Original Article

Glycyrrhizin ameliorates melanoma cell extravasation into mouse lungs by regulating signal transduction through HMGB1 and its receptors

Keiichi Hiramoto,[1] Yurika Yamate,[1] Kenji Goto,[1] Shiho Ohnishi,[1] Akihiro Morita,[1] Nobuji Yoshikawa,[2] and Shosuke Kawanishi[1,*]

[1]Department of Pharmaceutical Sciences, Suzuka University of Medical Science, 3500-3 Minamitamagaki-cho, Suzuka, Mie 513-8670, Japan
[2]Matsusaka R&D Center, Cokey Co., Ltd., Matsusaka, Mie 515-0041, Japan

(Received 7 August, 2020; Accepted 1 October, 2020; Published online 9 March, 2021)

Metastasis, which accounts for the majority of all cancer-related deaths, occurs through several steps, namely, local invasion, intravasation, transport, extravasation, and colonization. Glycyrrhizin has been reported to inhibit pulmonary metastasis in mice inoculated with B16 melanoma. This study aimed to identify the mechanism through which glycyrrhizin ameliorates the extravasation of melanoma cells into mouse lungs. Following B16 melanoma cell injection, mice were orally administered glycyrrhizin once every two days over 2 weeks; lung samples were then obtained and analyzed. Blood samples were collected on the final day, and cytokine plasma levels were determined. We found that glycyrrhizin ameliorated the extravasation of melanoma cells into the lungs and suppressed the plasma levels of interleukin-6, tumor necrosis factor-α, and transforming growth factor-β. Furthermore, glycyrrhizin ameliorated the lung tissue expression of high mobility group box-1 protein (HMGB1), receptor for advanced glycation end products (RAGE), Toll-like receptor (TLR)-4, RAS, extracellular signal-related kinase, NF-κB, myeloid differentiation primary response 88, IκB kinase complex, epithelial-mesenchymal transition markers, and vascular endothelial growth factor-A. Our study demonstrates that glycyrrhizin ameliorates melanoma metastasis by regulating the HMGB1/RAGE and HMGB1/TLR-4 signal transduction pathways.

***Key Words*: glycyrrhizin, melanoma, high mobility group box-1 protein, epithelial-mesenchymal transition, receptor for advanced glycation end product**

Metastatic cancer is associated with the highest rate of cancer-related deaths. Metastasis occurs through several steps, namely, local invasion, intravasation, transport, extravasation, and colonization.(1) Identification of the mechanisms that underlie cancer cell extravasation could lead to the development of new therapeutic strategies to mitigate metastasis. Evidence suggests that epithelial-mesenchymal transition (EMT) influences extravasation,(2) and metastases can undergo EMT, thereby exacerbating extravasation.(3) Acquisition of the EMT phenotype by tumor cells not only increases their migration and invasion potentials, thereby facilitating their ability to infiltrate blood vessels and produce circulating tumor cells (CTCs), but also promotes CTC survival in the bloodstream and their ability to extravasate out of the circulatory system.(4,5) For example, melanoma can induce invasive phenotypes through EMT.(6)

Licorice, an important herb, has been used in both western and eastern medicine. Glycyrrhizin, a major active constituent of licorice (*Glycyrrhiza glabra*), is composed of one glycyrrhetinic acid and two glucuronic acid molecules. Glycyrrhizin has numerous pharmacological effects, including anti-inflammatory, anti-viral, anti-carcinogenic, and hepatoprotective activities, and it has been widely used in Asia as injections or oral tablets to treat patients with allergy and chronic hepatitis. Further, it has several biological activities, including anti-carcinogenic activities.(7,8)

Skin melanoma arises from unregulated melanocyte growth, forming tumors, and invading neighboring tissues. Although melanoma is one of the least common skin cancer types, it accounts for 79% of all skin cancer-related deaths, and it is an aggressive cancer that disseminates from small primary tumors through the blood or lymph and metastasizes to various sites.(9,10) The increasing trends in recreational unprotected sun exposure and the depletion of stratospheric ozone have resulted in an increase in the incidence and mortality rates of malignant melanoma.(11) While previous studies have investigated alternative therapeutic strategies for melanoma, no study has yet investigated the therapeutic value of glycyrrhizin against melanoma.(12)

The protein high mobility group box 1 (HMGB1), released during necrosis from the nucleus into the cytoplasmic and extracellular space of cancer cells,(13,14) is involved in cell proliferation, activation of angiogenesis, cell motility, and inflammatory condition.(15,16) Released HMGB1 interacts with the cell-surface receptor for advanced glycation end products (RAGE), which is a primary signaling pathway triggering the onset of various diseases.(17) HMGB1 binds to RAGE thus activating several signaling molecules including NF-κB, extracellular signal-regulated kinase (ERK1/2), and p38.(18) HMGB1 can bind to Toll-like receptor (TLR)-2 and TLR-4, activating the expression and release of pro-inflammatory cytokines such as tumor necrosis factor (TNF) and interleukin (IL)-6.(19) Therefore, the inhibition of HMGB1 activity during treatment may positively affect antitumor therapy.(20) Glycyrrhizin has been reported to inhibit HMGB1, including the box A domain of HMGB1.(21)

This study aimed to identify the mechanism through which glycyrrhizin ameliorates the extravasation of melanoma cells into the lungs of mice and how HMGB1 exerts its effects. Therefore, we established a mouse model by intravenously

*To whom correspondence should be addressed.
E-mail: kawanisi@suzuka-u.ac.jp

doi: 10.3164/jcbn.20-125

injecting melanoma cells. This model is useful for cancer metastasis studies and drug discovery.

Materials and Methods

Experimental animals. The homograft study was conducted using specific-pathogen-free (SPF) male, 8-week-old C57BL/6j mice (SLC, Hamamatsu, Japan), which were housed in individual cages in an air-conditioned room at 23 ± 1°C under SPF conditions with a 12-h light/dark cycle. The B16 mouse melanoma cell line established from these tumors were used at passages 5 to 15. All cells were cultured in Eagle's minimal essential medium (Sigma-Aldrich, Darmstadt, Germany) supplemented with 10% total value of serum and L-glutamine. Cells were routinely examined to ensure that they remained free from mycoplasma and murine viruses. For tumor injection, sub confluent monolayers were harvested following a 1-mm treatment with 0.25% trypsin-0.02% ethylenediamine-tetraacetic acid (Sigma-Aldrich). The trypsinized cells were washed and resuspended in phosphate-buffered saline (PBS; Ca^{2+}, Mg^{2+}, free-phosphate buffered saline: Sigma-Aldrich). The animals were randomly assigned to one of the four study groups (n = 6 per group): Control, glycyrrhizin treatment (group A), B16 melanoma treatment (group B), and B16 melanoma + glycyrrhizin treatment (group C). The B16 melanoma cells were intravenously injected into the tail vein (1×10^5 cells/mouse). Mice were examined for tumor metastasis at 2 weeks following injection. This study was conducted in accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of Suzuka University of Medical Science (approval number: 34). All surgeries were performed under pentobarbital anesthesia, and all efforts were made to minimize animal suffering.

