Acceleration of wound healing by growth hormone-releasing hormone and its agonists

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Despite the well-documented action of growth hormone-releasing hormone (GHRH) on the stimulation of production and release of growth hormone (GH), the effects of GHRH in peripheral tissues are incompletely explored. In this study, we show that GHRH plays a role in wound healing and tissue repair by acting primarily on wound-associated fibroblasts. Mouse embryonic fibroblasts (MEFs) in culture and wound-associated fibroblasts in mice expressed a splice variant of the receptors for GHRH (SV1). Exposure of MEFs to 100 nM and 500 nM GHRH or the GHRH agonist JI-38 stimulated the expression of α-smooth muscle actin (αSMA) based on immunoblot analyses as well as the expression of an αSMA-β-galactosidase reporter transgene in primary cultures of fibroblasts isolated from transgenic mice. Consistent with this induction of αSMA expression, results of transwell-based migration assays and in vitro wound healing (scratch) assays showed that both GHRH and GHRH agonist JI-38 stimulated the migration of MEFs in vitro. In vivo, local application of GHRH or JI-38 accelerated healing in skin wounds of mice. Histological evaluation of skin biopsies showed that wounds treated with GHRH and JI-38 were both characterized by increased abundance of fibroblasts during the early stages of wound healing and accelerated reformation of the covering epithelium at later stages. These results identify another function of GHRH in promoting skin tissue wound healing and repair. Our findings suggest that GHRH may have clinical utility for augmenting healing of skin wounds resulting from trauma, surgery, or disease.

smooth muscle | actin | fibroblast proliferation | growth hormone

Growth hormone-releasing hormone (GHRH) is produced by the hypothalamus and acts on the pituitary, stimulating the production and release of growth hormone (GH) (1). Much evidence suggests that besides its hypophyseal action, GHRH plays a role in extrapituitary tissues and importantly in cancers stimulating tumor growth by paracrine and/or autocrine mechanisms (2, 3). Conversely, antagonistic analogs of GHRH potently inhibit the growth of various experimental human cancers in vitro and in vivo, suggesting that GHRH antagonists could serve as another specific class of anticancer agents (4).

In addition to the hypothalamus, expression of GHRH has been reported in several nonhypothalamic tissues including placenta, ovaries, testes, lymphocytes, and others (5–14), but the physiological significance of this ectopic production of GHRH remains poorly elucidated. However, it has been suggested that locally produced GHRH promotes follicular maturation by paracrine modulation of the stimulatory action of follicle stimulating hormone on granulosa cell function (12), whereas in Leydig cells, GHRH contributes to spermatogenesis (9). Recent evidence also demonstrates that this neuropeptide can induce cardiac repair after myocardial infarction, by mechanisms involving a direct action on the cardiomyocytes (15, 16). Lately, it was also reported that GHRH agonists stimulate the proliferation and reduce apoptosis of pancreatic islet cells (17).

The extrapituitary effects of GHRH in peripheral tissues, including cancers, are mediated, at least in part, by the splice variant of GHRH receptor (SV1). SV1 is derived by the alternative splicing of the RNA encoding the pituitary GHRH receptor (18). Contrary to the relatively restricted pattern of expression of GHRH receptor, SV1 is expressed in several extrapituitary tissues, including cancers, and exhibits both ligand-dependent and ligand-independent activity (19, 20).

Normal human fibroblasts respond to agonistic analogs of GHRH by increased proliferation, an effect that was apparent only in early passage cultures of fibroblasts and was abolished as the cultured cells approached senescence (21). These findings are consistent with a report that GHRH antagonists inhibit telomerase activity (22). Given the responsiveness of fibroblasts to GHRH and recent evidence suggesting a role for GHRH in cell migration (23), we tested the hypothesis (24) that GHRH plays a role in promoting skin wound healing and repair, a complex process in which concerted proliferation, migration, and reorganization of fibroblasts play an essential role (25). Our results provide evidence for a role of GHRH in wound healing, suggesting the clinical application of GHRH agonists in conditions involving skin tissue repair.

