Activation of growth hormone releasing hormone (GHRH) receptor stimulates cardiac reverse remodeling after myocardial infarction (MI)

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Both cardiac myocytes and cardiac stem cells (CSCs) express the receptor of growth hormone releasing hormone (GHRH), activation of which improves injury responses after myocardial infarction (MI). Here we show that a GHRH-agonist (GHRH-A; JI-38) reverses ventricular remodeling and enhances functional recovery in the setting of chronic MI. This response is mediated entirely by activation of GHRH receptor (GHRHR), as demonstrated by the use of a highly selective GHRH antagonist (MIA-602). One month after MI, animals were randomly assigned to receive: placebo, GHRH-A (JI-38), rat recombinant GH, MIA-602, or a combination of GHRH-A and MIA-602, for a 4-wk period. We assessed cardiac performance and hemodynamics by using echocardiography and micromanometry derived pressure-volume loops. Morphometric measurements were carried out to determine MI size and capillary density, and the expression of GHRHR was assessed by immunofluorescence and quantitative RT-PCR. GHRH-A markedly improved cardiac function as shown by echocardiographic and hemodynamic parameters. MI size was substantially reduced, whereas myocyte and nonmyocyte mitosis was markedly increased by GHRH-A. These effects occurred without increases in circulating levels of growth hormone and insulin-like growth factor I and were, at least partially, nullified by GHRH antagonism, confirming a receptor-mediated mechanism. GHRH-A stimulated CSCs proliferation ex vivo, in a manner offset by MIA-602. Collectively, our findings reveal the importance of the GHRH signaling pathway within the heart. Therapy with GHRH-A although initiated 1 mo after MI substantially improved cardiac performance and reduced infarct size, suggesting a regenerative process. Therefore, activation of GHRHR provides a unique therapeutic approach to reverse remodeling after MI.

heart failure | cardiac stem cells | cardiac regeneration

Mortality from cardiovascular disease has decreased over time as therapeutic advances have become more elaborate. Despite this progress, no current treatment fully reverses the primary cause of impaired heart function, the loss of cardiomyocytes. Because the worldwide prevalence of heart failure (HF) continues to increase, any intervention that improves this condition would promise a beneficial clinical outcome and should be further explored (1, 2).

Expression of mRNA for growth hormone releasing hormone (GHRH) has been detected in extrapituitary tissues, including the heart (3, 4), consistent with widespread biologic signaling beyond the hypothalamic pituitary axis. Recently, it was shown that the heart harbors GHRH receptors (GHRHRs) that can be activated by GHRH (1–44) (5) and synthetic GHRH agonists (6), suggesting that the ischemic heart is also a target of GHRH signaling. However, our current knowledge on the role of GHRH signaling in the heart is still limited. Granata et al. (5) reported that GHRH (1–44) promoted survival of cardiomyocytes and protected rat hearts from ischemia-reperfusion injury. Subsequently, we demonstrated that a potent GHRH agonist (GHRH-A; JI-38) stimulated substantial cardiac repair after acute ischemic injury in a rodent model independently of growth hormone (GH) or insulin-like growth factor I (IGF-I) (6). JI-38 is a synthetic analog of human GHRH with high activity and greater metabolic stability due to incorporation of amino acid substitutions that increase the peptide's resistance to degradation (7).

Experimental and clinical studies aimed at developing either GH or IGF-I therapeutically have met so far with mixed results. In addition, compounds in this class can produce side effects such as fluid retention, hypertension, arrhythmias, risk of diabetes, and increased body weight. Accordingly, activation of the cardiovascular GHRH/GHRHR axis has the potential to exert beneficial effects that are based on direct receptor action and can eliminate the side effects of GH or IGF-I (8, 9).

Whereas our previous results demonstrated the efficacy of GHRH-A at preventing remodeling, it remains important to assess whether this effect could actually reverse remodeling of the chronically injured heart. Therefore, we tested the hypothesis that activation of GHRHR in the heart using a potent GHRH-A can reverse ventricular remodeling and improve cardiac performance after chronic cardiac injury. We used a selective GHRH antagonist (MIA-602) to inhibit or block these actions to test whether the effects of the agonist are fully receptor mediated.

