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ORIGINAL CONTRIBUTION

Targeted myocardial delivery of GDF11 gene rejuvenates the aged mouse heart and enhances myocardial regeneration after ischemia–reperfusion injury

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Abstract Ischemic cardiac injury is the main contributor to heart failure, and the regenerative capacity of intrinsic stem cells plays an important role in tissue repair after injury. However, stem cells in aged individuals have reduced regenerative potential and aged tissues lack the capacity to renew. Growth differentiation factor 11 (GDF11), from the activin-transforming growth factor β superfamily, has been shown to promote stem cell activity and rejuvenation. We carried out non-invasive targeted delivery of the GDF11 gene to the heart using ultrasound-targeted microbubble destruction (UTMD) and cationic microbubble (CMB) to investigate the ability of GDF11 to rejuvenate the aged heart and improve tissue regeneration after injury. Young (3 months) and old (21 months) mice were used to evaluate the expression of GDF11 mRNA in the myocardium at baseline and after ischemia/reperfusion (*I*/*R*) and myocardial infarction. GDF11 expression decreased with age and following myocardial injury. UTMD-mediated delivery of the GDF11 plasmid to the aged heart after *I*/*R* injury effectively and selectively increased GDF11 expression in the heart, and improved cardiac function and reduced infarct size. Over-expression of GDF11 decreased senescence markers, p16 and p53, as well as the number of $p16^{+}$ cells in old mouse hearts. Furthermore, increased proliferation of cardiac stem cell antigen 1 ($Sca\text{-}1^{+}$) cells and increased homing of endothelial progenitor cells and angiogenesis in old ischemic hearts occurred after GDF11 over-expression. Repetitive targeted delivery of the GDF11 gene via UTMD can rejuvenate the aged mouse heart and protect it from *I*/*R* injury.

Keywords GDF11 · Heart · Ischemia–reperfusion · Rejuvenation · Regeneration

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Introduction

Aging, a process associated with the progressive functional decline of organs and tissues, is inevitable. Many clinical abnormalities associated with high morbidity and mortality are more prevalent in an aging population. Thus, determining ways of reversing or slowing the aging process is critical [15]. A model of heterochronic parabiosis, in which young and old mice are joined together with one circulatory system, has been used to demonstrate that factors circulating in a young mouse can rejuvenate stem cells in an old mouse [3]. Loffredo

and colleagues attributed the rejuvenating effects on the aged heart to circulating growth differentiation factor 11 (GDF11), a member of the activin-transforming growth factor β (TGF-β) superfamily. In their study, old mice that received treatment with GDF11 attained the same benefits as those that received heterochronic parabiosis; specifically, the reversal of age-related cardiac hypertrophy [17]. Further research has revealed that GDF11 reverses age-related skeletal muscular dysfunction [21] as well as impairments in cognitive function and synaptic plasticity [27], and that GDF11 remodels aged mouse cerebral vasculature and enhances olfactory neurogenesis [14]. These findings suggest that GDF11 can promote stem cell activity and rejuvenate the function of multiple organs in aged mice.

However, recent studies have reported conflicting data on the age-related change in circulating GDF11 and myostatin levels, as well as the influence of GDF11 on muscle regeneration [4, 20, 22]. Is GDF11 truly a specific anti-aging factor, and if so, how does it exert its effects? In addition, the widespread expression of GDF11 suggests that it has at least several biological functions and that an increase in GDF11 level in the blood might result in detrimental clinical effects. For example, GDF11 has been implicated in the inhibition of erythroid maturation and in ineffective erythropoiesis in β-thalassemia [24]. Therefore, the currently adopted therapy of systemic delivery of GDF11 through intraperitoneal injection of recombinant protein may not be an ideal method for rejuvenation of a specific organ. Instead, targeted delivery of GDF11 to the organ of interest may be a more suitable method for clinical applications.

In the current study, we evaluated the effects of targeted delivery of the GDF11 gene on rejuvenation of the aged mouse heart using ultrasound-targeted microbubble destruction (UTMD) and cationic microbubble (CMB). This technique has been shown to provide non-invasive, safe, repeatable, and targeted gene delivery to the infarcted myocardium [6–8]. We demonstrated that GDF11 expression decreased with age and after myocardial injury and that UTMD-mediated delivery of GDF11 specifically increased GDF11 levels in the heart. Furthermore, we showed that repetitive targeted delivery of the GDF11 plasmid improved cardiac function and reduced infarct size as well as stimulated cardiac stem cell (CSC) proliferation and decreased the expression of senescence markers in old mouse hearts after ischemia/reperfusion (*I*/*R*) injury.

Methods

Please see the Online Resource for a detailed methodology.

CMB solutions and plasmid DNA

The CMB was prepared according to our previous methodology [23]. For the in vitro transfection of GDF11 plasmid, HEK293 (human embryonic kidney) cells were used.

Animal models

Young (3 mo) and old (21 mo) C57BL/6 mice were obtained from Charles River Laboratories (Saint-Constant, QC, Canada), and *I*/*R* injury was generated under general anesthesia with isoflurane at a maintenance dose of 2% by occluding the left anterior coronary artery for 45 min followed by reperfusion, as previously described [6]. Myocardial infarction (MI) was produced by ligating the left anterior coronary artery permanently under general anesthesia, as previously described [5]. All animal procedures were approved by the Animal Care Committee of the Toronto General Research Institute, according to the NIH Guide for the Care and Use of Laboratory Animals (National Academy Press 1996).

UTMD delivery

Mice were sedated with 2% isoflurane, and the plasmid-MB solution was infused into the tail vein at a rate of 1.2 mL/h. Simultaneously, an ultrasound beam was delivered with an M3S transducer using a Vivid 7 system (GE Healthcare, Milwaukee, WI) operating in the second harmonic mode (transmit: 1.6 MHz; receive: 3.2 MHz) with an electrocardiograph (ECG) trigger at every fourth end-systole for 20 min. The depth was set at 2 cm, and the transducer was adjusted with a gel interface so that the focus was positioned at the myocardial level. A mechanical index of 1.3 was employed. Each ultrasound burst was seen to eliminate a large number of the MBs in the myocardium, and a pulsing interval of four cardiac cycles allowed replenishment of the MBs before the next ultrasound burst.

Experimental design and timeline

The GDF11 and empty plasmids were delivered to the heart by UTMD twice, 3 and 6 days after *I*/*R*. For the GDF11 dosing and injections with the recombinant GDF11, we followed the protocol used in previous reports [4, 22]. Animals were given a single daily intraperitoneal injection of either rGDF11 (R&D Systems) at 0.1 mg/kg or vehicle (60 mM NaAcetate Buffer, pH 5.0 and 10% Trehelose) for a total of 25 days, starting 3 days prior to and ending 21 days after *I*/*R*. For the cardiac function study, echocardiography was performed before 9 and

21 days after *I/R*. To analyze the endogenous levels of GDF11 mRNA, hearts were collected for RT-PCR analysis before surgery, 3 days after *I/R* and 7 days after MI, respectively. To evaluate the effectiveness of GDF11 plasmid delivery with UTMD, hearts were collected for RT-PCR analysis before and 3 days after two UTMD-mediated plasmid deliveries (9 days after *I/R*). For histology and immunostaining, hearts were collected 12 days after the two UTMD-mediated plasmid deliveries (21 days after *I/R*). Thus, we did not measure the acute effect of GDF11 on scar size, but rather its longer-term effect.

Statistical analyses

Data are expressed as mean ± standard deviation. Analyses were performed using GraphPad Prism software (v. 5) with the critical α-level set at $p < 0.05$. Comparisons among groups (mRNA and protein expression, infarct size, left ventricular systolic and diastolic function) were made using one-way ANOVA. When F values were significant for the main effects or the interaction, differences between groups were specified with Tukey's multiple range post hoc test. Student's *T* test was used for comparison of GDF11 and empty vector groups.

