Controlled delivery of fibroblast growth factor-1 and neuregulin-1 from biodegradable microparticles promotes cardiac repair in a rat myocardial infarction model through activation of endogenous regeneration

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Abstract

Acidic fibroblast growth factor (FGF1) and neuregulin-1 (NRG1) are growth factors involved in cardiac development and regeneration. Microparticles (MPs) mediate cytokine sustained release, and can be utilized to overcome issues related to the limited therapeutic protein stability during systemic administration. We sought to examine whether the administration of microparticles (MPs) containing FGF1 and NRG1 could promote cardiac regeneration in a myocardial infarction (MI) rat model. We investigated the possible underlying mechanisms contributing to the beneficial effects of this therapy, especially those linked to endogenous regeneration. FGF1- and NRG1-loaded MPs were prepared using a multiple emulsion solvent evaporation technique. Seventy-three female Sprague-Dawley rats underwent permanent left anterior descending coronary artery occlusion, and MPs were intramyocardially injected in the peri-infarcted zone four days later. Cardiac function, heart tissue remodeling, revascularization, apoptosis, cardiomyocyte proliferation, and stem cell homing were evaluated one week and three months after treatment. MPs were shown to efficiently encapsulate FGF1 and NRG1, releasing the bioactive proteins in a sustained manner. Three months after treatment, a statistically significant improvement in cardiac function was detected in rats treated with growth factor-loaded MPs (FGF1, NRG1, or FGF1/NRG1). The therapy led to inhibition of cardiac remodeling with smaller infarct size, a lower fibrosis degree and induction of tissue revascularization. Cardiomyocyte proliferation and progenitor cell recruitment was detected. Our data support the therapeutic benefit of NRG1 and FGF1 when combined with protein delivery systems for cardiac regeneration. This approach could be scaled up for use in pre-clinical and clinical studies.

Keywords: FGF1, NRG1, PLGA microparticles, myocardial infarction, cardiac repair

1. Introduction

Ischemic heart disease is the leading cause of morbidity and mortality worldwide [1]. Thus, there has been great interest in novel therapeutic options, such as gene (reviewed in [2]) and stem cell therapy (reviewed in [3]), or even direct administration of pro-angiogenic cytokines [4]. In the case of growth factor-based therapy, although pre-clinical studies and initial clinical trials had suggested beneficial effects [5, 6], double-blinded clinical trials with large cohorts of patients failed to validate the efficacy [7-9]. These negative findings may have resulted from issues related to growth factor selection, monotherapy instead of combinatorial therapy, and/or timing of growth factor delivery. Moreover, the therapeutic benefit of directly administered growth factors can be limited by the short circulating half-life and high instability of these proteins after injection. In this context, new strategies involving injectable biocompatible and biodegradable microparticles (MPs), which mediate sustained release of cytokines, might offer valuable approaches for overcoming these limitations [10].

Poly(lactic-*co*-glycolic acid) (PLGA) is a biopolymer that is FDA-approved for use as a drug delivery platform due to its excellent biocompatibility, high safety profile, and suitable biodegradation [11]. PLGA MPs were already shown to be useful for growth factor delivery [12, 13]. Moreover, we demonstrated the efficacy of treating infarcted hearts with PLGA MPs loaded with vascular endothelial growth factor (VEGF), which induced neovascularization and reduced cardiac remodeling after myocardial infarction (MI) in rats [14]. Indeed, many pre-clinical and clinical studies aimed at repairing infarcted heart tissue have explored pro-angiogenic cytokine administration as a means to promote tissue revascularization.

In addition to mediators of angiogenesis, the list of potential therapeutics for cardiac regeneration has continued to grow, and the use of factors involved in cardiac development, stem cell homing, cardiac differentiation/proliferation, or direct cardioprotection could lead to novel approaches for repairing damaged heart (reviewed in [4]). In this regard, *in vitro* studies have shown that adult cardiomyocytes do not proliferate under resting conditions, but may divide in response to extracellular mitogens, such as periostin [15], acidic fibroblast growth factor (FGF1) [16], and neuregulin-1 (NRG1) [17]. These findings have supported a new paradigm, which suggests that the heart might be capable of repair and regrowth in response to extracellular mitogens. Consistent with this idea, it is known that FGF1 regulates cardiac remodeling by exerting a protective and proliferative effect after MI [18, 19]. On the other hand, neuregulins play crucial roles in the adult cardiovascular system by inducing structural organization of sarcomeres, cell integrity, cell–cell adhesion [20], cell survival [21, 22] and angiogenesis [23]. In fact, several studies using animal models of heart failure have demonstrated the therapeutic benefits of neuregulins, which improved cardiac performance, attenuated disease markers, and prolonged animal survival [24, 25]. Furthermore, phase I and II clinical trials for chronic heart failure in humans confirmed the favorable effects mediated by neuregulins [26, 27], highlighting the therapeutic potential of these growth factors in cardiac repair.

In this study, we have examined the efficacy of novel MP-based delivery of NRG1 and FGF1 in a rat model of MI. Notably, the use of MPs prevented issues related to growth factor stability, facilitating sustained treatment in the damaged tissues. As a result, we observed significant improvement in cardiac function upon MP-mediated delivery of these factors to infarcted hearts. Finally, we investigated the underlying mechanisms contributing to this positive effect, especially those linked to endogenous regenerative capacity.

