Profound Actions of an Agonist of Growth Hormone–Releasing Hormone on Angiogenic Therapy by Mesenchymal Stem Cells

QunChao Ma,* Xiangyang Xia,* Quanwei Tao, Kai Lu, Jian Shen, Qiyuan Xu, Xinyang Hu, Yaoliang Tang, Norman L. Block, Keith A. Webster, Andrew V. Schally, Jian’an Wang, Hong Yu

Objective—The efficiency of cell therapy is limited by poor cell survival and engraftment. Here, we studied the effect of the growth hormone–releasing hormone agonist, JI-34, on mesenchymal stem cell (MSC) survival and angiogenic therapy in a mouse model of critical limb ischemia.

Approach and Results—Mouse bone marrow–derived MSCs were incubated with or without 10⁻⁸ mol/L JI-34 for 24 hours. MSCs were then exposed to hypoxia and serum deprivation to detect the effect of preconditioning on cell apoptosis, migration, and tube formation. For in vivo tests, critical limb ischemia was induced by femoral artery ligation. After surgery, mice received 50 μL phosphate-buffered saline or with 1×10⁶ MSCs or with 1×10⁶ JI-34–reconditioned MSCs. Treatment of MSCs with JI-34 improved MSC viability and mobility and markedly enhanced their capability to promote endothelial tube formation in vitro. These effects were paralleled by an increased phosphorylation and nuclear translocation of signal transducer and activator of transcription 3. In vivo, JI-34 pretreatment enhanced the engraftment of MSCs into ischemic hindlimb muscles and augmented reperfusion and limb salvage compared with untreated MSCs. Significantly more vasculature and proliferating CD31⁺ and CD34⁺ cells were detected in ischemic muscles that received MSCs treated with JI-34.

Conclusions—Our studies demonstrate a novel role for JI-34 to markedly improve therapeutic angiogenesis in hindlimb ischemia by increasing the viability and mobility of MSCs. These findings support additional studies to explore the full potential of growth hormone–releasing hormone agonists to augment cell therapy in the management of ischemia.

Key Words: angiogenesis effects ■ growth hormone–releasing hormone ■ mesenchymal stromal cells

Cell-based therapies show promise to promote regeneration and angiogenesis and reverse or rescue tissues injured by ischemia or infarct. In this context, various kinds of progenitor cells have been tested clinically, including cardiac progenitor cells, endothelial progenitor cells, induced pluripotent stem cells, embryonic stem cells, bone marrow mononuclear cells, mesenchymal stem cells (MSCs), with mixed outcomes. MSCs have the advantages of immune privilege, stemness, and ease of handling relative to other cell types and have become prominent vehicles for a wide range of indications. The mechanism of MSC action is thought to be primarily paracrine. When exogenously administered, MSCs can facilitate the formation of new capillaries and medium-sized arteries. However, therapeutic angiogenesis by MSCs is limited by poor survival especially in the hostile microenvironment of ischemic tissue. Many different approaches have been attempted to improve the efficacy of MSC therapy, including pretreating with various chemicals or polypeptides, pre-conditioning with physiological stimuli such as hypoxia, or combination with other cells including endothelial progenitor cells and endothelial cells. Optimal conditions have not been achieved.

Growth hormone–releasing hormone (GHRH) is a neuropeptide produced by the hypothalamus. It is carried to the anterior pituitary through the portal vessels and stimulates the release of growth hormone after binding to the GHRH receptor (GHRH-R) on cell membranes. Previous studies have shown that GHRH and its receptor are also expressed on cells...
in normal human tissues, for example, liver, kidney, lung, and prostate, as well as many tumors. GHRH and its agonists have been used to treat disorders of the endocrine system, as well as to promote cell proliferation and functional recovery in extrapituitary tissues. Compared with the native GHRH, synthetic agonists are much more potent and longer acting because of increased stability. One such GHRH agonist, JI-38, has been used to accelerate wound healing primarily by acting on wound-associated fibroblasts. The systemic administration of GHRH agonists has been shown to stimulate the recovery of cardiac function after infarction or ischemia/reperfusion injury by reducing apoptosis of cardiomyocytes, enhancing the recruitment of endogenous cardiac stem cells, stimulating angiogenesis in heart, and ameliorating cardiac remodeling.

Because GHRH-R is expressed in MSCs, we hypothesized that pretreatment of MSCs with such agonists may improve therapeutic properties of MSCs. Here, we demonstrate that JI-34, a potent GHRH agonist, indeed improves MSC proliferation and homing and, thus, augments therapy by promoting angiogenesis in ischemic skeletal muscle of mouse hindlimb.

**Materials and Methods**

Materials and Methods are available in the online-only Data Supplement.

**Results**

**GHRH-R Is Expressed on Mouse Bone Marrow–Derived MSCs**

GHRH-R expression on mouse bone marrow–derived MSCs was detected by Western blot (Figure 1A), flow cytometry (Figure 1B), and immunofluorescence staining (Figure 1A in the online-only Data Supplement). The results from both Western blot and immunofluorescence staining demonstrate that MSCs express levels of GHRH-R that are similar to the brain, whereas skeletal muscle was negative for GHRH-R expression. Flow cytometry showed that the mean fluorescence intensity of GHRH-R on MSCs was 224.3±34.6 (Figure 1B), which was not significantly changed after cells were treated with GHRH agonist, JI-34 (Figure 1B and IC in the online-only Data Supplement). These results were further confirmed by Western blot assay (Figure ID in the online-only Data Supplement). These data confirm that GHRH-R is expressed on MSCs.

