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# Anti-inflammatory effect of HGF responses to oral traumatic ulcers using an HGF-Tg mouse model

Xinhong WANG<sup>1)\*</sup>, Liting YAN<sup>1,2)\*</sup>, Yinghua TANG<sup>1)</sup>, Xiaoxi HE<sup>1)</sup>, Xiaomin ZHAO<sup>1)</sup>, Weijia LIU<sup>1)</sup>, Zhicong WU<sup>1)</sup> and Gang LUO<sup>1)</sup>

<sup>1)</sup>Department of Oral Medicine, Affiliated Stomatology Hospital of Guangzhou Medical University, Guangzhou Key Laboratory of Basic and Applied Research in oral Regenerative Medicine, No. 39 Huangsha Road, Liwan District, Guangzhou, Guangdong, 5101182, P.R. China

<sup>2)</sup>Wuxi Stomatology Hospital, No. 6 Jiankang Road, Liangxi District, Wuxi, Jiangsu, 214001, P.R. China

**Abstract:** Hepatocyte growth factor (HGF) has been implicated in inhibiting diverse types of inflammation. Oral traumatic ulceration (OTU) is a common disease of the oral mucosa, and inflammation is the main process for ulcer healing. This study aimed to explore the expression of HGF in oral ulcers and its role in ulcer inflammation. The saliva of 14 recurrent aphthous stomatitis (RAS) patients, 18 OTU patients and 17 healthy controls was collected. Traumatic ulcers of the left mucosa were observed in 42 wild-type (WT) and 42 HGF-overexpressing transgenic (HGF-Tg) mice. Histological scores, inflammatory cell expression and serum cytokine expression were measured and analyzed on the 5th day. The HGF protein level in ulcer-affected human saliva was 9.3-fold higher than that in healthy saliva. The HGF protein levels in RAS and OTU saliva were 14- and 5.7-fold higher, respectively, than those in healthy saliva. Traumatic ulcers enhanced HGF expression in ulcer-affected oral mucosa and in the blood of C57BL/6 mice by 1.21- and 1.40-fold, respectively. In HGF-Tg mouse traumatic ulcers, HGF expression was 1.34-fold higher than that in wild-type mice. HGF-Tg mice had lower weight loss, less ulcer area and lower histopathology scores than WT mice. The results from immunohistochemistry, flow cytometry and serum cytokine analysis showed that HGF-Tg animals presented fewer Ly6G-positive neutrophils and higher levels of circulating inflammatory cytokines. HGF overexpression alleviated weight loss, ulcer area and inflammation, suggesting the role of HGF in promoting the healing of oral ulcers.

**Key words:** cytokine, HGF, inflammation, oral ulcer

## Introduction

Traumatic ulcers of the oral mucosa are relatively frequent and may be induced by many underlying etiologic factors [1–3]. Oral traumatic ulcers feature discontinuity of the epithelium and inflammatory granulation tissue under damaged epithelium. Healing happens in a spontaneous manner that may vary from 1 to 2 weeks if the source of the trauma is removed. Chronic nonhealing ulcers are trapped in a prolonged inflammatory cycle and fail to proceed to the normal progression of healing [4].

The infiltration of inflammatory cells following the level of inflammatory cytokines is essential for the tissue repair and wound healing of ulcers [5, 6].

The HGF protein is essential for embryonic development, which is mainly expressed by mesenchymal cells [7]. It is abnormally expressed in various cancers and plays a role in the regulation of the biological behavior of cancer [8]. Moreover, HGF expression increases following a variety of injuries and autoimmune inflammatory diseases. Level of HGF are increased in epithelium, mesenchymal and endothelium cells in injured tissues,

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Corresponding author: G. Luo. email: gangluo2020@163.com

\*These authors contributed equally to this paper.

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so HGF is essential for self-repair after injuries [7–9]. It has been postulated that cells of the immune system contribute to regulating the expression of HGF and c-Met following injury and inflammation [10, 11]. HGF has been reported to inhibit the secretion of inflammatory cytokines and promote the secretion of anti-inflammatory cytokines [12–14].

In recent years, the application of HGF has been considered a new potential clinical drug in ulcer healing based on preclinical and clinical studies. The therapeutic effect of HGF has been examined in acute or chronic inflammation/fibrosis models [15, 16]. HGF has been reported to modulate the inflammatory environment and enhance wound healing and regeneration in animal models of inflammation-associated diseases, including a skin inflammation model [17], multiple sclerosis model [18], pulmonary artery hypertension model [19] and bronchiolitis obliterans model [20]. HGF can mediate wound healing by accelerating the regeneration of epithelial cells [21, 22] and by maintaining normal immune function [17]. Clinical data have shown that HGF gene therapy can significantly decrease the size of ulcers in patients with critical limb ischemia [23, 24].

As described above, HGF promotes wound healing by inhibiting inflammation in injured tissues. In oral mucosal tissues, elevated expression of HGF can increase oral mucosal fibroblast (OMF) proliferation in vitro, which is required for the wound healing properties of OMF [25]. Based on the existing knowledge, we hypothesized that HGF will alleviate inflammation and promote healing of oral ulcers in vivo. However, the effect of HGF on ulcer immune cells during oral traumatic ulcers is still a mystery.

In this study, we aimed to analyze the expression pattern of HGF in traumatic ulcer-affected oral mucosa tissue and tested the effects of HGF on ulcer area, inflammatory cells and cytokine expression in traumatic injury of the oral mucosa. Because of the short biological half-life of internal HGF protein, intravenous medicine delivery cannot ensure the effective function of HGF in traumatized tissues; thus, we established HGF-overexpressing transgenic (HGF-Tg) mice whose HGF expression level was higher than that of wild-type (WT) mice [26].

## Materials and Methods

### Collection of human saliva

Human saliva (5–10 ml) was obtained from healthy controls (n=17), patients with recurrent aphthous stomatitis (RAS) (n=14) and patients with oral traumatic ulceration (OTU) (n=18) without any systemic disease. The clinical characteristics of the patients and controls are

summarized in Supplementary Table 1. Human saliva was used to analyze HGF protein expression by ELISA.

### Animals

Wild-type C57BL/6J (WT) and HGF-overexpressing transgenic C57BL/6J (HGF-Tg) mice were used in this study. HGF-Tg mice were generated according to a previous report [26]. Briefly, the vector pRP[Exp]-CAG>mHgf[ORF045947] was injected into fertilized oocytes (C57BL/6J background) by pronuclear injection (Cyagen Biosciences) and HGF-overexpression transgenic mice (HGF-Tg) were established and verified by the following primers: HGF (forward): 5'-TC-TAGAGCCTCTGTAACCATGTTC-3', HGF (reverse): 3'-CCTGA TACACCTGTTGGCACAC T-5'. control Rgs (forward): 5'-CAACCACTTACAA GAGACCCGTA -3', control Rgs (reverse): 3'-GAGCCCTTAGAAATAACGTTT ACC-5'. In total, 42 WT and 42 HGF-Tg mice aged 8–10 weeks were used in this experiment. The mice were kept under 12-h/12-h light/dark cycles with free access to food and water in the Central Animal Laboratory of Guangzhou Medical University.

