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Repeated and targeted transfer of angiogenic plasmids into the infarcted rat heart via ultrasound targeted microbubble destruction enhances cardiac repair

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Aims Ultrasound-targeted microbubble destruction (UTMD) uses ultrasound energy to selectively deliver genes into the myocardium using plasmids conjugated to microbubbles. We hypothesized that repeated delivery of stem cell-mobilizing genes could boost the ability of this therapy to enhance cardiac repair and ventricular function after a myocardial infarction.

Methods and results Beginning 7 days after coronary artery ligation, stem cell factor (SCF) and stromal cell-derived factor (SDF)-1α genes were administered to adult rats using 1, 3, or 6 UTMD treatments (repeat 1, 3, and 6 groups) at 2-day intervals (control = 6 treatments with empty plasmid). Cardiac function (echocardiography) and myocardial perfusion (myocardial contrast echocardiography) were assessed on Days −7, 0, and 24 relative to the first treatment. Histological and biochemical assessments were performed on Day 24. Multiple UTMD treatments were associated with an increased presence of myocardial SCF and SDF-1α proteins and their receptors (vs. control and Repeat 1). All UTMD recipients exhibited increased vascular densities and smaller infarct regions (vs. control), with the highest ventricular densities in response to multiple treatments. Myocardial perfusion and ventricular function at Day 24 also improved progressively (vs. control) with the number of UTMD treatments.

Conclusions Targeted ultrasound delivery of SCF and SDF-1α genes to the infarcted myocardium recruited progenitor cells and increased vascular density. Multiple UTMD treatments enhanced tissue repair, perfusion, and cardiac function. Repeated UTMD therapy may be applied to tailor the number of interventions required to optimize cardiac regeneration after an infarction.

Keywords Myocardial infarction • Heart failure • Gene therapy • Echocardiography • Angiogenesis

Introduction

After an extensive myocardial infarction (MI), ventricular dysfunction may progress despite stenting or bypassing the infarct artery.[1,2] Novel gene therapies—in particular, non-invasive approaches that induce stem cell homing to the damaged heart—may improve function by promoting regional perfusion and tissue regeneration in the infarcted myocardium.[3,4]

Ultrasound-targeted microbubble destruction (UTMD) non-invasively and selectively delivers genes to the infarct via microbubble carriers that release plasmid DNA when they are targeted with an ultrasound beam. Intravenously administered lipid

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microbubbles are used for the clinical evaluation of myocardial perfusion, and have also been used successfully for cardiac gene delivery in animals.[5,6] We previously found that UTMD-mediated gene therapy improved myocardial perfusion and cardiac function following an MI in mice[7] and, in an accompanying editorial, Villanueva[8] suggested that the 'potentially powerful advantages of UTMD-mediated gene therapy warrant pragmatic studies to optimize protocols.'

We hypothesized that multiple treatments would boost the magnitude of cardiac repair by prolonging the effects of gene expression beyond those of a single delivery. Therefore, this study assessed whether the extent of cardiac repair achieved in response to UTMD-mediated gene transfer into the infarcted rat myocardium was enhanced by repeated treatments. The therapeutic genes introduced were for stem cell factor (SCF) and stromal cell-derived factor (SDF)-1α, because these proteins stimulate endogenous stem cell mobilization and homing in response to an MI.[9,10] We also examined whether repeated UTMD treatments could avoid myocardial injury.[11]

Methods

Plasmid DNA and microbubble solutions

We used pcDNA3 plasmids containing mouse SCF and human SDF-1α genes that were expressed using a cytomegalovirus (CMV) promoter (or empty pcDNA3 plasmids, for controls). Plasmid DNA (0.6 mg/kg body weight, as previously reported for UTMD-mediated gene delivery to the rat myocardium[6,7,12]) was incubated at room temperature for 20 min with lipid microbubbles (0.12 mL/rat, DEFINITY®) that were diluted with saline to a total volume of 0.5 mL/rat.

Animal model

Under general anaesthesia, MI was generated by coronary artery ligation in adult Sprague–Dawley rats (weight 200–225 g) as we previously described.[10] The Animal Care Committee of the Toronto General Research Institute approved all animal procedures. Experiments were performed according to the *Guide for the Care and Use of Experimental Animals* from the Canadian Council on Animal Care.

Experimental design and timeline

Seven days after MI, echocardiography was performed to measure infarct size; rats with akinetic left ventricular wall lengths of <0.8 or >1.2 cm were excluded from the study. The remaining rats were randomly assigned to 1–4 groups ($n = 6$ per group): control received 6 UTMD treatments with empty plasmids; repeat 1, repeat 3, and repeat 6 received 1, 3, or 6 treatments, respectively, with SCF and SDF-1α plasmid DNA. Hearts were collected at Day 24 after the first UTMD treatment.

Ultrasound-targeted microbubble destruction (gene delivery)

Ultrasound-targeted microbubble destruction treatments were administered at 2-day intervals between Day 0 (1 week after MI) and Day 10. Rats were sedated (2% Isoflurane) and the plasmid-microbubble solution was infused into the tail vein at a speed of 1.5 mL/h. During infusion, an ultrasound beam was delivered with a 15L8 transducer directed to the heart for 20 min (mechanical index = 1.6; 1 burst of ultrasound every 1800–2000 ms), and the heart was scanned repeatedly from base to apex (~3 min cycle). Contrast pulse sequencing technology was used to detect microbubble destruction. We used a high mechanical index and time-triggered ultrasound to achieve efficient microbubble destruction and increase the biological transfer. Previous studies associated this high mechanical index with a small troponin T leak,[13] but found no histological evidence of inflammation or myonecrosis, and no echocardiographic evidence of left ventricular dysfunction.