**GHRH Agonist Preconditioning Enhances Proliferation and Survival of MSCs**

To investigate the effects of JI-34 on cell proliferation and viability, we treated MSCs with increasing concentrations of JI-34 for 48 hours and quantified resulting cell numbers. Figure 1C shows that the proliferation of MSCs was optimally enhanced (114±8%) by JI-34 at a concentration of 1×10⁻⁸ mol/L. The viability of MSCs after culture under hypoxia (0.5% O₂) with serum deprivation for 48 hours was significantly increased by JI-34 pretreatment when compared with the untreated MSCs (Figure 1D). Augmentation of MSC proliferation and viability was optimal at JI-34 concentration of 1×10⁻⁸ mol/L; therefore, this concentration was used for further studies.

Apoptosis of MSCs after culture under the hypoxia with serum deprivation condition for 48 hours was determined by flow cytometric analysis of Annexin V⁺ cells (Figure 1E). The rate of apoptosis was reduced from 50.3%±9.1 to untreated cells to 32.9%±7.5 when the cells were pretreated with JI-34, whereas cytoprotection by JI-34 treatment was blocked by cotreatment with GHRH antagonist, MIA-602 (Figure 1F).

**JI-34 Increased Phosphorylation and Translocation of STAT3 in MSCs**

Signal transducer and activator of transcription 3 (STAT3) plays an important role in the promotion of angiogenesis, cell survival, and proliferation. To evaluate potential downstream GHRH-R signaling, the activation of STAT3 was examined. Treatment of MSCs with JI-34 resulted in an increase of STAT3 phosphorylation (Figure 2A). The ratio of phosphorylated STAT3 compared with total STAT3 (P-STAT3/T-STAT3) was significantly increased by 5 minutes of treatment with JI-34 and reached a plateau at 30 minutes (Figure 2B). Immunofluorescence staining of STAT3 (Figure 2C) revealed that the majority of STAT3 was in the cytoplasm in control cultures but translocated to the nucleus after treatment with JI-34. Translocation was blocked by GHRH antagonist, MIA-602 (Figure 2D). To further investigate the possible targets of JI-34, the expression of c-myc, a downstream target of STAT3, was analyzed. The expression of c-myc transcripts in MSCs was increased 200-fold by JI-34 treatment, and this was blocked by GHRH-R antagonist, MIA-602 (Figure 2D in the online-only Data Supplement). Similarly, c-myc protein was upregulated by JI-34 (Figure IIB in the online-only Data Supplement).

**JI-34 Enhances Mobility and Proangiogenic Activity of MSCs In Vitro**

In vitro transwell migration assays (Figure 3A) showed that the mobility of MSCs was significantly increased after the cells were treated with JI-34, and this was blocked by GHRH antagonist, MIA-602 (Figure 3B). To further characterize the possible paracrine effect of MSCs treated with JI-34, MSC-conditioned medium was used to culture human umbilical vein endothelial cells and endothelial tube formation was measured using a Matrigel assay (Figure 3C) as described in Materials and Methods. Tube formation was significantly enhanced by conditioned media from JI-34–treated MSCs (Figure 3D). When the GHRH antagonist, MIA-602, was present during the treatment, the effect of JI-34 on probe tube formation of human umbilical vein endothelial cell was abolished. Contributions of altered gene expression in JI-34–treated MSCs were assessed by quantifying a panel of transcriptional targets.
of angiogenesis-related genes (Table 1 in the online-only Data Supplement). The mRNA levels of vascular endothelial growth factor-A and stromal-derived factor-1 were increased by 9-fold and 2.5-fold, respectively, by treatment with JI-34 (Figure 3F). This effect was blocked by GHRH antagonist, MIA-602, or WP1066, a STAT3 inhibitor. Meanwhile, we also observed that the protein expression level of hepatocyte growth factor, vascular endothelial growth factor, and stromal-derived factor-1 was increased in MSC pretreated with JI-34, and this effect could be abolished by the addition of MIA-602 (Figure 3E).

In addition, we studied the effects of another novel GHRH-R agonist, MR-409. Compared with JI-34, MR-409 is superior in stability and activity.18 MSC proliferation and cell survival were significantly enhanced by treatment with MR-409 at $10^{-8}$ mol/L (Figure III in the online-only Data Supplement). MSC migration was enhanced by MR-409, and the effect was also suppressed by MIA-602 or STAT3 inhibitor, WP1066 (Figure IV in the online-only Data Supplement). These results indicate that treatment with MR-409 parallels that of JI-34.

**GHRH Agonist Augments Survival of MSCs In Vivo**

To investigate homing and engraftment of MSCs in ischemic muscle, transplantations of sex-mismatched and green fluorescent protein (GFP)-labeled cells were performed, and the number of retained donor cells in a recipient was examined by detecting the male-specific Sry and GFP genes on days 3, 7, and 14 after injection of cells into ischemic muscles. Significantly more donor cells were detected in the ischemic muscle of mice injected with MSCs preconditioned with JI-34 compared with untreated MSCs 3 and 7 days after cell transplantation, and a similar, but nonsignificant, trend was observed on day 14 post-transplantation (Figure 4A; Figure V in the online-only Data Supplement). This was confirmed by
tracking Dil-labeled MSCs (Figure VI in the online-only Data Supplement). In addition, the proliferation of transplanted MSCs was detected by Ki67/Dil costaining at day 7. No Ki67/Dil double-positive cells were detected in any groups, which indicated that JI-34 preconditioning did not promote the proliferation of MSCs in ischemic muscle (Figure VIIA in the online-only Data Supplement).

Figure 2. Activation of signal transducer and activator of transcription 3 (STAT3) in mesenchymal stem cell (MSC) by JI-34 pretreatment. A, Western blot analysis of STAT3 activation in lysates from MSC. Dynamic changes of STAT3 phosphorylation were observed at different time points. B, Expression of phosphorylated STAT3 was quantified as a ratio of phosphorylated STAT3 compared with total STAT3 by integrated optical density measurement (n=3). *P<0.05 vs control. C, Immunofluorescence staining for subcellular localization of STAT3 (green) in MSC cultured without additional agent (control) or with JI-34, JI-34+MIA-602, or MIA-602. Scale bars: 50 μm. D, Quantification of nuclear-localized STAT3 in C by a ratio of fluorescence in nucleus to cytoplasm (n=3 in each group). *P<0.05 vs control.