Results

The expression profile of GDF11 in young and old mice using *I/R* and MI models

For the *I/R* model, GDF11 mRNA levels at baseline in old mice were significantly lower than those in young mice (Fig. 1a). The expression levels of GDF11 mRNA in the ischemic hearts of young and old mice 3 days after *I/R* were significantly upregulated compared to their respective baselines, and the magnitude of increase expression was lower in the old ischemic hearts.

For the MI model, 7 days after MI, GDF11 mRNA levels in the border zone of young mouse hearts were significantly higher than levels in the scar region of young hearts and levels in sham hearts (Fig. 1b). However, GDF11 mRNA levels in the border zone of old mouse hearts were significantly lower than levels in the scar region of the same hearts. These observations provide the first evidence that endogenously expressed GDF11 mRNA was increased in the border zone of young mice and decreased in the border region of old mice after MI. Both injury models showed a change in GDF11 expression post-injury compared with baseline levels suggesting that GDF11 may play an active role in ischemic remodeling.

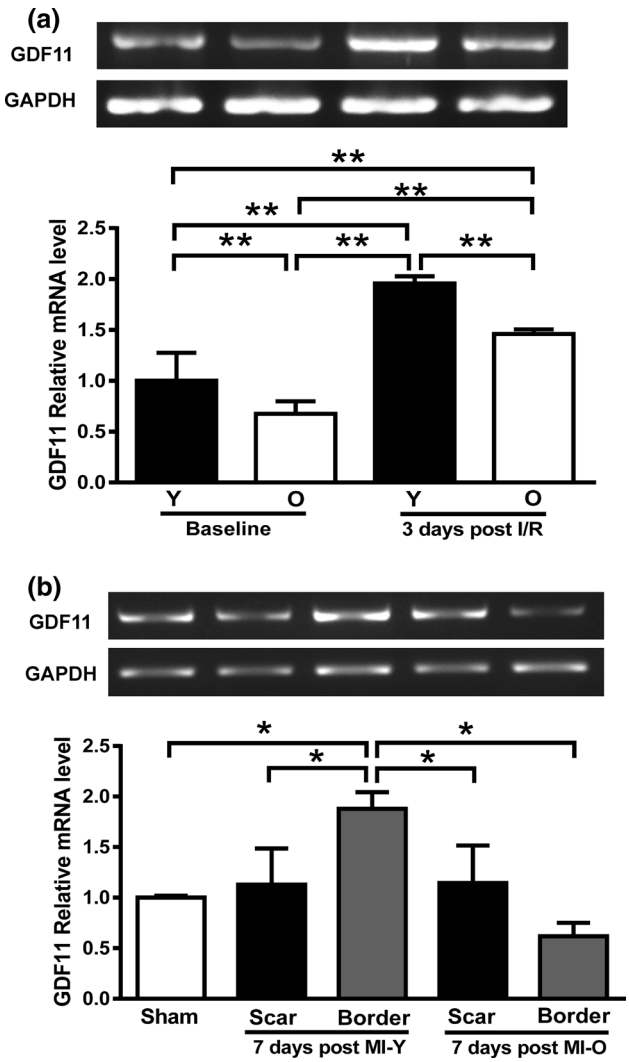


Fig. 1 GDF11 expression in mouse hearts before and after injury. GDF11 mRNA expression levels in mouse hearts following ischemia–reperfusion (*I/R*) injury (**a**) or myocardial infarction (MI) (**b**) were measured by RT-PCR. The basal mRNA expression levels of GDF11 were significantly lower in old than young mice ($n = 6$/group). Three days after *I/R* injury, the GDF11 mRNA expression levels in the hearts of young and old mice increased significantly compared to baseline ($n = 6$/group). The hearts of young mice had the highest expression level of GDF11 mRNA after *I/R* injury (**a**). The mRNA levels of GDF11 in the border zone of young mice 7 days after MI were the highest of all the groups with or without MI (**b**, $n = 5$–7/group). *$p < 0.05$, **$p < 0.01$

UTMD-mediated delivery of GDF11 increased the expression of GDF11 specifically in the hearts of old mice

We first verified the function of the GDF11 plasmid using the standard method of in vitro cationic lipid-mediated transfection with HEK293 cells. For this purpose, HEK293 cells were transfected either with GDF11 or empty plasmid DNA. Untransfected HEK293 cells served as a control.

Total RNA was extracted from control and transfected cells. As shown in Fig. 2a, transfected GDF11 mRNA expression was only detected in the GDF11 plasmid-transfected cells, but not in the control cells or the empty plasmid-transfected cells when a GDF11 plasmid-specific primer was used. Next, to demonstrate that the GDF11 plasmid indeed overexpressed the functional protein, Western blotting was used to detect the secreted GDF11 dimers and monomers in the cell culture supernatant of the transfected cells. As shown in Fig. 2b, the expression of mature/active dimers and monomers of the GDF11 protein was detected in the cell culture supernatant of the GDF11 plasmid-transfected cells, but not in the control or the empty plasmid transfected HEK293 cells. These results indicate that the GDF11 plasmid effectively expressed and secreted the functional GDF11 dimers and monomers.

To boost the expression of GDF11 in aged mouse hearts, UTMD was adapted to repeatedly deliver mouse GDF11 plasmid to ischemic hearts on both 3 and 6 days after *I/R*. GDF11 mRNA levels in old ischemic hearts were measured by RT-PCR 9 days after *I/R*. The GDF11 plasmid-mediated mRNA expression was only detected in animals receiving UTMD-mediated delivery of the GDF11 plasmid (old + GDF11), but not in young animals that only received *I/R* or old animals receiving UTMD-mediated delivery of the empty plasmid (old + empty-V) when a GDF11 plasmid-specific primer was used (Fig. 2c). Furthermore, endogenous GDF11 mRNA expression was significantly higher in the hearts of the old + GDF11 group than the old + empty-V group (Fig. 2d, e), whereas there were no significant differences in liver, spleen and kidney expression levels between the two groups (Fig. 2f). These results clearly demonstrate that UTMD-mediated delivery of GDF11 effectively increased the expression of GDF11 specifically in the hearts, but not in the other organs, of old mice.