Results

Mouse Embryonic Fibroblasts (MEFs) Express SV1. First, we evaluated whether MEFs express the receptor for GHRH SV1. Primary MEFs were exposed to GHRH and agonistic GHRH analog JI-38 (24, 26) at 100 nM and 500 nM for 24 h, and SV1 expression was then assessed by immunoblot analysis. As shown in Fig. 1*A*, MEFs expressed SV1 and the levels of SV1 expression were up-regulated by GHRH at 500 nM. Immunohistochemical analysis of 4-mm skin wounds, 5 d after skin wound incision revealed a mosaic pattern for anti-SV1 immunoreactivity confirming that at least a portion of fibroblasts within a dermal wound express SV1.

GHRH Induces the Expression of α-Smooth Muscle Actin (αSMA) in Fibroblasts. αSMA is an actin isoform that confers tensional and contractile activity (25, 26). Typically, SMA is selectively expressed in smooth muscle cells of adult animals but is induced in activated fibroblasts (myofibroblasts) during wound healing, within the stroma of many tumors, and in general under conditions where there is extensive tissue reorganization and regeneration (27, 28). Immunoblot analysis showed that GHRH and the agonist JI-38 stimulated SMA expression in MEFs (Fig. 1*A*). Of interest, this effect was significant at a concentration of 100 nM, but not 500 nM, of either peptide. A similar pattern of expression was found for focal adhesion kinase (FAK), an enzyme that plays a role in cell migration and invasion (29). The activation of αSMA in fibroblasts after GHRH treatment was confirmed in lung fibroblasts isolated from transgenic

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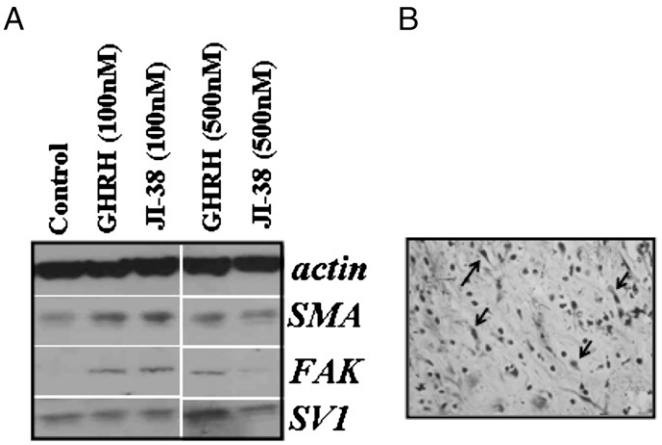

Fig. 1. Expression of SV1 in fibroblasts. (*A*) Western blot analysis of MEFs cultured for 24 h in the presence of GHRH or JI-38 and blotted for SV1, SMA, and FAK. Results showed that 100 nM GHRH and the GHRH agonist JI-38 induced SMA and FAK expression in MEFs. β-actin was used as a loading control. (*B*) Immunohistochemical analysis of expression of the GHRH receptor SV1 in mouse 4-mm skin wounds, 5 d after skin incision. Results showed a mosaic pattern of anti-SV1 immunoreactivity as evidenced by the brown precipitant (arrows).

mice bearing a reporter β-galactosidase (bGal) transgene under the regulation of the αSMA promoter/enhancer (30). Cells from these mice display bGal positivity when αSMA expression is activated (30). As shown in Fig. 2, under normal culture conditions the fraction of bGal-positive cells is minimal, but after an exposure to GHRH or agonist JI-38, it is markedly increased ≈3-fold compared with the untreated controls.

GHRH Promoted Cell Migration of MEFs in Vitro. Subsequently we subjected MEFs to scratch wound migration assays in vitro, after exposure to GHRH and JI-38 at 100 nM. In a scratch assay (31), after exposure to GHRH and JI-38, migration of MEFs was considerably increased compared with the migration of control MEFs (Fig. 3*A*). Consistent with this finding, migration through 8-μm pore transwells was significantly ($P < 0.05$) increased in MEFs exposed to GHRH and JI-38 at 100 nM, by ≈2.5- and 3-fold, respectively, as compared with the controls (Fig. 3*B*). A considerable, notwithstanding not significant, stimulation of cell migration, by ≈2-fold as compared with the controls, was also evidenced at 500 nM GHRH or JI-38. To rule out the possibility that the results of these two migration assays were simply due to the increased proliferation, MEFs were exposed to the peptide analogs and cell proliferation was evaluated. As shown in Fig. 3*C*, the proliferation of MEFs was stimulated after exposure to 100 nM GHRH, whereas 500 nM GHRH and 100 nM and 500 nM JI-38 induced no considerable effect in the rate of cell proliferation.