Troponin I in blood

To assess tissue damage resulting from UTMD, we measured plasma troponin I protein levels in each group before MI, immediately before the first UTMD treatment (7 days later), and 1, 3, or 7 days after the last UTMD treatment was completed (or after MI in the control animals). In order to isolate the effects of the UTMD procedure, all treatments delivered an empty plasmid (no gene transfer), and the control animals underwent MI, but not UTMD treatments (no plasmid delivery). We used an ELISA kit according to the manufacturer's instructions. Briefly, plasma samples (four-fold dilution; or standards or control samples) were incubated for 1 h at room temperature with anti-troponin I antibody, and then washed with phosphate buffer. Next, to permit colour development, 100 μL of tetramethylbenzidine (TMB) reagent was added to each sample and incubated for 20 min at room temperature. Finally, the samples were read at 450 nm with a microtitre well reader. Troponin I concentrations were extrapolated from the standard curve and expressed as nanogram per millilitre.

Table 1 Two-dimensional echocardiographic measurements of left ventricular size and function

Group	LVDd (cm)		LVDs (cm)		LVEF (%)	
	Day −7 (before MI)	Day 7 after last treatment (MI or UTMD)	Day −7 (before MI)	Day 7 after last treatment (MI or UTMD)	Day −7 (before MI)	Day 7 after last treatment (MI or UTMD)
MI control	0.59 ± 0.06	0.65 ± 0.04	0.29 ± 0.04	0.41 ± 0.02	76.11 ± 3.13	59.40 ± 5.68
Repeat 1	0.58 ± 0.08	0.62 ± 0.06	0.28 ± 0.02	0.40 ± 0.06	76.70 ± 2.66	58.26 ± 5.35
Repeat 3	0.61 ± 0.06	0.65 ± 0.04	0.29 ± 0.02	0.42 ± 0.05	76.77 ± 1.92	59.65 ± 5.06
Repeat 6	0.64 ± 0.06	0.68 ± 0.02	0.31 ± 0.02	0.43 ± 0.03	76.50 ± 1.78	60.41 ± 2.80

LVDd, left ventricular end-diastolic dimension; LVDs, left ventricular end-systolic dimension; LVEF, left ventricular ejection fraction; MI, myocardial infarction.
Animals in the MI control group underwent MI only, with no subsequent UTMD treatment; those in the Repeat 1, 3, and 6 groups underwent MI followed by 1, 3, or 6 UTMD treatments, respectively, with empty plasmids (delivered at 2-day intervals beginning at 7 days after MI).

Myocardial perfusion

Myocardial perfusion was evaluated using myocardial contrast echocardiography on Days 0 and 24 after the first UTMD treatment.[14] Rats were sedated, and a microbubble solution was injected through the tail vein. Digital images were used to measure signal intensity offline at 1800–2000 ms pulsing interval (plateau intensity). In this study, the anterior wall represented the infarct region, and the posterior wall represented the non-infarct (normal) region. The intensity ratio was calculated (signal intensity in the anterior wall divided by that in the posterior wall) to estimate myocardial blood volume.

Cardiac function, infarct wall length, and thickness

Using echocardiography, cardiac function (left ventricular ejection fraction) was evaluated at Days −7 (before MI), 0, and 24 (after first UTMD treatment), and infarct wall length and thickness were measured at Day 24, as we previously described.[10,15] Echocardiography also assessed the effects of repeated UTMD treatments (empty plasmid; no gene transfer) on cardiac morphometry (left ventricular end-diastolic dimension and end-systolic dimension) and function (ejection fraction). These measurements were carried out in each group before MI, and 7 days after the last UTMD treatment (or 7 days after MI in MI-only control animals).

Myocardial stem cell factor and stromal cell-derived factor-1α protein expression and cells expressing stem cell factor or stromal cell-derived factor-1α receptors

Stem cell factor and SDF-1α protein levels and cells expressing SCF or SDF-1α receptors (c-kit or CXCR4, respectively) were measured (ELISA and flow cytometry using specific antibodies, respectively) in left ventricular samples from Day 24 after the first UTMD treatment. These procedures are described in detail in the Supplementary material online, *Methods*.

Transgene mRNA expression in the myocardium

We used RT–PCR to measure SCF and SDF-1α mRNA expression in left ventricular samples from Day 24 after the first UTMD treatment. These procedures are described in detail in the Supplementary material online, *Methods*.

Immunohistochemistry

Alpha-smooth muscle actin (α-SMA) positive structures (including or excluding smooth muscle cells located adjacent to blood vessel walls) and Factor VIII positive endothelial cells were identified immunohistochemically in the (separated) infarct and border regions of ventricular sections obtained at Day 24 after the first UTMD treatment. These procedures are described in detail in the Supplementary material online, *Methods*.

Statistical analyses

Data are expressed as mean ± standard deviation. Analyses were performed using SPSS software (v. 12.0), with the critical α-level set at $P < 0.05$. Statistical tests were as follows: Comparisons among groups (mRNA intensity, protein or receptor expression, myofibroblast recruitment, infarct size, or thickness) were made using one-way analysis of variance (ANOVA). Repeated measures ANOVA tested the main effects and interactions of gene treatment and time relative to UTMD on troponin I levels, myocardial perfusion, and cardiac function (% ejection fraction). Two-way ANOVA tested the main effects and interactions of gene treatment and myocardial region on vascular density. A correlation analysis tested the relationship between SCF or SDF-1α levels and the percentages of cells expressing the respective receptor. When *F*-values were significant, differences between the groups were specified with Tukey's multiple range post-tests.