### Oral mucosa traumatic ulcer protocol

Traumatic ulcers of the oral mucosa were generated using the method described by Cavalcante in 2011 [5]. Briefly, the mice were anesthetized with 0.1–0.2 ml of pentobarbital sodium (0.1%) via intraperitoneal injection, and the oral mucosa was sterilized using 0.12% chlorhexidine gluconate. Ulceration was performed on the left oral mucosa using a number 15 scalpel blade; a 3-mm marker was used for standardization. The protocol was performed by the same investigator (Xiaoxi He) for standardization. We chose the 5th day as the sacrifice day because the mice lost weight during the first 5 days [5].

### Clinical and histological evaluation of traumatic ulcer

The mice were weighed from day zero to the sacrifice day (5th day), and weight loss = (final weight-initial weight). The larger (L) and minor (M) diameters of the ulcer were measured on the 3rd and 5th days. The area of the ulcer was calculated ( $\text{area} = \pi \times L \times M$ ). All measurements were performed by Xiaoxi He. The mucosa containing the ulcer was collected and fixed in 10% formalin after the mice were sacrificed. After being embedded in paraffin, the tissues were sectioned into 4- $\mu\text{m}$  slices that were then mounted on slides and stained with hematoxylin and eosin (HE) for inflammation score evaluation (from 0 to 4 according to published evaluation criteria) [2]. Blood collection and sample collection were performed separately on the 5th day.

### ELISA analysis for protein expression

ELISA was used to analyze the protein levels in human OTU saliva, mouse oral mucosa tissue protein extract and mouse serum. Mouse mucosal ulcer tissues were stored at  $-80^{\circ}\text{C}$  for use. Frozen mucosal ulcer tissues were solubilized in lysis buffer at  $4^{\circ}\text{C}$  for 20 min and then centrifuged at  $4^{\circ}\text{C}$  for 20 min ( $1,500 \times g$ ). The supernatant was collected, and the protein contents were detected by Coomassie Plus Protein Lysate Reagent (Pierce, Rockford, IL, United States). Mouse serum was separated by centrifugation (2,000 rpm, 5 min). The protein level of HGF was measured in mice (Mouse HGF ELISA Kit, RayBio<sup>®</sup>, Norcross, GA, USA) and human (Human HGF ELISA Kit, RayBio<sup>®</sup>) HGF ELISA kits, as instructed by the manufacturer's protocol.

### Serum cytokine analysis

Serum cytokines were tested in duplicate alongside a standard with the Mouse Cytokine Array Q5 (QAM-CYT-5, RayBio) according to the manufacturer's instructions. The fluorescent signal intensity was measured using an InnoScan 300 Microarray Scanner (Parc d'Activités Activestrel, 31390 Carbonne, France) at a wavelength of 532 nm and a resolution of  $10 \mu\text{m}$ . All results were analyzed using Q-Analyzer software for QAM-cyt-5. Healthy wild-type mice ( $n=3$ ), WT mice with ulcers ( $n=5$ ) and HGF-Tg mice with ulcers ( $n=8$ ) were included in this analysis.

### Flow cytometry analysis

Cell suspensions were prepared from mouse ulcer tissues and immune cells from WT and HGF-Tg mouse blood for flow cytometry analysis. Cells were washed and resuspended in PBS ( $1 \times 10^6/\text{ml}$ ), and cells were stained for 30 min at  $4^{\circ}\text{C}$  with the following antibodies: CD45-APC-Cy7 (Lot: 7096638, BD, Franklin Lakes, NJ, USA), CD4-PE (Lot: 7138675, BD), CD8-FITC (Lot: 4329219, Invitrogen, Carlsbad, CA, USA), CD11b-PerCy5.5 (Lot: 7066558, BD), Ly6G-FITC (Lot: 7052879, BD), CD19 (Lot: 550992, BD) and CD3 (Lot: 10203, BioLegend, San Diego, CA, USA). The cells were sorted by flow cytometry (FACS Aria III, BD), and the data were analyzed using FlowJo software (FACS Diva software, BD).

### Immunohistochemical staining for CD45 and Ly6G

Slides bearing  $4\text{-}\mu\text{m}$  tissue slices were deparaffinized and rehydrated and then processed according to a standard immunohistochemical staining manual. Briefly, endogenous peroxidase was blocked with 3%  $\text{H}_2\text{O}_2$ , and then, the samples were incubated for 1 h at room temperature with primary antibodies against CD45 (dilution 1:100, Lot: 7096638, BD) and Ly6G (dilution 1:100,

bs-2576R, Bioss, Boston, MA, USA), after which they were incubated with HRP secondary antibodies and substrate. The score of the Ly6G-positive cells was calculated according to Li with a minor change [27]: the average positive cell ratios were photographed and counted in 3–5 fields ( $400\times$ ) and then scored as follows: 0: 0% positive cells; 1: 1–33% positive cells; 2: 34–66% positive cells; and 3: 67–100% positive cells. CD45 was qualified by the average positive cell number that was counted in 3–5 fields ( $400\times$ ).