Results

As illustrated in *SI Appendix*, Fig. S1, body weights (BW) were similar for all treatment groups at baseline. BW at week 8 was increased by treatment with rat recombinant GH (rrGH) ($P < 0.01$), GHRH-A, and GHRH (A+Ant) ($P < 0.05$) in comparison with placebo and MIA-602; however, heart weight (HW), HW/BW, and HW/tibia length (HW/TL) ratios did not change.

GH and IGF-I Levels. The circulating levels of GH (*SI Appendix*, Fig. S2*A*) were similar in all groups, except the rrGH group. As expected, administration of rrGH substantially increased serum levels of GH ($P < 0.0001$ vs. all other groups). Surprisingly, IGF-I (*SI Appendix*, Fig. S2*B*) was increased in both rrGH and GHRH (A+Ant) groups ($P < 0.0001$ vs. placebo, GHRH-A, and MIA-602 groups) but without increases in GH level in the latter one.

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The authors declare no conflict of interest.

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Expression of GHRHR. The expression of GHRHR in isolated cardiac myocytes assessed by immunostaining (*SI Appendix*, Fig. S3) was substantially increased in GHRH-A and rrGH groups [$P <$ 0.05 vs. placebo, GHRH (A+Ant), and MIA-602]. In addition, RT-PCR (*SI Appendix*, Fig. S4) also revealed an overexpression of GHRHR in GHRH-A and rrGH groups ($P < 0.05$ vs. placebo).

Impact of GHRHR Activation on Ventricular Remodeling. Baseline echocardiography documented similar parameters of LV dimension and function in all groups (Fig. 1 and *SI Appendix*, Table S1). Importantly, at the time of treatment (30 d after MI), cardiac function was markedly impaired relative to baseline, indicating the development of ventricular dysfunction after MI; all echocardiographic parameters revealed similar degrees of global LV functional deterioration and ventricular chamber enlargement in all groups at the time of treatment.

Between the 4 and 8 wk evaluation, LVEDD (Fig. 1*A*) and LVESD (Fig. 1*B*) progressively increased in the placebo, rrGH, and GHRH (A+Ant) groups. This increase was prevented by GHRH-A (*SI Appendix*, Table S1). Moreover, the reduction in ejection fraction (EF) (Fig. 1*C*) due to MI was restored toward normal by ≈22% with the administration of GHRH-A [$P < 0.05$ vs. all other groups. Administration of MIA-602 blocked the favorable effects of GHRH-A on ventricular chamber size and EF.

Impact of GHRHR Activation on Cardiovascular Performance. Fig. 2*A* and *SI Appendix*, Table S2 and Fig. S5 summarize analyses of hemodynamic parameters derived from pressure-volume loops in steady-state and loading conditions just before euthanasia. Load-dependent parameters of systolic function such as stroke volume (SV) and cardiac output (CO) were markedly increased by treatment with GHRH-A [$P < 0.01$ vs. placebo, GHRH (A+Ant), and MIA-602 groups]. The improvement in cardiac performance was, at least partially, due to significant reduction in ventricular afterload (Ea), $P < 0.05$ vs. placebo and MIA-602.