Results

Repeated ultrasound-targeted microbubble destruction did not produce significant myocardial injury

Left ventricular size and function (left ventricular end-diastolic dimension, end-systolic dimension, ejection fraction) did not

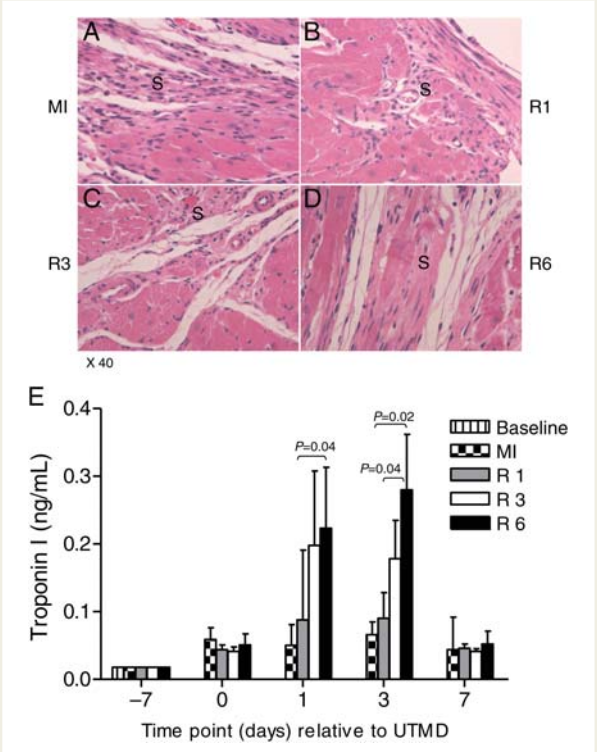

Figure 1 Circulating troponin I protein levels before and after myocardial injury and ultrasound-targeted microbubble destruction. (*A–D*) Representative microphotographs (×40) illustrating haematoxylin and eosin staining of myocardial tissue sections obtained at 7 days after myocardial infarction in control animals that underwent coronary ligation but not ultrasound-targeted microbubble destruction (no plasmid delivery) (MI; *A*), or at 1 day after the last ultrasound-targeted microbubble destruction treatment in animals that received 1, 3, or 6 treatments with empty plasmids (R1, R3, R6, respectively; *B–D*). S, infarct (scar) region. (*E*) Troponin I protein levels in the blood for each group at several time points relative to ultrasound-targeted microbubble destruction: before myocardial infarction (−7), before the first ultrasound-targeted microbubble destruction treatment (7 days after infarction; 0), and 1, 3, and 7 days after the last ultrasound-targeted microbubble destruction treatment (or after MI in controls). Baseline, reference level for normal tissue (no infarct). $n = 6$ per group.

differ among control, repeat 1, repeat 3, and repeat 6 groups before MI. At 7 days after the last UTMD with empty plasmid, ventricular dimensions and ejection fraction in the treated groups were statistically similar to those at 7 days after MI in a control group that did not receive UTMD (*Table 1*).

To monitor myocardial injury after UTMD, we measured troponin I levels in the blood before and after treatment. ANOVA revealed significant main effects ($P < 0.0001$) and a significant interaction effect ($P = 0.0002$) of the number of gene treatments and the number of days after UTMD on circulating troponin levels. The pre-MI baseline level was <0.02 ng/mL, which is the normal reference value in our laboratory. At 7 days after MI (before the first UTMD treatment), troponin I levels increased to $\sim$0.05 ng/mL, perhaps in response to the coronary ligation (*Figure 1*). Levels increased further on Days 1 and 3 after the last UTMD treatment, and were significantly higher at Day 3 in the repeat 6 vs. repeat 1 or control groups ($P = 0.04$, $P = 0.02$, respectively). However, troponin I levels returned to within the pre-UTMD range by Day 7 in all groups. Histological study of haematoxylin and eosin-stained cardiac sections revealed no