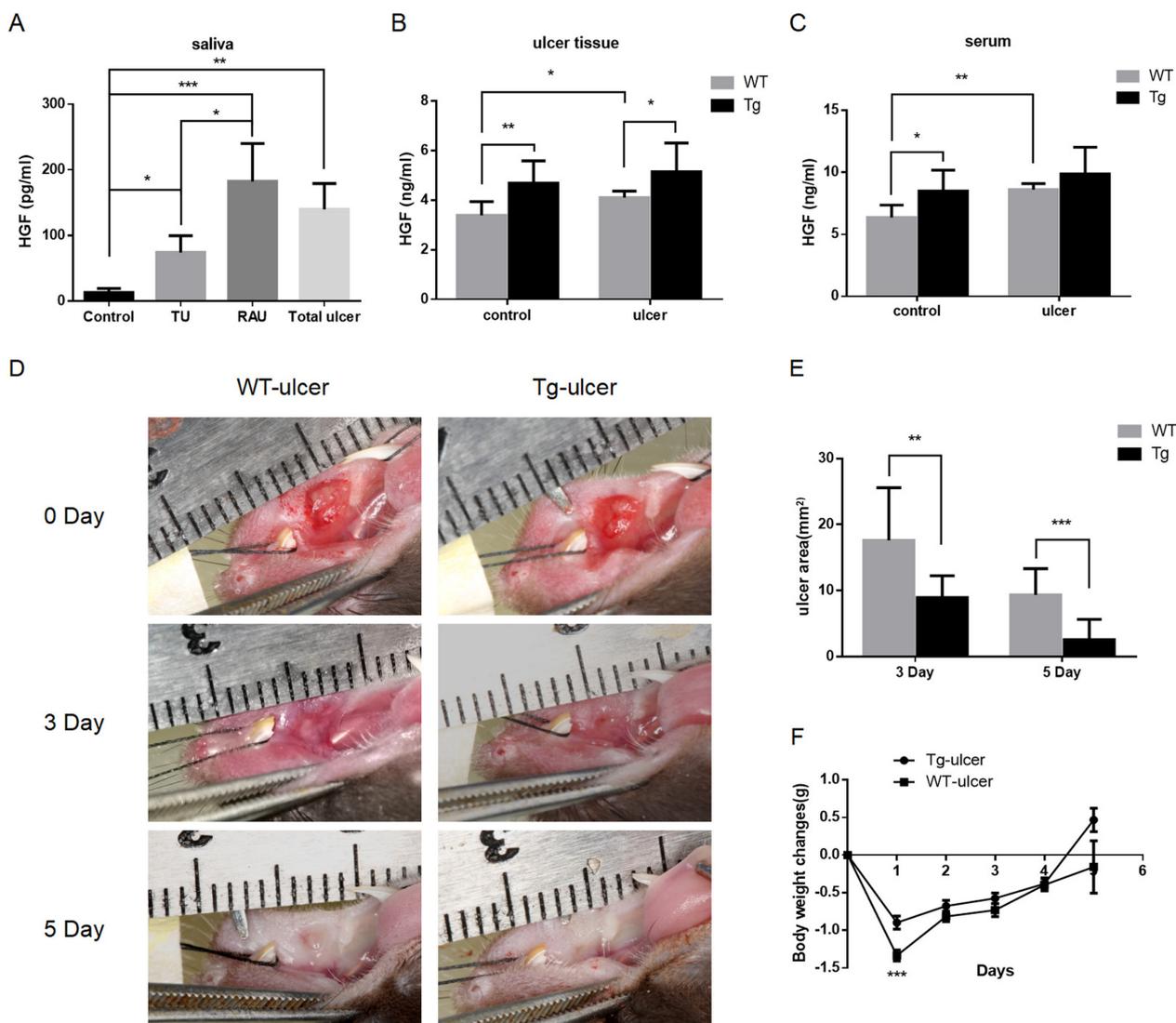
### Statistical analysis

SPSS 19.0 (IBM, New York, NY, USA) was used to calculate the statistical significance between groups. The unpaired *t*-test, Mann-Whitney *U* test and ANOVA were used to compare the differences between groups. Quantitative data are expressed as the mean  $\pm$  SD. GraphPad Prism 6 software (La Jolla, CA, USA) was used to plot the comparison of means, and  $P \leq 0.05$  was considered a statistically significant difference.

## Results

### Oral traumatic ulcers amplify HGF expression in oral mucosa

First, we examined the expression of HGF in human saliva of 14 RAS patients, 18 OTU patients and 17 healthy individuals. The HGF protein level in ulcer-affected human saliva was 9.3-fold higher compared with healthy saliva. The HGF protein levels in RAS and OTU saliva were 14 and 5.7-fold higher separately compared with that in healthy saliva. The HGF protein level in RAS-affected human saliva was 2.5-fold higher compared with that in OTU patients (Fig. 1A). The HGF protein level in the normal oral mucosa of HGF-Tg mice was 1.38-fold higher than that in WT mice (Fig. 1B). The HGF protein level in the oral ulcers of HGF-Tg mice was 1.34-fold higher than that in WT mice (Fig. 1B). The HGF protein level in oral ulcers of WT mice was 1.21-fold higher than that in healthy oral mucosa (Fig. 1B). At the same time, HGF protein expression was 1.1-fold higher in oral ulcers of HGF-Tg mice than in HGF-Tg healthy mucosa (Fig. 1B). The serum HGF protein level in HGF-Tg mice was 1.33-fold higher than the serum HGF protein level in WT mice (Fig. 1C). The serum HGF protein level of the WT oral ulcer mice was 1.40-fold higher than that of the WT healthy mice (Fig. 1C). The serum HGF protein level in oral ulcers of HGF-Tg mice was 1.11-fold higher than that in WT mice (Fig. 1C). At the same time, serum HGF protein expression was 1.16-fold higher in oral ulcers of HGF-Tg mice than in HGF-Tg healthy mucosa (Fig. 1C).



**Fig. 1.** Ulcer-induced HGF alleviates ulcer area and weight loss in mice. Expression of HGF in (A) saliva of OTUs (n=18), RAS patients (n=14) and healthy individuals (n=17) and (B) mucosal tissue in healthy and ulcerated WT or HGF-Tg mice (n=7). (C) Serum level of HGF protein in healthy and ulcerated WT or HGF-Tg mice (n=5). (D) Ulcer area of the buccal mucosa in WT and HGF-Tg mice, and (E) quantitative data for the ulcer area analyzed from macroscopic observation on the 3rd day (n=12) and the 5th day (n=18). (F) Weight loss of WT and HGF-Tg mice (n=34). Significant differences among the groups, \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .

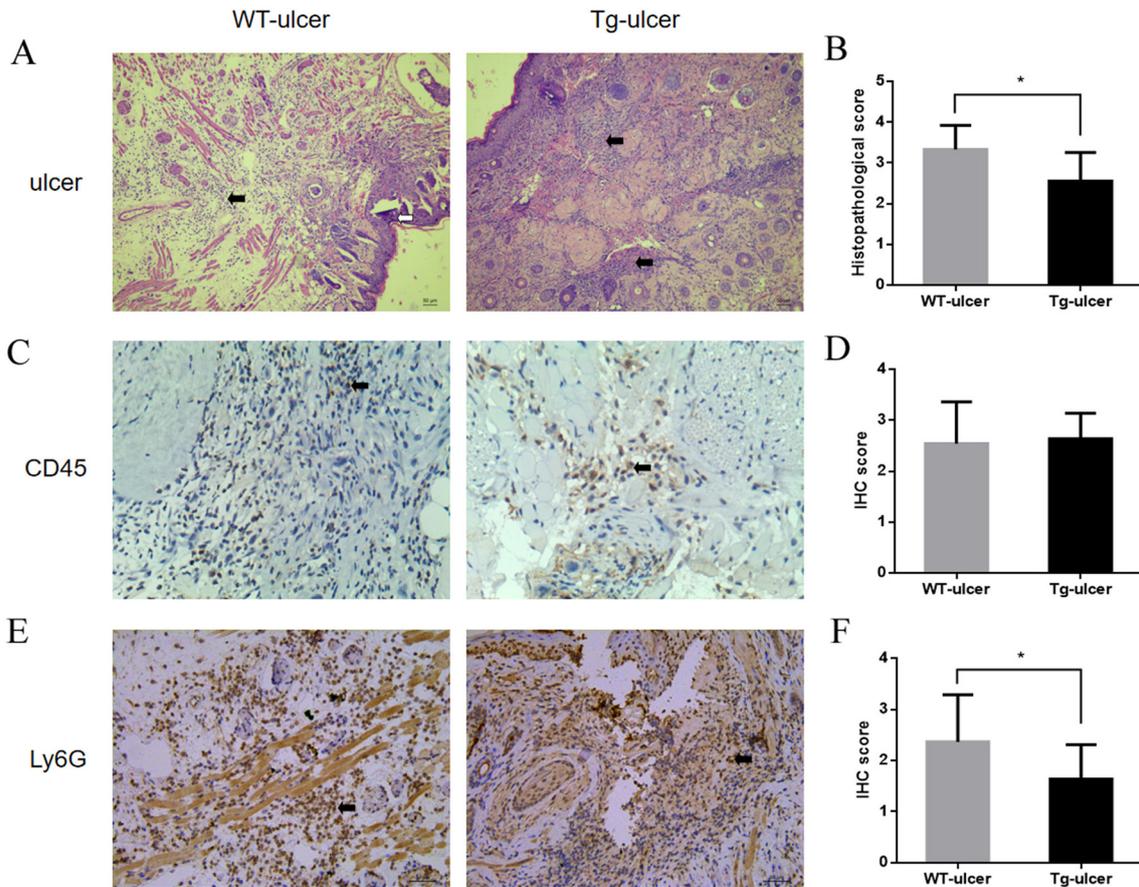
### High HGF expression alleviates weight loss and the ulcer area of oral ulcers

Area analysis of the ulcer showed that the ulcer area of all the groups showed a decrease on the 3rd and 5th days compared with day zero, and HGF-Tg mice showed a significantly lower ulcer area on the 3rd and 5th days compared with the WT group (Figs. 1D and 1E). All mice were alive on the sacrifice day, and there was a significant decrease in weight variation from the 1st to 4th day compared with day zero in both groups. HGF-Tg mice showed weight gain, and WT mice were close to the baseline weight value on the 5th day. Although the WT mice showed more weight loss than the HGF-Tg mice from the 1st to 5th day, only the 1st day was significantly different (Fig. 1F).