Figure 3. JI-34 pretreatment enhanced mesenchymal stem cell (MSC) migration and proangiogenic effect. A, Representative images of migration of MSC in a transwell assay. Scale bars: 200 μm. B, Quantification of migration of MSC. Cells that migrated to the lower chamber were counted (n=4 in each group). *P<0.05 vs others. C, Representative images showing tube formation of human umbilical vein endothelial cell on Matrigel cultured with conditioned media from the specified MSC. Scale bars: 100 μm. D, Quantification of tube formation in C by measuring branch lengths of formed tube. Only length >200 μm was counted. n=5, *P<0.05 vs others. E, Western blot and quantification of proangiogenic cytokines expressions in MSCs treated with JI-34 or MIA-602 for 24 hours. F, Real-time polymerase chain reaction was performed to detect the effect of JI-34 on mRNA expression for VEGF and SDF-1 within MSC (n=4). *P<0.05 vs control. GAPDH indicates glyceraldehyde 3-phosphate dehydrogenase; HGF, hepatocyte growth factor; SDF-1, stromal-derived-factor-1; and VEGF, vascular endothelial growth factor-A.
online-only Data Supplement). The apoptosis of engrafted MSCs was evaluated by terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate–nick end labeling and DiI costaining at day 3. Our results showed that compared with MSCs, apoptotic MSCs were less in MSC-JI group; however, the difference was not significant (Figure VIIB and VIIC in the online-only Data Supplement).

Pretreatment With JI-34 Enhances MSC Therapy in Ischemic Hindlimb

Reperfusion of ischemic mouse hind limbs was measured using laser Doppler perfusion imaging at intervals after femoral artery ligation (Figure 4B). Mice that received JI-34–conditioned MSCs recovered perfusion significantly faster than those that received untreated MSC or control groups (Figure 4C). Toe necrosis in ischemic limbs was also reduced in the JI-34–treated MSC group compared with that in the untreated MSC or control groups (Figure 4D and 4E).

Pretreatment of MSCs With JI-34 Augments Angiogenesis and Muscle Regeneration In Vivo

Muscle regeneration was analyzed by measuring central-localized nuclei in muscle cells of recovered tissues (Figure 5A). Administration of MSCs treated with agonist significantly increased the number of regenerating myofibers in ischemic gastrocnemius muscles 21 days after surgery (Figure 5B).

To investigate whether the recovery of blood perfusion was associated with angiogenic activity, capillary density of ischemic muscles was analyzed by CD31 immunostaining (Figure 5C). Capillary densities were significantly higher in mice that received preconditioned MSCs compared with those that received phosphate-buffered saline (PBS) or untreated MSCs on day 21 after injection (Figure 5D). Arteriole density in the ischemic muscles was also determined by immunostaining of smooth muscle \(\alpha\)-actin (Figure 5C). Transplantation of MSCs preconditioned with JI-34 also resulted in the highest arteriole density of ischemic muscle (Figure 5E). However, no direct differentiation of MSCs into endothelial cells was observed (Figure 5F).

Enhanced Angiogenesis Is Associated With Increased Proliferation of Endothelial Cells

To further explore the mechanism of augmented angiogenesis by MSCs, proliferation of endothelial cells was analyzed by immunostaining for Ki67 and CD31 in the ischemic muscles on day 7 after cell transplantation (Figure 6A). A robustly increased percentage of Ki67-positive cells (Figure 6B) and greater percentage of Ki67/CD31 double-positive cells (Figure 6C) was observed in mice that received JI-34–treated MSCs compared with those that received PBS or untreated MSCs. Furthermore, we performed real-time polymerase chain reaction to analyze the proangiogenic cytokines content in ischemic muscle. Results showed increased proangiogenic cytokines expression in MSC-JI group compared with other
groups (Figure VIII in the online-only Data Supplement). These data, together with vascular density assessment, indicate that transplantation of MSCs preconditioned with JI-34 promotes endothelial cell proliferation and neovascularization in ischemic muscle.

Transplantation of MSCs Preconditioned With JI-34 Enhances Recruitment of CD34+ Cells

To determine whether treatment with JI-34 enhanced MSC-mediated recruitment of endogenous progenitor cells, CD34-positive cells in ischemic muscles from each group at day 3

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**Figure 5.** Transplantation of pretreated mesenchymal stem cell (MSC) promotes angiogenesis and muscle regeneration in vivo. A, Representative hematoxylin and eosin–stained sections of ischemic muscles from each group at 21 days; scale bar: 50 μm, myocytes with centralized nuclei were considered as regenerating myofibers. B, Quantification of regenerating myofibers by counting the myocytes with centralized nuclei as a percentage of total myocytes in a field (n=5). *P<0.05 vs PBS and MSC. C, Immunofluorescent staining of CD31 and smooth muscle α-actin (α-SMA) in cryosections of muscles obtained from mice at day 21 after surgery. Endothelial cells were stained with CD31, and smooth muscle cells were stained with α-SMA. Scale bars: 100 μm. D and E, Quantification of CD31-positive endothelial cells and α-SMA-positive arteriole density (n=4 in sham group; n=5 in PBS group, and other group n=6). *P<0.05 vs PBS and MSC. F, Differentiation of MSCs in vivo. MSCs or MSCs preconditioned with JI-34 were stained with DiI (red) and injected into ischemic muscle. The muscles were harvested 21 days later, and cryosections stained with 4′,6-diamidino-2-phenylindole (DAPI) for nuclei (blue) and antibody against CD31 (green) for endothelial cells. No colocalized DiI with CD31 staining was observed. Scalar bar: 100 μm. PBS indicates phosphate-buffered saline.
postsurgery were quantified (Figure 6D). As expected, JI-34–treated MSCs resulted in significantly more CD34+ cells compared with all other groups (Figure 6E).