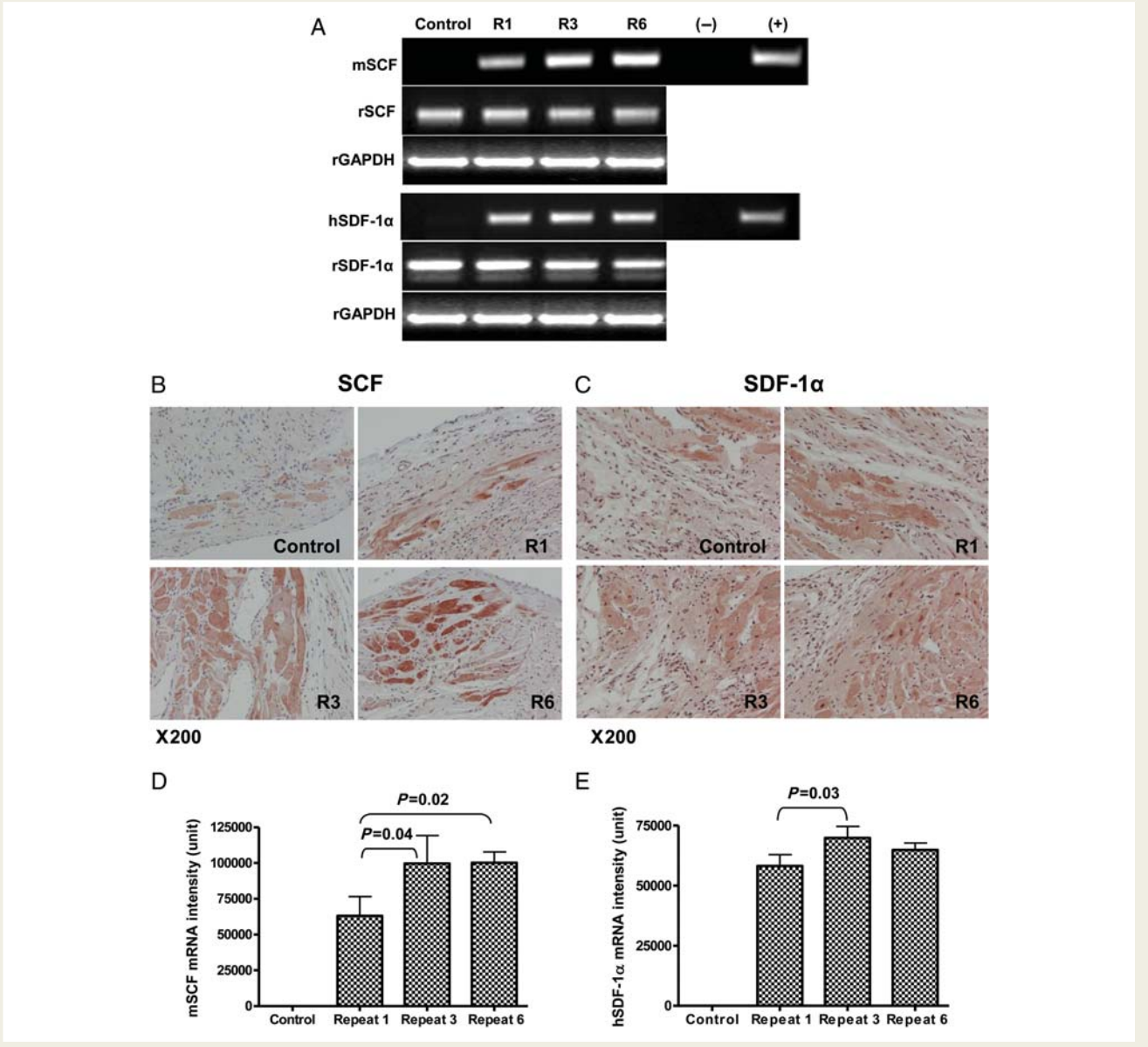

Figure 2 Myocardial expression of transfected stem cell factor and stromal cell-derived factor-1α mRNA expression by RT–PCR at Day 24. (*A*) mRNA expression measured in myocardial tissue from control animals and those that received 1, 3, or 6 ultrasound-targeted microbubble destruction treatments (R1, R3, R6). (−), negative control; (+), positive control; m, mouse; r, rat; h, human; rGAPDH, rat housekeeping gene. (*B* and *C*) Representative micrographs (×200) illustrating immunohistochemical expression of stem cell factor protein (*B*) or stromal cell-derived factor-1α protein (*C*) in myocardial tissue sections from each group. (*D* and *E*) mSCF (*D*) or hSDF-1α (*E*) mRNA intensity in control animals and those that received 1, 3, or 6 ultrasound-targeted microbubble destruction treatments (repeat 1, repeat 3, repeat 6). $n = 6$ per group.

microscopic evidence of vascular damage or microvascular or epicardial vessel thrombosis in the infarct or non-infarct regions in any of the rats (*Figure 1*).

Effects of multiple ultrasound-targeted microbubble destruction delivery of stem cell factor and stromal cell-derived factor-1α genes

Increased myocardial expression of stem cell factor and stromal cell-derived factor-1α mRNA and proteins

We measured myocardial expression of the transfected (exogenous) SCF and SDF-1α mRNA and total SCF and SDF-1α protein levels in the hearts at Day 24 after the first UTMD treatment. We recorded no expression of delivered mouse SCF or human SDF-1α mRNA in the control groups. In the repeat groups, we observed dose-sensitive increases in mRNA expression (*Figure 2*). In agreement with the mRNA data, both SCF levels and SDF-1α levels were significantly higher in the repeat 1, repeat 3, and repeat 6 groups than in the control group (*Figure 3A* and *B*). Among treated groups, levels of both proteins increased significantly after multiple treatments (for repeat 3 or repeat 6 vs. repeat 1), and there was a further increase in SCF levels after six treatments ($P = 0.02$ vs. repeat 3) (*Figure 3A*).

Increased c-kit and CXCR4 positive cells in the myocardium

Ultrasound-targeted microbubble destruction increased SCF and SDF-1α protein levels in the heart following an MI. To evaluate the biological function of these proteins, we quantified stem cell