### Higher expression of HGF alleviates inflammation of oral ulcers

Microscopic analysis of the cheek trauma of HGF-Tg mice on the 5th day revealed the presence of less ulceration associated with moderate fibrosis and chronic inflammatory processes. In the WT ulcer mice, we found a persistence of ulcers and mixed inflammatory infiltrates (neutrophils and mononuclear cells). The HGF-Tg group exhibited less ulceration and inflammation than the WT group (Figs. 2A and 2B).

To characterize the impact of HGF on oral mucosal ulcer tissue and circulating inflammatory cells, we performed flow cytometry analysis 5 days after oral traumatic ulcer induction. Blood T cells were gated by CD3<sup>+</sup>/CD8<sup>+</sup> or CD4<sup>+</sup>, B cells were gated by CD19<sup>+</sup>, and



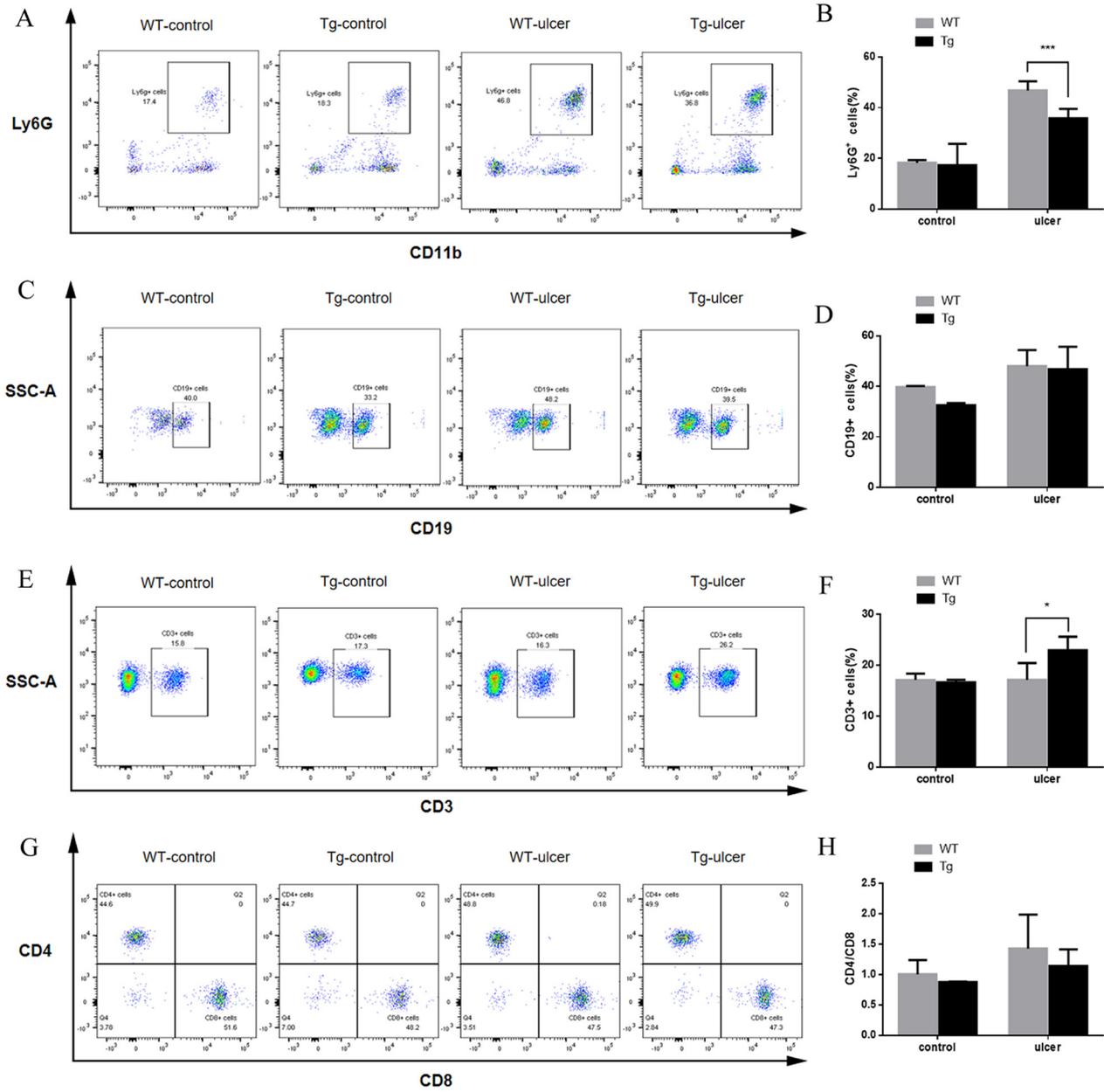
**Fig. 2.** HGF overexpression alleviates inflammation of ulcer tissues on the 5th day. (A) HE-stained histological images of ulcers in the WT and HGF-Tg mice. (B) Quantitative data of the histological scores from HE staining (n=18). Representative immunohistochemical staining of CD45 (C) and Ly6G (E) in ulcer sections. (D) Quantitative data of CD45 from immunohistochemical staining. (F) Quantitative data of Ly6G from immunohistochemical staining. The difference significance among the groups, \* $P < 0.05$ .

neutrophils were gated by CD11b<sup>+</sup>/Ly6G<sup>+</sup>. The T cells of the ulcer tissues were gated by CD45<sup>+</sup>/CD8<sup>+</sup> or CD4<sup>+</sup>, neutrophils were gated by CD45<sup>+</sup>/CD11b<sup>+</sup>/Ly6G<sup>+</sup>, and B cells were gated by CD19<sup>+</sup>. We found that fewer neutrophils (Figs. 3A and 3B) were visualized in the blood of HGF-Tg mice than in the blood of WT mice. In contrast, more T cells were visualized in the blood of HGF-Tg mice than in the blood of WT mice (Figs. 3E and 3F). There was no significant difference in terms of blood B cells between HGF-Tg and WT mice (Figs. 3C and 3D). Flow cytometry revealed that there was no significant difference in the CD4<sup>+</sup>/CD8<sup>+</sup> ratio between the groups (Figs. 3G and 3H). In this study, we also detected inflammatory cells in the oral ulcer tissue by flow cytometry. Fewer neutrophils were also found in the ulcer tissue of HGF-Tg mice than in that of WT mice (Fig. 4). However, CD4<sup>+</sup>T, CD8<sup>+</sup>T and B cells could not be detected in ulcer tissue by flow cytometry because the number of cells was less than the lowest detection limit.