2. Materials and methods

All animal procedures were approved by the University of Navarra Institutional Committee on Care and Use of Laboratory Animals as well as the European Community Council Directive Ref. 86/609/EEC. An expanded Methods section is available in the Supplementary Material.

2.1. Materials

Recombinant human FGF-1 and NRG-1 were supplied from ImmunoTools GmbH (Friesoythe, Germany). PLGA with a monomer ratio (lactic acid/ glycolic acid) of 50:50 Resomer® RG 503H (M_w: 34 kDa) was provided by Boehringer-Ingelheim (Ingelheim, Germany). Polyethylene glycol (PEG; M_w: 400), human serum albumin (HSA), bovine serum albumin (BSA), dimethylsulfoxide (DMSO) and sodium azide were provided by Sigma-Aldrich (Barcelona, Spain). Dichloromethane and acetone were obtained from Panreac Quimica S.A. (Barcelona, Spain). Poly(vinyl alcohol) (PVA) 88% hydrolyzed (M_w: 125,000) was obtained from Polysciences, Inc. (Warington, USA). Murine HL-1 cardiomyocyte-cell line (kindly donated by Dr. Claycomb, Louisiana State University Medical Center, USA) was used in the *in vitro* assays. Claycomb medium was provided by SAFC Biosciences (Lenexa, KS, USA) and 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H tetrazolium (MTS) was purchased from Promega (Madison, USA). Rabbit polyclonal anti-human FGF-1 antibody (ab9588) was supplied by Abcam (Cambridge, UK) and horseradish-peroxidase conjugated donkey anti-rabbit (NA934V) were purchased from GE Healthcare. Goat polyclonal anti-human NRG-1 antibody (sc-1793) and horseradish-peroxidase-conjugated donkey anti-goat IgG (sc-2020) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). ECL™ anti-rat IgG horseradish peroxidase-linked whole antibody was from Amersham Biosciences (Buckinghamshire, UK). Monoclonal anti-alpha smooth muscle actin-Cy3 (C6198) was provided by Sigma (St. Louis, MO, USA), anti-caveolin-1 and rat anti mouse CD45 (550539) from BD Pharmingen (Heidelberg, Germany). Rabbit polyclonal anti-human c-Kit antibody (A4502) was supplied from Dako (Carpinteria, CA, USA), monoclonal anti-human Ki-67 antibody (RM9106) was purchased from Thermo Fisher Scientific (Fremont, CA, USA) and mouse monoclonal cardiac troponin I antibody (ab19615) was obtained from Abcam (Cambridge, UK). DAPI nucleic acid stain was supplied from Molecular Probes-Invitrogen (Carlsbad, CA, USA) and TOPRO-3 was from Molecular Probes.

2.2. Preparation and characterization of MPs containing FGF1 and NRG1

FGF1- and NRG1-loaded PLGA MPs were prepared through a solvent extraction/evaporation method using the Total Recirculation One Machine System (TROMS) [14]. Particle size and size distribution were measured by laser diffractometry. Cytokine encapsulation efficiency and *in vitro* release from MPs was quantified by western blot. Bioactivity of MP-released proteins was evaluated *in vitro* by determining HL-1 cardiomyocyte proliferative capacity following growth factor treatment.

2.3. MI model and intramyocardial administration of MPs

Seventy-three female Sprague-Dawley rats underwent permanent left anterior descending coronary artery occlusion to induce MI. Among the surviving animals (n=57), only those with a left ventricular ejection fraction (LVEF) below 50% (n=46) at 2 days post-MI were included in the study. Four days post-MI, rats were divided into four groups, and the chests were reopened. Two milligrams of FGF1-loaded MPs

(FGF1-MP; 1,740 ng of FGF1), NRG1-loaded MPs (NRG1-MP; 1,300 ng of NRG1), a mixture of MPs loaded with FGF1 and NRG1 (FGF1/NRG1-MP; loaded with the same doses), or control non-loaded MPs (NL-MP) were injected with a 29-gauge needle into four regions surrounding the border of the infarct. At 1 week (n=6 rats) and 3 months (n=40 rats) post-injection the animals were sacrificed.

2.4. Morphometric and histological studies

Heart function was assessed 3 months post-treatment. In addition, heart tissue remodeling, revascularization, cardiac proliferation, and endogenous stem cells were investigated. All results obtained from the growth factor-treated groups were compared to the NL-MP-injected control group.

2.5. Statistical analysis

Results are expressed as mean ± SEM. Statistics were calculated using Prism 5.0 software (Graphpad Software Inc., San Diego, CA, USA). *P* values <0.05 were considered significant.

3. Results

3.1. FGF1 and NRG1 induce adult cardiomyocyte proliferation and survival in vitro

The effect of FGF1 and/or NRG1 on adult cardiomyocyte proliferation and apoptosis was studied *in vitro*. Treatment of HL-1 cardiomyocytes with different doses of FGF1 or NRG1 (alone or in combination) led to a statistically significant increase in cell proliferation (Figure 1A) (*P*<0.01). HL-1 cell apoptosis could be induced by hypoxia and serum deprivation, and addition of both FGF1 and NRG1 resulted in a statistically significant decrease in the apoptotic phenotype (Figure 1B and 1C).