Glycyrrhizin treatment. Following B16 melanoma cell injection, approximately 15 mg/kg of glycyrrhizin (Cokey Co., Matsusaka, Japan), dissolved in PBS (pH 7.4), was orally administered once every two days over 2 weeks. Control animals were administered PBS.[22] To evaluate treatment toxicity, mice were orally administered glycyrrhizin for 18 consecutive days; the toxic dose low was 90 mg/kg, which indicates that this dose is safe.[23]

Lung preparation and staining. We obtained the lung samples 2 weeks after study initiation. The lung specimens were fixed in phosphate-buffered paraformaldehyde (4%); embedded in frozen Tissue Tek, an optimal cutting temperature compound; and cut into 5-μm sections. The sections were stained with hematoxylin-eosin in accordance with the established procedures to enable histological analysis of the lung. To assess dopa-positive melanocyte expression, specimens were incubated at 37°C for 3 h in PBS containing 0.1% L-dopa (Sigma-Aldrich). Then, the specimens were washed in PBS for microscopic examination.[24]

Western blot analysis of the lungs. The lung samples were homogenized in lysis buffer (Kurabo, Osaka, Japan) and centrifuged at 8,000 × g for 10 min; the supernatant fluid was collected. Western blot analysis was performed as previously described.[22] The membranes were incubated at room temperature for 1 h with primary antibodies against HMGB1 (1:1,000; Abcam, Cambridge, MA), T-cell immunoglobulin and mucin domain 3 (TIM-3) (1:1,000; Cell Signaling Technology Inc., Danvers, MA), RAGE (1:1,000; Abcam), TLR-2 (1:1,000; Abcam), TLR-4 (1:1,000; Cell Signaling Technology Inc.), RAS (1:1,000; BD Biosciences, Franklin Lakes, NJ), p-extracellular signal-regulated kinase (p-ERK) (1:1,000, Bioss Antibodies Inc., Woburn, MA), p-NF-κB (1:1,000; Cell Signaling Technology Inc.), myeloid differentiation primary response (MyD) 88 (1:1,000; R&D Systems Inc., Minneapolis, MN), inhibitor of κB kinase (IKK) γ/NF-κB essential modulator protein (NEMO) (IKKγ/NEMO) (1:1,000; Abcam), EMT antibody sample kit (Snail, 1:1,000; vimentin, 1:1,000; N-cadherin, 1:1,000; β-catenin, 1:1,000; Cell Signaling Technology Inc.), vascular endothelial growth factor (VEGF)-A (1:1,000; BioLegend, San Diego, CA), or β-actin as a loading control (1:5,000; Sigma-Aldrich). The immune complex on the membranes was visualized using horseradish peroxidase-conjugated secondary antibody (Novex, Frederick, MD) and detected with ImmunoStar Zeta reagent (Wako, Osaka, Japan). The images of the membranes were acquired using a multi-grade software program (Fuji-film, Greenwood, SC).

Measurement of the plasma levels of HMGB1, IL-6, TNF-α, and TGF-β. Blood samples were collected from mice on the final day. The plasma HMGB1, IL-6, TNF-α, and TGF-β levels were determined using an enzyme-linked immunosorbent assay kit (HMGB1: Arigo Biolaboratories, Hsinchu City, Taiwan; IL-6, TNF-α, and TGF-β: R&D Systems) in accordance with the manufacturer's instructions.

Statistical analyses. All data are presented as the means ± SD. Data were entered into Microsoft Excel 2010. One-way analysis of variance (ANOVA) followed by Tukey's post-hoc test was used for analysis. All statistical analysis were performed using SPSS ver. 20 (IBM, Armonk, NY), and $p<0.05$ was considered statistically significant.

Results

Effects of glycyrrhizin treatment on the infiltration and proliferation of melanoma cells. Glycyrrhizin was administered once every two days; 2 weeks following treatment, the infiltration and proliferation of melanoma cells were observed through a microscope. Compared with the melanoma group, the melanoma + glycyrrhizin group showed an improvement in melanoma symptoms (Fig. 1). Tyrosine induces melanin production in dopa-positive cells. Since the proportion of dopa-positive cells is increased in melanoma, these cells serve as a parameter for this disease.[25]

Effects of glycyrrhizin treatment on the plasma levels of IL-6, TNF-α, and TGF-β in mice treated with melanoma cells. The plasma levels of IL-6, TNF-α, and TGF-β increased in mice treated with melanoma cells and decreased in the melanoma + glycyrrhizin treatment group (Fig. 2).

Effects of glycyrrhizin treatment on the expression and plasma levels of HMGB1 in lungs treated with melanoma cells. We examined the expression and plasma levels of HMGB1 (Fig. 3A) in the lungs (Fig. 3B). The HMGB1 plasma levels in the lungs were increased following melanoma cell treatment, but these levels decreased following glycyrrhizin administration (Fig. 3).

Effects of glycyrrhizin treatment on the expression of TIM-3, RAGE, TLR2, and TLR4 in lungs treated with melanoma cells. Next, we measured the expression of TIM-3, RAGE, TLR2, and TLR4, which are all glycyrrhizin receptors; the expression of these receptors was increased upon melanoma cell treatment. RAGE and TLR4 expression decreased following glycyrrhizin administration compared with the melanoma group (Fig. 4B and D). However, the expression of TIM-3 and TLR2 remained unchanged in the melanoma + glycyrrhizin and melanoma groups (Fig. 4A and C).

Effects of glycyrrhizin treatment on the expression of RAS, p-ERK, and p-NF-κB in lungs treated with melanoma cells. We examined the signal transducers downstream to RAGE when combined with glycyrrhizin. Melanoma cell treatment increased signal transducer levels, while melanoma + glycyrrhizin treatment restored them to the control group levels (Fig. 5).