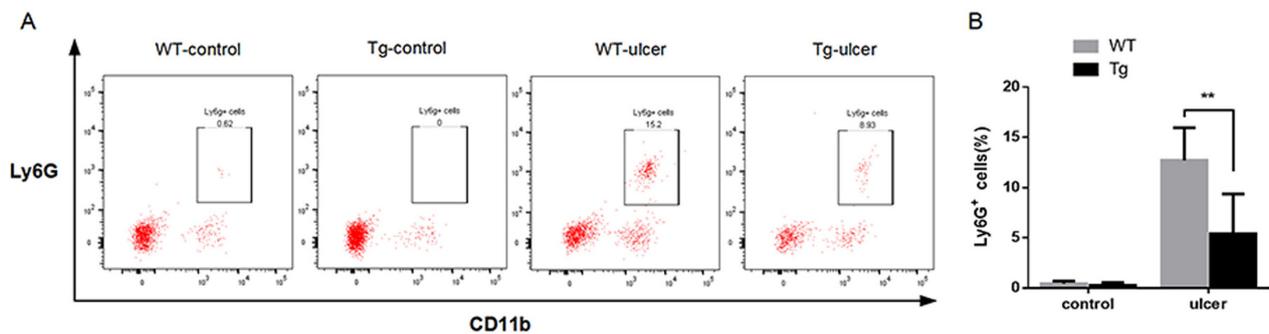
Similarly, we investigated Ly6G and CD45 expression in ulcer tissues using immunohistochemical staining.

There was more Ly6G-positive neutrophil infiltration in the WT mice, and it was significantly lower in the HGF-Tg group (Figs. 2E and 2F). The number of CD45-positive cells showed no difference between HGF-Tg ulcers and WT ulcers (Figs. 2C and 2D).

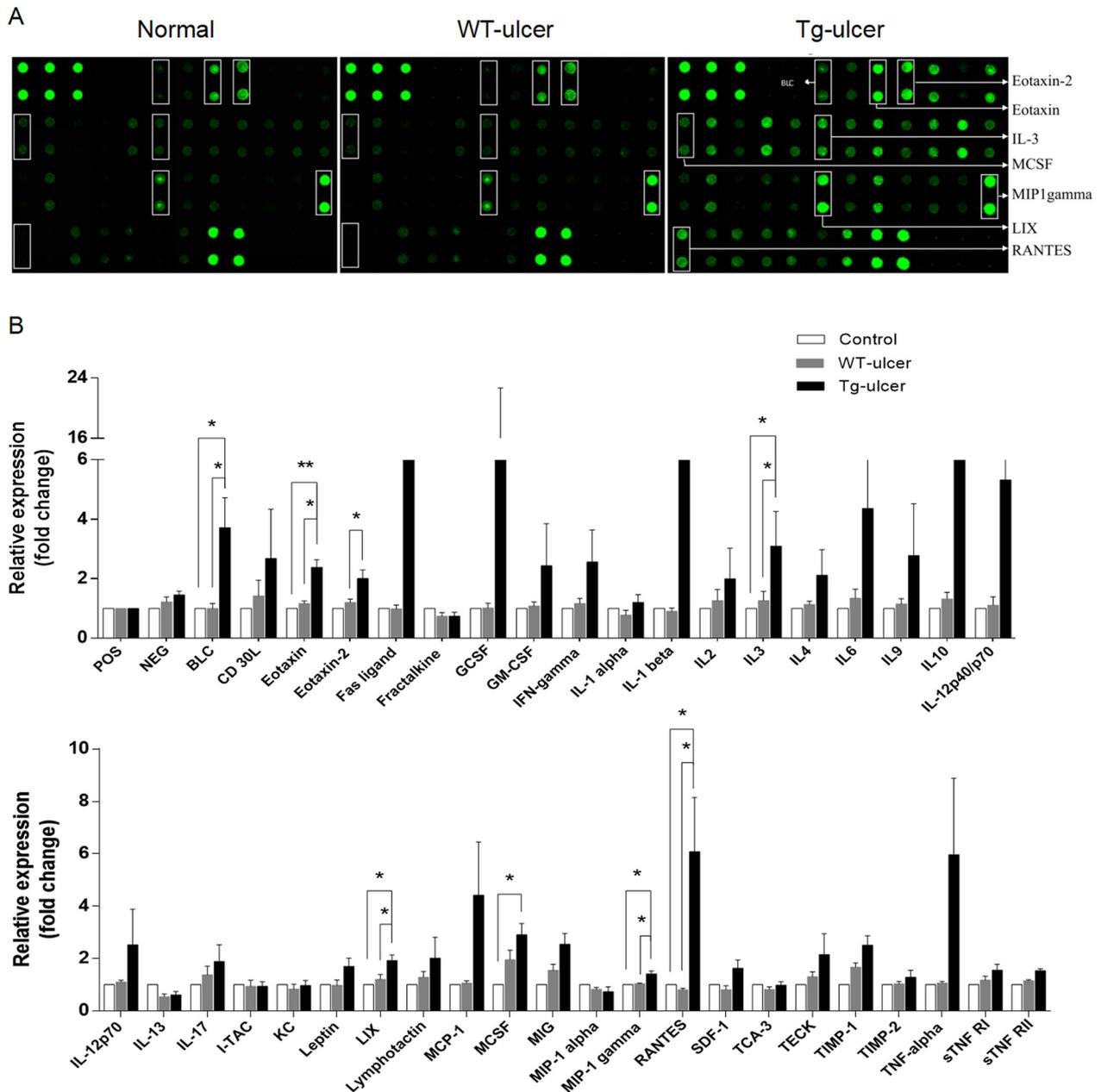
We further analyzed blood inflammatory cytokines using a RayBiotech antibody array experiment. In this assay, compared with the WT ulcer group, we detected 38 greater and 2 lesser (MIP-1 alpha, fractalkine) amounts of serum cytokine production in the ulcers of the HGF-Tg mice (Supplementary Table 2). Eotaxin, MIP-1 gamma, BLC, Eotaxin-2, RANTES, Lix and IL-3 were upregulated by more than 1.4-fold in the ulcers of the HGF-Tg mice compared to in the ulcers of the WT mice (Supplementary Table 2 and Fig. 5). When comparing the HGF-Tg group with the control group, eotaxin, MIP-1 gamma, BLC, MCSF, RANTES, Lix and IL-3 showed more than 1.5-fold expression over that in the WT group (Supplementary Table 2, Fig. 5). Only IL-6 was significantly lower in the WT ulcer group than that in the WT control group (Supplementary Table 2, Fig. 5).



**Fig. 3.** HGF overexpression alleviates serum neutrophils and ulcer T cells on the 5th day. Inflammatory cells were isolated from the blood of the WT (n=10) and HGF-Tg mice (n=10) and stained for Ly6G (A), CD19 (C), CD3 (E) and CD4/CD8 (G). Quantitative data for Ly6G (B), CD19 (D), CD3 (F) and CD4/CD8 (H). Significant differences among the groups, \* $P < 0.05$ , \*\*\* $P < 0.001$ .



**Fig. 4.** HGF overexpression alleviates ulcer tissue neutrophils. Inflammatory cells on the 5th day. Inflammatory cells were isolated from the ulcer tissue of the WT (n=8) and HGF-Tg mice (n=7) and stained for Ly6G (A), (B) Quantitative data for tissue Ly6G. Significant differences among the groups, \*\* $P < 0.01$ .