**Reduction of Cell Apoptosis In Vivo by MSCs Preconditioned With JI-34**

To determine the mechanism of cell protection by preconditioned MSCs, antiapoptotic proteins were analyzed by Western blot (Figure 7A). Significantly more B-cell lymphoma-2 protein was detected in the ischemic muscles transplanted with MSCs treated with JI-34 compared with those transplanted with untreated MSCs or PBS (Figure 7B). These data implicate the activation of antiapoptotic and inhibition of proapoptotic pathways as mechanisms of cytoprotection by JI-34 in vivo.

**Discussion**

In this study, we demonstrated that GHRH agonist, JI-34, significantly enhanced the viability and mobility of MSCs. After treatment with the agonist, the production of cytokines from MSCs was augmented, and the therapeutic effects in a mouse model of critical limb ischemia were significantly augmented compared with untreated MSCs. JI-34 agonist bound to a GHRH-R on MSCs and activated downstream STAT3/c-myc signaling and antiapoptotic pathways. These effects of agonist JI-34 were blocked by the GHRH antagonist, MIA-602, confirming an essential role for GHRH-R binding. MSCs pretreated with JI-34 displayed improved survival in ischemic muscles relative to untreated MSCs. Administration of MSCs treated with JI-34 into ischemic limbs significantly enhanced local angiogenesis, resulting in better reperfusion and limb salvage. More capillaries and mature vessels were observed in the ischemic muscles that received JI-34–treated MSCs. The effect of MSCs in vivo was associated with enhanced EC proliferation and recruitment of CD34+ progenitor cells. Transdifferentiation of MSCs into vascular cells was rarely observed, indicating a primary paracrine role of MSCs in promoting angiogenesis.
Previous work has shown that systemic administration of GHRH agonists stimulates proliferation of cells in peripheral tissues. Dioufa et al\textsuperscript{19} reported that the GHRH agonist, JI-38, improved wound healing by activating wound-associated fibroblasts through GHRH-R binding. It has been shown that rat cardiomyocytes express pituitary-type GHRH-R, and administration of exogenous GHRH was cardioprotective by preventing apoptosis and reducing the cardiac scar size. This was attributed to activation of extracellular regulated protein kinases1,2, phosphoinositide 3-kinase/protein kinase B and adenylate cyclase/cyclic adenosine monophosphate/protein kinase A signaling pathways.\textsuperscript{17} Subcutaneous injection of GHRH agonist, JI-38, into rats with acute myocardial infarction improved angiogenesis and cardiac remodeling.\textsuperscript{24} The effects of JI-38 include the augmentation of cardiac precursor cell proliferation without elevating systemic growth hormone levels.\textsuperscript{20} In addition, GHRH agonist, MR403, was shown to increase viability and proliferation of islet cells, thereby improving the survival of cultured insulinoma cells, suggesting promise for improved islet transplantation.\textsuperscript{25,26} In this study, we observed that JI-34 preconditioning increased MSC proliferation and survival. However, MSCs preconditioned with high concentration of JI-34 (10^{-7} \text{ mol/L}) did not exhibit obvious protective effect. We speculated that JI-34 at low concentration will promote cell proliferation and survival but causes cytotoxicity at high concentration. All GHRH analogs exhibited higher biological activity and are more stable than crude GHRH.\textsuperscript{14} The biological activities of both JI-38 and MR403 are similar or virtually identical.\textsuperscript{14,18} To our knowledge, this study is the first to describe a positive therapeutic benefit of MSCs by pretreatment with a GHRH agonist.

Splice variants (SVs) of GHRH-R have been detected in many extrapituitary tissues, including prostate,\textsuperscript{27} pancreatic islet,\textsuperscript{26} and heart.\textsuperscript{17} It has been demonstrated that functional SVs can replace the functions of GHRH pituitary-type receptor.\textsuperscript{28} In this study, we detected the expression of 39 kDa GHRH-R SV1 in mouse MSCs (Figure 1). Previous studies provided evidence that SV1 plays a pivotal role in regulating cell proliferation and survival.\textsuperscript{29} GHRH agonists seem to exert their actions in extrapituitary tissues through direct binding to SV1 without activation of the canonical downstream pathway of growth hormone/insulin-like growth factor axis.\textsuperscript{20} Consistent with previous studies, our in vitro data exhibited similar effects of agonist, JI-34, on enhancement of MSC proliferation and viability, whereas the expression of SV1 was not changed after the JI-34 treatment, indicating that the effects of JI-34 on MSCs were not mediated by upregulation of the SV1 receptor. GHRH can also activate Janus kinase 2/signal transducer and activator of transcription 3 pathway.\textsuperscript{30} GHRH binds with its receptor and induces STAT3 phosphorylation at tyrosine.\textsuperscript{19,21} Activation of STAT3 has been shown to play a critical role in regulation of angiogenesis and activation of cell survival pathway.\textsuperscript{23,31} Upon activation mediated by JI-34, STAT3 translocates from cytoplasm into the nucleus to regulate gene expression. Our results illustrate that JI-34 preconditioning corresponds to STAT3 activation and possibly its downstream proangiogenic and cell survival pathway. Our data suggested that STAT3 is an important part of GHRH pathway and participates in preconditioning-mediated cytoprotection actions.