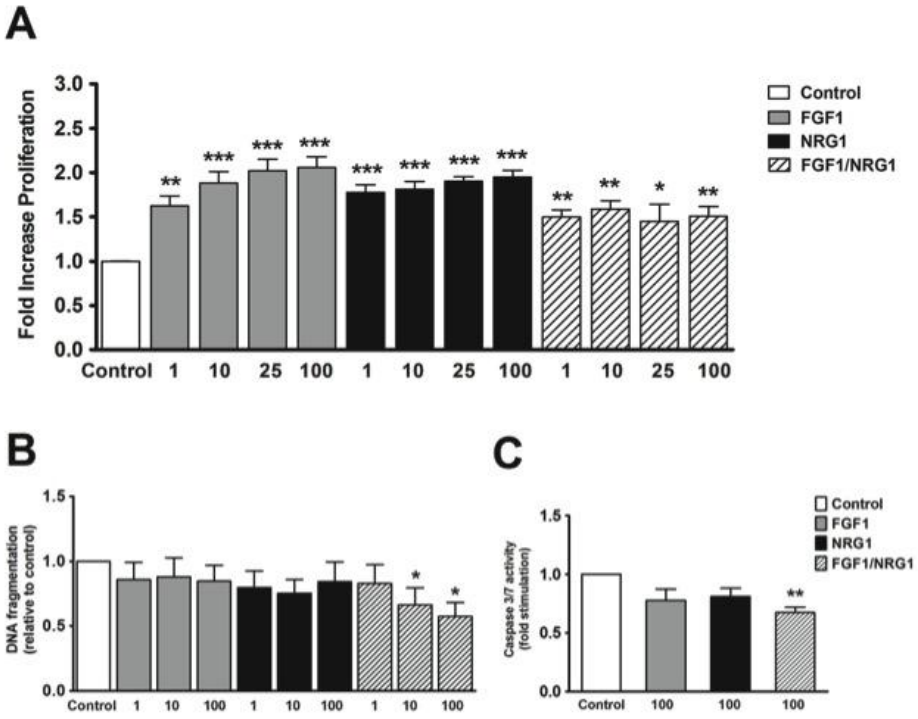

Figure 1: FGF1 and NRG1 effects on cardiomyocyte proliferation and apoptosis *in vitro*

(A) HL-1 cardiomyocyte proliferation following treatment with free FGF1 or free NRG1 (1, 10, 25, and 100 ng/ml) administered alone or in combination (FGF1/NRG1). **(B,C)** HL-1 cell apoptosis in the presence of FGF1, NRG1, or FGF1/NRG1 was measured by ELISA detection of histone-associated DNA

fragmentation **(B)** or detection of caspase-3/7 activity **(C)**. Data are expressed as mean ± SEM from three independent experiments (**P*<0.05, ***P*<0.01, and ****P*<0.001 *vs.* control).

3.2. Formulation and characterization of FGF1- and NRG1-loaded MPs

MPs prepared by TROMS had a spherical shape with an average size of 5.1±1.4 µm (Figure 2A). Encapsulation efficiency was found to be 87.4±2.3% for FGF1 and 65.5±5.1% for NRG1, which corresponded to final loading of 874.1±23.4 ng of FGF1 and 655.3±50.1 ng of NRG1 per mg of polymer. *In vitro* growth factor release profiles revealed an initial burst effect, which indicated that both factors displayed very similar release rates from day 7 to day 28, with 65% of NRG1 and almost 70% of FGF1 being released within 28 days (Figure 2B). Also, we assessed the bioactivity of the MP-released cytokines by induction of HL-1 proliferation. NRG1 and FGF1, either released from the particles or as free cytokines, induced 1.5–1.7 fold increase in HL-1 proliferation in comparison with controls (NL-MP or no cytokine), indicating that both cytokines retained their biological activity after encapsulation into PLGA MPs (Figure 2C).