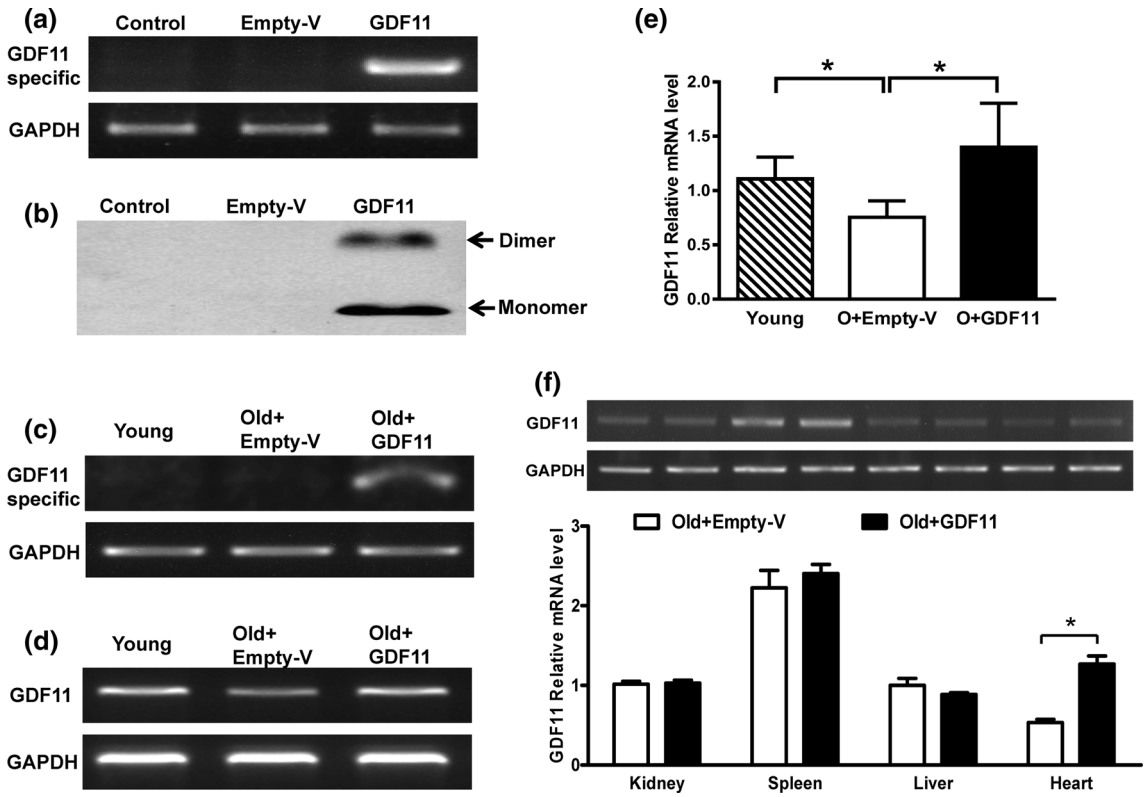


Fig. 2 In vitro and in vivo expression of GDF11 mRNA and protein. **a** HEK 293 cells were transfected with GDF11 and empty plasmids by Cationic Lipid Mediated Transfection. A GDF11 plasmid-specific primer was used to detect the transfected GDF11 mRNA expression. **b** The expression of mature/active dimer and monomer of GDF11 protein was detected in the cell culture supernatant of the GDF11 plasmid-transfected HEK293 cells. **c** The GDF11 and empty plasmids were delivered to the heart by UTMD twice (3 and 6 days after *I/R*). GDF11 plasmid-specific mRNA was detected by RT-PCR using the specific primer for the GDF11 plasmid. **d** The expression of endogenous GDF11 mRNA in ischemic mouse hearts was measured by RT-PCR 9 days after *I/R*. **e** The endogenous GDF11 mRNA expression levels in the group receiving UTMD-mediated delivery of GDF11 plasmid (old + GDF11) were significantly higher than the group receiving the empty vector (old + empty-V, $n = 5$–7/group). **f** GDF11 mRNA and protein in the ischemic mouse hearts, liver, spleen and kidney were measured by RT-PCR and Western blot, respectively, 9 days after *I/R*. The expression of GDF11 mRNA in hearts increased significantly in the old + GDF11 group compared to the old + empty-V group, while there was no significant difference in expression levels in the liver, spleen and kidney between the two groups ($n = 5$/group). *$p < 0.05$

UTMD-mediated delivery of GDF11 improved cardiac function in old mice after *I/R*

Cardiac systolic and diastolic functions were determined by echocardiography (Fig. 3a, b) before and 9 and 21 days after *I/R*. Four groups of animals were compared: young animals receiving *I/R* (Young), old animals receiving UTMD-mediated delivery of empty plasmid (old + empty-V), old animals receiving UTMD-mediated delivery of GDF11 plasmid (old + GDF11), and old animals receiving the daily injection of recombinant GDF11 for 25 days (GDF11 protein). Fractional shortening was similar in all 4 groups before *I/R* (day 0) at approximately 40% and decreased to approximately 34% 9 days following *I/R* (3 days after two gene deliveries; Fig. 3c). Fractional shortening was significantly lower in the old + empty-V group compared with the other three groups 21 days after *I/R*. The decline in fractional shortening was partially reversed by UTMD-mediated delivery of GDF11 plasmid (old + GDF11 group), and this decline was comparable to that observed in the group receiving daily injections of recombinant GDF11 protein (GDF11 protein group). Transmitral peak velocity of early-diastole (*E*)/velocity of early diastole of the mitral annulus (*E′*), an index of diastolic function, was correlated with the impairment in diastolic function (Fig. 3d). *E*/*E′* was significantly higher in the old + empty-V group compared with the other three groups 21 days after *I/R*. GDF11 therapy either with UTMD-mediated GDF11 plasmid delivery or with GDF11 protein injection attenuated the increase in *E*/*E′* 21 days after *I/R*.

Twenty-one days after *I/R*, invasive pressure–volume measurements (Fig. 4a) demonstrated that ejection fraction (Fig. 4b), dP/dt max (Fig. 4c), and dP/dt min (Fig. 4d) were significantly higher in the young, old + GDF11 plasmid and GDF11 protein groups than the old + empty-

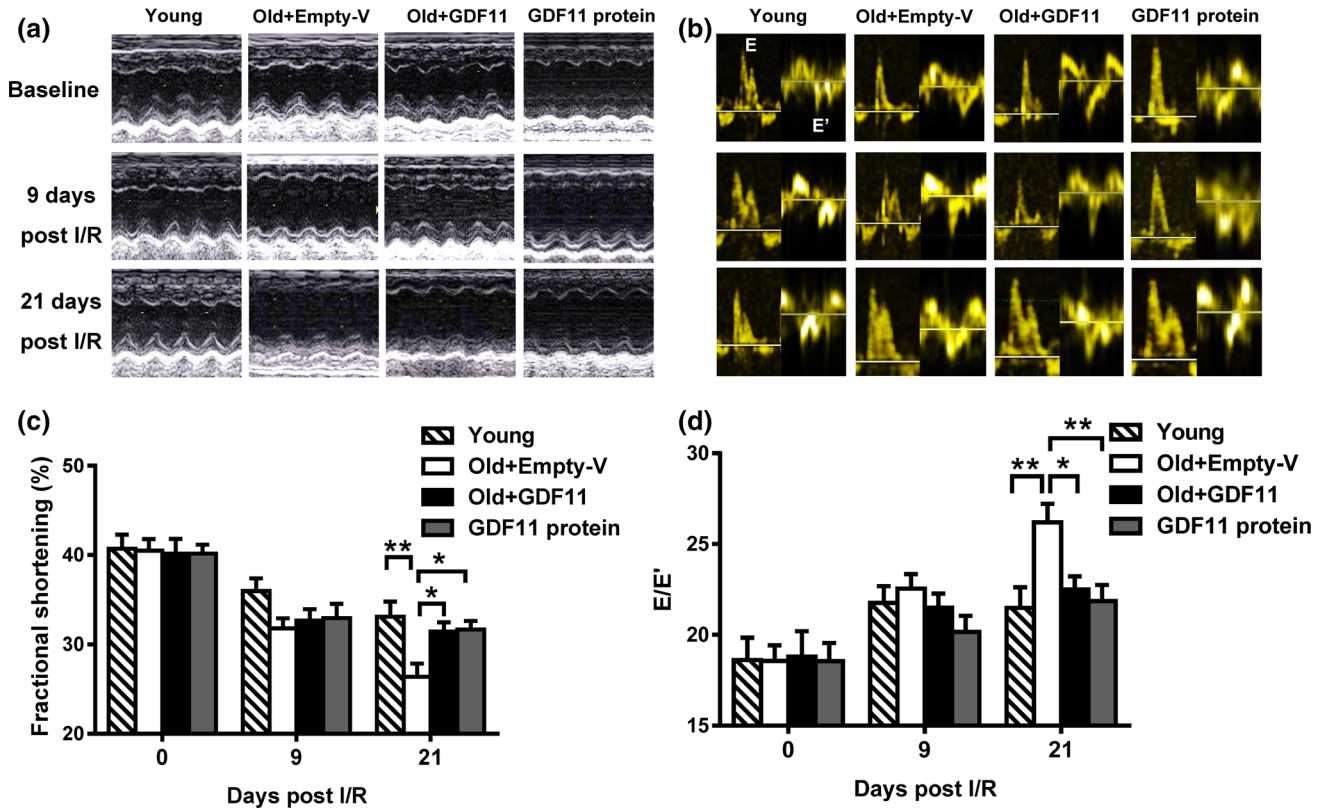
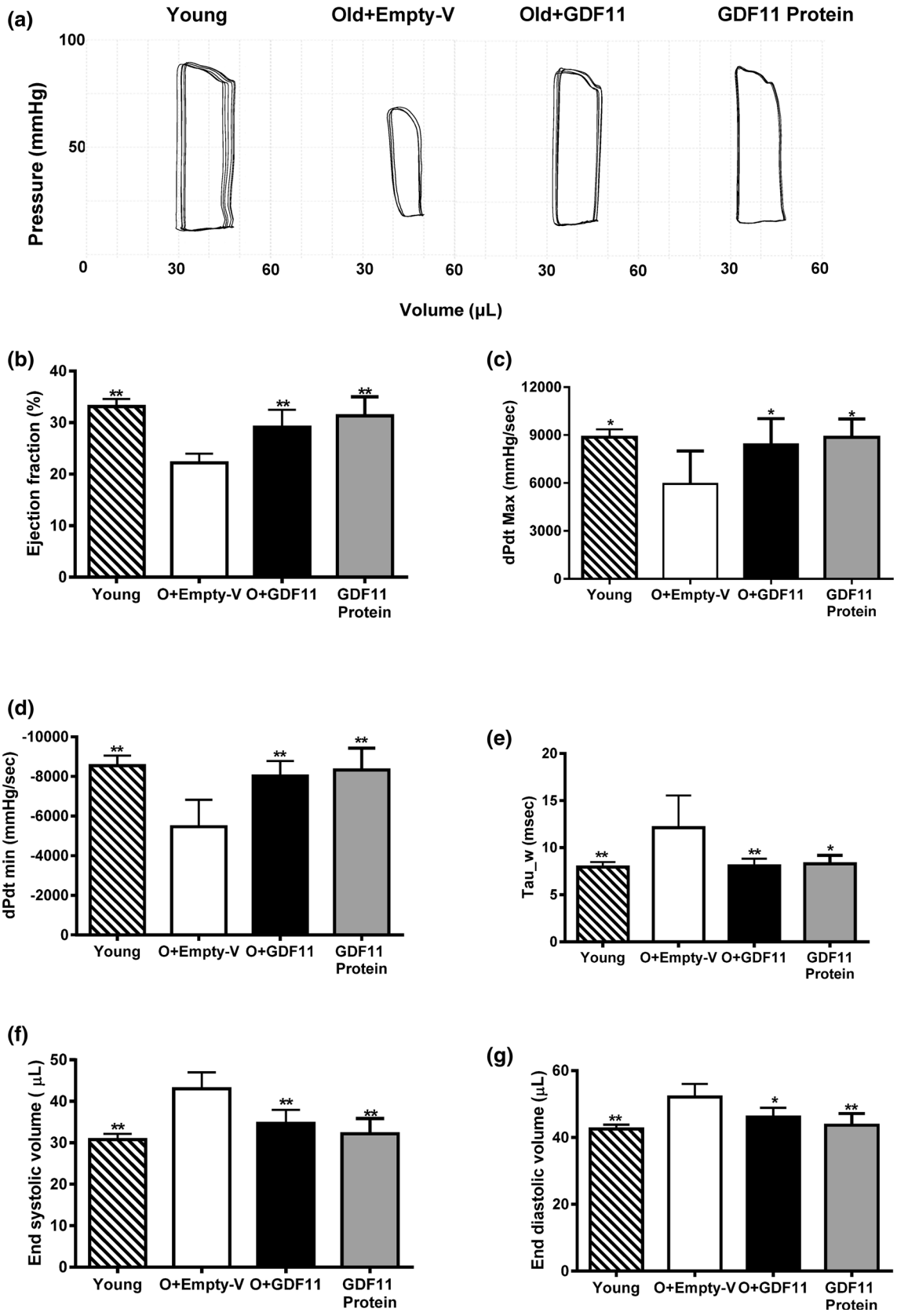


