solomon SD, Inzucchi SE, Kober L, Kosiborod MN, Martinez FA, Ponikowski P, Sabatine MS, Anand IS, Belohlavek J *et al.*: **Dapagliflozin in patients with heart failure and reduced ejection fraction**. *N Engl J Med* 2019, **381**:1995-2008.

5. Murry CE, Reinecke H, Pabon LM: **Regeneration gaps: observations on stem cells and cardiac repair**. *J Am Coll Cardiol* 2006, **47**:1777-1785.

6. Bergmann O, Bhardwaj RD, Bernard S, Zdunek S, Barnabe-Heider F, Walsh S, Zupicich J, Alkass K, Buchholz BA, Druid H *et al.*: **Evidence for cardiomyocyte renewal in humans**. *Science* 2009, **324**:98-102.

7. Senyo SE, Steinhauser ML, Pizzimenti CL, Yang VK, Cai L, Wang M, Wu TD, Guerquin-Kern JL, Lechene CP, Lee RT: **Mammalian heart renewal by pre-existing cardiomyocytes**. *Nature* 2013, **493**:433-436.

8. Braga L, Ali H, Secco I, Giacca M: **Non-coding RNA therapeutics for cardiac regeneration**. *Cardiovasc Res* 2021, **117**:674-693.

9. Zacchigna S, Martinelli V, Moimas S, Colliva A, Anzini M, Nordio A, Costa A, Rehman M, Vodret S, Pierro C *et al.*: **Paracrine effect of regulatory T cells promotes cardiomyocyte proliferation during pregnancy and after myocardial infarction**. *Nat Commun* 2018, **9**:2432.

10. Ali H, Braga L, Giacca M: **Cardiac regeneration and remodelling of the cardiomyocyte cytoarchitecture**. *FEBS J* 2020, **287**:417-438.

11. Tian Y, Liu Y, Wang T, Zhou N, Kong J, Chen L, Snitow M, Morley M, Li D, Petrenko N *et al.*: **A microRNA-Hippo pathway that promotes cardiomyocyte proliferation and cardiac regeneration in mice**. *Sci Transl Med* 2015, **7**:279ra238.

12. Volinia S, Calin GA, Liu CG, Ambs S, Cimmino A, Petrocca F, Visone R, Iorio M, Roldo C, Ferracin M *et al.*: **A microRNA expression signature of human solid tumors defines cancer gene targets**. *Proc Natl Acad Sci U S A* 2006, **103**:2257-2261.

13. He L, Thomson JM, Hemann MT, Hernando-Monge E, Mu D, Goodson S, Powers S, Cordon-Cardo C, Lowe SW, Hannon GJ *et al.*: **A microRNA polycistron as a potential human oncogene**. *Nature* 2005, **435**:828-833.

14. Gruszka R, Zakrzewska M: **The oncogenic relevance of miR-17-92 cluster and its paralogous miR-106b-25 and miR-106a-363 clusters in brain tumors**. *Int J Mol Sci* 2018, **19**:879.

15. Mehlich D, Garbicz F, Wlodarski PK: **The emerging roles of the polycistronic miR-106b approximately 25 cluster in cancer — a comprehensive review**. *Biomed Pharmacother* 2018, **107**:1183-1195.

16. Eulalio A, Mano M, Dal Ferro M, Zentilin L, Sinagra G, Zacchigna S, Giacca M: **Functional screening identifies miRNAs inducing cardiac regeneration**. *Nature* 2012, **492**:376-381.

17. Diez-Cunado M, Wei K, Bushway PJ, Maurya MR, Perera R, Subramaniam S, Ruiz-Lozano P, Mercola M: **miRNAs that induce human cardiomyocyte proliferation converge on the Hippo pathway**. *Cell Rep* 2018, **23**:2168-2174.

18. • Torrini C, Cubero RJ, Dirkx E, Braga L, Ali H, Prosdocimo G, Gutierrez MI, Collesi C, Licastro D, Zentilin L *et al.*: **Common regulatory pathways mediate activity of microRNAs inducing cardiomyocyte proliferation**. *Cell Rep* 2019, **27**:2759-2771.e5.
Description of the main pathways leading to microRNA-stimulated cardiomyocyte proliferation, in particular showing the essential involvement of the Hippo pathway and of the Yap transcriptional cofactor in the regulation of this process.

19. Zacchigna S, Zentilin L, Giacca M: **Adeno-associated virus vectors as therapeutic and investigational tools in the cardiovascular system**. *Circ Res* 2014, **114**:1827-1846.

20. •• Gabisonia K, Prosdocimo G, Aquaro GD, Carlucci L, Zentilin L, Secco I, Ali H, Braga L, Gorgodze N, Bernini F *et al.*: **MicroRNA therapy stimulates uncontrolled cardiac repair after myocardial infarction in pigs**. *Nature* 2019, **569**:418-422.