MSCs are multipotent stem cells that have been widely used for cell-mediated therapy for various clinical indications including cardiovascular.\textsuperscript{32} Transplantation of MSCs has also been used to reduce foot necrosis and to increase perfusion of lower limbs in patients with limb ischemia.\textsuperscript{33} Such therapy is limited by poor retention and engraftment of the donor cells.\textsuperscript{34} The hostile microenvironment of ischemia leads to massive apoptosis and impaired function of the transplanted cells. Indeed, it has been reported that only 0.35±0.05% of transplanted cells survive 4 weeks after limb transplantation.\textsuperscript{35}

To overcome these limitations, cell preconditioning by physical, chemical, pharmacological, or genetic modification before transplantation has emerged to augment cell function and therapy.\textsuperscript{36} Our previous study showed that hypoxia preconditioning markedly increased the viability and mobility of MSCs through a leptin-mediated mechanism and enhanced therapeutic efficacy of MSCs in a mouse myocardial infarction model.\textsuperscript{11} Moreover, preconditioning with chemotactic factor can enhance survival and improve biological function of transplanted cells.\textsuperscript{37,38}

Here for the first time, we found that preconditioning MSCs with GHRH agonist, JI-34, significantly protected MSCs from apoptosis induced by serum deprivation and hypoxia stress in vitro consistent with antiapoptosis as a mechanism of cytoprotection and improved therapy by JI-34.\textsuperscript{39} In agreement with this, Jaszberenyi et al\textsuperscript{40} recently reported that GHRH antagonists increased the expression of proapoptosis protein B-cell lymphoma-2–associated agonist of cell death in U-87 MG glioblastoma cells as reverse evidence. By transplantation of sex-mismatched and GFP-labeled MSCs and subsequent quantification of Sry gene and GFP gene expression from the injected cells, we found that JI-34 treatment markedly improved MSC survival and retention in ischemic limbs and 7 days after cell transplantation. These data, together with our in vitro and in vivo data verified that treating MSCs with JI-34 augments cell homing and maximized the therapeutic effect of transplantation and enhanced generation of cytoprotective and angiogenic cytokines, including vascular endothelial growth...
factor and stromal-derived factor-1. In support of this, Gomez et al.22 found that GHRH agonists augmented vascular endothelial growth factor-A production in MSCs. However, it is important to note that in our hands, the improved short-term survival by JI-34 treatment was not sustained over a longer period (14 days). Moreover, we failed to detect any MSC-derived endothelial cell or α-smooth muscle cell 21 days after transplantation. Therefore, MSC transdifferentiation may not contribute significantly to therapy in this model. Rather, the effects of MSCs are primarily paracrine, affecting both cell activity and recruitment of progenitor cells. We found an increased proangiogenic cytokines expression in the ischemic limb muscle receiving preconditioned MSCs. The latter is also supported by a significant augmentation of CD34+ cells found in the ischemic muscle that received JI-34–treated MSCs relative to controls.

In conclusion, our data demonstrate that preconditioning of MSCs with GHRH agonist, JI-34, enhances the survival and proliferation of MSCs, increases the secretion of proangiogenic factors, and augments the therapeutic potential of MSCs to promote angiogenesis in ischemic tissue. These results support clinical testing of GHRH agonists as agents to improve MSC therapy for cardiovascular indications including critical limb ischemia.

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Disclosures

None.

References

Clinical trials of mesenchymal stem cell (MSC) therapy have shown promise for the treatment of cardiovascular disease, including heart failure and critical limb ischemia. However, poor cell survival and engraftment into host tissues have limited efficacy of MSC therapy. Here, we show that pretreatment of MSCs with JI-34, an agonist of growth hormone–releasing hormone, markedly augmented the activities of signal transducer and activator of transcription 3 and significantly enhanced cytoprotection and mobility of MSCs in vitro and in vivo. JI-34–pretreated MSCs sustained better engraftment and superior proangiogenic ability when transplanted into ischemic limbs. Our findings support a role for growth hormone–releasing hormone agonists in preconditioning of stem cells, before transplantation, to enhance cell activation and homing, and related therapeutic activity.
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Methods and Materials

Animal care
The male and female C57BL/6 mice were obtained from College of Life Science, Zhejiang Chinese Medical University (Hangzhou, Zhejiang, China). All mice were housed in stainless steel cages with sawdust bedding. They were kept at 23 ± 1 °C, humidity 55 ± 5%, under a 12 hours dark/light cycle and were allowed unlimited food and water. All procedures were approved by the Animal Ethics Committee of Zhejiang University, which complies with the Guide for the Care and Use of Laboratory Animals (2011).

Isolation and culture of MSCs
Bone marrow derived MSCs were obtained from 4-week old C57BL/6J mice as described previously1. After exposing the bone marrow cavity by cutting off the both ends of bones, bone marrow cells were obtained by flushing the cavity of femurs and tibias with PBS containing 100U/mL penicillin, and 100 U/mL streptomycin (Genom, Hangzhou, China). After being centrifuged at 1000 rpm for 5 minutes, cells were resuspended and cultured in a 10-cm culture dish with 10 mL DMEM/F12 culture medium (Corning, USA) supplemented with 10% fetal bovine serum (FBS) (Pufei, Australia), L-Glutamine 200 mM (Gibco, Life Science, Brazil), 10U/mL penicillin, and 10U/mL streptomycin (Genom). Culture medium was replaced after 24 hours, then changed every 3 days. Cells were subcultured to 80%-90% confluence and passed after dissociation with 0.25% Trypsin & 0.02% EDTA (Genom). For normal oxygen conditions (21%O₂, 5%CO₂, 37°C) cells were incubated in a standard humidified CO₂ incubator (HERACELL 150i, Thermo SCIENTIFIC, Germany). Characterization of MSCs was performed by flow cytometric analysis of surface markers CD29, SCA-1, CD44, CD31, and FLK-1 (Supplemental Figure IX).

