Hybrid nano-delivery systems for micrornas in cardiac regenerative medicine: design, characterisation and preclinical testing in *in vitro* 2d and 3d tissue models

Valeria Chiono[1,2,3], Letizia Nicoletti[1,2,3], Camilla Paoletti[1,2], Martina Coletto[1,2], Giovanni Paolo Stola[1,2,3], Gerardina Ruocco[1,2], Alice Zoso[1,2], Elena Marcello[1,2], Irene Carmagnola[1,2] and Clara Mattu[1,2].

[1]Politecnico di Torino, Corso Duca Degli Abruzzi 24, Turin, Italy
[2]Interuniversity Center for the Promotion of the 3Rs Principles in Teaching and Research, Centro3R, Pisa, Italy.
[3] PoliRNA Srl, Via Vincenzo Vela 42, Turin, Italy.

valeria.chiono@polito.it

Cardiovascular diseases are the leading cause of death. Among them, myocardial infarction (MI) causes the irreversible loss of cardiomyocytes, replaced by a dysfunctional scar tissue, mainly populated by cardiac fibroblasts, which often leads to heart failure [1]. Recently, specific microRNAs (miRNAs) have been identified for post-MI treatment, being able either to induce the proliferation of cardiomyocytes, or to trigger the direct reprogramming of cardiac scar fibroblasts into cardiomyocytes, or to reduce fibrotic response [2-4]. However, gold-standard lipid nanoparticles (LNPs), clinically approved for different RNA therapies, such as the vaccination against Covid-19 and the treatment of liver-related diseases, suffer from poor biological half-life and main accumulation in non-target organs, mainly the liver [4]. Hence, safe, precise and efficient nanoparticles for the *in vivo* delivery of miRNAs to cardiac tissues are currently missing and needed for future therapeutic applications of miRNAs.

To this purpose, firstly we have developed new lipoplexes, based on [2-(2,3-didodecyloxypropyl)-hydroxyethyl] ammonium bromide (DE) and L-alpha-dioleoylphosphatidylethanolamine (DOPE), showing improved biocompatibility and ability for intracellular miRNA delivery respect to commercial transfection agents [4]. Then, for *in vivo* applications, we have designed and patented novel ligand-functionalized polymer-lipid hybrid nanoparticles (f-HNPs/miRNAs), having: (i) a lipidic core for high miRNA loading; (ii) a synthetic polymer shell, improving biological half-life and allowing controlled and sustained miRNA release; (iii) specific selected ligands, grafted on the nanoparticle surface, for active cell targeting. The f-HNPs/miRNAs showed higher miRNA encapsulation efficiency (99% vs 65%) and biocompatibility (80-100% vs. 50-60%) and similar transfection ability than commercial transfection agents in 2D cell models. In parallel, reliable *in vitro* models replicating human cardiac scar tissue were engineered for more reliable preclinical validation of nanomedicine approaches. To reproduce cardiac fibrotic tissue hallmarks (extracellular matrix architecture, composition, and stiffness), electrospun scaffolds with biomimetic architecture, biochemical properties and surface stiffness were prepared, based on randomly-oriented poly(ε-caprolactone) PCL nanofiber mats, surface grafted with human type I collagen (C1) and fibronectin (F) [5]. Human cardiac fibroblasts (CFs) underwent phenotype switch into myofibroblasts upon 7 days culture on the biomimetic scaffolds, without the need for transforming growth factor-beta (TGF-β). Model reliability and predictivity was validated upon antifibrotic drug treatment [5], showing the downregulation of fibrotic markers (α-SMA expression, and cell-produced C1 and F). The model was then exploited for the study of biocompatibility, internalization ability and efficacy of f-HNPs/miRNAs, loaded with negmiR, siRNA-Cy5 and a set of four reprogramming miRNAs, respectively. Design of complex models including other cells, e.g., cardiomyocytes, is ongoing to assess precision cell delivery. Finally, *in vivo* preclinical trials in a mouse model of chronic MI showed significantly improved heart biodistribution of f-HNPs/siRNA-Cy5 than control unfunctionalized HNPs/siRNA-Cy5, minimizing liver, spleen and kidney accumulation. *In vivo* acute toxicity tests in mouse models showed the safety of f-HNPs/miRNA. Cardiac ejection fraction was enhanced 30 days post-MI treatment with reprogramming miRNAs by f-HNPs.

Hence, f-HNPs/miRNA showed safety and target efficiency in 2D and 3D human fibrotic cardiac tissue models and in an *in vivo* mouse model of MI. By changing miRNAs and surface functionalization, other applications of f-HNPs are under exploration, such as the induction of cardiomyocyte proliferation.

This work was supported by: the European Union's ERC projects “BIORECAR” (772168; Horizon 2020) and “POLIRNA” (101113522; Horizon Europe); PoC Transition project “NanoCARD” (Fondazione Compagnia di San Paolo – Fondazione Links); NODES PoC project “NAPTER” supported by MUR – M4C2 1.5 of PNRR funded by the European Union - NextGenerationEU (ECS00000036); project “NANOMIRC” (Bando a Cascata CN00000041, CN RNA & Gene Therapy, Spoke 4, PNRR M4C2 1.4).

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