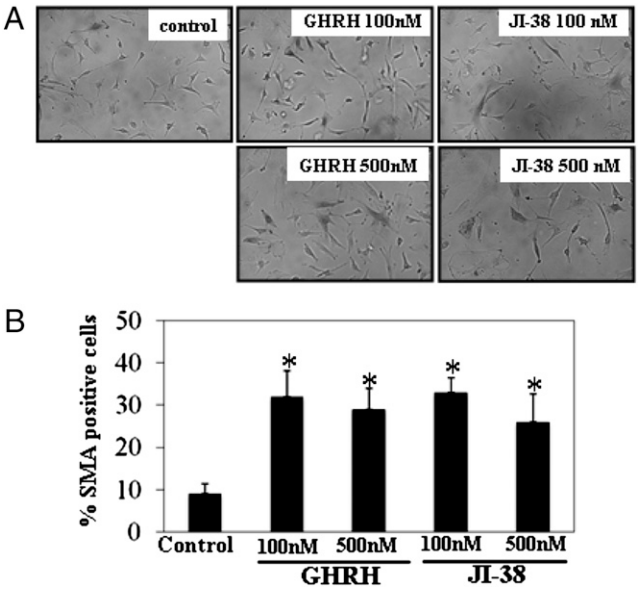

Fig. 2. bGal staining in lung fibroblasts from transgenic mice bearing an αSMA promoter-bGal reporter transgene, exposed to GHRH or JI-38 at 100 nM and 500 nM. (*A*) bGal staining (blue) shows a mosaic pattern of positivity, implying the induction of αSMA in a fraction of the fibroblasts. (*B*) Graphical presentation of the bGal-positive fibroblasts indicates that GHRH and JI-38 treatment resulted in a nearly 3-fold increase in the fraction of αSMA-lacZ–positive cells.

GHRH and GHRH Agonist JI-38 Accelerated Wound Healing in Vivo. To evaluate the effect of GHRH on wound healing in vivo, wild-type mice were subjected to 4-mm skin biopsies and the healing of the inflicted wounds in the presence or absence of GHRH and JI-38 at 100 nM was observed daily. To avoid variation in the wound-healing process between different experimental animals, a control wound was always included in each mouse, thus permitting paired analysis. As shown in Fig. 4, a considerable acceleration of wound healing was observed in the presence of 100 nM GHRH or JI-38 as compared with the controls that was evidenced as soon as in day 3 and resulted in nearly complete healing ≈10 d after incision (Fig. 4*B*). Histological analyses were performed on skin wounds at days 5 and 8 and showed increased content of fibroblasts, particularly in the wounds treated with GHRH and, to a lesser extent, in the JI-38 treated wounds, as compared with the controls on day 5 (Fig. 5). Quantification of the fibroblast content in the wound area showed that fibroblast density was increased by ≈80% and 40% in the GHRH and JI-38 treated wounds ($P < 0.05$; Fig. 6), respectively. On day 8, reformation of the covering epithelium was almost complete after treatment with GHRH and skin resembled the normal, before the wound state. We noted that both histologically and macroscopically, GHRH was more potent than JI-38 at equimolar concentrations. At day 8, the epidermis had been regenerated in all three groups, but in GHRH-treated wounds the epidermis, as well as the dermis, were almost normal with a diffuse mild inflammatory infiltration. In JI-38–treated wounds, the stroma was dense and rich in fibroblasts, indicating a more advanced stage of healing as compared with the control ($P < 0.05$). Van Gieson staining at day 8 (Fig. 5) indicated the nearly normally arranged collagen fibers in the lamina propria of the GHRH-treated samples. In the controls and JI-38–treated specimens, collagen fibers (red) could not be seen, implying delayed healing.