**Fig. 5.** HGF overexpression triggers upregulation of blood inflammatory factors on the 5th day. (A) Representative images of antibody arrays against 39 cytokines. (B) Significantly increased concentrations of serum inflammatory factors. Significant differences among the groups, \* $P < 0.05$ , \*\* $P < 0.01$ .

## Discussion

Recent studies have described the indispensable contribution of HGF to suppress inflammation in wound healing [13, 28]. However, the expression pattern of HGF in inflammatory oral mucosa ulcers is still unknown. In this study, we detected upregulation of HGF protein in the oral traumatic ulcer tissue of mice compared with healthy oral mucosa. Our results also indicated the higher expression of HGF in saliva during oral traumatic ulceration in humans. At the same time, oral traumatic ulcer conditions increased the level of serum HGF

in WT mice. Although oral ulcer is an oral mucosa disease, the inflammatory nature of the disease makes it relevant to various systemic effects, including body weight and quality of life [5, 29]. These results suggest the possible role of increased HGF on the systemic effects of oral traumatic ulcers.

Moreover, high HGF expression has been shown to mediate anti-inflammatory action and regeneration in a variety of inflammatory and traumatic models, including oral tissues [14, 30–34]. HGF is upregulated in injured tissues or organs, probably due to the need for tissue repair and anti-inflammation toward immune cells [7].

In this study, we found that upregulated HGF inhibited the clinical process and tissue inflammatory reactions of ulcers. This finding was confirmed by histological observations, which showed a decreased ulcer score and inflammatory cells in HGF-Tg mice, suggesting the possible role of HGF in alleviating inflammation and improving wound healing in oral traumatic ulcers.

Ulceration in the oral cavity can cause weight loss in animal models, which is probably due to high infection, the slow healing process and large ulcer area [1]. HGF gene therapy has been reported to decrease the size of ulcers in patients with critical limb ischemia [23]. Yuge *et al.* showed that HGF gene therapy prevented weight loss in sulfate-induced colitis in mice [35]. Additionally, HGF mice improved body regain after subcutaneous transplantation of diabetic rats [36]. Our results also showed that the ulcer area and ulcer-induced weight loss were significantly lower in HGF-Tg mice. This therapeutic effect of HGF is probably associated with increased epithelial proliferation, an anti-inflammatory effect, and a reduction in the number of infiltrating immune cells [13, 19, 35, 37, 38].

Our findings indicated that the effect of HGF on the healing of oral wounds involves decreasing the number of neutrophils in connective tissue of wound sites as well as circulating neutrophils. Neutrophils are the major infiltrating cells in wounds, especially in the early phase of damage [39]. One study has shown that HGF-specific c-Met knockout mice show significantly severe neutrophil infiltration in nonalcoholic fatty liver disease (NAFLD) [40]. HGF treatment can decrease the lymphocytes of the spleen in a mouse model [20]. In contrast, Bevan *et al.* reported that HGF/SF strongly improved skin wound repair in db/db mice by recruiting immune cells to the site of the wound through the repair process [41]. We also found that circulating T cells were higher in HGF-Tg mice. Thus, HGF mediates different immune reactions in different disease models and different stages of the same disease, and these dynamic changes have improved the understanding of HGF-mediated anti-inflammation.

The systemic inflammatory process is indispensable for the healing of oral ulcers, and severe inflammation may delay the healing of the epithelium [1]. Inflammatory cytokines mediate complex functions in traumatic ulcers, depending on their proportional concentrations [18, 41–43]. HGF may be a new drug target to treat metabolic disease by inhibiting inflammatory cytokines in the adipose tissue of HGF-Tg mice [44]. The anti-inflammatory roles of HGF have been described, including inhibiting pro- and inflammatory cytokines, such as TNF- $\alpha$ , IFN- $\gamma$ , TGF- $\beta$  and iNOS, increasing anti-inflam-

matory cytokines, such as RANTES, IL-4, and IL-10, and blocking NF- $\kappa$ B. All these roles could promote the wound healing process [20, 45]. However, some reports have shown that HGF reduced wound damage with higher levels of inflammatory cytokines and lower immune cells [35, 46]. This difference can be explained by the selection of different stages of disease for the analysis of immune cell and cytokine expression [35]. We measured the effect of HGF on 40 inflammatory cytokines on the 5th day after the model was established. Almost all cytokines had a higher fold-change of fluorescent signal intensity in the HGF-Tg group than in the WT group, except for fractalkine and MIP-1 alpha. These results are similar to those of previous studies [35, 46]. The levels of LIX, IL3, eotaxin, eotaxin-2, MIP-1, BLC, RANTES and MCSF were significantly higher in HGF-Tg mice than in WT mice after injury. Eotaxin, LIX, eotaxin-2, MIP-1, BLC, and RANTES are chemokines that are early signals from traumatic tissues to systemic immune cells [42]. This suggests that HGF promotes a stronger signal for the recruitment of inflammatory cells into traumatic tissue in the early stages of inflammation [35, 42]. The levels of the colony-stimulating factors, LIX and IL3, were also significantly higher in the HGF-Tg mice, possibly suggesting better tissue recovery after injury [42]. We also observed that almost all the pro- and anti-inflammatory cytokines increased in HGF-Tg mice, although the difference showed no statistical significance. Therefore, HGF can contribute to anti-inflammatory effects and wound repair by adjusting cytokine production [8].

In this study, we revealed a higher expression of HGF in the clinical saliva of oral traumatic ulceration patients. We further detected higher HGF expression in the ulcer tissue and serum of mice with traumatic ulcers of the oral mucosa. This result suggests HGF as a possible diagnostic marker of oral ulcers. The HGF-Tg ulcer mouse model unveiled the role of HGF in the prevention of neutrophils. The healing process of wounds is a complex, programmed event of genetic and biological series, and HGF is one of the potential growth factors that participate in the whole process of wound healing [47]. A limitation of this study is that we did not analyze the role of HGF in the re-epithelialization of wounds. Reepithelialization is important for ulcer healing, and HGF can promote skin and corneal wound healing by promoting the migration and proliferation of keratinocytes in the epithelium in an inflammatory environment [13, 48]. Another limitation of this study is that we did not analyze the mechanism of HGF-mediated anti-inflammation during the ulcer healing process. Our ongoing study will investigate the precise mechanism of HGF in the repair

process of oral ulcers.

In conclusion, we observed that HGF-Tg ulcer mice had less weight loss and ulcer area, less Ly6G-positive neutrophil infiltration, and higher levels of circulating cytokines, with a positive effect on the healing of oral traumatic ulcers.

### Ethics Statement

This animal experimentation was approved by the ethics committee of Affiliated Stomatology Hospital of Guangzhou Medical University (date of approval: 2016.2.26, approval number: 2016-067), and written informed consent was obtained from all patients and controls.

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### Conflicts of Interest

All authors declare no conflicts of interest.

### Author Contributions

Xinhong Wang designed and collected data and wrote the manuscript. Liting Yan performed the experiments and wrote the paper. Yinghua Tang, Xiaoxi He, Zhao Xiaomin, Liu Weijia and Wu Zhicong performed the experiments and analyzed the results. Gang Luo designed and conceived the study.