Fig. 3 UTMD-mediated delivery of GDF11 improved cardiac function in old mice after *I/R* injury. Echocardiography was performed before and 9 and 21 days after ischemia/reperfusion (*I/R*) injury in young mice (young), old mice that received ultrasound-targeted microbubble destruction (UTMD)-mediated delivery of empty plasmid (old + empty-V), old mice that received UTMD-mediated delivery of GDF11 plasmid (old + GDF11), or old mice that received a daily dose of recombinant GDF11 for 25 days (GDF11 protein). Representative M-mode (**a**) and pulse wave Doppler and tissue Doppler (**b**) images taken at baseline and 9 and 21 days after *I/R*. **c** Fractional shortening was significantly lower in the old + empty-V group compared with the other three groups 21 days after *I/R*. The decline in fractional shortening was partially reversed by UTMD-mediated delivery of GDF11 plasmid (old + GDF11 group) and this decline was comparable to that observed in the group receiving daily injections of recombinant GDF11 protein (GDF11 protein group, $n = 5$–7/group). **d** The E/E' increased significantly in the old + -empty-V group compared with the other three groups 21 days after *I/R*. GDF11 therapy either with UTMD-mediated GDF11 plasmid delivery or with GDF11 protein injection attenuated the increase in *E*/*E′* 21 days after *I/R*; $n = 5$–7/group. $^{*}p < 0.05$, $^{**}p < 0.01$


(a)
Young
Old+Empty-V
Old+GDF11
GDF11 Protein
Pressure (mmHg)
Volume (µL)
(b)
Ejection fraction (%)
(c)
dPdt Max (mmHg/sec)
(d)
dPdt min (mmHg/sec)
(e)
Tau_w (msec)
(f)
End systolic volume (µL)
(g)
End diastolic volume (µL)
Young
O+Empty-V
O+GDF11
GDF11 Protein

◀**Fig. 4** Hemodynamic changes in old mice after *I/R* injury and GDF11 therapy. Twenty-one days after ischemia/reperfusion (*I/R*) injury, invasive pressure–volume (P–V) measurements were performed in four groups: young mice, old mice that received ultrasound-targeted microbubble destruction (UTMD)-mediated delivery of empty plasmid (O + Empty-V), UTMD-mediated delivery of GDF11 plasmid (O + GDF11) or a daily dose of recombinant GDF11 injection for 25 days (GDF11 protein). **a** Representative P–V loops of mouse hearts 21 days after *I/R*. The ejection fraction (**b**), d*P*/d*t* max (**c**) and d*P*/d*t* min (**d**) were significantly higher in the young, the O + GDF11 and the GDF11 protein groups than the O + Empty-V group. The Tau-w (**e**), end systolic volume (**f**) and end diastolic volume (**g**) were significantly lower in the young, O + GDF11 and GDF11 protein groups than the O + Empty-V group ($n = 5$–7/group). $^{*}p < 0.05$, $^{**}p < 0.01$

V group. Conversely, Tau-w (Fig. 4e), end systolic volume (Fig. 4f), and end diastolic volume (Fig. 4g) were significantly lower in the young, old + GDF11 plasmid and GDF11 protein groups than the old + empty-V group. Taken together, these results indicate that interventions with GDF11, either through UTMD-mediated plasmid delivery (old + GDF11) or protein injection, effectively protected the aged mouse heart from *I/R* injury.

UTMD-mediated delivery of GDF11 decreased infarct size in old mice after *I/R*

Twelve days after two UTMD-mediated plasmid deliveries (21 days after *I/R*), a smaller scar size was observed in both the young and old + GDF11 groups than the old + empty-V group (Fig. 5a, b). Histological examination of mouse hearts was performed 21 days after *I/R* and the myocardial sections were stained with H&E and Masson's trichrome. The old + empty-V group was found to have larger infarcts and greater collagen deposition in the infarct region compared to both the young and the old + GDF11 groups (Fig. 5c, d).