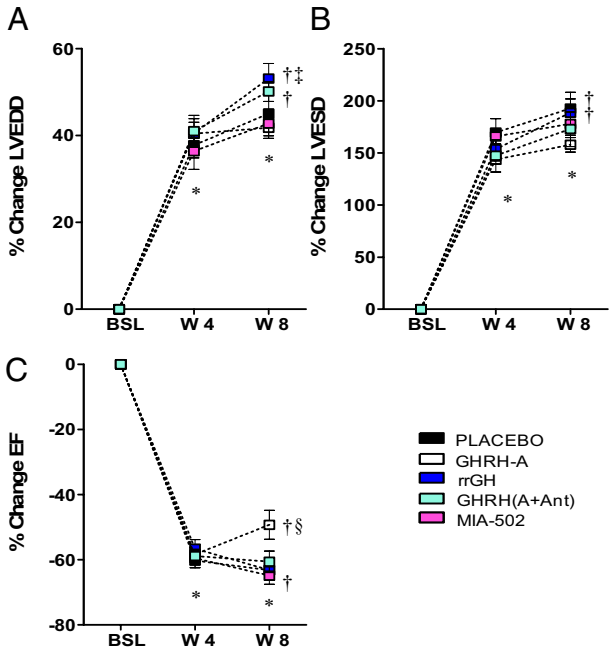

Fig. 1. Impact of treatments on ventricular remodeling. Graphs correspond to changes over time in LV end-diastolic (LVEDD) (*Upper Left*), end-systolic (LVESD) diameters (*Upper Right*), and ejection fraction (EF) (*Lower*). All values represent mean ± SEM (n = 7–10), *$P < 0.05$ vs. baseline (BSL), same group; †$P < 0.05$ vs. wk 4 (W4), same group; ‡$P < 0.05$ vs. all other groups at week 8 (W8), except GHRH (A+Ant). §$P < 0.05$ vs. all other groups at wk 8.

In addition, LV end-diastolic pressure (LVEDP) was also reduced by therapy with GHRH-A ($P < 0.05$ vs. placebo). In agreement with our echocardiographic data, GHRH-A led to a sustained improvement of myocardial function, as determined by EF. Moreover, preload recruitable stroke work (PRSW) trended to be higher only in the GHRH-A group ($P = 0.0547$).

Impact on Scar Size, Capillary Density, and Cell Survival. MI size was similar in all groups (Fig. 2*B*), except in rats treated with GHRH-A, which showed a significant scar reduction ($P < 0.05$ vs. all other groups). Capillary density (*SI Appendix*, Fig. S6) at the MI border zone was increased in all treated groups, but to a greater extent in the GHRH-A group ($P < 0.0001$ vs. placebo and MIA-602), whereas at the areas remote to MI, there were no differences. Apoptotic cells were detected by TUNEL assay (*SI Appendix*, Fig. S7). Overall, none of the treatments significantly reduced the expression of apoptotic cells at the chronic stage of MI.

Impact on Cellular Division, Proliferation, and Differentiation. The number of cardiac stem cells (c-kit^{pos}) was not different among groups but there was a trend for them to be higher after GHRH-A therapy and, as shown in Fig. 3, the majority of c-kit^{pos} cells were not bone marrow derived; these cells were CD45^{neg} and tryptase^{neg} and were clearly distinguishable from resident cardiac mast cells (c-kit^{pos} CD45^{pos}). Thus, similar to our previous study, the quantity of mast cells was minute.

In addition, the presence of cellular mitosis was determined by the nuclear localization of phospho-histone H_3 (pH_3). Our results showed that the expression of pH_3^{pos} cells, including myocytes and nonmyocytes, was significantly higher in the rats treated with GHRH-A and rrGH at the infarct border zone (Fig. 4).

Next, we determined the impact of GHRH-A activity on cardiac stem cells (CSCs) division in vitro by incorporation of the thymidine analog EdU during S phase of the cell cycle. Our data (Fig. 5) showed an increase in the proliferation of CSCs after pretreatment with GHRH-A ($P < 0.05$ vs. placebo, Student's t test), whereas other treatments did not show a difference. Importantly, we also confirmed by fluorescence-activated cell sorting (FACS) analysis that CSCs express the GHRH-R (*SI Appendix*, Fig. S8).

As illustrated in *SI Appendix*, Fig. S9, the expression of the transcription factor GATA-4 at the border zone was increased by therapy with GHRH-A or rrGH [$P < 0.05$ vs. placebo, MIA-602, and GHRH (A+Ant) groups].