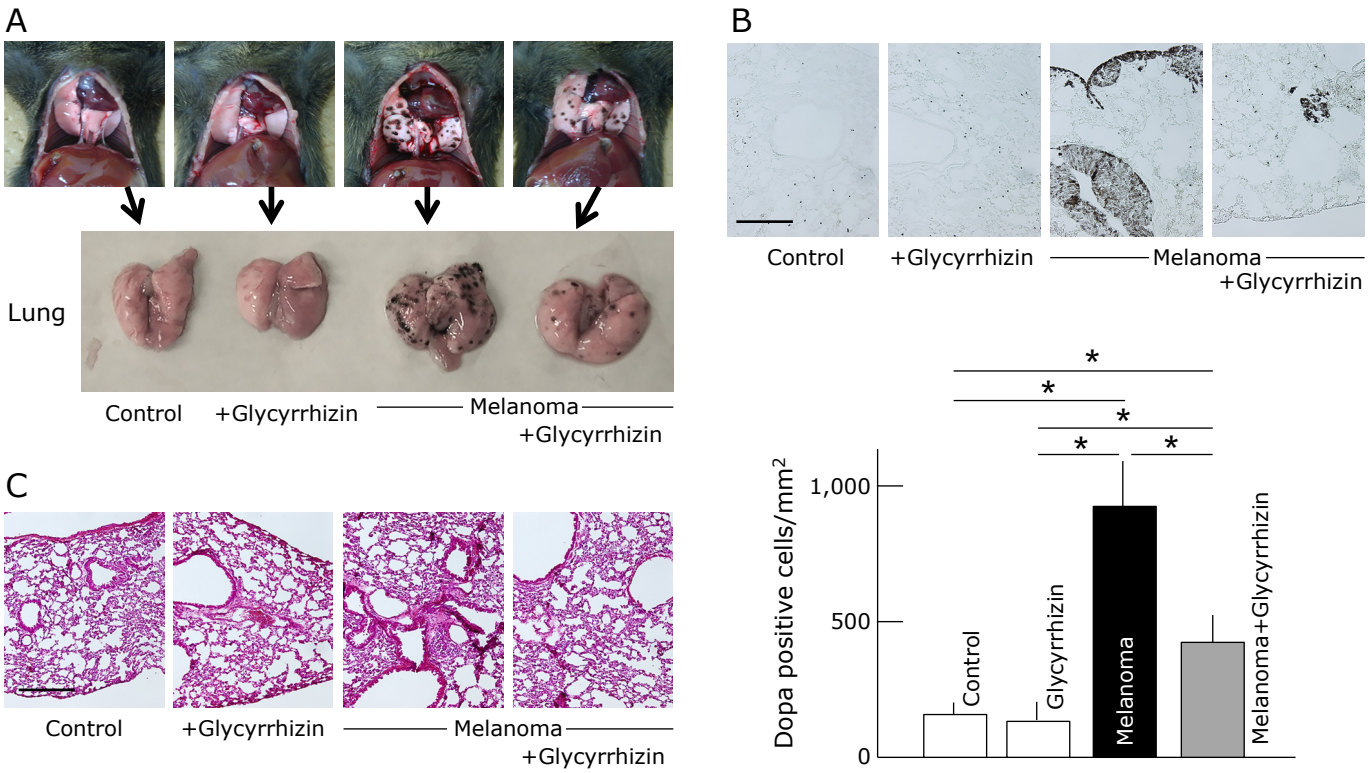

Fig. 1. Effect of glycyrrhizin treatment on infiltration and proliferation of melanoma in the lungs, 2 weeks after study initiation, (A) Macroscopic images of the lungs. The number of dopa-positive cells (B) are shown. Histological analysis of lung sections (scale bar = 100 μm) (C). Values are expressed as the mean ± SD derived from six animals. *p<0.05.

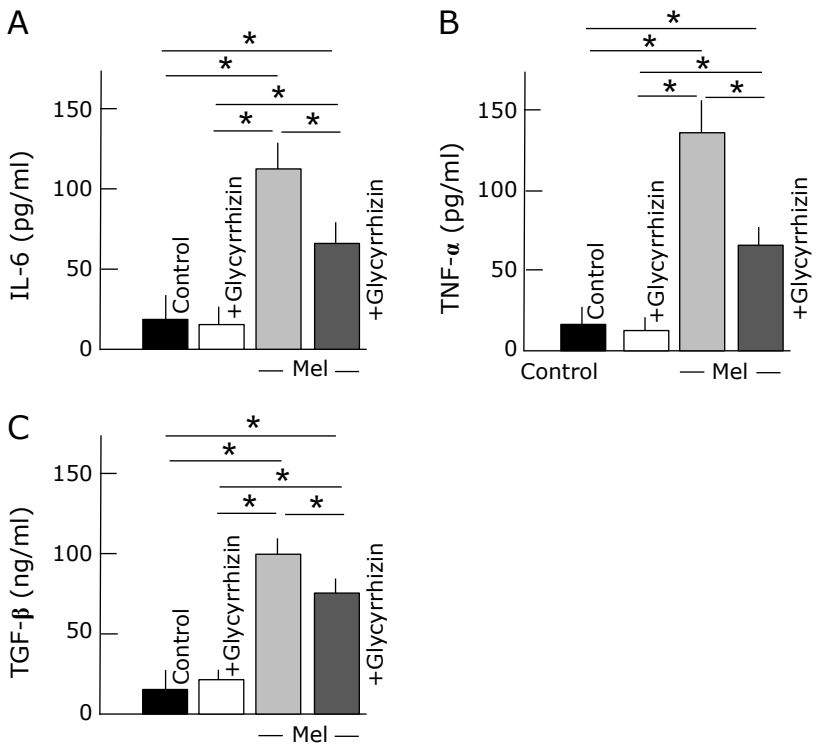

Fig. 2. Effects of glycyrrhizin treatment on the plasma levels of IL-6 (A), TNF-α (B), and TGF-β (C) 2 weeks following study initiation. We measured the levels of IL-6, TNF-α, and TGF-β in the plasma of mice using an ELISA kit. The values are expressed as the mean ± SD derived from six animals. *p<0.05.

doi: 10.3164/jcbn.20-125

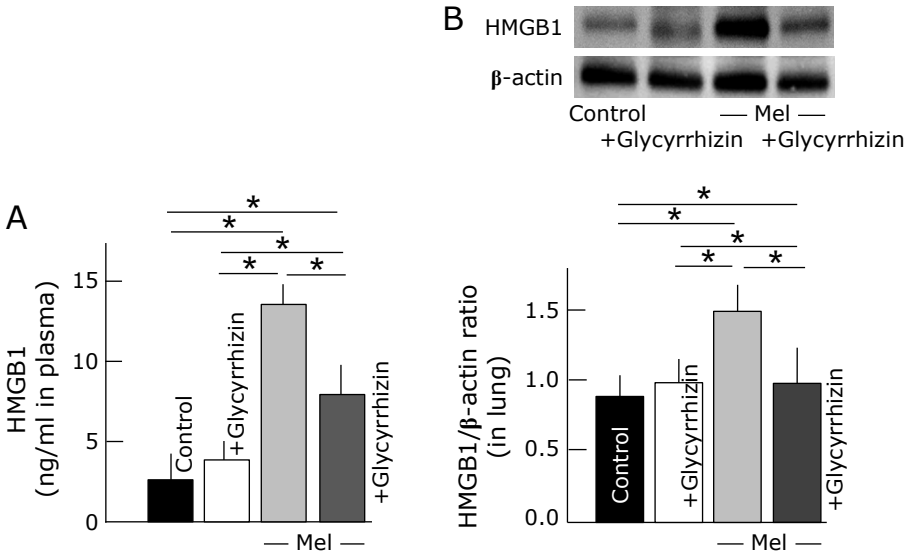

Fig. 3. Effects of glycyrrhizin on HMGB1 expression in the lungs of the model mice. The values are expressed as the mean ± SD derived from six animals. *p<0.05.