UTMD-mediated delivery of GDF11 stimulated the proliferation of CSCs in old mouse hearts after *I/R*

To evaluate the changes in the number of CSCs over the 6 day-period following the delivery of GDF11, c-kit^{+} and Sca-1^{+} CSCs were quantified in the ischemic region of both young and old mouse hearts. The number of c-kit^{+} (Fig. 6a) and Sca-1^{+} (Fig. 6b) CSCs increased by approximately 149 and 238%, respectively, in the old + GDF11 group relative to the old + empty-V group 9 days after *I/R* ($p < 0.01$). Young hearts still had the highest number of c-kit^{+} (Fig. 6a) and Sca-1^{+} (Fig. 6b) CSCs. These results suggest that a larger proportion of myocyte-committed c-kit^{+} and Sca-1^{+} CSCs may be reactivated in aging myopathy. To confirm the proliferation of CSCs, young and old mice received BrdU injections for 3 consecutive days, and *I/R* was performed one day later. Immunostaining for BrdU and Sca-1 confirmed the proliferation of CSCs. In comparison to hearts from the old + empty-V group, the number of BrdU^{+} CSCs increased 116% following UTMD-mediated delivery of GDF11 (Fig. 6c) which was comparable to the number of BrdU^{+} CSCs in young hearts, indicating that GDF11 also has the ability to stimulate CSC proliferation in the infarcted regions of the aged heart.

UTMD-mediated delivery of GDF11 increased homing of EPCs and angiogenesis in old mouse hearts after *I/R*

To determine whether homing of endothelial progenitor cells (EPCs) occurred after the delivery of GDF11, KDR and CD34 were used as bio-markers for EPCs, and KDR/CD34 double-positive cells were quantified in the ischemic regions of both young and old mouse hearts. Approximately, 2.3-fold increase in the number of KDR^{+}/CD34^{+} cells (Fig. 7a) was observed in the old + GDF11 group relative to the old + empty-V group 9 days after *I/R* which was comparable to the number of KDR^{+}/CD34^{+} cells in young hearts. This result suggests that GDF11 treatment may stimulate the homing of a large number of EPCs to the ischemic myocardium. To determine whether increased homing of EPCs resulted in increased angiogenesis, isolectin B4 and SMA were stained and quantified in the ischemic region of young and old hearts to evaluate capillary and arteriolar densities, respectively. The capillary (Fig. 7b) and arteriolar (Fig. 7c) densities were expressed as the percentage of isolectin^{+} and SMA^{+} pixels per 0.2 mm^{2} area, respectively. The capillary and arteriolar densities in the border zone increased significantly in both the young and old + GDF11 groups relative to the old + empty-V group 9 days after *I/R*. This effect was sustained up to 21 days after *I/R* in both the young and old + GDF11 groups relative to the old + empty-V group. These findings indicate that GDF11 can stimulate angiogenesis in the aged ischemic heart.

UTMD-mediated delivery of GDF11 rejuvenated senescent hearts

We evaluated the mRNA expression levels of the senescence marker oncogenes p16 and p53 by RT-PCR 21 days after *I/R*. Relative to the old + empty-V group, the mRNA levels of p16 and p53 decreased an average of 2.1- and 2.4-fold, respectively, in the old + GDF11 group (Fig. 8a, b). The decrease in p16 and p53 mRNA levels in the old + GDF11 group was comparable to that of the young group. Western blot analysis revealed a significant decrease

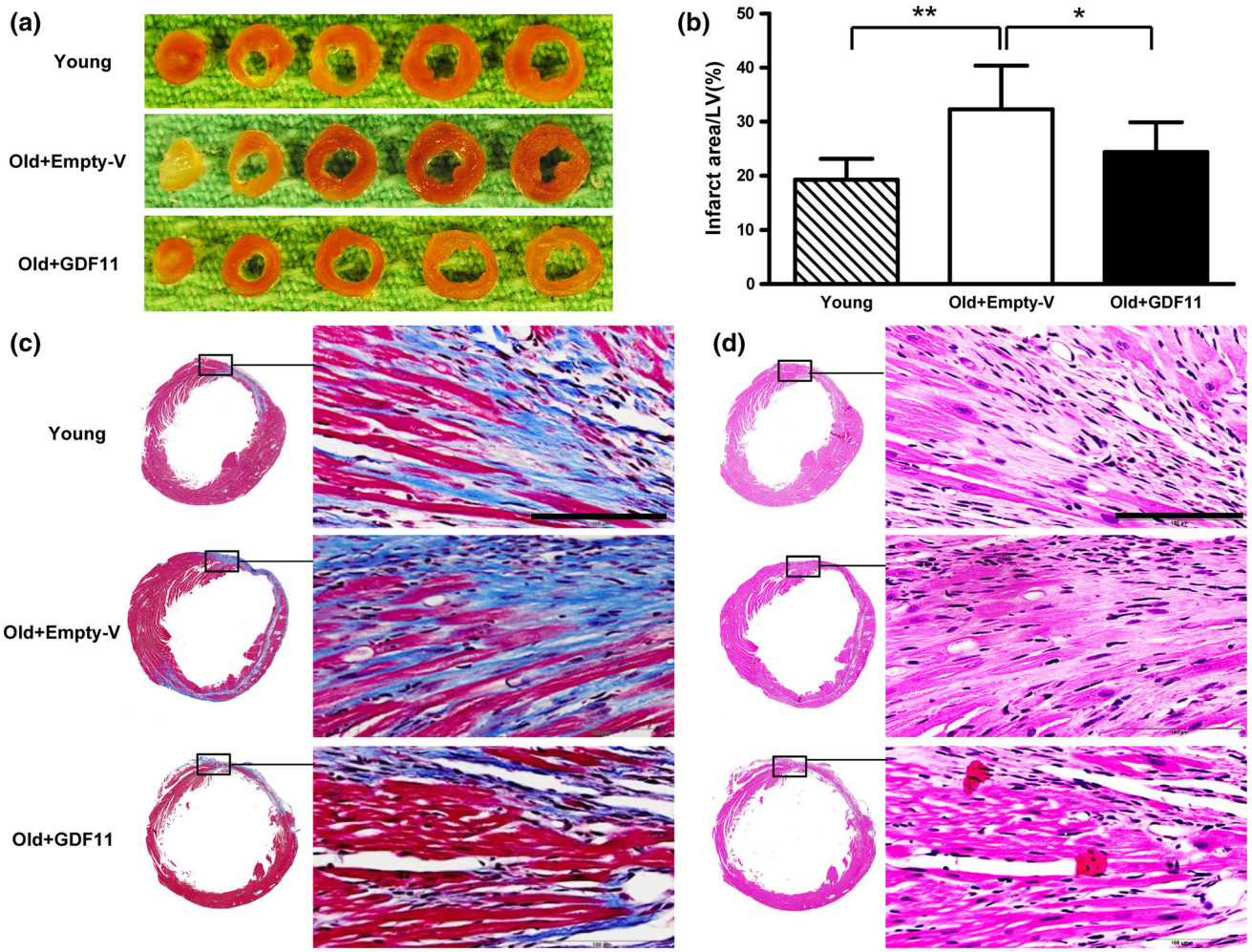


Fig. 5 UTMD-mediated delivery of GDF11 decreased infarct size of old mouse hearts after *I/R* injury. A smaller scar area was observed in hearts from both the young and old mice that received ultrasound-targeted microbubble destruction (UTMD)-mediated delivery of GDF11 plasmid (old + GDF11 group) than in hearts from old mice that received UTMD-mediated delivery of empty plasmid (old + empty-V group) 21 days after *I/R* (**a**, **b**). Representative photomicrographs (*scale bar* 100 μm) of whole sectioned hearts and mid-papillary transverse sections stained with Masson's trichrome (**c**) and H&E (**d**) to depict scar area 21 days after *I/R*. ($n = 6$/group), $*p < 0.05$, $**p < 0.01$

in p16 protein expression in both the young old + GDF11 groups relative to the old + empty-V group (Fig. 8c). Immunolabeling of $p16^+$ cells showed a nuclear localization of the protein (Fig. 8d). Quantification of $p16^+$ cells showed a significant decrease in the number of $p16^+$ cells in both the young and old + GDF11 groups compared to the old + empty-V group (Fig. 8e). These results indicate that UTMD-mediated delivery of GDF11 rejuvenated the senescent hearts of aged mice.