Effects of Treatments at the Molecular Level. Gene expression analysis after treatments revealed increased levels of several genes (*SI Appendix*, Fig. S4). Real-time PCR values of the relative expression of mRNA for anti- and proapoptotic genes (Bcl_2 and Bax, respectively) were significantly increased in all treated groups in comparison with the placebo group. Stromal derived factor 1 (SDF-1) was up-regulated only in GHRH-A and GHRH (A+Ant) groups, whereas stem cell factor was overexpressed in GHRH-A, MIA-602, and GHRH (A+Ant) groups. Therapy with GHRH-A and rrGH also increased the expression of vascular endothelial factor A (VEGF-A) mRNA. Interestingly, mRNA for IGF-I was only up-regulated in the combination group. Cyclin A_2 expression was highly increased by GHRH-A therapy and, importantly, down-regulated by MIA-602 treatment.

mRNA for GHRH was up-regulated in all treated groups, but not in the rrGH-treated rats. However, mRNA for GHRHR showed overexpression in the groups treated with GHRH-A, rrGH, and GHRH (A+Ant) but not with MIA-602.

Discussion

The results of this study indicate that the administration of GHRH-A in a chronic stage of myocardial injury can reverse ventricular remodeling and improve cardiac performance.

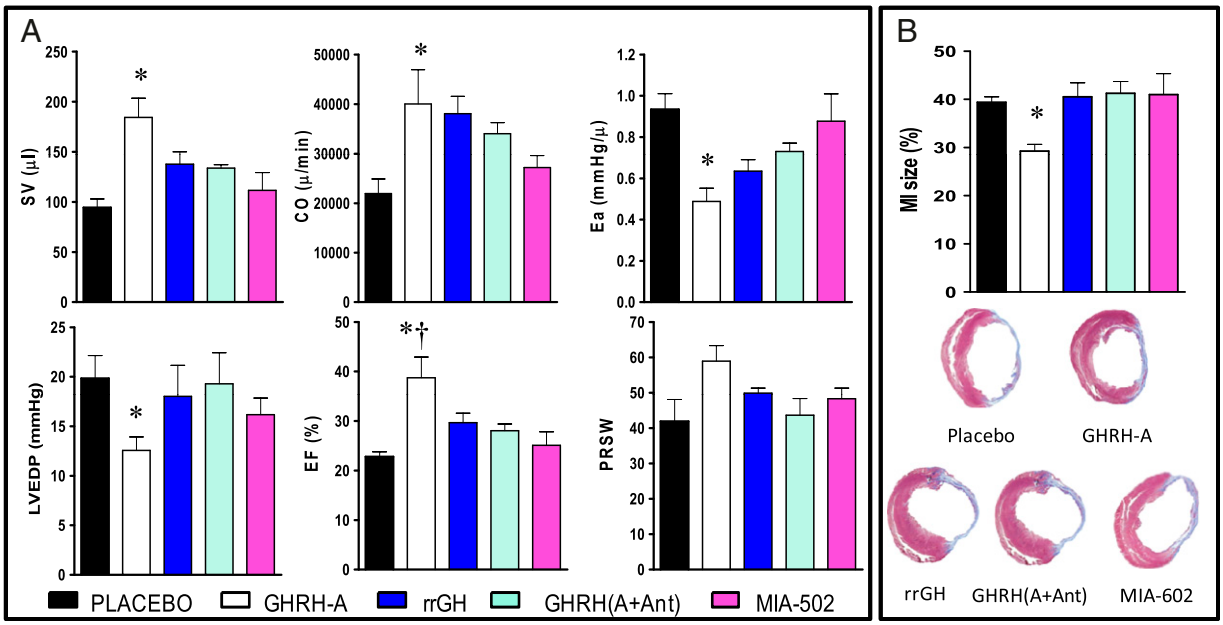

Fig. 2. Hemodynamic parameters derived from pressure-volume loops (*A*) and infarct size (*B*). *A* shows stroke volume (SV), cardiac output (CO), arterial elastance (Ea), $^{*}P < 0.05$ vs. placebo and MIA-602; LV end-diastolic pressure (LVEDP), $^{*}P < 0.05$ vs. placebo (Student's *t* test); ejection fraction (EF), $^{*}P < 0.01$ vs. placebo and MIA-602; $^{\dagger}P < 0.05$ vs. GHRH (A+Ant); preload recruitable stroke work (PRSW). All values represent mean ± SEM (n = 5–7). (*B*) MI size was significantly reduced by GHRH-A therapy, $^{*}P < 0.05$ vs. all groups (n = 7–10). At the bottom, representative Masson's trichrome-stained sections of each group at midventricular level.