Discussion

Wound healing is a complex process in which fibroblasts play an essential role. During healing, the orchestrated proliferation and migration of the resident dermal fibroblasts occurs and is followed by induction of expression of smooth muscle cell contractile proteins including αSMA, their modulation into myofibroblasts, and the reorganization of the extracellular matrix. This process eventually results in skin tissue regeneration (29, 30, 32).

The principal action of the hypothalamic neuropeptide GHRH is considered to be the stimulation of synthesis and release of GH from the pituitary. However, there is increasing evidence implicating GHRH in a wide additional range of physiological and pathological processes including carcinogenesis, immune function, follicular maturation, Leydig cell differentiation, cardiac repair during myocardial infraction, and others (2, 9, 14–16). These findings, in combination with the well-documented findings showing that there is extrahypothalamic, local in situ production

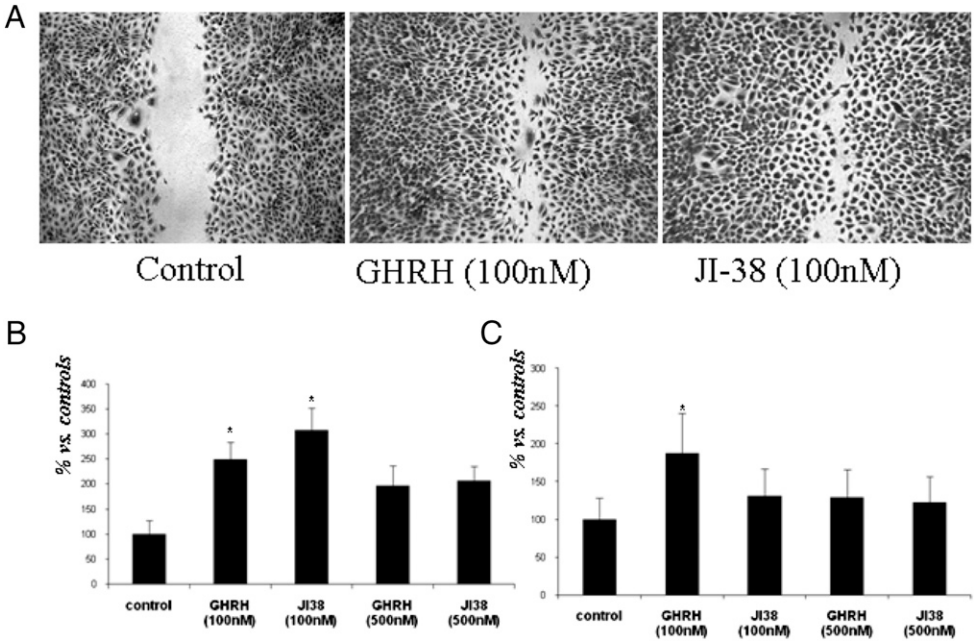

Fig. 3. GHRH and JI-38 induce migration of cultured MEFs. (*A*) Scratch migration assay of MEFs exposed to 100 nM GHRH or JI-38 showed increased migration of cells as compared with controls. (*B*) GHRH and JI-38 at 100 nM significantly increased migration of MEFs in an 8-μm-pore transwell system. (*C*) GHRH stimulated proliferation of MEFs but only at a concentration of 100 nM. $*P < 0.05$.

of GHRH by several peripheral tissues suggest a pleiotropic action for this peptide hormone with other targets remaining to be identified (2, 3). The present study provides evidence showing involvement of GHRH in dermal wound healing. Of particular significance, we provide compelling evidence that GHRH accelerated in vivo healing of skin wounds in mice. Moreover, results of our in vitro studies suggest that this acceleration may be due, at least in part, to augmentation of migration of wound-associated fibroblasts and activation of αSMA, which is well documented to increase contractility of myofibroblasts, a process important in wound retraction and repair (30). Finally, we present evidence showing that fibroblasts express the GHRH receptor SV1, which is significant because this finding identifies receptors that potentially mediate the direct effects of GHRH in this cell type.