Discussion

GDF11 has been implicated as a serum factor involved in the rejuvenating power of young blood [3, 17, 21] and several studies have shown that GDF11 can rejuvenate the function of multiple organs in old mice [14, 17, 21]. However, recent findings have questioned the purported age-related decrease in circulating GDF11 and the influence of GDF11 on rejuvenation [4, 22].

The aim of the current study was to evaluate the expression pattern of GDF11 in the aged mouse heart and to explore the potential therapeutic role of this growth factor in cardiac rejuvenation and tissue regeneration after myocardial injury. We found that GDF11 mRNA levels at baseline in old mice were significantly lower than levels in young mice. Moreover, we found that the expression of GDF11 mRNA in the ischemic hearts of young and old mice 3 days after *I/R* injury was significantly higher than baseline expression. However, the magnitude of increase was smaller in old mice. Therefore, we postulated that interventions that increase

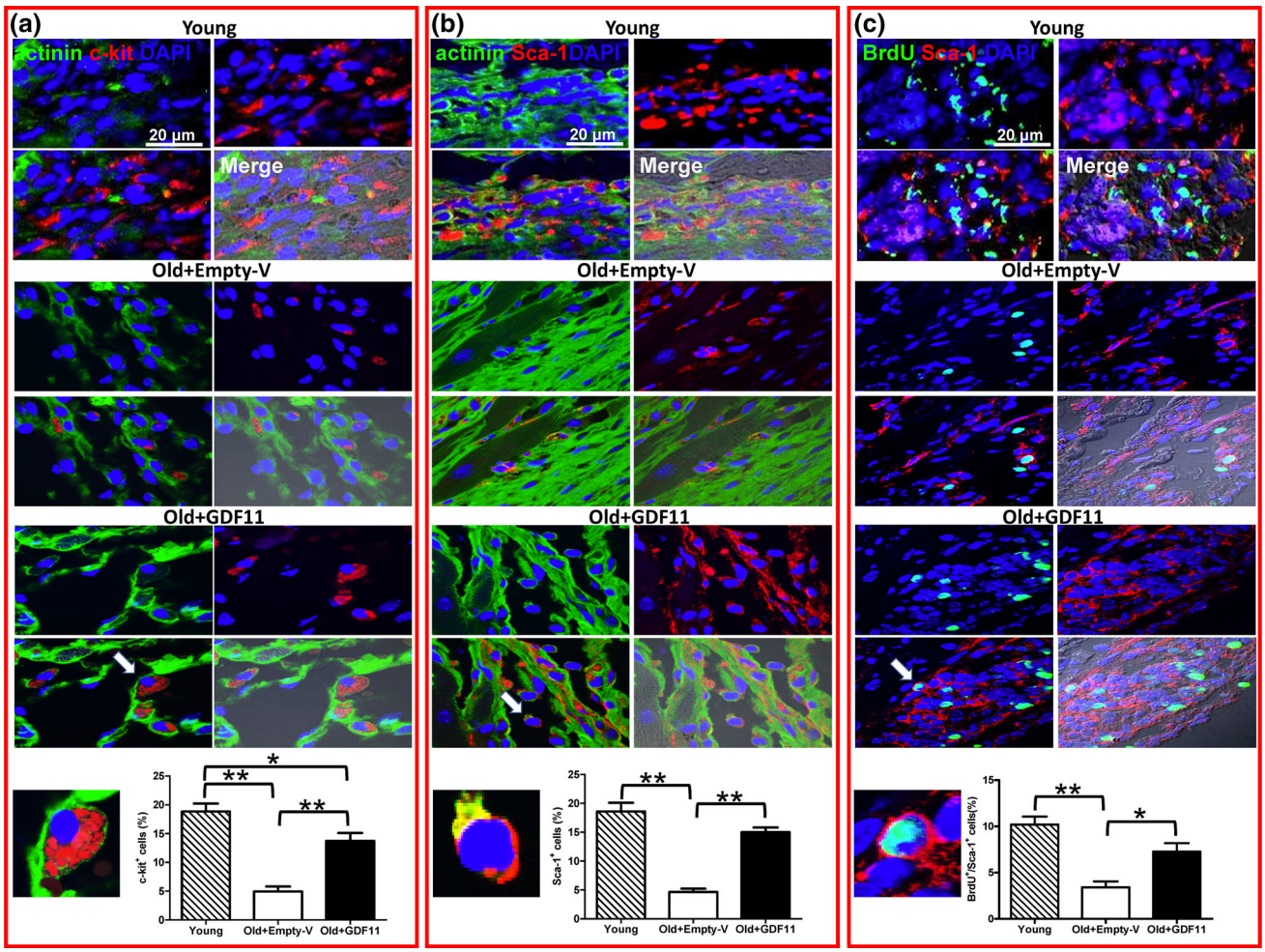
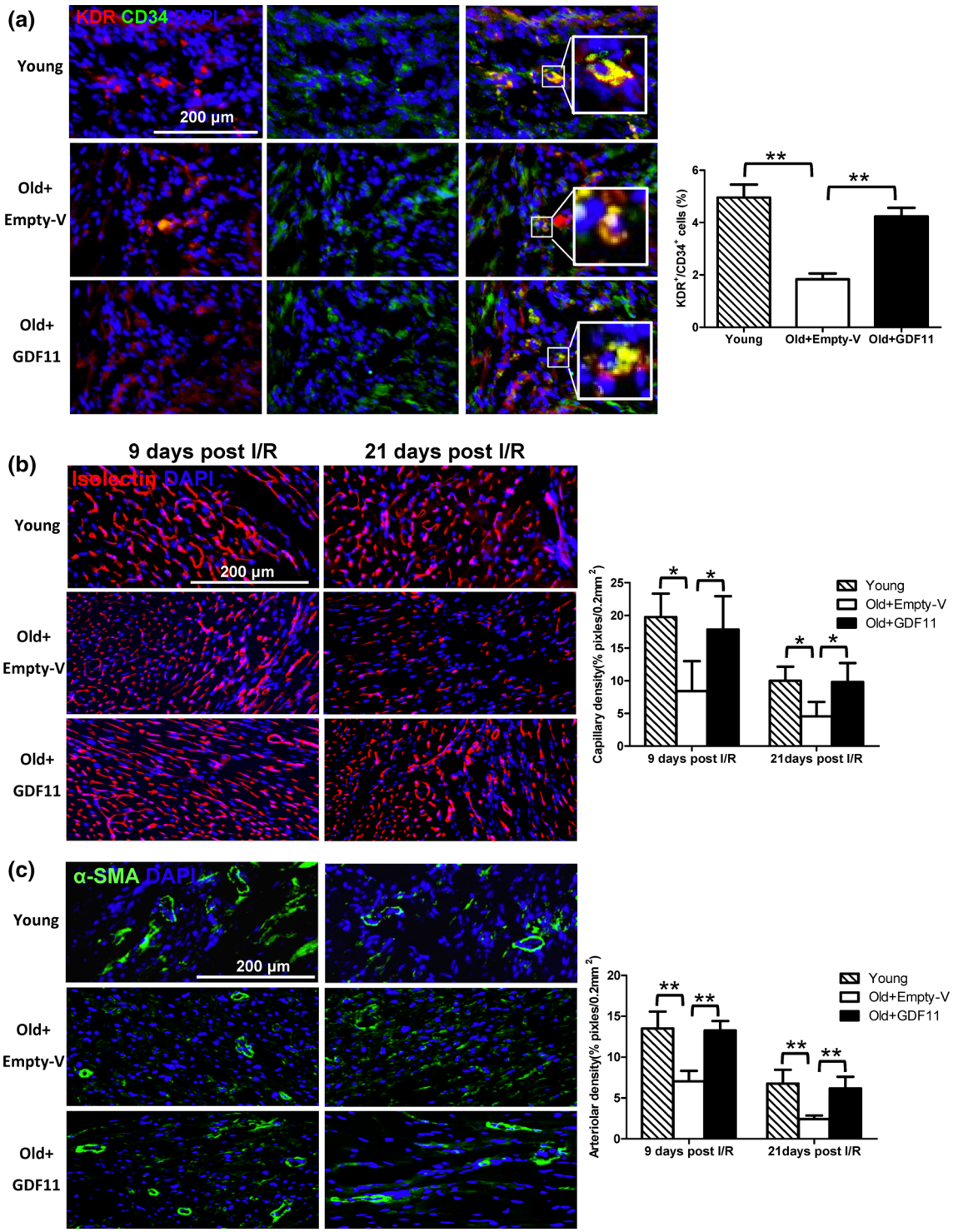