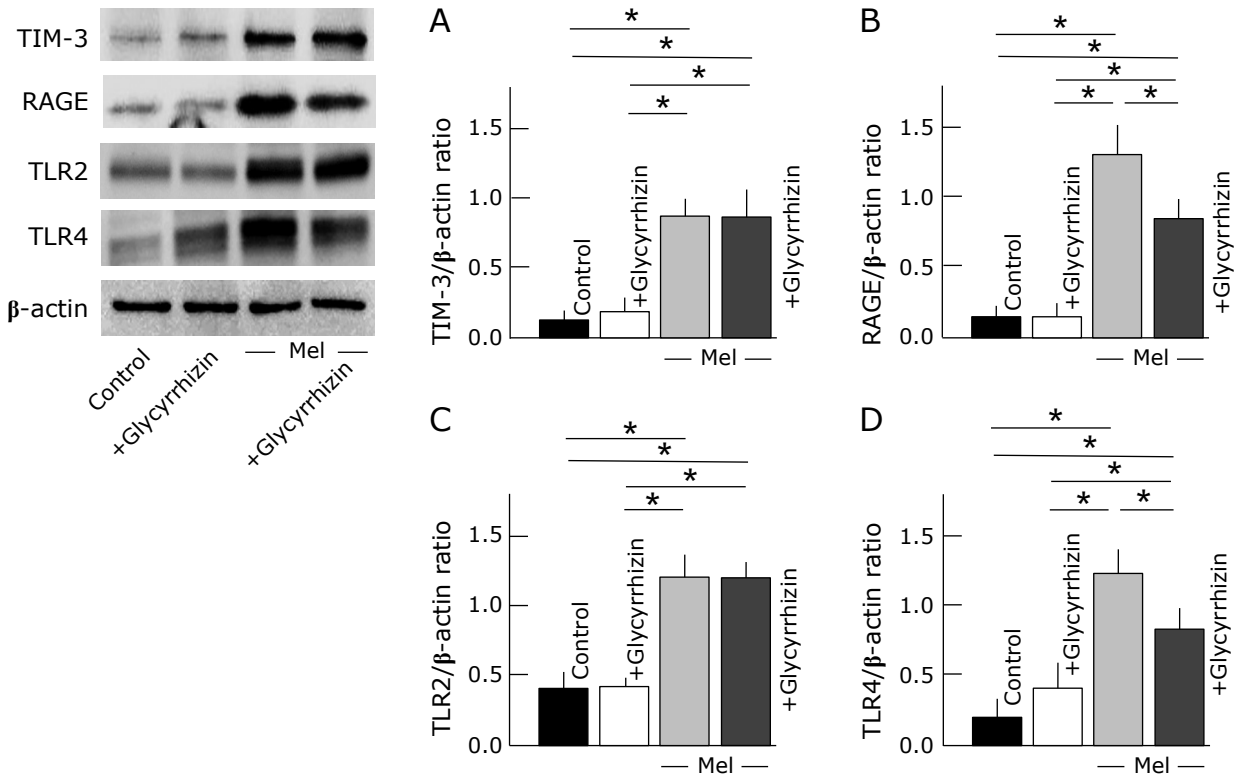

Fig. 4. Effects of glycyrrhizin treatment on the expression of HMGB1 receptors (TIM-3 (A), RAGE (B), TLR2 (C), and TLR4 (D)) in the lungs of the model mice. The values are expressed as the mean ± SD derived from six animals. *p<0.05.

Effects of glycyrrhizin treatment on the expression of MyD88 and IKKγ/NEMO in lungs treated with melanoma cells. We examined the signal transducer downstream to TLR4 when combined with glycyrrhizin. The expression of MyD88 and IKKγ/NEMO was increased following melanoma cell treatment, while it was decreased following melanoma + glycyrrhizin treatment (Fig. 6A and B).

Effects of glycyrrhizin treatment on the expression level of EMT in lungs treated with melanoma cells. We examined the expression of EMT (vimentin, snail, N-cadherin, and β-catenin), which was increased following melanoma cell treatment and decreased following melanoma + glycyrrhizin treatment (Fig. 7A–D).

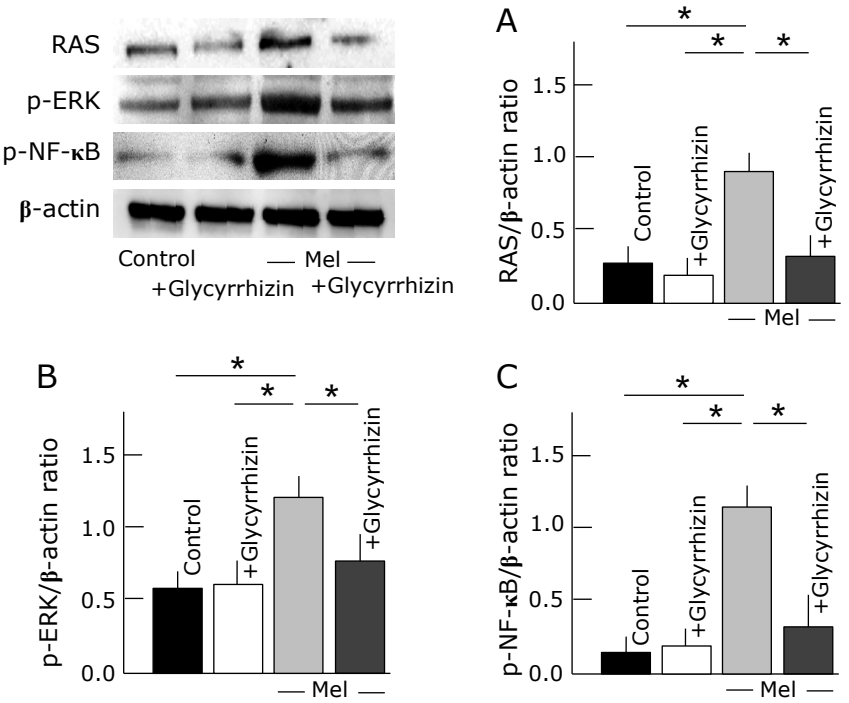

Fig. 5. Effects of glycyrrhizin treatment on the expression of RAS (A), p-ERK (B), and p-NF-κB (C) in the lungs of the model mice. The values are expressed as the mean ± SD derived from six animals. *p<0.05.

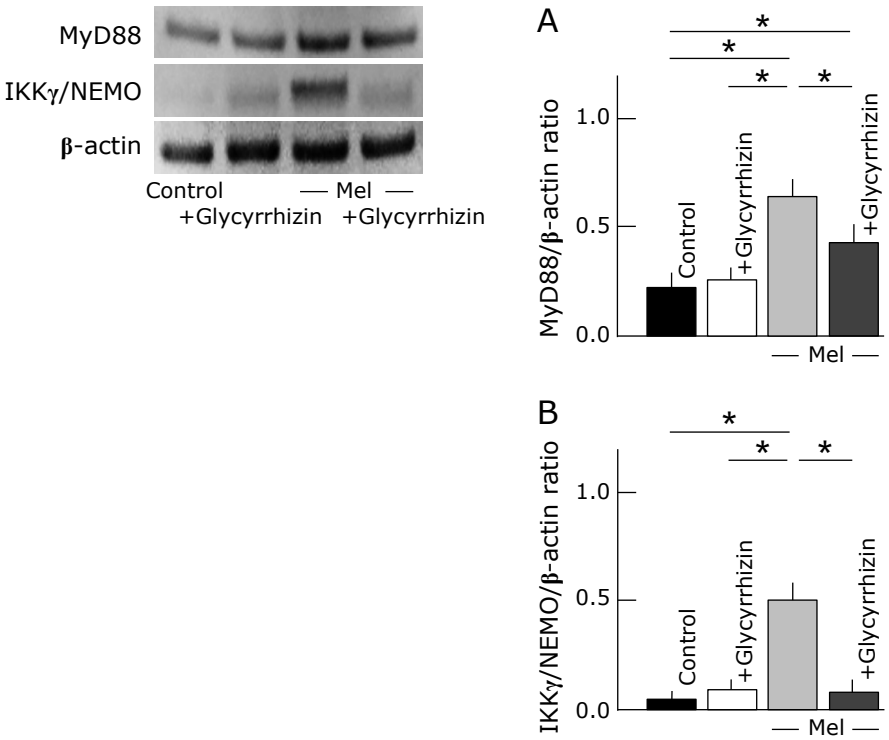

Fig. 6. Effects of glycyrrhizin treatment on the expression of MyD88 (A) and IKKγ/NEMO (B) in the lungs of the model mice. The values are expressed as the mean ± SD derived from six animals. *p<0.05.