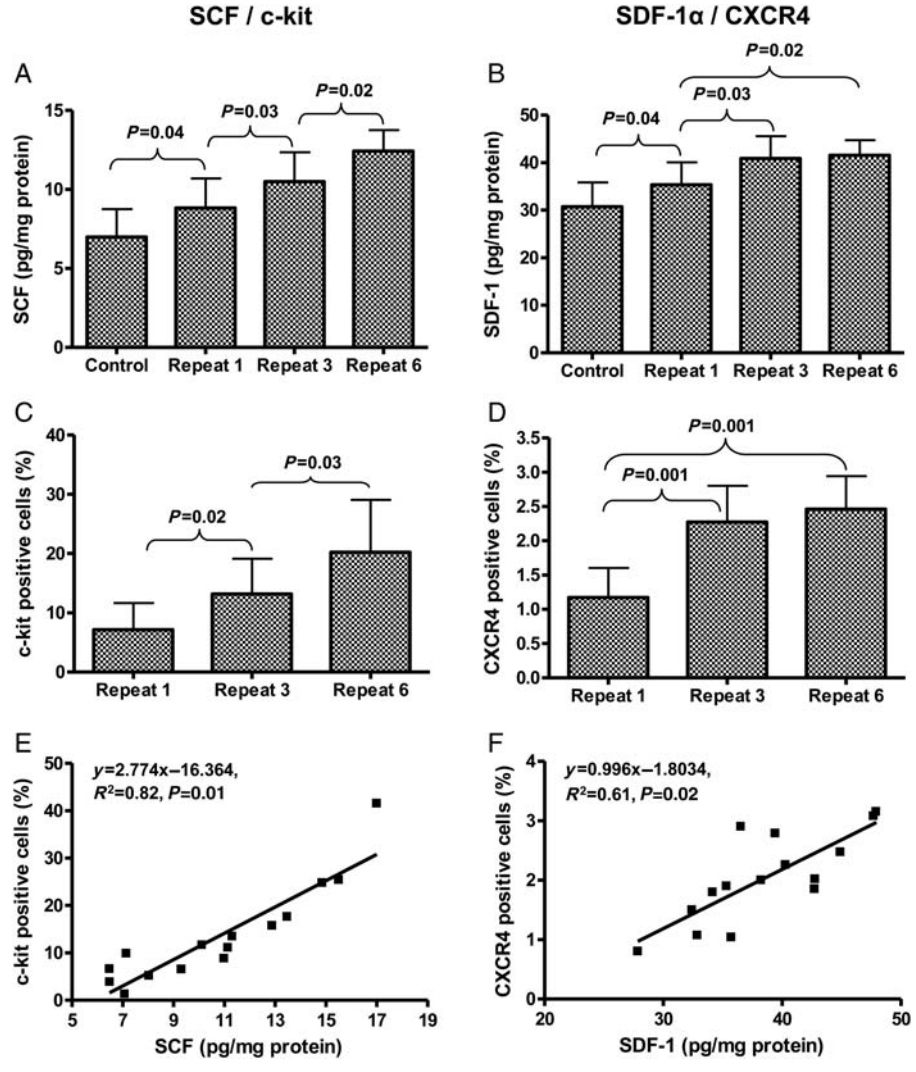

Figure 3 Myocardial stem cell factor and stromal cell-derived factor-1α protein levels and c-kit or CXCR4 (receptor) positive cells by flow cytometry at Day 24. (*A* and *B*) Cardiac stem cell factor (*A*) or stromal cell-derived factor-1α (*B*) protein levels in control animals and those that received 1, 3, or 6 ultrasound-targeted microbubble destruction treatments (repeat 1, repeat 3, repeat 6). (*C* and *D*) C-kit (*C*) or CXCR4 (*D*) positive cells (positive cells as a percentage of total cells in the heart) in each group. $n = 6$ per group. (*E* and *F*) Correlation between stem cell factor (*E*) or stromal cell-derived factor-1α (*F*) levels and receptor positive cells in the whole heart.

homing using flow cytometry with antibodies against the SCF and SDF-1α receptors (c-kit and CXCR4). At Day 24 after the first UTMD treatment, c-kit positive cells, and CXCR4 positive cells were significantly more numerous in the hearts of rats that received multiple treatments rather than a single gene delivery ($P = 0.02$ or $P = 0.001$ for repeat 3 vs. repeat 1 in *Figure 3C* and *D*, respectively), with a further increase in the number of c-kit positive cells after six treatments ($P = 0.03$ vs. repeat 3). Correlations between SCF or SDF-1α levels and the percentages of cells expressing the respective receptor were significant ($P = 0.01$ and $P = 0.02$, respectively) (*Figure 3E* and *F*). Thus, increased myocardial protein expression of SCF and SDF-1α in response to UTMD delivery enhanced stem cell homing to the damaged myocardium.

Increased myofibroblast recruitment to the infarcted myocardium

Myofibroblasts play an important role in wound healing after an MI.[16] Immunohistochemical quantification of myofibroblasts in the infarcted myocardium at Day 24 after the first UTMD treatment evaluated the effect of cytokine expression on wound healing. We found that the area of the infarct region containing cells expressing α-SMA at Day 24 (excluding smooth muscle cells in blood vessel walls) increased after gene transfer and increased further after repeated UTMD treatments ($P = 0.03$ for repeat 1 vs. control; $P = 0.04$ for repeat 3 vs. repeat 1; $P = 0.01$ for repeat 6 vs. repeat 3) (*Figure 4*). These results suggest that increased SCF and SDF-1α expression boost myofibroblast recruitment to the infarct.

Increased regional blood vessel density and myocardial perfusion

At Day 24 after the first UTMD treatment, immunohistochemistry identified small vascular structures in the heart (mainly capillaries; Factor VIII positive), and larger vascular structures with smooth muscle cells located adjacent to blood vessel walls (α-SMA positive). The density of both types of vessels increased significantly after gene transfer and increased further after repeated UTMD treatments in both infarct and border regions (*Figure 5*). The greater the number of UTMD treatments, the greater the number of vascular structures (capillaries and arterioles) induced.

We used myocardial contrast echocardiography to assess myocardial perfusion by comparing the ratio of the signal intensity in the anterior wall (infarct region) and that in the posterior wall (non-infarct region) over 24 days after the first UTMD treatment. Intensity ratios increased significantly following UTMD treatments ($P = 0.01$ for repeat 1 vs. control) and were highest following six