Cell proliferation
Cell proliferation was examined using Cell Count Kit-8 (CCK-8) (Bestbio, Shanghai, China) according to the manufacturer’s introduction. MSCs were cultured on a 96-well plate with an initial 2000 cells in 200 μL DMEM/F12 medium with 10% FBS per well. GHRH agonist, JI-34, at final concentrations of 10^-7 - 10^-9 mol/L, was added to the culture. After 24 hours incubation, 10 μL CCK-8 solution was added into each well for 4 hours incubation at 37°C. Culture medium was discarded after the incubation, and then 100 μL dimethyl sulfoxide (DMSO) was added into each well. The absorbance at 450 nm was measured by microplate reader (Bio-Rad, Berkeley, CA, USA).

Cell survival
For in vitro cell survival assay, either pretreated or non-treated MSCs were cultured in serum free medium under hypoxia (0.1% O₂, 5% CO₂) at 37°C for 48 hours. For control, MSCs were plated in complete culture medium with normoxia (21% O₂, 5% CO₂) condition for the same period. The apoptosis of MSCs was measured by a commercial FITC-AnnexinV Apoptosis Detection Kit (BD Biosciences, Franklin Lakes, NJ, USA) using flow cytometry according to the manufacturer’s instructions. MSC viability was evaluated using CCK-8 as described above.

Flow cytometric analysis
At room temperature, MSCs at 3rd passage were dissociated by 0.125% trypsin (HyClone). Cells
were re-suspended in PBS and incubated with the following antibodies in the dark for 30 mins: CD29 (Ebioscience), CD44 (Ebioscience), Sca-1 (Ebioscience), FLK-1 (Ebioscience), CD31 (BD Biosciences), and GHRH-R (Abcam). For GHRH-R detection, cells were further stained with Alexa fluor 488 conjugated antibody (Invitrogen) for 30 mins at room temperature. Non-specific mouse IgG-FITC and IgG-PE were used as controls. After incubation, the cells were washed twice with PBS, and analyzed by flow cytometry (BD Biosciences).

**Tube formation assay**
Matrigel assay was used to analyze the ability of MSC-conditioned media to promote tube formation of human umbilical vein endothelial cells (HUVECs) in vitro. MSCs were grown in DMEM with 10% FBS for 3 days, then medium was replaced with fresh DMEM/F12, without serum, containing 10⁻⁸ mol/L JI-34, and cultured for 24 hrs. Then the medium was again replaced with fresh DMEM/F12 with 2% FBS without agonist. After culture for another 24 hours, the supernatant was collected for tube formation assay. Matrigel (50 μL/well, BD Biosciences) was put into 96-well plate at 4°C, then incubated for 30 minutes at 37°C. HUVECs (10000 cells/well) suspended in 100 μL conditional medium collected from MSCs were plated on the Matrigel. After culture for 4-6 hours, images were taken using an Olympus microscope. The tube formation was quantified by analyzing the total tube length in each well with Image-Pro Plus (MediaCybernetics, USA).

**Migration assay**
MSCs were cultured with fresh medium with or without 10⁻⁸ mol/L JI-34 for 24 hours, and trypsinized and counted. Cell suspension (20000 cells in 200 μL DMEM medium adding 1% FBS per well) was seeded onto the apical surface of the inserts (in triplicate) of Falcon FluoroBlok 24-wellinsert plates (6.5 mm diameter) with 8 μm pores (BD Biosciences). In each basal chamber, 500 μL DMEM with 10% FBS were added. Following 24 hours incubation at 37 °C, 5% CO₂, the non-migrating cells attached to the upper side of the filter were carefully removed with a cotton swab and the migrating cells were fixed with 4% paraformaldehyde then stained with Hoechst 33258 (Invitrogen, molecular probes, USA) 1:5000 solution for 20 minutes. Photography was done with an inverted Olympus® IMT-2 fluorescence microscope. Five random visual fields were captured for each insert, and cell number was counted using Image-Pro software.

**Real time RT-PCR**
Total RNA was extracted using TRIzol (Invitrogen, Life Technology) according to the manufacturer’s protocol. Total RNA (1 μg) was used for reverse transcription to synthesize cDNA using the PrimeScript™ 1st Strand cDNA Synthesis Kit (TaKaRa, Dalian, China), and SYBR® Premix Ex TaqTM II (Tli RNaseH Plus) (TaKaRa, Dalian, China) was applied for the real time RT-PCR process on an ABI PRISM 7500fast Detection System (Applied Biosystems, Carlsbad, CA, USA). PCR conditions were settled at 95 °C 5 min for holding stage; 95°C 15s, 60°C 32s, 95°C 15 s for cycling stage with 40 cycles; 72 °C 1 min for melting stage with 0.5°C stepping rate. The sequence of PCR primers were listed in Support Information Table S1. Each reaction was carried out in triplicate and target genes were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or 18s rRNA for quantification. PCR data were calculated by the comparative ΔΔct methods.
**Western Blot**

Protein extraction and immunoblot methods were performed as described. For cytokines detection assay, MSCs were cultured with fresh medium containing 10^8 mol/L JI-34 with or without 10^{-7} MIA-602 for 24 hrs, then cell lysates were collected. After quantification by BCA protein assay (Bio-Rad, Berkery, CA, USA) with 5X loading buffer, 40μg of proteins were resolved in 10-12% SDS-PAGE. After protein transfer, the nitride cellular membrane was incubated with specific antibodies including anti-GHRHR(1:500) (ab76263, Abcam, USA), anti-STAT3 (#4904, Cell Signaling Technology, USA), anti-p-STAT3 (#9145, Cell Signaling Technology), anti-Bcl-2 (#2827, Cell Signaling Technology), anti-Bcl-xL (#2764, Cell Signaling Technology), anti-c-myc (sc-40, Santa Cruz Technology, USA), anti-cleaved caspase-3 (#9664, Cell Signaling Technology), SDF-1(Ab25117, Abcam, USA) HGF (ab83760, Abcam, USA) VEGF (ab46154, Abcam, USA), anti-GAPDH (KC-5G5, KANGCHEN, China), anti-β-actin (KC-5A08, KANGCHEN, China), at 4°C overnight, then incubated with secondary goat-anti-rabbit IgG conjugated with horse radish peroxidase (HRP) (sc-2004, Santa Cruz Technology), or goat-anti-mouse IgG conjugated with HRP (sc-2005, Santa Cruz Technology) at room temperature for 2 hours. After being washed three times by PBS-T, the membranes were infiltrated with ECL (Bio-Rad, USA) and visualized by ChemiDoc™ MP Imaging System (Bio-Rad, USA).