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### References

- Silva PGB, de Codes ÉBB, Freitas MO, Martins JOL, Alves APNN, Sousa FB. Experimental model of oral ulcer in mice: Comparing wound healing in three immunologically distinct animal lines. *J Oral Maxillofac Pathol*. 2018; 22: 444. [Medline] [CrossRef]
- Oliveira BV, Barros Silva PG, Nojosa JS, Brizenno LA, Ferreira JM, Sousa FB, et al. TNF-alpha expression, evaluation of collagen, and TUNEL of *Matricaria recutita* L. extract and triamcinolone on oral ulcer in diabetic rats. *J Appl Oral Sci*. 2016; 24: 278–290. [Medline] [CrossRef]
- Fitzpatrick SG, Cohen DM, Clark AN. Ulcerated lesions of the oral mucosa: clinical and histologic review. *Head Neck Pathol*. 2019; 13: 91–102. [Medline] [CrossRef]
- Hanson SE, Bentz ML, Hematti P. Mesenchymal stem cell therapy for nonhealing cutaneous wounds. *Plast Reconstr Surg*. 2010; 125: 510–516. [Medline] [CrossRef]
- Cavalcante GM, Sousa de Paula RJ, Souza LP, Sousa FB, Mota MRL, Alves APNN. Experimental model of traumatic ulcer in the cheek mucosa of rats. *Acta Cir Bras*. 2011; 26: 227–234. [Medline] [CrossRef]
- Mesquita KC, Dantas TS, de Barros Silva PG, de Queiroz Rodrigues MI, Alves APNN, Mota MRL, et al. Abatacept treatment impairs the cell migration and wound healing of oral ulcers in rats: Role of interleukin (IL)-1 $\beta$ , -6 and -10 and CD8/CD30 cells: Influence of abatacept treatment on oral wound healing: Experimental model on rats. *Life Sci*. 2020; 243: 117243. [Medline] [CrossRef]
- Nakamura T, Mizuno S. The discovery of hepatocyte growth factor (HGF) and its significance for cell biology, life sciences and clinical medicine. *Proc Jpn Acad, Ser B, Phys Biol Sci*. 2010; 86: 588–610. [Medline] [CrossRef]
- Ilangumaran S, Villalobos-Hernandez A, Bobbala D, Ramananthan S. The hepatocyte growth factor (HGF)-MET receptor tyrosine kinase signaling pathway: Diverse roles in modulating immune cell functions. *Cytokine*. 2016; 82: 125–139. [Medline] [CrossRef]
- Miyagi H, Thomasy SM, Russell P, Murphy CJ. The role of hepatocyte growth factor in corneal wound healing. *Exp Eye Res*. 2018; 166: 49–55. [CrossRef]
- Gallo S, Sala V, Gatti S, Crepaldi T. Cellular and molecular mechanisms of HGF/Met in the cardiovascular system. *Clin Sci (Lond)*. 2015; 129: 1173–1193. [Medline] [CrossRef]
- Bendinelli P, Matteucci E, Dogliotti G, Corsi MM, Banfi G, Maroni P, et al. Molecular basis of anti-inflammatory action of platelet-rich plasma on human chondrocytes: mechanisms of NF- $\kappa$ B inhibition via HGF. *J Cell Physiol*. 2010; 225: 757–766. [Medline] [CrossRef]
- Rong SL, Wang XL, Wang YC, Wu H, Zhou XD, Wang ZK, et al. Anti-inflammatory activities of hepatocyte growth factor in post-ischemic heart failure. *Acta Pharmacol Sin*. 2018; 39: 1613–1621. [Medline] [CrossRef]
- Omoto M, Suri K, Amouzegar A, Li M, Katikireddy KR, Mittal SK, et al. Hepatocyte growth factor suppresses inflammation and promotes epithelium repair in corneal injury. *Mol Ther*. 2017; 25: 1881–1888. [Medline] [CrossRef]
- Ishibashi H, Tonomura H, Ikeda T, Nagae M, Sakata M, Fujiwara H, et al. Hepatocyte growth factor/c-met promotes proliferation, suppresses apoptosis, and improves matrix metabolism in rabbit nucleus pulposus cells in vitro. *J Orthop Res*. 2016; 34: 709–716. [Medline] [CrossRef]
- Imamura R, Matsumoto K. Hepatocyte growth factor in physiology and infectious diseases. *Cytokine*. 2017; 98: 97–106. [Medline] [CrossRef]
- Sanada F, Fujikawa T, Shibata K, Taniyama Y, Rakugi H, Morishita R. Therapeutic angiogenesis using HGF plasmid. *Ann Vasc Dis*. 2020; 13: 109–115. [Medline] [CrossRef]
- Baek JH, Birchmeier C, Zenke M, Hieronymus T. The HGF receptor/Met tyrosine kinase is a key regulator of dendritic cell migration in skin immunity. *J Immunol*. 2012; 189: 1699–1707. [Medline] [CrossRef]
- Bai L, Lennon DP, Caplan AI, DeChant A, Hecker J, Kranso J, et al. Hepatocyte growth factor mediates mesenchymal stem cell-induced recovery in multiple sclerosis models. *Nat Neurosci*. 2012; 15: 862–870. [Medline] [CrossRef]
- Pang Y, Liang MT, Gong Y, Yang Y, Bu PL, Zhang M, et al. HGF reduces disease severity and inflammation by attenuating the NF- $\kappa$ B signaling in a rat model of pulmonary artery hypertension. *Inflammation*. 2018; 41: 924–931. [Medline] [CrossRef]
- Cao XP, Han DM, Zhao L, Guo ZK, Xiao FJ, Zhang YK, et al. Hepatocyte growth factor enhances the inflammation-alleviating effect of umbilical cord-derived mesenchymal stromal cells in a bronchiolitis obliterans model. *Cytotherapy*. 2016;