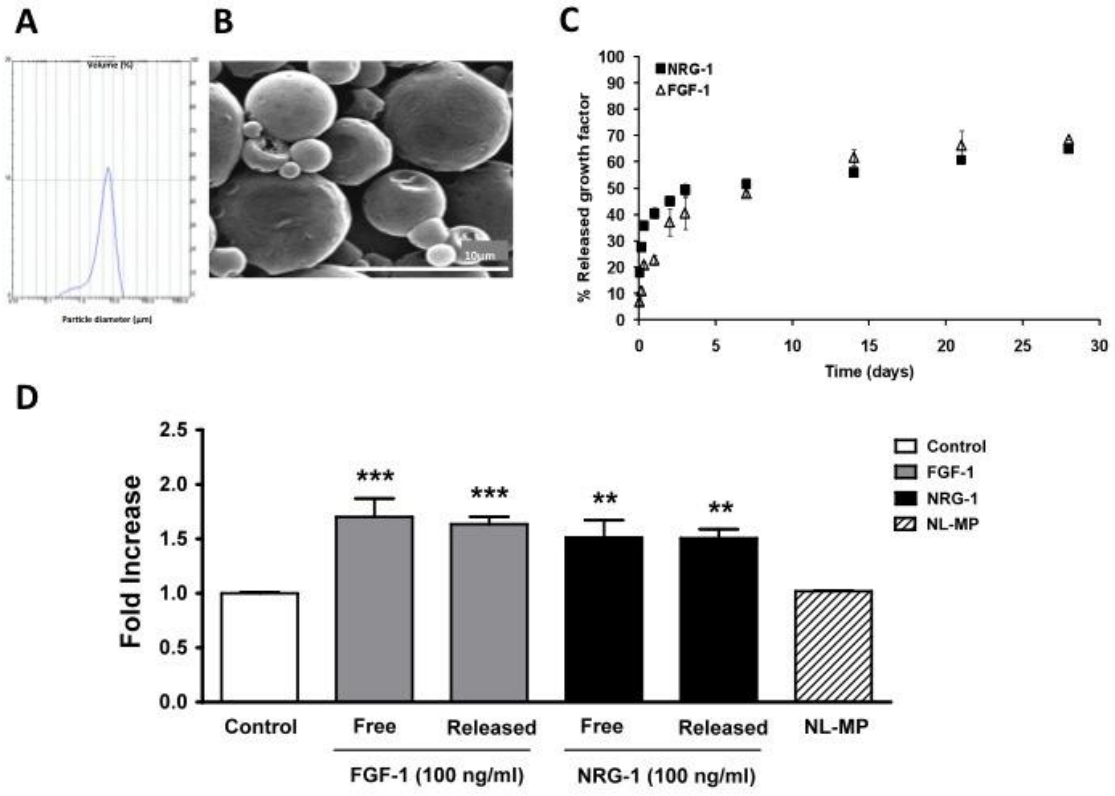

Figure 2: Characterization of MPs loaded with FGF1 and NRG1

(A) Particle size distribution and (B) Scanning electron microscopy images of MPs loaded with FGF1. **(**C**)** FGF1 and NRG1 *in vitro* release from MPs. **(**D**)** HL-1 cells were cultured in the presence of either MP-released or free FGF1 and NRG1 (100 ng/ml) and cell proliferation was assessed at 72 hours using an MTS assay. Data are expressed as mean ± SEM from three independent experiments (***P*<0.01 and ****P*<0.001).

3.3. Treatment with FGF1- and NRG1-loaded MPs improved cardiac function in a rat model of acute MI

Next, we investigated the effect of the growth factor-loaded MPs on cardiac repair using a rat model of acute MI. Four days after inducing MI, rats were treated with FGF1-MP, NRG1-MP, FGF1/NRG1-MP, or control (NL-MP). Three months after transplant, we observed significant improvement in LVEF of animals treated with FGF1-MP and

NRG1-MP compared to those injected with NL-MP (Table 1). Moreover, absolute changes in LVEF (3 months post-infarction LVEF – baseline infarction LVEF) were significantly greater in the rats treated with FGF1-MP (15.0 ± 4.9%, P<0.05), NRG1-MP (18.0 ± 5.7%, P<0.05) or FGF1/NRG1-MP (13.0 ± 1.9%, P<0.05) when compared with the NL-MP group (1.1 ± 3.6%) and similar among the three growth factor-loaded MP treatments (P=NS).. Left ventricular end-systolic and end-diastolic diameters and volumes were higher in the NL-MP group, consistent with left ventricular (LV) chamber dilatation and progression of myocardial dysfunction (see Table 1; LVEDV, LVESV, LVEDD, and LVESD). In contrast, a statistically significant improvement in LVEDV and LVESV as well as LV mass was observed in FGF1-MP, NRG1-MP, and FGF1/NRG1-MP treated animals in comparison with NL-MP, indicating a beneficial effect on heart remodeling (Table 1).

Three months following transplantation, infarct size was significantly reduced in animals treated with growth factor loaded MPs in comparison with NL-MP (NL-MP: 16.8±2.8%; FGF1-MP: 11.9±3.8%, *P*<0.01; NRG1-MP: 12.3±3.6%, *P*<0.01; FGF1/NRG1-MP: 11.7±3.8%, *P*<0.01) (Figure 3A), but no differences were detected among the growth factor treated rats. Similarly, fibrosis (collagen deposition) was significantly reduced in animals treated with FGF1-MP, NRG1-MP, or FGF1/NRG1-MP when compared with the NL-MP group (Figure 3B), whereas LV thickness was significantly increased in the animals treated with FGF1-MP (2.06±0.18mm, *P*<0.05), NRG1-MP (1.67±0.07mm, *P*=0.05), and FGF1/NRG1-MP (1.93±0.14mm, *P*<0.05) compared with control (NL-MP: 1.55±0.14mm).

Table 1: Cardiac function data by echocardiography

	NL-MP	FGF1-MP	NRG1-MP	FGF1/NRG1-MP
LVEF				
Baseline	38.0±2.6	29.2±2.9	30.1±4.1	38.4±4.7
Day 90	39.1±3.0	44.2±4.0*	48.1±3.1*	51.4±4.9**
FS				
Baseline	14.4±1.9	11.9±1.3	12.4±2.0	16.2±2.2
Day 90	16.8±1.5	19.4±2.1*	21.4±1.7*	23.0±2.8**
LV mass				
Baseline	1.35±0.04	1.48±0.07	1.63±0.16	1.46±0.09
Day 90	1.78±0.07**	1.39±0.07	1.41±0.06	1.44±0.06
LVEDV				
Baseline	1.151±0.119	1.364±0.098	1.690±0.219	1.454±0.190
Day 90	1.916±0.194**	1.225±0.170	1.154±0.088*	1.177±0.110
LVESV				
Baseline	0.756±0.083	0.963±0.073	1.159±0.153	0.912±0.134
Day 90	1.186±0.158*	0.721±0.131	0.614±0.058*	0.585±0.072**
LVEDD				
Baseline	0.800±0.031	0.855±0.023	0.912±0.053	0.868±0.043
Day 90	0.965±0.037**	0.810±0.045	0.788±0.039	0.809±0.028
LVESD				
Baseline	0.685±0.030	0.753±0.022	0.796±0.044	0.729±0.624
Day 90	0.804±0.039*	0.658±0.049	0.642±0.021*	0.624±0.030*