Concurrently, infarct size was significantly reduced and, more importantly, none of these effects were accompanied by increases in circulating levels of GH or IGF-I, and were, for the most part, abrogated by the GHRH-antagonist (MIA-602). Our in vivo and in vitro findings confirm that the effects observed are receptor mediated, and importantly suggest that the effects result from a multifactorial mechanism involving cell-cycle reentry, angiogenesis, and likely cardiac stem cell activation. Together these data support the possibility of a unique therapeutic principle for chronic ischemic HF.

To determine the cardioprotective mechanisms of GHRH-A, capillary density, rate of apoptosis, cell cycle activity (mitosis), and expression of cardiac c-kit^{pos} cells and genes related to these pathways were studied (6, 10, 11). Our previous study in acute MI (6) showed that beneficial functional effect of GHRH-A therapy were mediated mainly through reduction of fibrosis, apoptosis, and recruitment of c-kit^{pos} cells—effects that were associated with myocardial regeneration. In the current study, we documented a similar reduction in scar size and improvement in EF. At a cellular level, we observed a trend in c-kit^{pos} cell increase in vivo, but documented that GHRH-A stimulated augmentation of CSCs proliferation ex vivo. Importantly, cell cycle reentry of myocytes was also augmented by GHRH-A in vivo, suggestive of an increase in the pool of transient-amplifying cells originating from CSCs (11). The direct stimulation of CSCs by GHRH-A is further supported by documentation of the GHRHR on these stem cells by FACS.

The origin of cardiac c-kit cells could represent either circulating cells likely originating in bone (12, 13) or cardiac stem cells

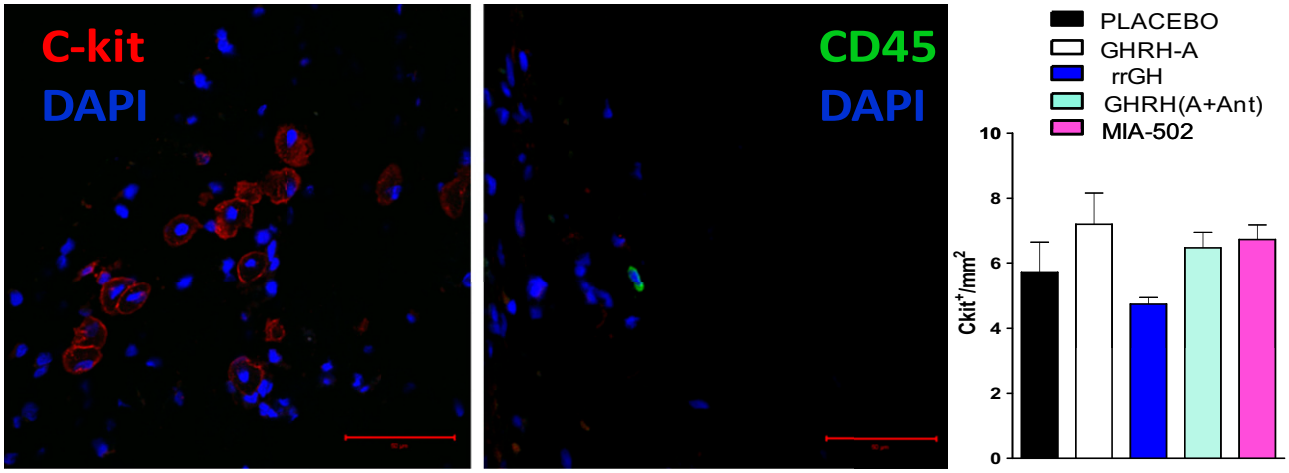

Fig. 3. Representative confocal micrograph image of c-kit^{pos} (red), CD45^{pos} (green), and nuclei (blue). (Scale bar: 50 μm.) (*Right*) Bar graph corresponds to expression of c-kit^{pos} cells in the heart (n = 3–4).