Exposure of MEFs to GHRH or its agonistic analog JI-38 caused stimulation in the expression of αSMA. This effect was greater at lower doses of GHRH and JI-38 (100 nM) and less pronounced at higher doses (500 nM). Because SV1 levels were similar or even stimulated by GHRH at 500 nM, it is not likely that the reduction in the potency of GHRH in stimulation of αSMA was due to the down-regulation of SV1 receptors by the treatment. As evidenced by the bGal staining, the pattern of αSMA positivity

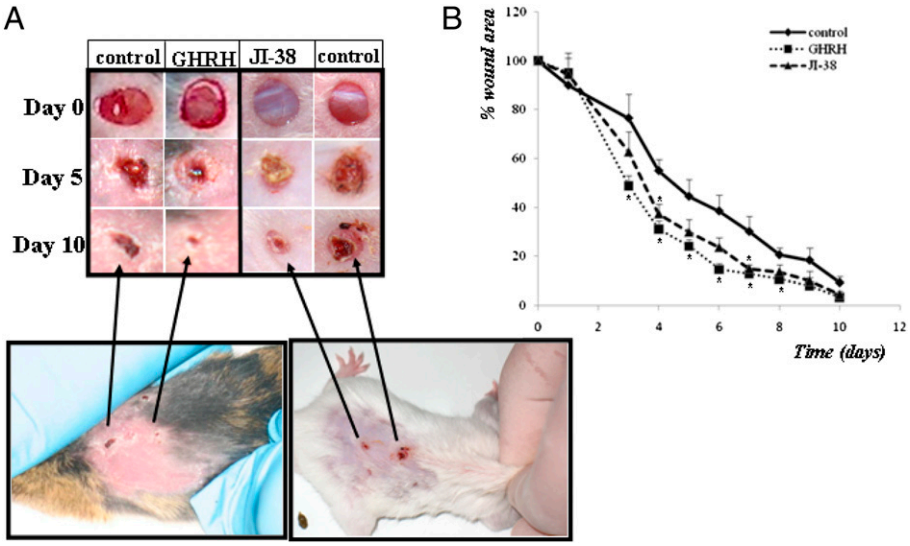

Fig. 4. Healing of 4-mm circular skin wounds in mice exposed to GHRH or GHRH agonist JI-38 twice per day at 100 nM. Four wounds were generated in each mouse and always included controls, exposed to vehicle alone. Time points and treatment regimens are indicated. (*A*) Representative microphotographs of control, GHRH, and JI-38-treated wounds on days 0, 5, and 10 after incisions. Relative wound area is shown in the graph (*B*). Experimental groups for wound-area measurements consisted of 5, 7, and 5 wounds, respectively for the control, GHRH, and JI-38-treated wounds. Data are expressed as mean ± SEM of percent wound area relative to wound area on day 0. $*P < 0.05$.