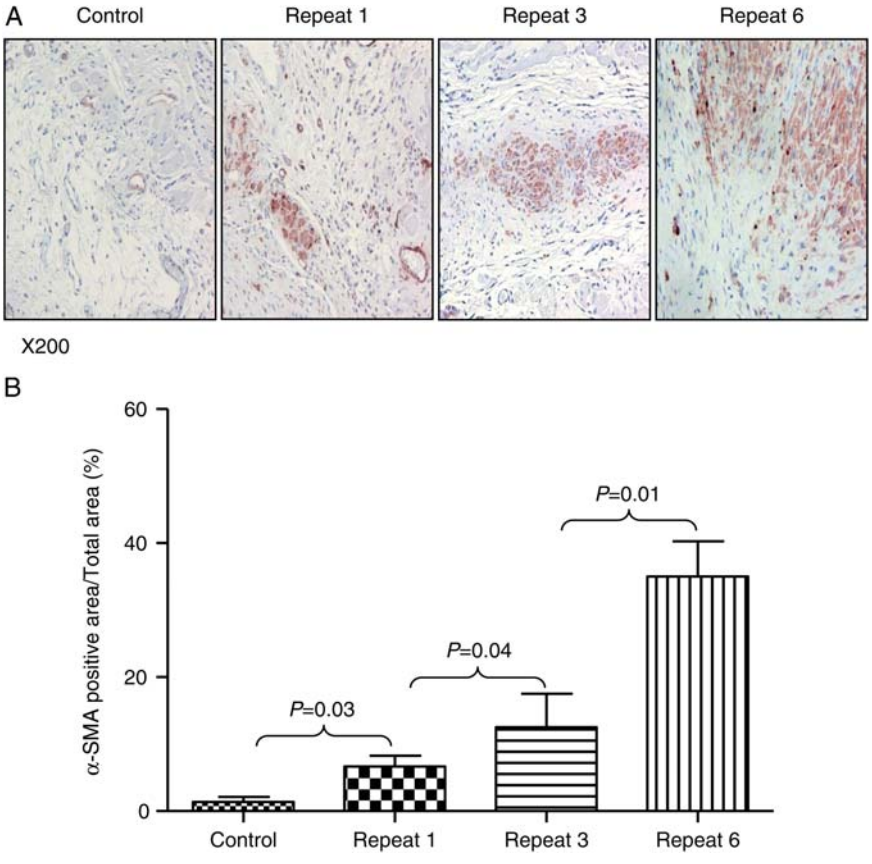

Figure 4 Myofibroblast recruitment into the infarct region at Day 24 by immunohistochemical staining. (*A*) Representative micrographs (×200) illustrating α-smooth muscle actin expression (recruited myofibroblasts) in the infarct region of myocardial tissue from control animals and those that received 1, 3, or 6 ultrasound-targeted microbubble destruction treatments (repeat 1, repeat 3, repeat 6). (*B*) α-smooth muscle actin positive structures (expressed as a percentage of the total area) in each group. $n = 6$ per group.

treatments ($P = 0.001$ for repeat 6 vs. repeat 3) (*Figure 6A*), demonstrating that functional blood flow improved with increasing frequency of SCF and SDF-1α gene delivery.

Improved cardiac systolic function over time

Ejection fraction was similar in control and treated groups before MI, and decreased in all groups 7 days after MI (before gene transfer) (*Figure 6B*). At 24 days after the first UTMD treatment, cardiac function remained depressed relative to pre-MI levels; however, the dysfunction was mitigated by gene transfer, with increasing functional recovery in response to increasing numbers of UTMD treatments ($P = 0.02$ for repeat 1 vs. control; $P = 0.01$ for repeat 3 vs. control; $P = 0.001$ for repeat 6 vs. control) (*Figure 6B*).

Decreased infarct size and increased wall thickness

We used echocardiography to measure the length and thickness of the infarcted (akinetic) ventricular muscle on the short-axis view of