Effects of glycyrrhizin treatment on the expression level of VEGF-A in lungs treated with melanoma cells. We assessed the expression of VEGF-A induced in another pathway associated with TLR4. The expression of VEGF-A increased following melanoma cell treatment and decreased following melanoma + glycyrrhizin treatment (Fig. 8).

doi: 10.3164/jcbn.20-125

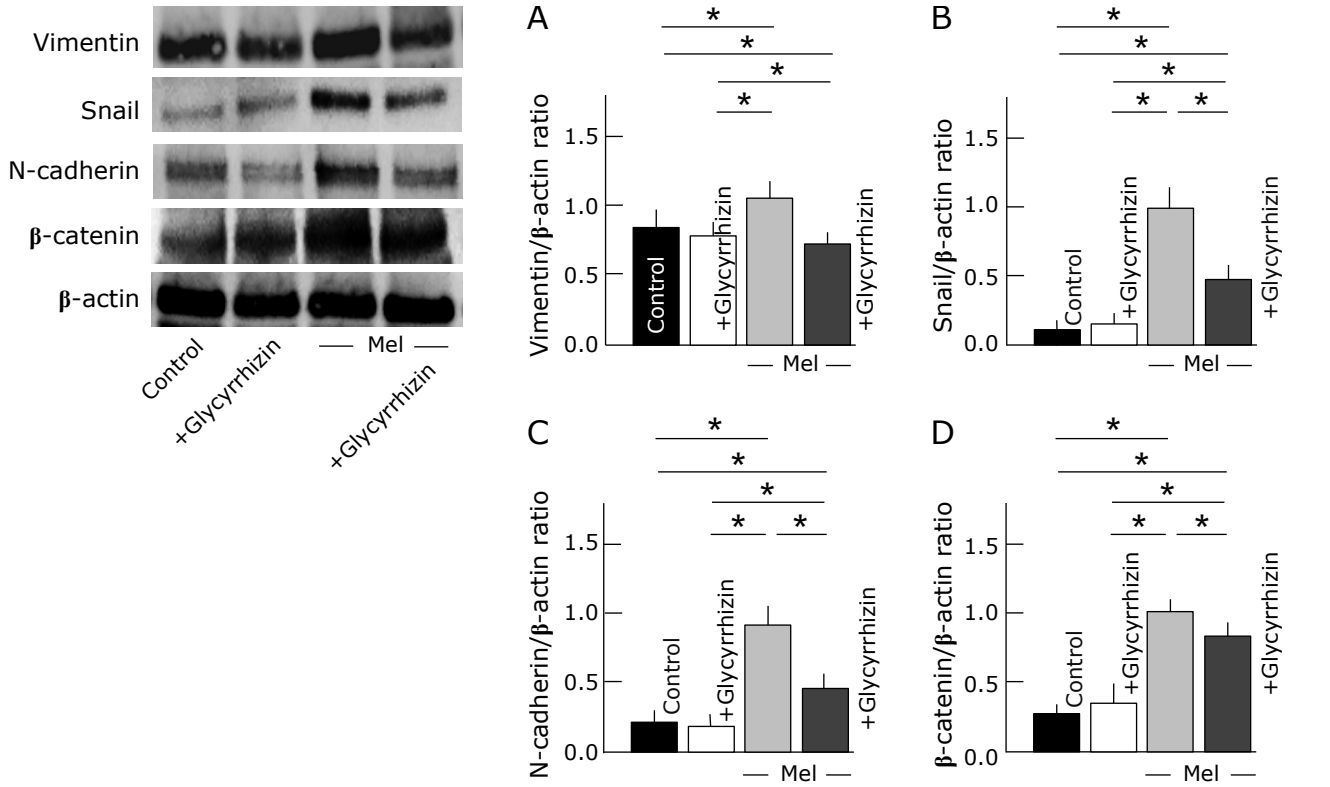

Fig. 7. Effects of glycyrrhizin treatment on the expression of EMT [vimentin (A), Snail (B), N-cadherin (C), and β-catenin (D)] in the lungs of the model mice. The values are expressed as the mean ± SD derived from six animals. *p<0.05.

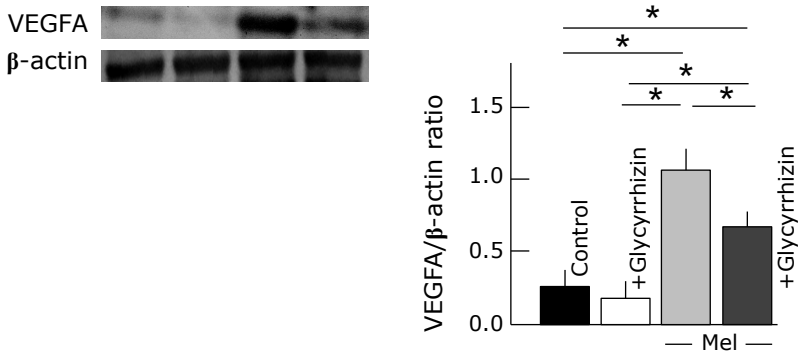

Fig. 8. Effects of glycyrrhizin treatment on VEGF-A expression in the lungs of the model mice. The values are expressed as the mean ± SD derived from six animals. *p<0.05.

Discussion

In this study, we confirmed the inhibitory effect of glycyrrhizin on experimental pulmonary metastasis in mice inoculated with B16 melanoma. However, the mechanism through which glycyrrhizin ameliorates the extravasation of melanoma cells into the lungs remains unclear. Further, this study demonstrated that glycyrrhizin inhibited the expression of HMGB1 and its receptors RAGE and TLR4, as well as RAS, ERK, NF-κB, and EMT markers, which are downstream to RAGE, and VEGF-A, MyD88, and IKKγ/NEMO, which are downstream to TLR4.

Glycyrrhizin targets HMGB1, which plays an important role in maintaining genome stability and autophagy activity during tumor growth.[26,27] HMGB1 can also be released into the extracellular space during necrosis, apoptosis, and autophagy.[28,29] In this study, necrosis and apoptosis were observed upon intravenous injection of cancer cells, which increased the release of HMGB1 to the extracellular space. Extracellular HMGB1 has cytokine, chemokine, and growth factor activities, thus acting as a protumor protein.[30] Once released, HMGB1 stimulates pro-inflammatory responses by binding to several cell surface receptors, including RAGE, TIM-3, TLR2, and TLR4. It then activates downstream signaling pathways, such as NF-κB, ERK1/2, and Akt, and the IL-6/signal transducer and activator of the transcription 3 (STAT3) pathway.[28,29,31] In this study, glycyrrhizin inhibited the expression of RAGE, which is a member of the immunoglobulin gene superfamily of cell surface molecules and the first reported receptor for HMGB1.[17,32] RAGE is expressed on immune cells, activated endothelial cells, vascular smooth muscle cells, and several cancer cells.[33]

Glycyrrhizin inhibited the expression of downstream RAS, ERK, and NF-κB by suppressing RAGE activity, along with the extravasation/proliferation of melanoma.

TLR4 expression was also inhibited in this study; TLR4s are typical pattern recognition receptors expressed on cells of the innate immune system. HMGB1 binds to TLR4 and mediates MyD88-dependent activation of the canonical IKK complex and nuclear translocation of NF-κB, which in turns induces the release of various pro-inflammatory cytokines, including TNF-α, IL-1, and IL-6.[34–36] Further, glycyrrhizin suppressed the expression of MyD88 and NF-κB and inhibited the signal of the HMGB1/TLR4 pathway and TLR4/VEGF-A expression. HMGB1 activates TLR4 and induces VEGF-A. Reymond *et al.*[36] have reported that myeloid cells, such as monocytes and macrophages, secrete VEGF locally, which stimulates cancer cell extravasation. VEGF, secreted by various types of primary tumors, can induce pulmonary hyperpermeability prior to carcinoma cell metastasis into the lungs, facilitating the extravasation of circulating tumor cells.[37] In this study, glycyrrhizin inhibited cancer cell infiltration by suppressing the expression of TLR4/VEGF-A.