LVEF: left ventricular ejection fraction (%); **FS:** fractional shortening (%); **LV mass:** left ventricular mass (g); **LVEDV:** left ventricular end-diastolic volume (ml); **LVESV:** left ventricular end-systolic volume (ml); **LVEDD:** left ventricular end-diastolic diameter (cm); **LVESD:** left ventricular end-systolic diameter (cm). Values (mean ± SEM); **P*<0.05, ***P*<0.01, *vs*. NL-MP.

Next, we evaluated the effect of growth factor loaded MPs on the number of arterioles/arteries (alpha smooth muscle actin [α-SMA]-coated vessels) and capillaries

(small caliber caveolin-1$^+$ vessels). The density of α-SMA$^+$ vessels was significantly greater in animals treated with FGF1-MP and/or NRG1-MP (Figure 4A). In addition, we observed a significant increase in the area of α-SMA$^+$ vessels after administration of any of the cytokine loaded MPs in comparison with the NL-MP (FGF1-MP: 5519±448 μm^2; NRG1-MP: 8489±803 μm^2; FGF1/NRG1-MP: 8064±925 μm^2; NL-MP: 3972±779 μm^2; P<0.05).

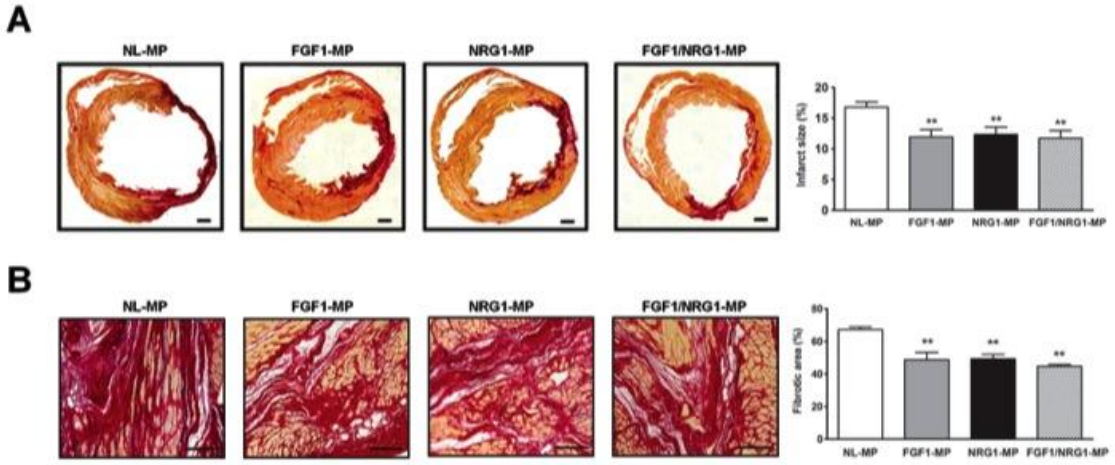

Figure 3: Cardiac remodeling was inhibited by treatment with growth factor-loaded MPs
Representative images and quantification of infarct size (% of left ventricular [LV] infarcted area *vs.* total LV area) **(A)** and fibrosis **(B)** as measured by Sirius Red staining 3 months after injection of growth factor-loaded MPs or NL-MP. Results are shown as mean ± SEM (**P<0.01 *vs.* NL-MP). Scale bars: 1 mm (A), 200 μm (B).

Also, significantly more capillaries were found in the infarcted and peri-infarcted zones of animals treated with NRG1-MP or FGF1/NRG1-MP in comparison with NL-MP, whereas FGF1-MP treatment alone did not yield a similar effect (Figure 4B). Notably, we observed a negative correlation between LVEF and the degree of fibrosis (R=–0.599; p=0.002), while there was a positive correlation of LVEF with vasculogenesis (the area occupied by α-SMA-coated vessels) (R=0.591; p=0.002) (Table 2).