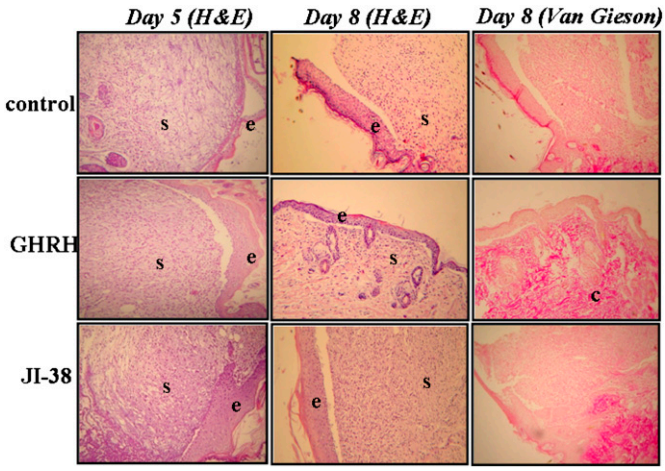

Fig. 5. Histology of 4-mm circular skin wounds in mice exposed to GHRH or GHRH agonist JI-38 twice per day at 100 nM. (*Left*) At day 5, a considerable increase in the fibroblasts was observed in the GHRH and JI-38–treated mice, as opposed to edematous and loose stroma of the controls. (*Center*) At day 8, the epidermis has been regenerated in all three cases. In GHRH-treated wounds the epidermis, and the dermis, were almost normal with a diffuse mild inflammatory infiltration. In JI-38–reated wounds, the stroma is dense and rich in fibroblasts, indicative for a more advanced stage of healing as compared with the control. Van Gieson staining at day 8 (*Right*) indicated the nearly normally arranged collagen fibers in the lamina propria of the GHRH-treated samples. In the controls and JI-38–treated specimens, collagen fibers (red) could not be seen, implying delayed healing. Collagen fibers in the bottom of these sections can be seen and correspond to the unwounded (normal) epithelium: s, stroma; e, epithelium; c, collagen fibers stained red by the Van Gieson staining.

was mosaic, whereas the increase by the treatment was due to elevation of the fraction of the αSMA-positive cells, rather than the overall intracellular up-regulation of αSMA in cultured cells. Although this observation requires a validation by additional experiments, it is consistent with the previously reported transient stimulation of αSMA in experimental wounds (32).

By affecting proliferation (20), migration, and αSMA expression, GHRH is implicated in various stages of the wound-healing process. It is of interest that different doses of GHRH or agonist JI-38 did not elicit analogous effect in terms of the specific cellular response measured. The reasons for this difference are unclear, but may represent complex integration of multiple signaling pathways with differing sensitivities, differences in local concentrations of GHRH versus JI-38 secondary to differences in local solubility, systemic absorption, diffusion, binding properties, or tachyphylaxis, and to differential receptor desensitization.

The fact that the agonist JI-38 was more potent than GHRH only at the transwell-based migration assay may be related to the fact that it was developed and characterized to be much more potent than GHRH, by using an in vivo growth hormone release assay after a s.c. administration (24, 26). The high activity of JI-38 and related agonists was due to the resistance to degradation by s.c. peptidases (24, 26). The response to these GHRH agonists is based on the pituitary GHRH receptor (24, 26). It is therefore possible that receptors, such as SV1, that may mediate the effects of GHRH in the fibroblasts, exhibit different sensitivity to the agonist than the pituitary type of GHRH receptor.

It has to be noted that skin wound healing in mice bears certain differences as compared with wound healing of the human skin. In example, humans have "tight" skin as opposed to mice that are considered loose-skinned animals, making the comparison in healing between these species difficult (33). Thus, our findings should be interpreted with caution. Taken this limitation into consideration, our results have identified a unique function for GHRH in the promotion of skin tissue repair. Our study suggests the possibility of therapeutic use of GHRH analogs for accelerating wound healing after traumatic injury, surgery, or disease.

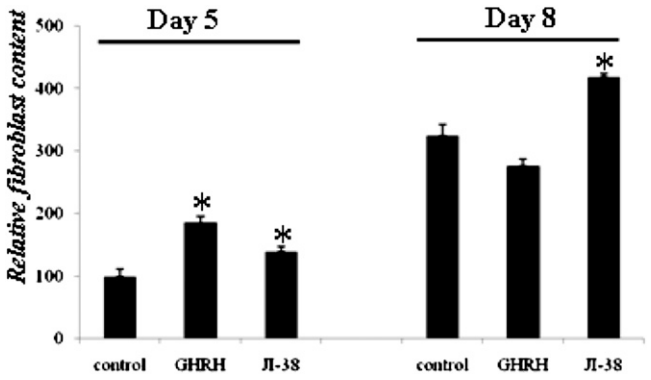

Fig. 6. Quantification of fibroblast content in 4-mm circular skin wounds in mice exposed to GHRH or GHRH agonist JI-38 twice per day at 100 nM on days 5 and 8. Fibroblast number was assessed by direct counting of fibroblast in at least three random optic fields in the wound areas under light microscope and expressed relative to the fibroblast content in the controls on day 5. Data are expressed as mean ± SEM. $*P < 0.05$.