The first evidence that and AAV vector expressing miR-199a can stimulate cardiac regeneraiton in a large animal with myocardial infarction.

21. da Costa Martins PA, Salic K, Gladka MM, Armand AS, Leptidis S, el Azzouzi H, Hansen A, Coenen-de Roo CJ, Bierhuizen MF, van der Nagel R *et al.*: **MicroRNA-199b targets the nuclear kinase Dyrk1a in an auto-amplification loop promoting calcineurin/NFAT signalling**. *Nat Cell Biol* 2010, **12**:1220-1227.

22. Yonezawa S, Koide H, Asai T: **Recent advances in siRNA delivery mediated by lipid-based nanoparticles**. *Adv Drug Deliv Rev* 2020, **154–155**:64-78.

23. Pala R, Anju VT, Dyavaiah M, Busi S, Nauli SM: **Nanoparticle-mediated drug delivery for the treatment of cardiovascular diseases**. *Int J Nanomed* 2020, **15**:3741-3769.

24. Kulkarni JA, Cullis PR, van der Meel R: **Lipid nanoparticles enabling gene therapies: from concepts to clinical utility**. *Nucleic Acid Ther* 2018, **28**:146-157.

25. • Gao F, Kataoka M, Liu N, Liang T, Huang ZP, Gu F, Ding J, Liu J, Zhang F, Ma Q *et al.*: **Therapeutic role of miR-19a/19b in cardiac regeneration and protection from myocardial infarction**. *Nat Commun* 2019, **10**:1802.
Demonstration of efficient stimulation of cardiomyocyte proliferation and caridac regeneration after administration of synthetic miR-19a/miR-19b RNA.

26. 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.

27. Filion MC, Phillips NC: **Toxicity and immunomodulatory activity of liposomal vectors formulated with cationic lipids toward immune effector cells**. *Biochim Biophys Acta* 1997, **1329**:345-356.

28. Ito Y, Kawakami S, Charoensit P, Higuchi Y, Hashida M: **Evaluation of proinflammatory cytokine production and liver injury induced by plasmid DNA/cationic liposome complexes with various mixing ratios in mice**. *Eur J Pharm Biopharm* 2009, **71**:303-309.

29. Meyer O, Kirpotin D, Hong K, Sternberg B, Park JW, Woodle MC, Papahadjopoulos D: **Cationic liposomes coated with polyethylene glycol as carriers for oligonucleotides**. *J Biol Chem* 1998, **273**:15621-15627.

30. Buyens K, Lucas B, Raemdonck K, Braeckmans K, Vercammen J, Hendrix J, Engelborghs Y, De Smedt SC, Sanders NN: **A fast and sensitive method for measuring the integrity of siRNA-carrier complexes in full human serum**. *J Control Release* 2008, **126**:67-76.

31. Deng S, Zhao Q, Zhen L, Zhang C, Liu C, Wang G, Zhang L, Bao L, Lu Y, Meng L *et al.*: **Neonatal heart-enriched miR-708 promotes proliferation and stress resistance of cardiomyocytes in rodents**. *Theranostics* 2017, **7**:1953-1965.

32. • Yang H, Qin X, Wang H, Zhao X, Liu Y, Wo HT, Liu C, Nishiga M, Chen H, Ge J *et al.*: **An in vivo miRNA delivery system for restoring infarcted myocardium**. *ACS Nano* 2019, **13**:9880-9894.
Delivery of pro-regenerative miR-199a-3p using lipid polymeric nanoparticles in hydrogel for intracardiac injection.

33. •• Adams D, Gonzalez-Duarte A, O'Riordan WD, Yang CC, Ueda M, Kristen AV, Tournev I, Schmidt HH, Coelho T, Berk JL *et al.*: **Patisiran, an RNAi therapeutic, for hereditary transthyretin amyloidosis**. *N Engl J Med* 2018, **379**:11-21.
Description of the clinical trial that later led to the market approval for patisiran, the first RNAi therapeutics to be ever approved.

34. •• Baden LR, El Sahly HM, Essink B, Kotloff K, Frey S, Novak R, Diemert D, Spector SA, Rouphael N, Creech CB *et al.*: **Efficacy and safety of the mRNA-1273 SARS-CoV-2 vaccine**. *N Engl J Med* 2021, **384**:403-416.
Results of the clinical trial conducted by Moderna using a SNALP for the delivery of a chemically modified mRNA coding for the SARS-CoV-2 coronavirus Spike protein.