- 18: 402–412. [Medline] [CrossRef]
21. Li JF, Duan HF, Wu CT, Zhang DJ, Deng Y, Yin HL, et al. HGF accelerates wound healing by promoting the dedifferentiation of epidermal cells through  $\beta$ 1-integrin/ILK pathway. *BioMed Res Int*. 2013; 2013: 470418. [Medline] [CrossRef]
  22. Hisadome M, Ohnishi T, Kakimoto K, Kusuyama J, Bando K, Kanekura T, et al. Hepatocyte growth factor reduces CXCL10 expression in keratinocytes. *FEBS Lett*. 2016; 590: 3595–3605. [Medline] [CrossRef]
  23. Morishita R, Shimamura M, Takeya Y, Nakagami H, Chujo M, Ishihama T, et al. combined analysis of clinical data on HGF gene therapy to treat critical limb ischemia in japan. *Curr Gene Ther*. 2020; 20: 25–35. [Medline] [CrossRef]
  24. Powell RJ, Simons M, Mendelsohn FO, Daniel G, Henry TD, Koga M, et al. Results of a double-blind, placebo-controlled study to assess the safety of intramuscular injection of hepatocyte growth factor plasmid to improve limb perfusion in patients with critical limb ischemia. *Circulation*. 2008; 118: 58–65. [Medline] [CrossRef]
  25. Dally J, Khan JS, Voisey A, Charalambous C, John HL, Woods EL, et al. Hepatocyte growth factor mediates enhanced wound healing responses and resistance to transforming growth factor- $\beta$ 1-driven myofibroblast differentiation in oral mucosal fibroblasts. *Int J Mol Sci*. 2017; 18: 1843–1857. [Medline] [CrossRef]
  26. Wang X, Tang Y, Shen R, Chen J, Chen G, Luo G, et al. Hepatocyte growth factor (HGF) optimizes oral traumatic ulcer healing of mice by reducing inflammation. *Cytokine*. 2017; 99: 275–280. [Medline] [CrossRef]
  27. Ng YL, Klopčič B, Lloyd F, Forrest C, Greene W, Lawrence IC, NgYL. Secreted protein acidic and rich in cysteine (SPARC) exacerbates colonic inflammatory symptoms in dextran sodium sulphate-induced murine colitis. *PLoS One*. 2013; 8: e77575. [Medline] [CrossRef]
  28. Song P, Han T, Xiang X, Wang Y, Fang H, Niu Y, et al. The role of hepatocyte growth factor in mesenchymal stem cell-induced recovery in spinal cord injured rats. *Stem Cell Res Ther*. 2020; 11: 178–192. [Medline] [CrossRef]
  29. Mays JW, Sarmadi M, Moutsopoulos NM. Oral manifestations of systemic autoimmune and inflammatory diseases: diagnosis and clinical management. *J Evid Based Dent Pract*. 2012; 12:(Suppl): 265–282. [Medline] [CrossRef]
  30. Sorour AE, Lönn J, Nakka SS, Nayeri T, Nayeri F. Evaluation of hepatocyte growth factor as a local acute phase response marker in the bowel: the clinical impact of a rapid diagnostic test for immediate identification of acute bowel inflammation. *Cytokine*. 2015; 71: 8–15. [Medline] [CrossRef]
  31. Avetisyan M, Wang H, Schill EM, Bery S, Grider JR, Hassell JA, et al. Hepatocyte growth factor and MET support mouse enteric nervous system development, the peristaltic response, and intestinal epithelial proliferation in response to injury. *J Neurosci*. 2015; 35: 11543–11558. [Medline] [CrossRef]
  32. Boldyreva MA, Bondar IV, Stafeev IS, Makarevich PI, Beloglazova IB, Zubkova ES, et al. Plasmid-based gene therapy with hepatocyte growth factor stimulates peripheral nerve regeneration after traumatic injury. *Biomed Pharmacother*. 2018; 101: 682–690. [Medline] [CrossRef]
  33. Guru S, Sam SE, Rajan S, Padmanabhan S. Comparative evaluation of salivary hepatocyte growth factor in smokers and non-smokers with chronic periodontitis. *J Investig Clin Dent*. 2018; 9: e12306. [Medline] [CrossRef]
  34. Ohnishi T, Suwa M, Oyama T, Arakaki N, Torii M, Daikuhara Y. Prostaglandin E2 predominantly induces production of hepatocyte growth factor/scatter factor in human dental pulp in acute inflammation. *J Dent Res*. 2000; 79: 748–755. [Medline] [CrossRef]
  35. Yuge K, Takahashi T, Khai NC, Goto K, Fujiwara T, Fujiwara H, et al. Intramuscular injection of adenoviral hepatocyte growth factor at a distal site ameliorates dextran sodium sulfate-induced colitis in mice. *Int J Mol Med*. 2014; 33: 1064–1074. [Medline] [CrossRef]
  36. Yang SY, Yang KC, Sumi S. Prevascularization-free primary subcutaneous transplantation of xenogeneic islets coencapsulated with hepatocyte growth factor. *Transplant Direct*. 2020; 6: e620. [Medline] [CrossRef]
  37. Li JF, Duan HF, Wu CT, Zhang DJ, Deng Y, Yin HL, et al. HGF accelerates wound healing by promoting the dedifferentiation of epidermal cells through  $\beta$ 1-integrin/ILK pathway. *BioMed Res Int*. 2013; 2013: 470418. [Medline] [CrossRef]
  38. Lu Z, Chang W, Meng S, Xu X, Xie J, Guo F, et al. Mesenchymal stem cells induce dendritic cell immune tolerance via paracrine hepatocyte growth factor to alleviate acute lung injury. *Stem Cell Res Ther*. 2019; 10: 372–397. [Medline] [CrossRef]
  39. Ahlfors E, Czerkinsky C. Contact sensitivity in the murine oral mucosa. I. An experimental model of delayed-type hypersensitivity reactions at mucosal surfaces. *Clin Exp Immunol*. 1991; 86: 449–456. [Medline] [CrossRef]
  40. Kroy DC, Schumacher F, Ramadori P, Hatting M, Bergheim I, Gassler N, et al. Hepatocyte specific deletion of c-Met leads to the development of severe non-alcoholic steatohepatitis in mice. *J Hepatol*. 2014; 61: 883–890. [Medline] [CrossRef]
  41. Bevan D, Gherardi E, Fan TP, Edwards D, Warn R. Diverse and potent activities of HGF/SF in skin wound repair. *J Pathol*. 2004; 203: 831–838. [Medline] [CrossRef]
  42. Clausen F, Marklund N, Hillered L. Acute inflammatory biomarker responses to diffuse traumatic brain injury in the rat monitored by a novel microdialysis technique. *J Neurotrauma*. 2019; 36: 201–211. [Medline] [CrossRef]
  43. Hutchinson PJ, O'Connell MT, Rothwell NJ, Hopkins SJ, Nortje J, Carpenter KL, et al. Inflammation in human brain injury: intracerebral concentrations of IL-1alpha, IL-1beta, and their endogenous inhibitor IL-1ra. *J Neurotrauma*. 2007; 24: 1545–1557. [Medline] [CrossRef]
  44. Muratsu J, Iwabayashi M, Sanada F, Taniyama Y, Otsu R, Rakugi H, et al. Hepatocyte growth factor prevented high-fat diet-induced obesity and improved insulin resistance in mice. *Sci Rep*. 2017; 7: 130–136. [Medline] [CrossRef]
  45. Benkhoucha M, Santiago-Raber ML, Schneiter G, Chofflon M, Funakoshi H, Nakamura T, et al. Hepatocyte growth factor inhibits CNS autoimmunity by inducing tolerogenic dendritic cells and CD25+Foxp3+ regulatory T cells. *Proc Natl Acad Sci USA*. 2010; 107: 6424–6429. [Medline] [CrossRef]
  46. Jeschke MG, Bolder U, Finnerty CC, Przkora R, Müller U, Maihöfer R, et al. The effect of hepatocyte growth factor on gut mucosal apoptosis and proliferation, and cellular mediators after severe trauma. *Surgery*. 2005; 138: 482–489. [Medline] [CrossRef]
  47. Valente S, Ciavarella C, Pasanisi E, Ricci F, Stella A, Pasquinelli G. Hepatocyte growth factor effects on mesenchymal stem cells derived from human arteries: a novel strategy to accelerate vascular ulcer wound healing. *Stem Cells Int*. 2016; 2016: 3232859. [Medline] [CrossRef]
  48. Miura Y, Ngo Thai Bich V, Furuya M, Hasegawa H, Takahashi S, Katagiri N, et al. The small G protein Arf6 expressed in keratinocytes by HGF stimulation is a regulator for skin wound healing. *Sci Rep*. 2017; 7: 46649. [Medline] [CrossRef]