Methods

Peptides, Cell Culture, Western Blot, and Histological Analysis. hGHRH(1-29) NH_2 was obtained from Sigma. Agonist JI-38 was synthesized as reported (24, 26). The peptides were dissolved in PBS at the indicated concentrations. Controls contained solvent alone. Fibroblasts were isolated by using standard methods and maintained in Dulbecco's modified Eagle medium containing 10% FBS and antibiotics/antimycotics. For all experiments, primary fibroblasts of <10 passages old were used. For Western blot analysis, antibodies for αSMA, FAK, and actin were obtained from Sigma, whereas for SV1, the antibody 2317/5 was used (34). The same antibody was also used for the immunohistochemistry as described (35). Histology was performed on standard 5-μm sections of paraffin-embedded material stained with hematoxylin/eosin. bGal staining was performed in lung fibroblasts, isolated from transgenic mice bearing an αSMA-β-galactosidase reporter transgene. After treatment with the peptides for 24 h, cells were washed (three times each for 5 min) with 0.1 M PBS (pH 7.4) and fixed with 0.05% glutaraldehyde in PBS, or 10 min at room temperature. After three more washes, cells were stained with 1 mg/mL of 5-bromo-4-chloro-3-indonyl β-D-galactopyranoside (X-gal), 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, and 2 mM $MgCl_2$ overnight at 37 °C.

Cell Proliferation and Migration Assays. The rate of cell proliferation was evaluated by the Trypan blue exclusion assay under inverted microscope. Cell migration was performed by the "scratch" assay as described, exposing cells to the peptides for

18 h (31). For the transwell-based migration assay, MEFs were seeded on 8-μm pore size transwells (Corning) and after 5 d exposure to the peptides at the indicated concentration, cell number was evaluated in the bottom compartment. All in vitro experiments were performed in triplicates and similar results were obtained.

Mice and Wound-Healing Assay. Wild-type mice of mixed C57BL6/FVB genetic background, originally obtained by Jackson Laboratories were maintained in our laboratory. Care of animals was in accord with University of Athens Institutional guidelines. The reporter αSMA-bGal mice have been described (36). Wound-healing assay in vivo was performed after administration of anesthesia (100 mg/kg ketamine, 10 mg/kg xylazine). Mice were shaved in the back, wiped with ethanol, and wounds were cut by using a biopsy punch (4 mm). The skin was grabbed at dorsal midline and placed over a cardboard backing on one side of the skin fold. The biopsy punch was pressed until both layers of the skin were punched and the cardboard was reached. Four wounds were performed per mouse, two on each side of the dorsal midline, at equal distance, so that the tension of the skin would be equal all over the mouse back and would not influence the contraction of the wounds. The experimental protocol was reviewed and approved by the University of Athens Animal Experimentation Committee. GHRH and JI-38 (100 nM) were applied twice daily in a volume of ≈50 μl. At least 10 wounds for each of the control (vehicle, PBS), GHRH, or JI-38 were observed. To avoid study variation due to the wound-healing variability between different experimental animals, each mouse was its own control. For wound area measurements, digital pictures of wounds were analyzed by the ImageJ (NIH) software. Wound size was monitored over time relative to a 4-mm-diameter circular paper cutout placed next to the wound as described (37). For histology, 4–6 wounds per time point per group were analyzed. Similar results were obtained among different mice, and histological sections for each group and representative are shown.

Van Gieson Elastic Stain for Collagen. Tissue sections from mice wounds were deparaffinized and rehydrated in distilled H_2o. These sections were then stained in orcein (Sigma) 1% wt/vol in 96% ethanol for 30 min at room temperature and washed in distilled H_2O. Then, the sections were stained in Weigert's iron chloride solution (A: 0.83% wt/vol Ferric chloride, 1.5% wt/vol Ferrous sulfate, 0.67% vol/vol 12 M HCl in distilled H_2O, and B: 1% wt/vol Hematoxylin in absolute ethanol mixed in a proportion of 3A:1B) for 8 min at room temperature and washed in distilled H_2O. Finally, sections were stained in picric-fuchsin solution (1.14% picric acid, 0.1% acid fuchsin in distilled H_2O) for 1 min at room temperature. Then, slides were dehydrated and then mounted.

Statistical Analysis. The data are presented as mean ± SEM. Statistical analysis was performed by using one-way ANOVA and Dunnett's test. The results were considered significant when $P < 0.05$.

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