35. •• Polack FP, Thomas SJ, Kitchin N, Absalon J, Gurtman A, Lockhart S, Perez JL, Pérez Marc G, Moreira ED, Zerbini C *et al.*: **Safety and efficacy of the BNT162b2 mRNA Covid-19 vaccine**. *N Engl J Med* 2020, **383**:2603-2615.
Results of the clinical trial conducted by Pfizer using a SNALP for the delivery of a chemically modified mRNA coding for the SARS-CoV-2 coronavirus Spike protein.

36. Kulkarni JA, Darjuan MM, Mercer JE, Chen S, van der Meel R, Thewalt JL, Tam YYC, Cullis PR: **On the formation and morphology of lipid nanoparticles containing ionizable cationic lipids and siRNA**. *ACS Nano* 2018, **12**:4787-4795.

37. Jayaraman M, Ansell SM, Mui BL, Tam YK, Chen J, Du X, Butler D, Eltepu L, Matsuda S, Narayanannair JK *et al.*: **Maximizing the potency of siRNA lipid nanoparticles for hepatic gene silencing in vivo**. *Angew Chem Int Ed Engl* 2012, **51**:8529-8533.

38. Cheng X, Lee RJ: **The role of helper lipids in lipid nanoparticles (LNPs) designed for oligonucleotide delivery**. *Adv Drug Deliv Rev* 2016, **99**:129-137.

39. Mui BL, Tam YK, Jayaraman M, Ansell SM, Du X, Tam YY, Lin PJ, Chen S, Narayanannair JK, Rajeev KG *et al.*: **Influence of polyethylene glycol lipid desorption rates on pharmacokinetics and pharmacodynamics of siRNA lipid nanoparticles**. *Mol Ther Nucleic Acids* 2013, **2**:e139.

40. Ryals RC, Patel S, Acosta C, McKinney M, Pennesi ME, Sahay G: **The effects of PEGylation on LNP based mRNA delivery to the eye**. *PLoS One* 2020, **15**:e0241006.

41. Chen S, Tam YYC, Lin PJC, Sung MMH, Tam YK, Cullis PR: **Influence of particle size on the in vivo potency of lipid nanoparticle formulations of siRNA**. *J Control Release* 2016, **235**:236-244.

42. Shi B, Keough E, Matter A, Leander K, Young S, Carlini E, Sachs AB, Tao W, Abrams M, Howell B *et al.*: **Biodistribution of small interfering RNA at the organ and cellular levels after lipid nanoparticle-mediated delivery**. *J Histochem Cytochem* 2011, **59**:727-740.

43. • Balwani M, Sardh E, Ventura P, Peiro PA, Rees DC, Stolzel U, Bissell DM, Bonkovsky HL, Windyga J, Anderson KE *et al.*: **Phase 3 trial of RNAi therapeutic Givosiran for acute intermittent porphyria**. *N Engl J Med* 2020, **382**:2289-2301.
Results of a clinical trial based on the administration of an siRNA conjugated with *N*-acetylgalactosamine (GalNAc) for specific hepatic cell targeting. These results later led to the clinical approval of the RNAi drug.

44. Scott LJ, Keam SJ: **Lumasiran: first approval**. *Drugs* 2021, **81**:277-282.

45. Ray KK, Landmesser U, Leiter LA, Kallend D, Dufour R, Karakas M, Hall T, Troquay RP, Turner T, Visseren FL *et al.*: **Inclisiran in patients at high cardiovascular risk with elevated LDL cholesterol**. *N Engl J Med* 2017, **376**:1430-1440.

46. Zhang MM, Bahal R, Rasmussen TP, Manautou JE, Zhong XB: **The growth of siRNA-based therapeutics: Updated clinical studies**. *Biochem Pharmacol* 2021:114432.

47. Adams D, Gonzalez-Duarte A, O'Riordan WD, Yang C-C, Ueda M, Kristen AV, Tournev I, Schmidt HH, Coelho T, Berk JL *et al.*: **Patisiran, an RNAi therapeutic, for hereditary transthyretin amyloidosis**. *N Engl J Med* 2018, **379**:11-21.

48. Wagner MJ, Mitra R, McArthur MJ, Baze W, Barnhart K, Wu SY, Rodriguez-Aguayo C, Zhang X, Coleman RL, Lopez-Berestein G *et al.*: **Preclinical mammalian safety studies of EPHARNA (DOPC nanoliposomal EphA2-targeted siRNA)**. *Mol Cancer Ther* 2017, **16**:1114-1123.

49. Schultheis B, Strumberg D, Kuhlmann J, Wolf M, Link K, Seufferlein T, Kaufmann J, Feist M, Gebhardt F, Khan M *et al.*: **Safety, efficacy and pharcacokinetics of targeted therapy with the liposomal RNA interference therapeutic Atu027 combined with gemcitabine in patients with pancreatic adenocarcinoma. A randomized phase Ib/IIa study**. *Cancers (Basel)* 2020, **12**.