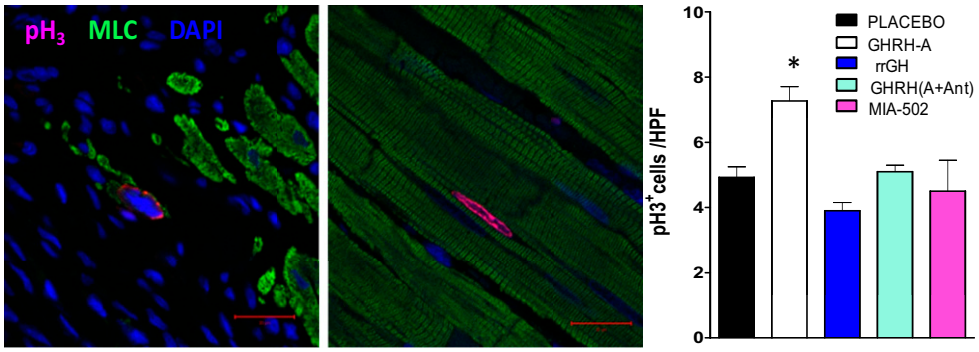

Fig. 4. Immunostaining analysis of mitosis in heart tissues by phospho-histone H_3 (pH_3). Representative confocal micrograph images of pH_3 (magenta), myosin light chain (MLC, green), and nuclei (DAPI, blue). (Scale bar: 20 μm.) Bar graph corresponds to expression of pH_3^{pos} cells at the border zone. Data represent mean ± SEM (n = 3), *P < 0.05 vs. placebo and rrGH groups.

(11, 14). Our costaining of these cells with CD45 was able to differentiate them from hematopoietic origin, and, also most likely cardiac mast cells (15). Thus, the present trend toward an increase in c-kit cells in vivo, accompanied by an increase in pH_3 cells, and the ex vivo results demonstrating that GHRH-A augments CSCs proliferation, all support the notion that GHRH-A's stimulate cardiac stem cell mediated tissue repair.

Our data also showed an increased expression of the transcription factor GATA-4 in response to GHRH-A, which is known to play important roles in regulating cell differentiation, proliferation, organ morphogenesis and, noted more recently, in regulation of apoptosis (16). Data by Rysa et al. (17) suggested that GATA-4 is an antiapoptotic factor required for adaptive responses and a key regulator of hypertrophy and hypertrophy-associated genes in the heart. Moreover, the reversal of reduced GATA-4 activity prevented adverse postinfarction remodeling through myocardial angiogenesis, antiapoptosis, and stem cell recruitment (17, 18).

We measured the expression level of various genes to gain further insights into the molecular underpinnings of our findings. Importantly, supporting the finding that GHRH-A stimulates cellular regeneration, we found increased levels of cyclin A_2 mRNA in these hearts, and that this effect was blocked in the group receiving MIA-602. Previous work by Cheng et al. (19) demonstrated that cyclin A_2 induces cardiac regeneration after MI and prevents HF through induction of a side population of cells with enhanced proliferative capacity.

We also tested the possibility that increased release of soluble factors (paracrine effects) contributed to the improvement in functional cardiac performance in the heart (20–23). This proposition is compatible with the results of Gnecchi et al. (24), which have implicated growth factors, such as VEGF-A, IGF-I, and basic fibroblast growth factor, in post-MI cardiac repair. In support of this paracrine hypothesis, our results revealed that the mRNA level for VEGF-A is significantly up-regulated in both GHRH-A and rrGH-treated rats relative to the placebo group. Higher expression of VEGF-A has also been associated with better collateral circulation development after ischemia (25, 26) and, consequently, better clinical outcome. More recently, Tang et al. (27) reported that activation of VEGF/SDF-1 signaling promotes myocardial repair at least in part through cardiac stem cell mobilization. SDF-1 is well known as a homing factor for a variety of stem cell populations and, therefore, for improving cardiac function post-MI (28, 29). This finding is also consistent with our results, which show up-regulation of SDF-1 in the GHRH-A and combination-treated groups.