HMGB1 has been shown to induce EMT (markers of vimentin, snail, N-cadhelin and β-catenin) cancer cells.[38] EMT is the process through which stationary epithelial cells become migratory and invasive mesenchymal cells, thus influencing the initiation and development of cancer metastasis.[39–41] In this study, glycyrrhizin suppressed the induction of EMT. HMGB1-induced EMT is mediated by RAGE.[42,43] HMGB1/RAGE axis signaling increases the secretion of primary growth factors associated with EMT, including TGF-β1, platelet-derived growth factor, and connective tissue growth factor.[43,44] Relevantly, Tsai and Yang have previously reported that platelets play a critical role in maintaining EMT activation in CTCs by providing the TGF-β signal. HMGB1 exacerbates TGF-β production and triggers Smad2/3 phosphorylation, which upregulates Snail 1, thereby inducing EMT.[45,46] Similarly HMGB1/RAGE axis signaling increases ERK1/2 phosphorylation, which in turn increases tumor cell invasion and metastasis.[46,47] HMGB1 binding to RAGE enhances NF-κB expression, phosphorylation, and nuclear translocation, all of which induce EMT and invasion.[42,46,48] Activated NF-κB increases the expression of Snail and IL-8, which promotes EMT.[42,49,50] Furthermore, the production of IL-6 and TNFα by RAGE/ERK/NF-κB signaling stimulates TGF-β secretion, which in turn increases EMT.[51–53]

The TLR4/MyD88 complex mediates NF-κB signaling-induced cancer cell invasion and EMT.[54] The results of this study show that glycyrrhizin controls the HMGB1/RAGE/TGF-β/EMT and HMGB1/RAGE/ERK/NF-κB/EMT systems, inhibiting the transposition and onset of melanoma. Moreover, glycyrrhizin inhibited the expression of TLR4. HMGB1 binding of TLR2 and TLR4 plays an important role in tumor metastasis.[6,55,56] TLR4 is overexpressed in melanoma, colon cancer, and breast cancer, while the expression of its downstream adaptor protein, MyD88, is elevated in melanoma and colon cancer.[57,58] HMGB1/TLR4-mediated activation of IKKγ/NEMO signaling contributes to NF-κB activation.[59,60] The IKKγ/NEMO-dependent NF-κB canonical pathway maintains RAGE expression.[59,60] Therefore, HMGB1 plays an important role in regulating EMT. HMGB1-RAGE/TLR4-induced EMT is mediated by Snail, NF-κB, and STAT3 through the activation of signaling pathways, including TGF-β, phosphatidylinositol 3-kinase/AKT, mitogen-activated protein kinase, and IKK.[42,46,48,49] The mechanism can be summarized as follows: HMGB1, binding to TLR4, mediates MyD88-dependent activation of the canonical IKK complex and nuclear translocation of NF-κB. This signaling induces the release of various pro-inflammatory cytokines and mediates Snail activation of EMT.[35,36,61] Glycyrrhizin may also inhibit the HMGB1/TLR4/EMT pathway; however, it does not influence the expression of TLR2 and TIM-3. TLR2 induces inflammatory cytokines, EMT, and TLR4.[42,56,62] Further, TIM-3 regulates B cell lymphoma, leukemia 2, and Bax and exerts an inhibitory effect on cancer.[63] However, the

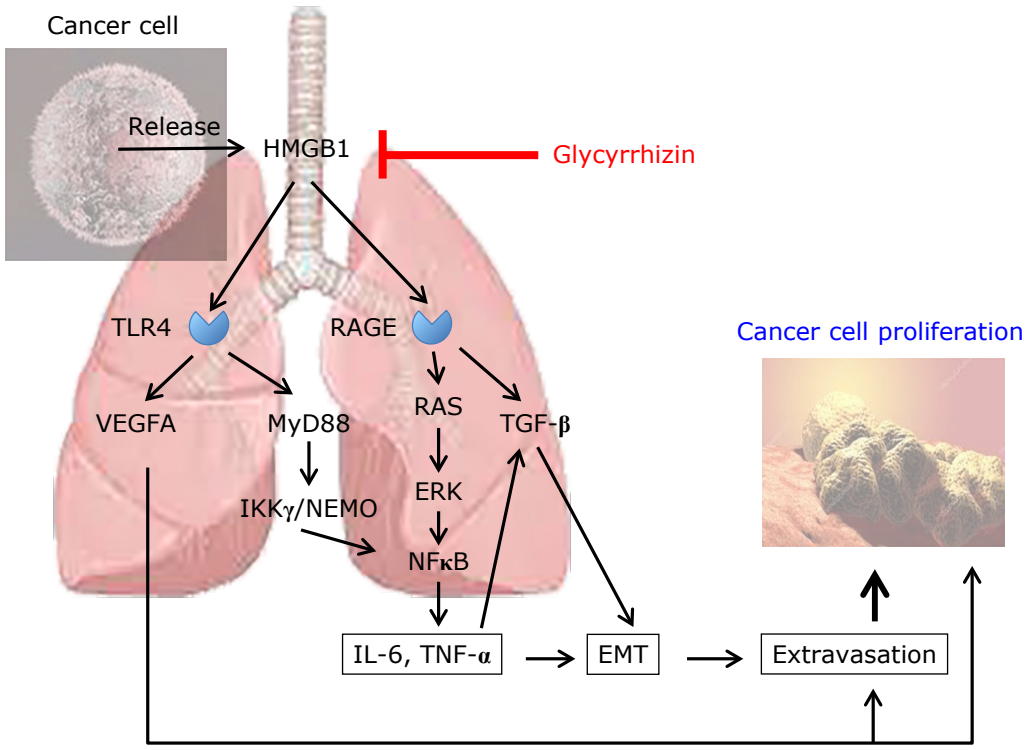

Fig. 9. Flowchart of the mechanism through which glycyrrhizin inhibits the infiltration and proliferation of melanoma.

doi: 10.3164/jcbn.20-125

association of glycyrrhizin, TLR2, and TIM-3 warrants further investigations.[63] In addition, as mechanisms underlying the inhibitory action of glycyrrhizin against melanoma, signaling pathways involving phosphatesitol-3-kinase (PI3K), p38, and protein kinase A (PKA),[64,65] along with the inhibition of ROS released from mitochondria have been previously reported.[66] In this study, we reported a glycyrrhizin-controlled signal transduction pathway consisting of HMGB1/RAGE and HMGB1/TLR4. However, whether it is also associated with other control pathways warrants further investigations. Therefore, identifying a control pathway through which melanoma is inhibited by glycyrrhizin is essential.

Glycyrrhizin suppresses the infiltration and proliferation of melanoma by inhibiting HMGB1 expression, which itself results in EMT and inflammatory cytokine suppression (Fig. 9). Glycyrrhizin suppresses HMGB1 expression, which is increased during anticancer treatment, thereby enhancing the therapeutic effect of the anticancer treatment. Collectively, these findings suggest that glycyrrhizin can be potentially used for cancer prophylaxis and treatment. Our findings should be validated in prospective trials.

Author Contributions

KH, YY, KG, SO, and AM performed experiments and analyzed the data; NY provided new tools and regents; SK conceived and supervised the study; KH and SK designed experiments and wrote the manuscript.

Acknowledgments

This study was supported by the JSPS KAKENHI under Grant (number 19K10585) and Cokey Co., Ltd. Under Grant (number 20190401).