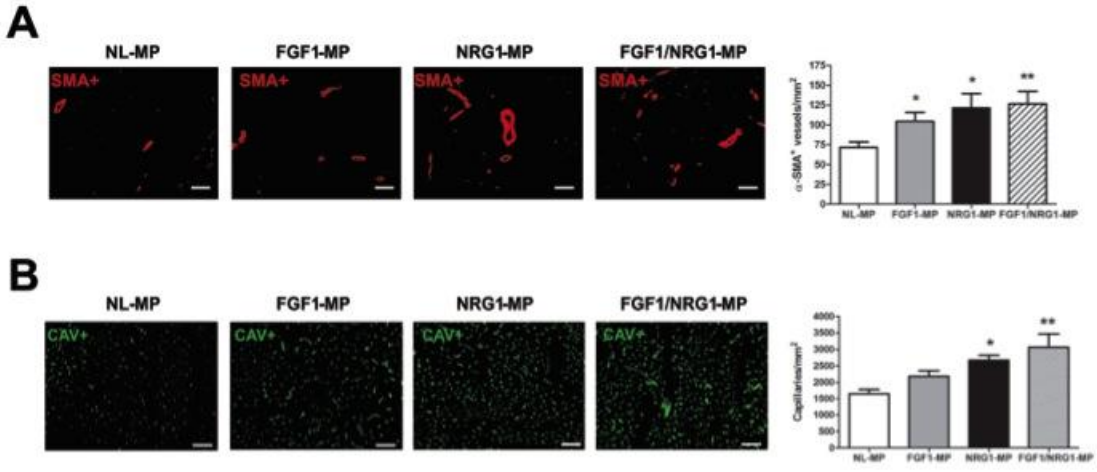

Figure 4: FGF1 and NRG1 released from MPs in the ischemic myocardium exerted arteriogenic and angiogenic effects
Representative images and quantification of α-SMA$^+$ vessel density (arteriogenesis) **(A)** and caveolin-1$^+$ vessels (capillary staining) **(B)** in infarcted and peri-infarcted zones 3 months after injection of growth

factor-loaded MPs or NL-MP. Results are shown as mean ± SEM (*P<0.05 and **P<0.01 *vs.* NL-MP control group). Scale bars: 50 µm.

Table 2: Pearson correlation analysis between functional echocardiographic parameters and histological results of growth factor loaded MP-treated hearts

	Fibrosis	Vessel Area	Apoptosis
Ejection fraction	-0.599(p=0.002)**	0.591(p=0.002)**	-0.364(p=0.081)
LVESV	0.543(p=0.006)**	-0.428(p=0.037)*	0.517(p=0.010)**

Data are shown as correlation coefficient (significance). **LVESV**: Left ventricular end-systolic volume (ml).

3.4. Apoptosis of cardiomyocytes is attenuated by growth factor-loaded MPs

Three months after treatment, there was a similar percentage of TUNEL-positive, apoptotic cardiomyocytes detected in the peri-infarcted zones of hearts treated with FGF1-MP, NRG1-MP, or control (NL-MP: 2.8±0.9%; FGF1-MP: 1.5±0.2%, P=NS; NRG1-MP: 1.8±0.6%; P=NS); however, there was a clear trend in cardiomyocyte protection observed when the combination of growth factors was used (NL-MP: 2.8±0.9% *vs.* FGF1/NRG1-MP: 1.1±0.3%; P=0.08). Therefore, consistent with the *in vitro* experiments (Figure 1B and 1C), these findings suggest that dual treatment with NRG1 and FGF1 has a protective effect.

3.5. Growth factor-loaded MPs promote cardiomyocyte proliferation

In vivo, NRG1 and FGF1 were reported to induce proliferation of cardiomyocytes.[19, 24] Thus, to determine whether growth factor loaded MPs could have a similar effect, expression of Ki67 was examined in myocyte-specific enhancer factor 2c $(MEF2c)^+$ and cardiac troponin-T $(cTnT)^+$ cardiomyocytes. We observed a significant increase in the number of $Ki67^+$ cardiomyocytes in the infarcted and peri-infarcted zones following treatment with NRG1-MP compared with NL-MP at 1 week (Figure 5A) and 3 months (Figure 5B) post-implantation (1.5- and 3.4-fold increases in $Ki67^+$ cardiomyocytes/mm^2 at 1 week and 3 months, respectively).

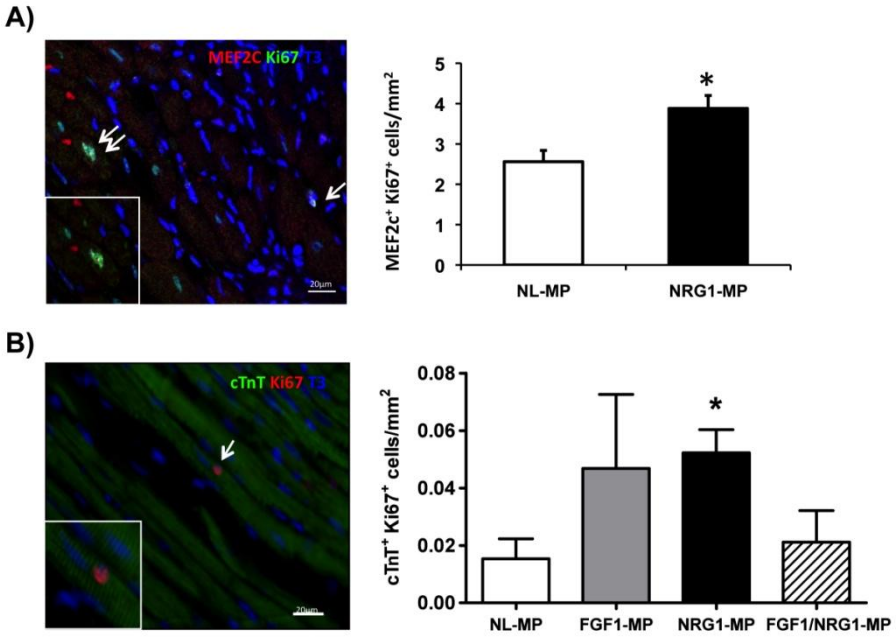

Figure 5: Growth factor-loaded MPs induced cardiomyocyte proliferation
Proliferating cardiomyocytes were measured 1 week ($MEF2c^+/Ki67^+$) after NRG1-MP or NL-MP injection **(A)** or 3 months ($cTnT^+/Ki67^+$) after FGF1-MP, NRG1-M, FGF1/NRG1-MP or NL-MP injection **(B)**. Representative images and quantification are shown. Data are represented as mean±SEM (**P*<0.05 and ***P*<0.01 *vs.* NL-MP control group). Scale bars: 20 µm.