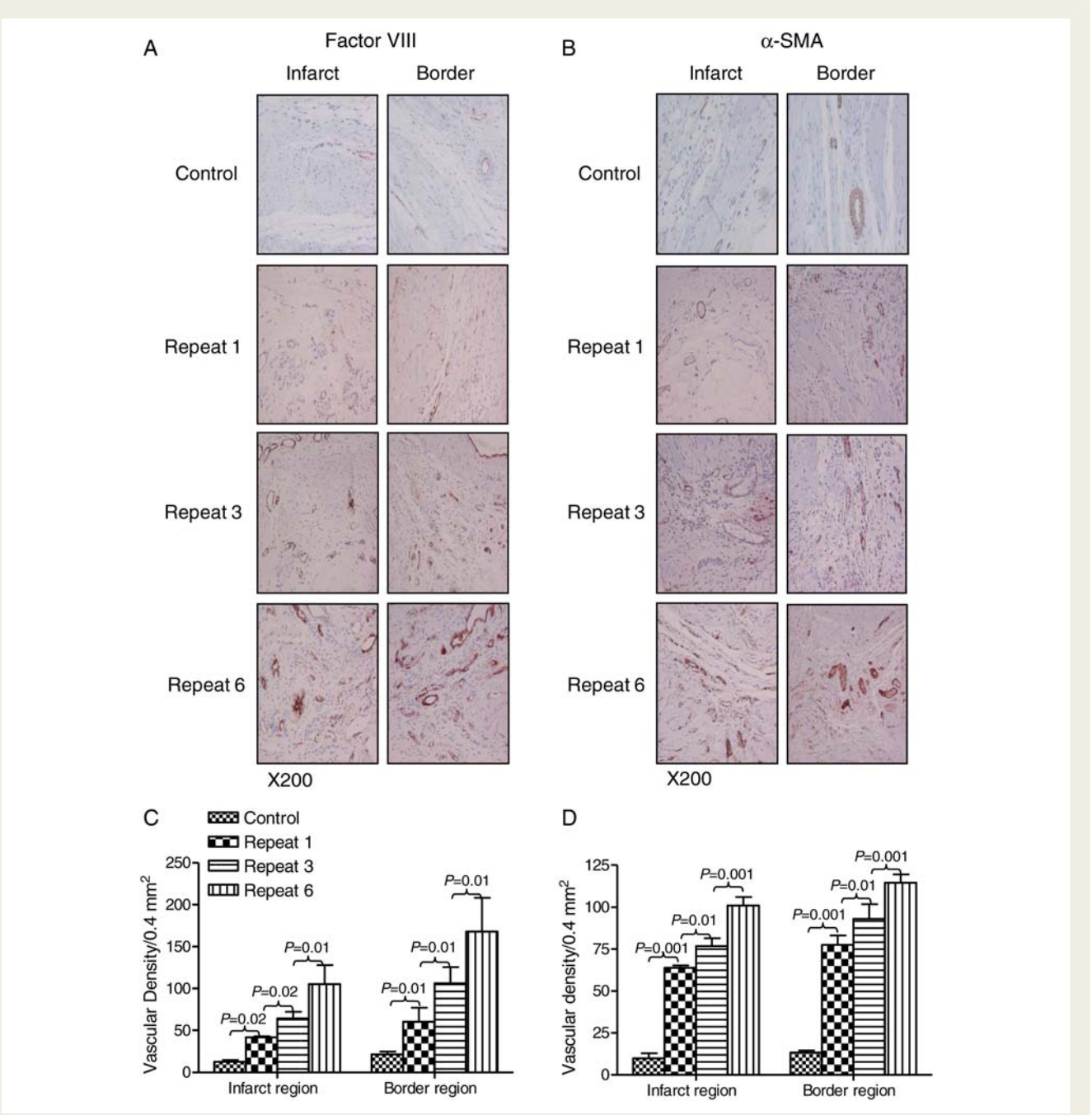

Figure 5 Vascular density at Day 24 by immunohistochemical staining. (*A* and *B*) Representative microphotographs (×200) illustrating Factor VIII (*A*) and α-smooth muscle actin (*B*) expression in the infarct and border regions of myocardial tissue from control animals and those that received 1, 3, or 6 ultrasound-targeted microbubble destruction treatments (repeat 1, repeat 3, repeat 6). (*C* and *D*) Vascular densities for each group and region are shown in (*C*) (Factor VIII) and (*D*) (α-smooth muscle actin). $n = 6$ per group.

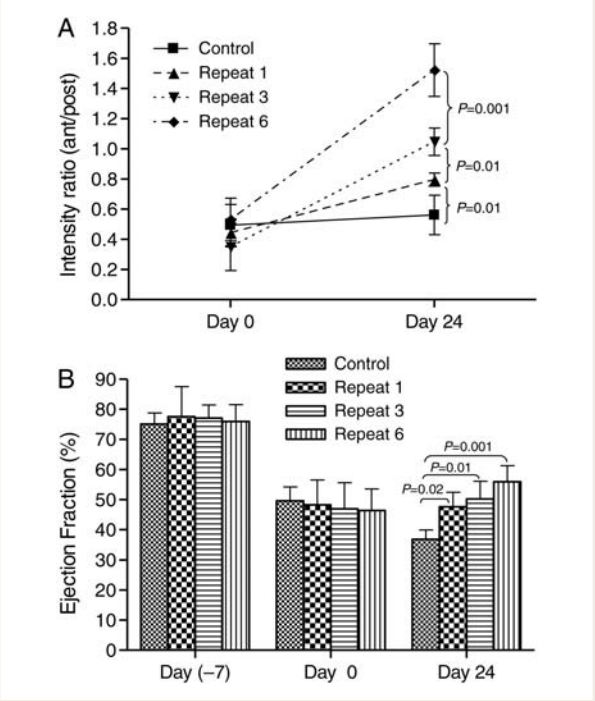

Figure 6 Myocardial perfusion and cardiac function over time. (*A*) In myocardial contrast echocardiography, the intensity ratio [ratio of signal intensity in anterior wall (infarct region) divided by posterior wall (non-infarct region) (ant/post)] before gene transfer (Day 0) and 24 days after the first ultrasound-targeted microbubble destruction treatment (Day 24) in control animals and those that received 1, 3, or 6 ultrasound-targeted microbubble destruction treatments (repeat 1, repeat 3, repeat 6). (*B*) By echocardiography, cardiac systolic function (% ejection fraction) before myocardial infarction (Day −7), and at Days 0 and 24 in each group. $n = 6$ per group.

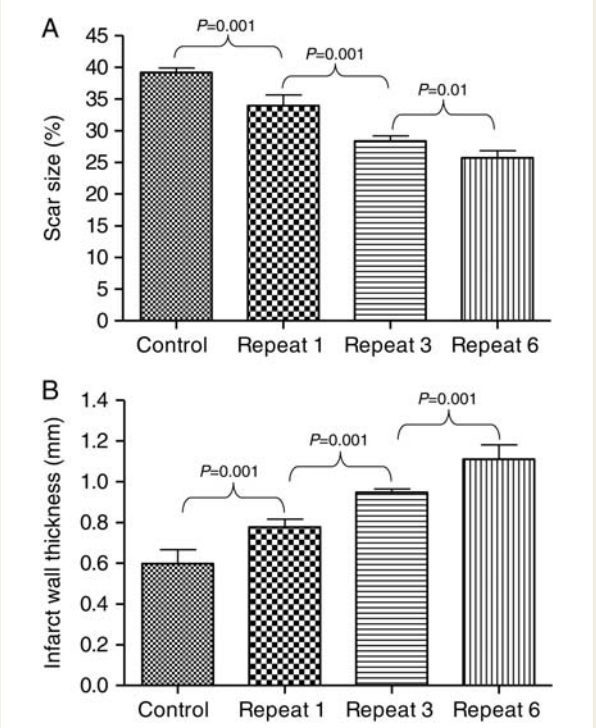

Figure 7 Infarct size and wall thickness at Day 24. (*A*) Scar size (infarct length expressed as a percentage of circumference; measured on the short-axis view of the left ventricle at the mid-papillary muscle level) in control animals and those that received 1, 3, or 6 ultrasound-targeted microbubble destruction treatments (repeat 1, repeat 3, repeat 6). (*B*) Wall thickness (in millimetre) of the mid-portion of the infarct in each group. $n = 6$ per group.

the left ventricle at the mid-papillary muscle level during end-diastole. An increasing number of UTMD treatments produced increasingly shorter ($P = 0.001$ for repeat 1 vs. control; $P = 0.001$ for repeat 3 vs. repeat 1; $P = 0.01$ for repeat 6 vs. repeat 3) and thicker ($P = 0.001$ for repeat 1 vs. control; $P = 0.001$ for repeat 3 vs. repeat 1; $P = 0.001$ for repeat 6 vs. repeat 3) infarcts (*Figure* 7).