Fig. 6 UTMD-mediated delivery of GDF11 stimulated proliferation of CSCs in old mice after *I/R* injury. Immunolabelling of myocardial sections for cardiac stem cell (CSC) factors c-kit (**a**) and Sca-1 (**b**) (*red*) and actinin (*green*) 9 days after *I/R*. Zoomed images show c-kit$^+$ or Sca-1$^+$ cells and nuclei were stained blue with DAPI. The number of c-kit$^+$ and Sca-1$^+$ cells in old mice that received ultrasound-targeted microbubble destruction (UTMD)-mediated delivery of GDF11 plasmid (old + GDF11 group) was significantly greater than in old mice that received UTMD-mediated delivery of empty plasmid (old + empty-V group, $n = 5$/group). Young hearts still had the highest number of c-kit$^+$ (**a**) and Sca-1$^+$ (**b**) CSCs. (**c**) Immunolabelling of myocardial sections for BrdU (proliferating cells; *green*) and Sca-1 (CSCs; *red*). Zoomed image shows BrdU/Sca-1 double-positive cells and nuclei are stained blue with DAPI. The number of BrdU$^+$/Sca-1$^+$ cells in the border zone was significantly higher in the old + GDF11 group than the old + empty-V group which was comparable to the number of BrdU$^+$ CSCs in young hearts ($n = 5$/group). *$p < 0.05$, **$p < 0.01$

the expression level of GDF11 in the old mouse heart may confer beneficial effects.

We employed UTMD to target the delivery of the GDF11 plasmid to the injured heart because this approach avoided the potential for deleterious effects on the function of other organs, which is a disadvantage of the currently used therapy of systemic GDF11 delivery through intraperitoneal injection of recombinant protein. Delivery of the GDF11 plasmid by UTMD 3 and 6 days after *I/R* injury selectively increased the expression of GDF11 in the heart with no impact on GDF11 expression in the liver, spleen or kidney. These findings indicate that UTMD-mediated delivery of GDF11 is a repeatable technique to produce a targeted increase in the expression of the GDF11 gene in the ischemic myocardium.

After a targeted increase in the expression of GDF11 in old mouse hearts, the aged hearts were protected against a subsequent *I/R* insult and the resultant infarct size was decreased. This result was further validated by elevating the circulating levels of GDF11 in old mice by systemic administration of recombinant GDF11 protein for 25 days. GDF11 therapy either with UTMD-mediated GDF11 plasmid delivery or with GDF11 protein injection attenuated the decline in cardiac function 21 days after *I/R* injury. Investigation of the underlying mechanism revealed that GDF11 treatment increased the proliferation of CSCs that were labeled as Sca-1$^+$, and these cells migrated to the border and scar zones of ischemic hearts. The increase in the number of CSCs stimulated the homing of EPCs and


(a)
KDR CD34 DAPI
Young
Old+
Empty-V
Old+
GDF11
200 μm
KDR+/CD34+ cells (%)
Young
Old+Empty-V
Old+GDF11
(b)
9 days post I/R
21 days post I/R
Isolectin DAPI
Capillary density(% pixles/0.2mm2)
9 days post I/R
21days post I/R
(c)
α-SMA DAPI
Arteriolar density(% pixles/0.2mm2)

◀**Fig. 7** UTMD-mediated delivery of GDF11 increased homing of EPCs and angiogenesis after *I/R* injury. **a** Immunolabelling of myocardial sections for KDR (*red*) and CD34 (*green*) shows endothelial progenitor cells (EPCs). Zoomed images show KDR/CD34 double-positive cells with nuclei stained *blue* with DAPI. The number of $KDR^+/CD34^+$ cells in the border zone was significantly greater in both the young and old mice that received ultrasound-targeted microbubble destruction (UTMD)-mediated delivery of GDF11 plasmid (old + GDF11 group) than in old mice that received UTMD-mediated delivery of empty plasmid (old + empty-V group, $n = 4$/group). Representative micrographs (magnification ×200) of isolectin staining for capillaries (*red*, **b**) and α-SMA staining for arterioles (*green*, **c**) with nuclei stained blue with DAPI. The capillary and arteriolar densities are expressed as the percentage of $isolectin^+$ (**b**) and SMA^+ (**c**) pixels per 0.2 mm^2 area, respectively. The capillary and arteriolar densities in the scar and border zones were significantly greater in both the young and old + GDF11 groups than the old + empty-V group 9 and 21 days after *I/R* injury ($n = 4$/group). $*p < 0.05$, $**p < 0.01$

subsequently increased capillary and arteriole densities in the border zone to aid repair of the ischemic myocardium. At the same time, targeted delivery of GDF11 decreased the expression of senescence markers p16 and p53 in old mouse hearts after *I/R* injury. These findings demonstrate that repetitive targeted delivery of the GDF11 plasmid via UTMD can rejuvenate the aged mouse heart and protect it from *I/R* injury.

Recent studies have questioned the age-related change in circulating GDF11 and myostatin levels, as well as the influence of GDF11 on muscle regeneration [4, 20, 22] since Loffredo et al. attributed the rejuvenating effects on the aged heart to circulating GDF11 [17]. Egerman et al. found that circulating GDF11, when measured using a new and more specific anti-GDF11 immunoassay could not be detected in the plasma from either old or young mice, but showed a trend toward higher levels in aged rats and humans [4]. Furthermore, treatment with GDF11 in vitro or injecting recombinant GDF11 in vivo inhibited myoblast differentiation and skeletal muscle regeneration. Similar to the observations made by Egerman et al., Smith et al. found the detection sensitivity to be too low to measure the serum