**Immunofluorescence staining for STAT3**

Localization of STAT-3 in response to JI-34 treatment was determined by immunofluorescence staining of STAT-3. MSCs were seeded on coverslips and incubated with or without JI-34 and MIA-602 for 45 min, washed with PBS and fixed with 1 % paraformaldehyde for 10 min. Cells were then permeabilized with 0.2 % Triton X-100 for 20 minutes. After washes, the cells were blocked for 30 min at room temperature with 5 % BSA, and incubated overnight at 4°C with the anti-STAT3 antibody (Cell Signaling Technology). The cells were further incubated with FITC-conjugated secondary antibody for 1 h. Cell nuclei were stained with Hoechst 33258 (Sigma) for 15 min and then mounted on slides. The mean fluorescent density of cytoplasmic and nucleus were analyzed using Image-Pro Plus 6.0 software. Quantification of STAT-3 nucleus fold change was based on comparison of the ratio of nuclear mean fluorescent density to cytoplasmic fluorescence density.

**Animal Model and cell delivery**

Male C57BL/6 mice (10-week old, weighting 22–25 g) were used for the hind limb ischemia model by ligation of femoral artery as described. In brief, a longitudinal incision from the groin to the knee-joint was used to expose the vessels after removing the hair. The femoral artery was separated from the vein and nerve, ligated by 6-0 silk (Ethicon, Sommerville, NJ), and excised. The skin incision was closed with 4-0 silk (Ethicon). No mice died during the surgery procedure. Immediately after surgery, the animals were randomly divided into four groups. Mice of the Sham group received excision without artery ligation and phosphate buffer saline (PBS) injection. The Control group received PBS (50μL). Mice of MSC and MSC-JI groups received MSCs (1x10^6) without or with JI-34 preconditioned. Cells were suspended in 50 μL PBS and were directly injected into the gastrocnemius muscle at four points with a 29 gauge needle. For the in vivo tracking of MSCs, MSCs either treated or non-treated with JI-34 at 1x10^6 cells/mL were labeled with 0.25 μg/μL 1,1'-dioctadecyl-3,3,3',3''- tetramethylindocarbocyanine (DiI, Invitrogen) in a dark CO2 incubator at 37 °C for 5 mins, then at 4°C for 15 mins. The cells were washed 5 times to remove unbounded DiI.
Finally the cells were kept on ice until they were implanted.

For in vivo tracking, Mice bone marrow MSCs at passage 5-6 were also transfected with GFP recombinant lentivirus ([plvx-IRS-ZsGreen1 (Cat: #632187, Clonetech) and packaging plasmids X-tremeGENE HP DNA Transfection Reagent (Cat: #06366546001, Roche)). In short, MSCs plated at 5×10^5 cells in a T25 flask and transduced with 2 x 10^7 lentivirus (40 MOI). Culture medium was replaced with fresh complete medium 6 hours later. Before transplantation, MSCGFP were treated with 10 nM JI-34 for 24h. A total 1x10^6 MSCGFP or JI-34 treated MSC (MSCGFP,JI) were transplanted after artery ligation as described previously.

**Laser Doppler perfusion images**

On days 0, 3, 7, 14 and 21 following surgery, limb blood flow was measured using a Laser LDPI analyzer (PeriCam PSI, PerimedAB, Sweden) as described previously^4. All mice were kept on a 37°C heating pad to maintain the body temperature. Perfusion index was defined as the ratio of the ischemic (right) to non-ischemic (left) limb’s blood flow using a LDPI win 3.13 program (Perimed AB).

**Histological analyses**

After 3, 7 and 21 days, mice were sacrificed and the gastrocnemius muscle were harvested and embedded in optimal cutting temperature compound (Sakura Finetek USA Inc., CA). Frozen sections were cut at 7μm thickness. To examine the capillary and arteriole densities and progenitors in ischemic muscles, sections were stained with rat anti-mouse CD31 (BD bioscience), goat anti-mouse smooth muscle α-actin (α-SMA) (Abcam) and rat anti-mouse CD34 (Abcam), respectively. Proliferating endothelial cells were detected by co-staining using rabbit anti-mouse Ki67 (Abcam) with rat anti-mouse CD31. Sections were further stained with Alexa fluor 488 or 549 conjugated secondary antibody (Invitrogen). After being mounted with Vectashield DAPI mounting medium, the samples were analyzed using a fluorescence confocal microscope (Leica). Results were presented as arterioles/mm^2 and capillaries/mm^2. For morphometric analysis, cryo-sections of ischemic limb were stained with hematoxylin and eosin. All images were taken under 200/400 magnification in 7 or 8 vision fields/section.

**Quantification of retained cells in ischemic muscle**

MSCs were harvested from young male C57BL/6J mice and cultured as described above. Male MSCs were transplanted into ischemic hindlimbs of female mice at 1x10^6 cells per mouse. Mice were sacrificed at day 3 and day 7, and gastrocnemius muscles were collected for DNA extraction (TAKARA MiNiBEST Universal Genomic DNA Extraction Kit). Quantification of sry gene was determined by real-time RT-PCR, and retention of male MSCs was calculated by the ct mean of sry according to the standard curve generated as follows. Whole genome DNA from 1x10^7 MSCs was collected in 30μL water; then had serial 10-fold dilution to 1 cell per tube. The male-specific gene sry was quantified by real-time RT-PCR with the absolute quantification method. A standard curve was developed according to the relative cell numbers and the ct mean of sry gene. Gene ID of sry was 21674 in NCBI database. The primer sequence of sry gene was in the Forward primer 5’TTTTGCCTCCCATAGTAGATTTTCT3’; and Reverse primer 5’TGTACCGCTCTGCAACCA3’.