Discussion

We demonstrated that UTMD successfully delivered the SCF and SDF-1α genes into the infarcted myocardium, and that repeated treatments enhanced the gene expression with minimal myocardial injury. The greatest improvements in stem cell recruitment, vascularity, tissue repair, and ventricular function were achieved when multiple, rather than single, UTMD treatments were given.

Normally up-regulated for about the first week after an MI, SCF and SDF-1α proteins, and their receptors stimulate bone marrow stem cell mobilization and cell homing to the site of injury,[9,10,16] which is an essential step in the regenerative process. We previously demonstrated that cells expressing the SCF receptor, c-kit, are key regulators of the angiogenic switch in the infarcted myocardium.[10] Meanwhile, SDF-1α facilitates adhesion and migration through its receptor, CXCR4.[17,18] The SDF-1α gene also confers enhanced vasculogenesis and angiogenesis *in vivo* through a VEGF/eNOS-related pathway.[19] We anticipated that delivering the SCF and SDF-1α transgenes beginning at 7 days after an MI would amplify the natural angiogenic response by producing a 'second wave' of the signals that induce cell homing. Indeed, we found that an elevation in SCF and SDF-1α protein levels was correlated with an accumulation of c-kit and CXCR4 positive cells in the myocardium, and these recruited cells were associated not only with increased angiogenesis, but also with myofibroblast accumulation in the infarct region and a limitation of infarct thinning and expansion.

Ultrasound-targeted microbubble destruction-mediated gene therapy may be a clinically useful improvement over conventional gene delivery approaches (which include the systemic delivery of naked DNA, viral vectors, or plasmids and are limited by poor targeting, transfection of healthy tissues, and vector immunogenicity) because it is a non-invasive treatment that targets gene delivery to a specific ischaemic tissue with limited toxicity or immunogenicity. When lipid microbubbles are positively charged in saline,[20] they can combine with negatively charged plasmid DNA, protecting it

from degradation in the blood. Transfection is facilitated when a directed ultrasound beam is used to burst the microbubbles, portions of which pass through the endothelial layer lining the vessels. Microbubble destruction also induces transient nano-cavities in the membranes of myocardial cells that allow transfection by mechanisms that include endocytosis.[21] Gene passage can be directly into the injured cells,[22] or between them, through gap junctions opened by the ultrasound.[23] The procedure can be safely repeated, and might also 'prime' the ischaemic myocardium for improved survival and engraftment of subsequently implanted cells.[4,15] The intravenous injection of plasmids and Definity microbubbles presents a low risk for DNA transfection into other organs[24] since destruction of the microbubbles causes degradation of the plasmids in the blood.

One concern associated with the UTMD-mediated gene transfer strategy is limited vascular access to the ischaemic tissue after coronary occlusion. In this study, the myocardium lacked a direct antegrade blood supply; however, microbubbles and their associated genes could still access the ischaemic region through the collateral circulation that develops after an MI[10]—particularly in ischaemic region venules and the border region where gene accumulation and transfer into the myocardium are most likely to occur.[25] In fact, delaying gene transfection until 7 days after MI may have facilitated gene transfer by allowing sufficient time for the establishment of a collateral circulation.

To address the possibility that UTMD could cause myocardial tissue damage (that may be aggravated by repeated ultrasound treatments), we measured circulating levels of cardiac-specific troponin I protein as an indicator of myocardial tissue injury after UTMD. While the highest number of treatments (repeat 6) was associated with a mild elevation in troponin I compared with control or repeat 1, the magnitude of any myocardial damage due to this small troponin leak was insufficient to produce detectable functional differences or histopathological evidence of vascular damage or thrombosis. Further, troponin I levels returned to control levels rapidly (by 7 days after UTMD), indicating a transient protein release pattern.

Since even small troponin leaks have been associated with an increased risk of cardiovascular events in patients (with unstable angina or after percutaneous coronary interventions),[26,27] the benefits and risks of UTMD should be carefully compared in clinical trials assessing repeated myocardial contrast echocardiography for angiogenic cytokine delivery. Future studies will also be required to define the optimal echocardiographic settings to deliver the genes while minimizing cardiac injury, and to define the most effective genes (or combination of genes) and doses. These factors will probably influence the optimal number of treatments; fortunately, contrast echocardiography permits repeated measurements of the effects of gene therapy. Some circumstances might require numerous gene transfers, and so the relationship between number of treatments and amount of microvascular injury will need to be established in clinical trials. It would also be useful to assess the effect of treatment timing and determine whether endogenous levels of the therapeutic cytokines are affected by minor tissue injury that may be induced by the ultrasound stimulus itself. This study extends the information previously published about UTMD gene transfer. Repeated delivery was more effective than single treatments and appeared to be safe since only a minor troponin leak was found with six UTMD applications.

In conclusion, the therapeutic value of UTMD-mediated SCF and SDF-1α gene transfer into the infarcted heart was enhanced by repeated (at least six) treatments. Thus, UTMD may permit a tailored approach to cardiac regeneration after an extensive infarct that delivers repeated treatments until myocardial perfusion and ventricular function have been restored.

Supplementary material

Supplementary material is available at *European Heart Journal* online.

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