Abbreviations

EMT	epithelial-mesenchymal transition
HMGB1	high mobility group box 1
MyD	myeloid differentiation primary response
NEMO	NF-κB essential modulator protein
RAGE	receptor for advanced glycation end products
TIM-3	T-cell immunoglobulin and mucin domain 3
TLR	Toll-like receptor
VEGF	vascular endothelial growth factor

Conflict of Interest

The authors report no conflicts of interest. The glycyrrhizin used by this experiment was supplied from the Cokey Co. free of charge.

References

1 Tsai JH, Yang J. Epithelial-mesenchymal plasticity in carcinoma metastasis. *Genes Dev* 2013; **27**: 2192–2206.
2 Fröse J, Chen MB, Hebron KE, *et al*. Epithelial-mesenchymal transition induces podocalyxin to promote extravasation via ezrin signaling. *Cell Rep* 2018; **24**: 962–972.
3 Lambert AW, Pattabiaman DR, Weinberg RA. Emerging biological principles of metastasis. *Cell* 2017; **168**: 670–691.
4 Sökeland G, Schumacher U. The functional role of integrins during intra- and extravasation within the metastatic cascade. *Mol Cancer* 2019; **18**: 12.
5 Liu H, Zhang X, Li J, Sun B, Qian H, Yin Z. The biological and clinical importance of epithelial-mesenchymal transition in circulating tumor cells. *J Cancer Res Clin Oncol* 2015; **141**: 189–201.
6 Pearlman RL, Montes de Oca MK, Pal HC, Afaq F. Potential therapeutic targets of epithelial-mesenchymal transition in melanoma. *Cancer Lett* 2017; **391**: 125–140.
7 Khan R, Rehman MU, Khan AQ, Tahir M, Sultana S. Glycyrrhizic acid suppresses 1,2-dimethylhydrazine-induced colon tumorigenesis in Wistar rats: alleviation of inflammatory, proliferation, angiogenic, and apoptotic markers. *Environ Toxicol* 2018; **33**: 1272–1283.
8 Ikeda K, Arase Y, Kobayashi M, *et al*. A long-term glycyrrhizin injection therapy reduces hepatocellular carcinogenesis rate in patients with interferon-resistant active chronic hepatitis C: a cohort study of 1249 patients. *Dig Dis Sci* 2006; **51**: 603–609.
9 Hartman KG, McKnight LE, Liriano MA, Weber DJ. The evolution of S100B inhibitors for the treatment of malignant melanoma. *Future Med Chem* 2013; **5**: 97–109.
10 Barbai T, Fejős Z, Puskas LG, Tímár J, Rásó E. The importance of microenvironment: the role of CCL8 in metastasis formation of melanoma. *Oncotarget* 2015; **6**: 29111–29128.
11 Braeuer RR, Watson IR, Wu CJ, *et al*. Why is melanoma so metastatic? *Pigment Cell Melanoma Res* 2014; **27**: 19–36.
12 Kobayashi M, Fujita K, Katakura T, Utsunomiya T, Pollard RB, Suzuki F. Inhibitory effect of glycyrrhizin on experimental pulmonary metastasis in mice inoculated with B16 melanoma. *Anticancer Res* 2002; **22**: 4053–4058.
13 Gauley J, Pisetsky DS. The translocateion of HMGB1 during cell activation and cell death. *Autoimmunity* 2009; **42**: 299–301.
14 Palumbo R, Sampaolesi M, De Marchis F, *et al*. Extracellular HMGB1, a signal of tissue damage, induces mesoangioblast migration and proliferation. *J Cell Biol* 2004; **164**: 441–449.
15 Mitola S, Belleri M, Urbinati C, *et al*. Cutting edge: extracellular high mobility group box-1 protein is a proangiogenic cytokine. *J Immunol* 2006; **176**: 12–15.
16 Srikrishna G, Freeze HH. Endogenous damage-associated molecules pattern molecules at the crossroads of inflammation and cancer. *Neoplasia* 2009; **11**: 615–628.
17 Sims GP, Rowe DC, Rietdijk ST, Herbst R, Coyle AJ. HMGB1 and RAGE in inflammation and cancer. *Annu Rev Immunol* 2010; **28**: 367–388.
18 Ibrahim ZA, Armour CL, Phipps S, Sukkar MB. RAGE and TLRs: relatives, friends or neighbors? *Mol Immunol* 2013; **56**: 739–744.
19 Yang WS, Han NJ, Kim JJ, Lee MJ, Park SK. TNF-α activates high-mobility group box 1 - Toll-like receptor 4 signaling pathway in human aortic endothelial cells. *Cell Physiol Biochem* 2016; **38**: 2139–2151.
20 Kawanishi S, Ohnishi S, Ma N, Hiraku Y, Murata M. Crosstalk between DNA damage and inflammation in the multiple steps of carcinogenesis. *Int J Mol Sci* 2017; **18**: 1808.
21 Mollica, L, De Marchis F, Spitaleri A, *et al*. Glycyrrhizin binds to high-mobility group box 1 protein and inhibits its cytokine activities. *Chem Biol* 2007; **14**: 431–441.
22 Jimbow K, Uesugi T. New melanogenesis and photobiological processes in activation and proliferation of precursor melanocytes after UV-exposure: ultrastructual differentiation of precursor melanocytes from Langerhans cells. *J Invest Dermatol* 1982; **78**: 108–115.
23 Horigome H, Hirano T, Oka K. Therapeutic effect of glycyrrhetinic acid in MRL lpr/lpr mice: implications of alteration of corticosteroid metabolism. *Life Sci* 2001; **69**: 2429–2438.
24 Yokoyama S, Hiramoto K, Koyama M, Ooi K. Skin disruption is associated with indomethacin-induced small intestine injury in mice. *Exp Dermatol* 2014; **23**: 659–663.
25 Aoki Y, Tanigawa T, Abe H, Fujiwara Y. Melanogenesis inhibition by an oolong tea extract in b16 mouse melanoma cells and UV-induced skin pigmentation in brownish guinea pigs. *Biosci Biotechnol Biochem* 2007; **71**: 1879–1885.
26 Bianchi ME, Crippa MP, Manfredi AA, Mezzapelle R, Rovere Querini P, Venereau E. High-mobility group box 1 protein orchestrates responses to tissue damage via inflamemation, innate and adaptive immunity, and tissue repair. *Immunol Rev* 2017; **280**: 74–82.
27 Bénéteau M, Zunino B, Jacquin MA, *et al*. Combination of glycolysis inhibition with chemotherapy results in an antitumor immune response. *Proc*