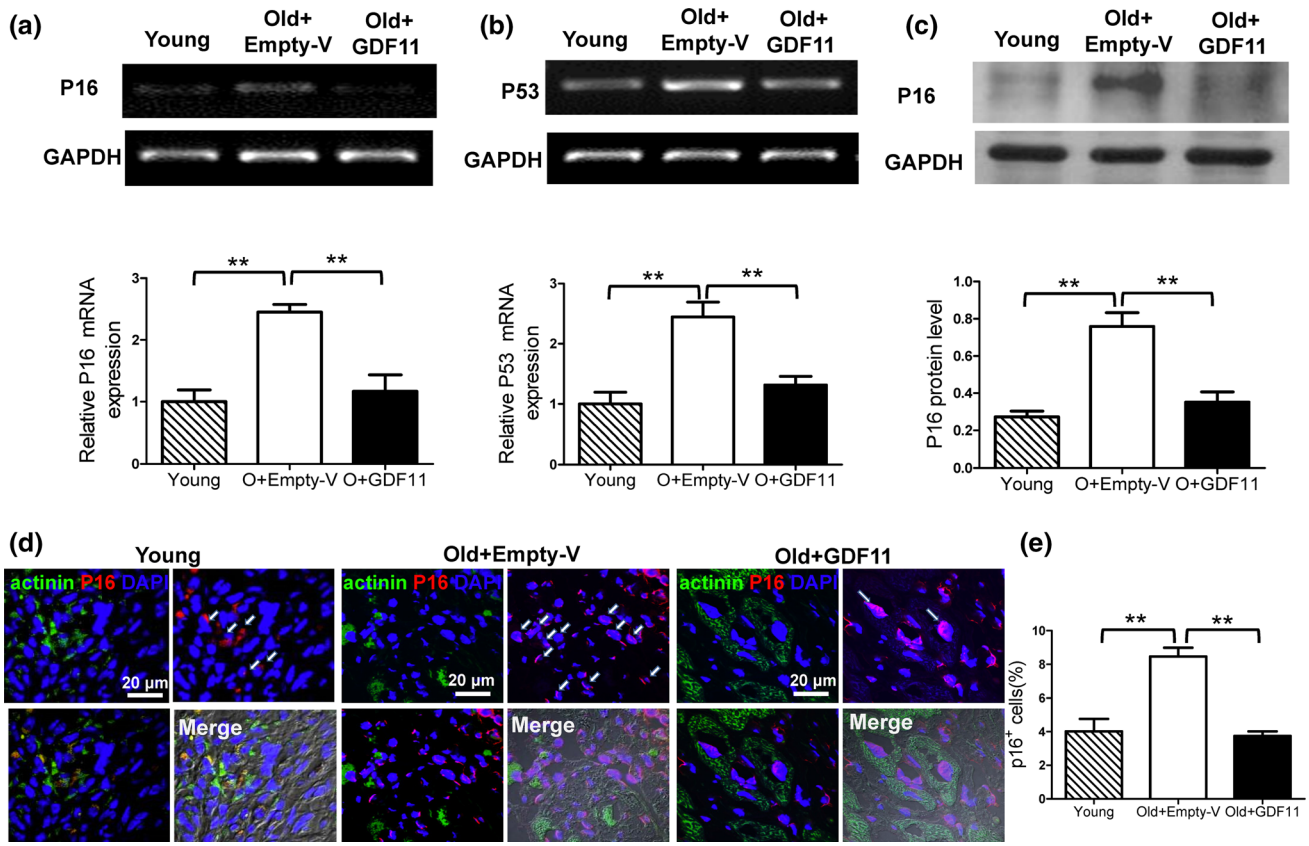


Fig. 8 Rejuvenation of the aged ischemic heart through UTMD-mediated delivery of GDF11. RT-PCR demonstrated that the expression levels of p16 (**a**) and p53 (**b**) mRNA were significantly lower in both the young and old mice that received ultrasound-targeted microbubble destruction (UTMD)-mediated delivery of GDF11 plasmid (old + GDF11 group) than in old mice that received UTMD-mediated delivery of empty plasmid (old + empty-V group, $n = 5$/group). **c** Western blot showed that p16 protein expression was significantly lower in both the young and old + GDF11 groups than the old + empty-V group ($n = 4$/group). **d** Representative micrographs of immunofluorescent staining for p16 (*red*) with nuclei stained *blue* with DAPI. White *arrows* indicate $p16^+$ cells. **e** The number of $p16^+$ cells was significantly lower in both the young and the old + GDF11 groups than in the old + empty-V group ($n = 5$/group). $**p < 0.01$

level of GDF11 in mice from either young or old groups [22]. Therefore, the premise of reduced circulating GDF11 as an underlying contributor to the aging process in older animals could not be validated in mice. Furthermore, when circulating GDF11 was elevated by daily injections of a recombinant form of GDF11 for 4 weeks, no significant effect could be detected in the aged mouse hearts in terms of weight, myocyte size, contractile function, and pathological gene induction [22]. However, recent research led by Loffredo et al. [17] and Poggioli et al. [19] supported the previous finding that circulating levels of GDF11/8 decline with age in mice, rats, horses and sheep. They also showed by Western blot analysis that the apparent age-dependent increase in GDF11 levels, as reported by Egerman et al. [4], is due to cross-reactivity of the anti-GDF11 antibody with an immunoglobulin which is known to increase with age. They found that GDF11 administration in mice rapidly activated SMAD2 and SMAD3 signaling in myocardium in vivo and decreased cardiac mass in both young (2 month old) and old (22 month old) mice in a dose-dependent manner after only nine days [19]. They confirmed an age-dependent decline in serum GDF11/8 levels in multiple mammalian species and that exogenous GDF11 rapidly activates SMAD signaling and reduces cardiomyocyte size [19].

Despite the controversy surrounding the level of circulating GDF11 and the influence of GDF11 on rejuvenation, we think that the currently adopted therapy of systemic delivery of GDF11 through intraperitoneal injection of recombinant protein may not be an ideal method for rejuvenation of a specific organ. Instead, targeted delivery of GDF11 to the organ of interest may be a more suitable method for clinical applications. We have previously proven this technique provides non-invasive, safe, repeatable, and targeted gene delivery to the infarcted myocardium [6, 7]. We demonstrated that UTMD-mediated delivery of GDF11 specifically increased GDF11 levels in the heart. We believe that targeted delivery of GDF11 resulted in efficient and specific elevation of GDF11 in the heart and rejuvenated the aged heart.

Previous in vitro and in vivo findings strongly suggest that cardiac-resident stem cells appear to function as intracardiac modulators of cardiac repair that become functionally deficient with increasing age [2, 13, 16, 25, 26]. CSCs in the young heart promote the generation of an adequate supply of differentiated progeny [12], while CSCs in the aged heart express increased levels of senescence-associated proteins which reduce the number of functionally competent CSCs [9, 13]. Endogenous cardiac repair could be limited by the decrease in functionally competent CSCs, but potentiated by improvement in functionally competent CSCs. Indeed, previous studies have shown that while premature senescence of cardiac progenitors and their progeny caused by doxorubicin treatment may be responsible for the decline in the regenerative capacity of the heart and may represent the cellular basis of doxorubicin-induced cardiomyopathy in humans [18], potentiation of cardiac progenitor cell reparative capacity by Notch activation decreased cellular senescence and enhanced preservation of cardiac function [10]. In the current study, UTMD-mediated delivery of GDF11 increased the number of cardiac c-kit$^+$ and Sca-1$^+$ stem cells by 1.5- and 2.4-fold, respectively, after *I/R* injury in old mice, indicating that a larger fraction of myocyte-committed c-kit$^+$ and Sca-1$^+$ cells was activated. Moreover, the fraction of BrdU$^+$ cells increased 1.2-fold following UTMD-mediated delivery of GDF11, indicating that GDF11 can also stimulate overall cardiac progenitor cell proliferation after *I/R* injury which may be one of the reasons responsible for the improved cardiac function.

Considering the role of c-kit signaling in the mobilization of EPCs [11], it is possible that this angiogenic switch is mediated by EPCs that are quickly recruited to areas of pathological angiogenesis [1]. Angiogenesis is an important aspect of granulation repair tissue. Our previous study showed that bone marrow c-kit$^+$ cells act as important regulators of the angiogenic switch in the infarcted myocardium, thereby driving efficient cardiac repair [5]. In this experiment, we showed that delivery of GDF11 resulted in a 2.3-fold increase in the number of homed EPCs and 2.1- and 1.9-fold increases in capillary and arteriole densities, respectively, in the border zone after *I/R* injury. Conversely, the expression levels of p16 and p53, two cell senescence markers, decreased on average by 2.1- and 2.4-fold, respectively, in mice that received UTMD-mediated delivery of the GDF11 plasmid. These findings indicate that UTMD-mediated delivery of GDF11 not only stimulated CSC proliferation, but also decreased the expression of senescence-associated proteins, in turn promoting the homing of EPCs and angiogenesis. These cellular and physiological changes induced by UTMD-mediated GDF11 therapy protected the aged heart from ischemic injury.

In conclusion, our findings show that GDF11 expression declines with age and the protective effects of UTMD-mediated delivery of GDF11 on the aged ischemic heart provide support for the classification of GDF11 as an anti-aging factor. UTMD-mediated delivery of the GDF11 plasmid produced a targeted increase in GDF11 levels in the heart without affecting levels in other organs. Increased GDF11 expression in the aged ischemic heart was associated with better cardiac function and a smaller infarct size as well as increased CSC proliferation and decreased cell senescence post-injury. Thus, UTMD-mediated targeted delivery of GDF11 effectively

rejuvenated the senescent heart and protected it from ischemic injury.

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Compliance with ethical standards

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

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