Surprisingly, mRNA for IGF-I was only increased in the combination group, whereas IGF-I levels in the serum were increased in both rrGH and combination-treated rats. We postulated that the discrepancies observed in the circulating levels of GH and IGF-I in the combination group can be explained by the fact that stimuli other than GH, such as prolactin (PRL), platelet-derived growth factor, or nutritional status, can also lead to an increase in IGF-I synthesis (30, 31). Dardenne et al. (32) reported that cells expressing GH receptor also express receptors for PRL. Because the effect of administration of GHRH antagonist was not completely abolished in the combination group, one could speculate that the interval (5 min) between the administration of GHRH antagonist (MIA-602) and agonist (GHRH-A) presumably was not adequate to block or attenuate the effects of GHRH-A (JI-38); however, in fact, GH level was not increased in this group.

The impact of GHRH-A's on the GH/IGF-I axis warrants mention. Physiologically, spikes in GH secretion result from pulsatile release of GHRH. We administered s.c. a GHRH-agonist analog twice daily for 4 wk, and elevations in circulating GH and IGF-I levels did not occur. However, the agonist would be expected to produce a GH spike starting 15–30 min after injection and lasting for 30–60 min (i.e., 60–90 min after injection), a measurement at 12–24 h would not detect this elevation.

The current study is limited because the pharmacokinetics of the agonist are not fully characterized. Our results clearly indicate that the half-life of JI-38 is adequate to stimulate the reported myocardial processes. Indeed, as our results indicate, the doses

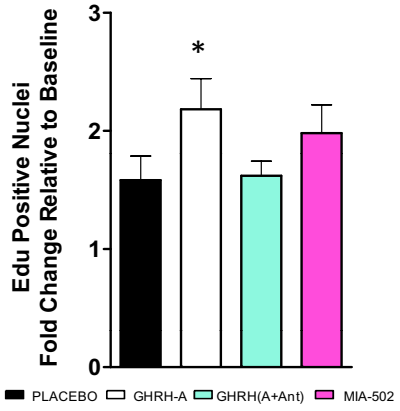

Fig. 5. Impact of GHRH-A activity in cell division of cardiac stem cells (CSCs). Values represent fold change (mean ± SEM) of incorporation of the thymidine analog EdU for 16 h relative to baseline. GHRH-A treatment significantly increased the rate of DNA synthesis relative to vehicle (DMSO), *P < 0.05, Student's t test (n = 5–8).

delivered failed to activate the endocrine GH–IGF-I axis. Additional work will be required to clarify the pharmacodynamics, pharmacokinetics, metabolism, and breakdown products of JI-38.

Collectively, our findings demonstrate that GHRH-A therapy, when initiated 1 mo after MI once ventricular remodeling has already occurred, substantially improves the degree of cardiac dysfunction and reduces infarct size, suggesting that the regenerative process is still potentially activated at this late stage, and these benefits are independent on the GH or IGF-I. Together our findings support the possibility that the use of potent GHRH agonists could have therapeutic benefits in patients with acute MI, chronic ischemic heart disease, and possibly a broader array of diseases of heart muscle.

Materials and Methods

Animal Model. All experiments involving rats were carried out in accordance with protocols reviewed and approved by the University of Miami Animal Care and Use Committee in compliance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health publication no. 85–23, revised 1996).

MI was induced by permanent ligation of the left coronary artery in female 6-mo-old Fisher-344 rats as described (33). Animals were randomly assigned to receive placebo (DMSO + propylene glycol), GHRH-agonist (GHRH-A [JI-38]; 50 μg/kg), GHRH-antagonist (MIA-602; 50 μg/kg), combination of GHRH-A plus GHRH-antagonist [GHRH (A+Ant), same dose as for each treatment alone] or rat recombinant GH (rrGH; 0.5 mg/kg) starting 4 wk after MI. All treatment was given s.c. twice daily for 4 wk.

Drugs. rrGH was supplied by Dr. A. F. Parlow from National Hormone and Pituitary Program (University of California-Harbor, Torrance, CA) and GHRH-A (JI-38) and GHRH-antagonist (MIA-602) were synthesized in the laboratories of A.V.S. (7, 9, 34). For additional information, see *SI Appendix, SI Materials and Methods*.

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