3.6. NRG1-MP induced c-Kit$^+$/CD45$^-$ progenitor cell recruitment during early myocardial repair

Finally, we investigated the effect of growth factor-loaded MPs on cardiac progenitor cell recruitment by measuring the number of c-Kit^+ cells in ischemic myocardium. Although the majority of c-Kit^+ cells in the peri-infarct and infarct zones were $CD45^-$ one week after treatment, c-$Kit^+/CD45^+$ cells represented the largest c-Kit^+ population at 3 months (Figure 6). Notably, NRG1-MP injection into the ischemic myocardium resulted in a significant 10-fold increase in c-$Kit^+/CD45^-$ progenitor cell recruitment compared to the control group during the early phase of myocardial repair (1 week after MP injection) (NL-MP: 4.88±1.14 c-$Kit^+/CD45^-$ cells/mm^2; NRG1-MP: 48.40±3.73 c-$Kit^+/CD45^-$ cells/mm^2, P<0.001), but not at 3 months post injection (Figure 6). In fact, the number of c-$Kit^+/CD45^-$ cells detected at 3 months was much lower than at 1 week. These results suggested a transient recruitment of cardiac progenitors shortly after injury. Moreover, when c-$Kit^+/CD45^+$ cells were quantified 1 week and 3 months after injection, we observed no significant differences between the groups that received growth factor-loaded MPs and NL-MP.

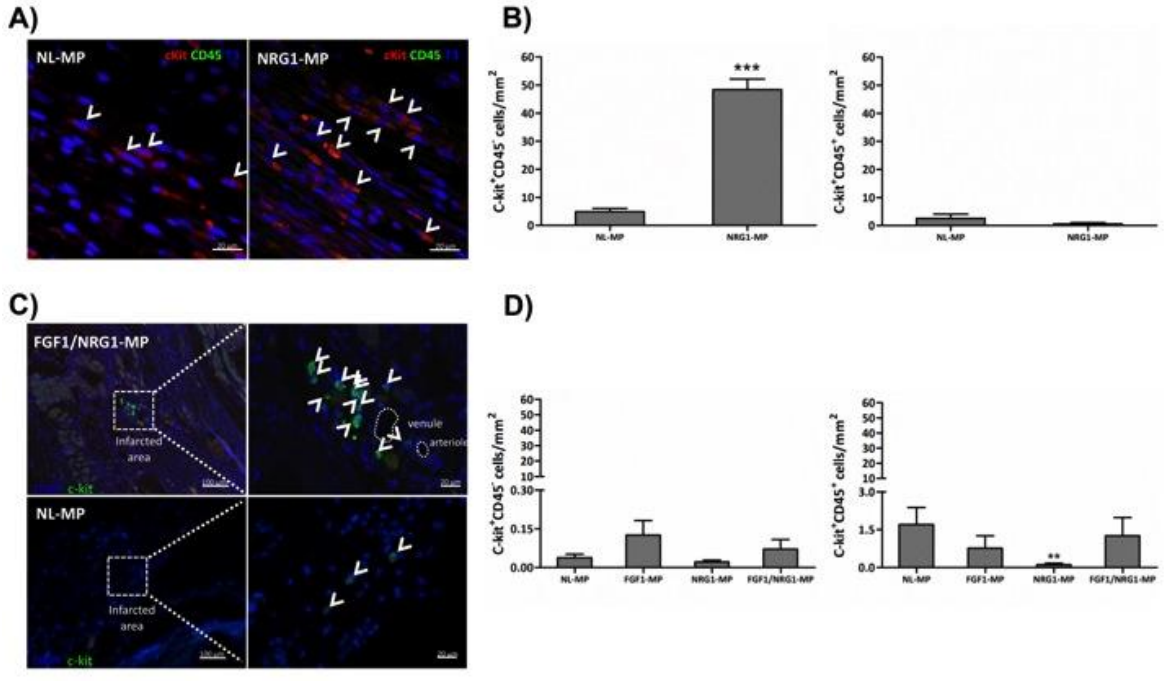

Figure 6: NRG1-MP treatment induced cardiac progenitor cell recruitment
Cardiac progenitor cell (c-$Kit^+/CD45^+$ and c-$Kit^+/CD45^-$) recruitment was assessed 1 week after injection of NRG1-MP or NL-MP **(A,B)** and 3 months after injection of NRG1-MP, FGF1-MP, NRG1/FGF1-MP or NL-MP **(C,D)**. Representative immunofluorescence images **(A,C)** and quantitation **(B,D)** are shown. Data are represented as mean±SEM (**P*<0.05 and ***P*<0.01 *vs.* NL-MP control group).

4. Discussion

Protein therapies have failed to show consistent benefits in clinical trials of ischemic cardiovascular diseases [4, 28]. Therefore, efforts aimed at understanding the factors limiting these strategies are fundamental for the successful development of future protein-based approaches. Our findings have suggested that PLGA MPs loaded with NRG1 and FGF1 can be used to improve cardiac function after acute MI by inducing an increase in vasculogenesis, inhibiting cardiac remodeling, and recruiting c-Kit$^+$ cardiac progenitors. Thus, the combination of this clinically applicable platform for drug delivery along with proteins involved in cardiac biology may represent a new therapeutic approach, which overcomes some of the classical drawbacks of protein therapy.