For detecting GFP positive cells in ischemic muscles, mice were sacrificed 7 days after surgery.
Ischemic gastrocnemius muscles were harvested then submitted to DNA extraction and PCR analysis to detect the presence of ZsGreen1-positive MSCs. Whole genome DNA from 1x10^5 ZsGreen1-positive MSCs was collected in 30μL water; then had serial 10-fold dilution to ten cells per tube. Purified DNA from each sample were amplified using SYBR Premix Ex Taq. The primers for ZsGreen1 were forward primer 5’ GCGAGAAGATCCATCCCGTG3’; and reverse primer 5’ ACTTCTGGTCTTGGCGTCG3’.

**Statistical analysis**

Results were expressed as means ± SD. Continuous variables were compared by the Student t test, and multiple comparisons were performed by one-way ANOVA with a Bonferroni correction. Statistical analyses were performed using Prism 6 (GraphPad Software Inc, USA). A value of \( p < 0.05 \) was accepted as statistically significant.

Profound actions of an agonist of growth hormone releasing hormone on angiogenic therapy by mesenchymal stem cells

Supplement Material

Supplementary Table I. Primer sequences.

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Supplementary Figure I. Detection of GHRH-R expression on MSCs. (A) Immunofluorescent staining of GHRH-R (green) on pituitary, skeletal muscle and MSCs. Nuclei were stained with DAPI (blue). Scale bars: 100 μm. (B, C) Flow cytometric analysis (n = 3) and (D) Western blot analysis of GHRH-R expression on MSCs treated with JI-34 or untreated. DAPI: 4′,6-diamidino-2-phenylindole.
Supplementary Figure II. c-myc expression on MSCs augmented with JI-34. (A) Real time PCR was performed to detect the effect of JI-34 on the expression of c-myc at mRNA level within MSCs. c-myc was upregulated by JI-34 treatment. *, *p < 0.05 vs. Control. (B) Protein expression level of c-myc after treatment with 10 nM JI-34 for 24h (JI). GAPDH: Glyceraldehyde 3-phosphate dehydrogenase.
Supplementary Figure III. Effect of MR-409 on proliferation and viability of MSCs. (A) Proliferation of MSCs treated with different concentration of MR-409 was compared with untreated control under normal culture condition. (n = 4) *, p < 0.05 vs. Control. (B) Survival of MSCs treated with different concentration of MR-409 was compared with that untreated controls under hypoxia and serum deprivation condition. (n = 4) *, p < 0.05 vs. Control.
Supplementary Figure IV. Effect of GHRH agonist, MR-409, on MSC migration. (A) MSCs migration capability was tested by a transwell assay followed by crystal violet staining of the migrated cells (×400) in the presence of following reagents: Control: none; MR+ MIA: MR-409 and GHRH antagonist, MIA-602; MR+WP1066: MR-409 and STAT3 inhibitor, WP1066. Scale Bar is 100 μm. (B) Quantification of migrated MSCs in A. n=4; *, p < 0.01 vs. control. #, p < 0.05 vs. MR-409.
Supplementary Figure V. MSC retention 7 days after transplantation. (A) Quantification of GFP (ZSgreen1) gene copies 7 days after transplantation. n = 4 in each group; *, p < 0.05 vs. MSC group. (B) The correlation between GFP DNA copies and the CT cycles. GFP: green fluorescent protein.
Supplementary Figure VI. Engraftment of the injected MSCs. MSCs (1x10^6) either untreated, or preconditioned with JI-34 were stained with red fluorescent dye Dil and then injected into left ischemic gastrocnemius muscle. The muscles were harvested 7 days or 21 days later. Cryo-sections were examined for retained MSCs after staining with DAPI for nuclei (blue). Scale bar: 100µm. Left panel: untreated MSC group, Right pane: MSCs preconditioned with JI-34. Dil: 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate. DAPI: 4',6-diamidino-2-phenylindole.
Supplementary Figure VII. MSC proliferation and apoptosis in vivo. (A) Representative pictures of immunostaining of Ki67+ cells (green) in the ischemic muscle at 7 days after surgery. MSCs were stained with Dil (Red) before transplantation. No Ki67+ MSCs were found in both 2 groups. (B) The survival of MSCs/pretreated MSCs 3 days after transplantation. TUNEL staining for detecting apoptosis (Green) of MSCs (Dil) and Hoechst 33258 for nuclei (blue). (C) Quantification of apoptotic MSCs (TUNEL+/Dil+). n = 3 in each group. Scale Bar: 100μm. TUNEL: Terminal deoxynucleotidyl transferase dUTP nick end labeling.
Supplementary Figure VIII. Pro-angiogenic factors expression in ischemic muscle. Gene expression of pro-angiogenic factors were detected by real-time PCR. (n = 3 in each group) *, p < 0.05 vs. PBS; #, p < 0.05 vs. MSC. PBS: Phosphate Buffered Saline; EGF: epidermal growth factor; VEGF: vascular endothelial growth factor; SDF-1: stromal cellderived factor – 1; 18S: 18S ribosomal RNA.
Supplementary Figure IX

A

B

CD29 99.9%

SCA-1 99.9%

CD44 96.5%

CD31 2.1%

FLK-1 0.1%
Supplementary Figure IX. Characterization of mouse bone marrow-derived MSCs. (A) The morphology of BM-MSCs at 3rd passage with fibroblastoid shape. Scale bar: 200 μm. (B) Representative cell surface markers of mouse BM-MSCs by flow cytometry. Mouse BM-MSCs are positive for CD29, SCA-1 and CD44 and negative for CD31 and FLK-1.