A number of studies have investigated systems that allow controlled delivery of therapeutic proteins, such as hydrogels, peptide nanofibers, liposomes, nanoparticles, and MPs. These strategies have been used mainly for the delivery of VEGF [14, 29-31], FGF1 [19] and FGF2 [32, 33]. Although hydrogels represent an appealing class of delivery vehicles, technical difficulties related to injection of the gelatin hydrogel into the thin ventricular wall of infarcted rat hearts have been reported [34]. Moreover, while liposomes have been shown to accumulate experimentally in areas of MI [30, 35, 36], their clinical application has been hindered because they are unstable and readily interact with high-density lipoproteins in blood. Also, self-assembling nanofiber scaffolds coated with VEGF were recently shown to improve cardiac function in rat and pig models of MI by inducing arteriogenesis and recruitment of endogenous myofibroblasts and cardiomyocyte-like cells [37]. In contrast to some of these systems, PLGA MPs are advantageous because they allow local drug delivery over an extended period of time. In fact, we have shown that these MPs persist in heart tissue for at least 90 days following implantation, favoring long-term growth factor therapy [38]. The importance of this sustained release is supported by the fact that previous studies analyzing intravenous injection of free NRG1 did not observe improvements in infarct size [22], supporting the benefit of using PLGA MPs for delivery. Furthermore, while MPs were implanted by direct injection in this study, the size of the particles employed should allow for delivery through a less invasive procedure, such as using a NOGA-guided catheter. Thus, future studies validating this system in larger models might pave the way for future clinical application of this strategy, which should be relatively straightforward since PLGA has already been approved for clinical use.

NRG1 regulates cardiovascular homeostasis during development and adulthood by stimulating recruitment and proliferation of cardiac progenitors and cardiomyocytes as well as inducing angiogenesis, vasculogenesis, and cardiac remodeling [24]. On the other hand, FGF1 is a potent cardiac mitogen capable of reducing damage-induced cardiac scarring [19]. Based on the putative activities of these two growth factors, we hypothesized that a combination therapy involving administration of both cytokines would be more beneficial than each individually; however, we did not observe a consistent synergistic effect *in vitro* or *in vivo*. Instead, we observed that the effect of both cytokines combined was lower than either alone. Thus, as both proteins may utilize similar signaling pathways [15, 17], it is possible that they compete, limiting their combined effect. Indeed, this important observation should be considered when designing new studies involving combination therapies. Interestingly, even though the cytokines showed an additive effect in preventing cardiomyocyte apoptosis *in vitro*, this finding did not translate *in vivo*.

Further elucidation of the mechanisms by which different growth factors contribute to cardiac repair should help to refine protein-based therapies, as cytokines with different functional mechanisms may act synergistically. Interestingly, when tissue

revascularization was assessed, a significant increase in arteriolar/arteries density and capillaries were found after treatment in each of the groups treated with growth factor-loaded MPs. These effects were only significant in those groups treated with NRG1, suggesting that at least *in vivo*, NRG1 has a greater vasculogenic effect in comparison with FGF1. While various populations of cardiac progenitor cells have been described, one of the most commonly employed cardiac progenitor markers is c-Kit, which identifies cells that participate in endogenous cardiac repair following MI [39]. Indeed, we identified a dramatic 10-fold increase in the number of c-Kit^{+}/CD45^{-} progenitor cells 7 days after injection of NRG1-loaded MPs, which coincided with the time when the most drug was released from MPs. Thus, this finding suggests a potential role for NRG1 in cardiac progenitor recruitment. Moreover, it has been reported that c-Kit^{+} progenitor cells can present a vascular phenotype, which might suggest that they participate in *de novo* vessel formation [40]. Nevertheless, we did not detect the recruitment of specific progenitors with a vascular phenotype.

In addition to cardiac progenitors, recent studies have suggested that adult cardiomyocytes can contribute directly to cardiac regeneration through either proliferation or dedifferentiation processes [41, 42]. Thus, targeting cardiomyocyte proliferation could also represent a useful therapeutic approach. Although our study was not designed to specifically investigate cardiomyocyte proliferation, we observed increased Ki67^{+} in cardiomyocytes 1 week and 3 months after injection of growth factor-loaded MPs, suggesting that a sustained release of NRG1 and FGF1 may increase the *in vivo* proliferative potential of myocytes. As observed in the case of c-Kit^{+} progenitors, the effect on myocyte proliferation was negligible at 3 months but was 100-fold higher 1 week after injection.

5. Conclusions

Taken together, our present findings demonstrate the therapeutic efficacy of combining sustained protein delivery platforms with growth factors for achieving cardiac regeneration. Specifically, we have successfully delivered FGF1 and NRG1 to ischemic myocardium using a slow-release polymer. This strategy significantly contributed to global myocardial function during post-MI remodeling by promoting angiogenesis and arteriogenesis, inducing cardiac proliferation, and eliciting stem cell recruitment. Further validation of this therapeutic approach in large preclinical models could pave the way for implementation of this strategy in patients with ischemic heart disease.

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