Natl Acad Sci U S A 2012; **109**: 20071–20076.
28 Kang R, Zhang Q, Zeh HJ 3rd, Lotze MT, Tang D. HMGB1 in cancer: good, bad, or both? *Clin Cancer Res* 2013; **19**: 4046–4057.
29 Tang D, Kang R, Zeh HJ 3rd, Lotze MT. High-mobility group box 1 and cancer. *Biochim Biophys Acta* 2010; **1799**: 131–140.
30 Ding J, Cui X, Liu Q. Emerging role of HMGB1 in lung diseases: friend or foe. *J Cell Mol Med* 2017; **21**: 1046–1057.
31 Hori O, Brett J, Slattery T, *et al*. The receptor for advanced glycation end products (RAGE) is a cellular binding site for amphoterin. Mediation of neurite outgrowth and co-expression of rage and amphoterin in the developing nervous system. *J Biol Chem* 1995; **270**: 25752–25761.
32 Yang S, Xu L, Yang T, Wang F. High-mobility group-1 and its role in angiogenesis. *J Leukoc Biol* 2014; **95**: 563–574.
33 Urbonaviciute V, Fürnrohr BG, Meister S, *et al*. Induction of inflammatory and immune responses by HNGB1-nucleosome complexes: implications for the pathogenesis of SLE. *J Exp Med* 2008; **205**: 3007–3018.
34 Park JS, Svetkauskaite D, He Q, *et al*. Involvement of toll-like receptors 2 and 4 in cellular activation by high mobility group box 1 protein. *J Biol Chem* 2004; **279**: 7370–7377.
35 Yu M, Wang H, Ding A, *et al*. HMGB1 signals through toll-like receptor (TLR) 4 and TLR2. *Shock* 2006; **26**: 174–179.
36 Reymond N, d'Água BB, Ridley AJ. Crossing the endothelial barrier during metastasis. *Nat Rev Cancer* 2013; **13**: 858–870.
37 Valastyan S, Weinberg RA. Tumor metastasis: molecular insights and evolving paradigms. *Cell* 2011; **147**: 275–292.
38 Thiery JP, Acloque H, Huang RY, Nieto MA. Epithelial-mesenchymal transitions in development and disease. *Cell* 2009; **139**: 871–890.
39 De Craene B, Berx G. Regulatory networks defining EMT during cancer initiation and progression. *Nat Rev Cancer* 2013; **13**: 97–110.
40 Zheng H, Kang Y. Multilayer control of the EMT master regulators. *Oncogene* 2014; **33**: 1755–1763.
41 Zhu L, Li X, Chen Y, Fang J, Ge Z. High-mobility group box 1: a novel inducer of the epithelial-mesenchymal transition in colorectal carcinoma. *Cancer Lett* 2015; **357**: 527–534.
42 Cheng M, Liu H, Zang D, *et al*. HMGB1 enhances the age-induced expression of CTGF and TGF-β via RAGE-dependent signaling in renal tubular epithelial cells. *Am J Nephrol* 2015; **41**: 257–266.
43 He M, Kubo H, Ishizawa K, *et al*. The role of the receptor for advanced glycation end-products in lung fibrosis. *Am J Physiol Lung Cell Mol Physiol* 2007; **293**: L1427–L1436.
44 Lynch J, Nolan S, Slattery C, Feighery R, Ryan MP, MeMorrow T. High-mobility group box protein 1: a novel mediator of inflammatory-induced renal epithelial-mesenchymal transition. *Am J Nephrol* 2010; **32**: 590–602.
45 Tafani M, Schito L, Pellegrini L, *et al*. Hypoxia-increased RAGE and P2X7R expression regulates tumor cell invasion through phosphorylateion of Erk1/2 and Akt and nuclear translocateion of NF-κB. *Carcinogenesis* 2011; **32**: 1167–1175.
46 Taguchi A, Blood DC, del Toro G, *et al*. Blockade of RAGE-amphoterin signallyng suppresses tumore growth and metastases. *Nature* 2000; **405**: 354–360.
47 Chen RC, Yi PP, Zhou RR, *et al*. The role of HMGB1-RAGE axis in migrateon and invasion of hepatocellular carcinoma cell lines. *Mol Cell Biochem* 2014; **390**: 271–280.
48 Chung HW, Jang S, Kim H, Lim JB. Combined targeting of high-mobility group box-1 and interleukin-8 to control micrometastasis potential lin gastric cancer. *Int J Cancer* 2015; **137**: 1598–1609.
49 Yu LX, Yan L, Yang W, *et al*. Platelets promote tumour metastasis via interaction between TLR4 and tumour cell-released high-mobility group box1 protein. *Nat Commun* 2014; **5**: 5256.
50 Kay AM, Simpson CL, Stewart JA Jr. The role of AGE/RAGE signaling in diabetes-mediated vascular calcification. *J Diabetes Res* 2016; **2016**: 6809703.
51 Chen Y, Tan W, Wang C. Tumor-associated macrophage-derived cytokines enhance cancer stem-like characteristics through epithetial-mesenchymal transition. *Onco Targets Ther* 2018; **11**: 3817–3826.
52 Serban AI, Stanca L, Geicu OI, Munteanu MC, Dinischiotu A. RAGE and TGF-β1 cross-talk regulate extracellular matrix turnover and cytokine synthesis in AGEs exposed fibroblast cells. *PLoS One* 2016; **11**: e0152376.
53 Wan G, Xie M, Yu H, Chen H. Intestinal dysbacteriosis activates tumor-associated macrophages to promote epithetial-mesenchymal transition of colorectal cancer. *Innate Immun* 2018; **24**: 480–489.
54 Ye K, Chen QW, Sun YF, Lin JA, Xu JH. Loss of BMI-1 dampens migration and EMT of colorectal cancer in inflammatory microenvironment through TLR4/MD-2/MyD88-mediated NF-κB signaling. *J Cell Biochem* 2018; **119**: 1922–1930.
55 Conti L, Lanzardo S, Arigoni M, *et al*. The noninflammatory role of high mobility group box 1/Toll-like receptor 2 axis in the self-renewal of mammary cancer stem cells. *FASEB J* 2013; **27**: 4731–4744.
56 Mittal D, Saccheri F, Vénéreau E, Pusterla T, Bianchi ME, Rescigno M. TLR4-mediated skin carcinogenesis is dependent on immune and radioresistant cells. *EMBO J* 2010; **29**: 2242–2252.
57 Rajput S, Volk-Draper LD, Ran S. TLR4 is a novel determinant of the response to paclitaxel in breast cancer. *Mol Cancer Ther* 2013; **12**: 1676–1687.
58 Kew RR, Penzo M, Habiel DM, Marcu KB. The IKKα-dependent NF-κB p52/ReIB noncanonical pathway is essentiall to sustain a CXCL12 autocrine loop in cells migrating in response to HMGB1. *J Immunol* 2012; **188**: 2380–2386.
59 Penzo M, Molteni R, Suda T, *et al*. Inhibitor of NF-kappa B kinases alpha and beta are both essential for high mobility group box 1-mediated chemotaxis. *J Immunol* 2010; **184**: 4497–4509.
60 Hoppe G, Talcott KE, Bhattacharya SK, Crabb JW, Sears JE. Molecular basis for the redox control of nuclear transport of the structural chromatin protein Hmgb1. *Exp Cell Res* 2006; **312**: 3526–3538.
61 Wu FH, Yuan Y, Li D, *et al*. Endothelial cell-expressed Tim-3 facilitates metastasis of melanoma cells by activating the NF-kappaB pathway. *Oncol Rep* 2010; **24**: 693–699.
62 Jube S, Rivera ZS, Bianchi ME, *et al*. Cancer cell secretion of the DAMP protein HMGB1 supports progression in malignant mesothelioma. *Cancer Res* 2012; **72**: 3290–3301.
63 Deng QP, Wang MJ, Zeng X, Chen GG, Huang RY. Effects of glycyrrhizin in a mouse model of lung adenocarcinoma. *Cell Physiol Biochem* 2017; **41**: 1383–1392.
64 Uto T, Ohta T, Yamashita A, Fujii S, Shoyama Y. Liquiritin and liquiritigenin induce melanogenesis via enhancement of p38 and PKA signaling pathways. *Medicines (Basel)* 2019; **6**: 68.
65 Zheng Y, Wang H, Yang M, *et al*. Prenylated flavonoids from roots of *Glycyrrhiza uralensis* induce differentiation of B16-F10 melanoma cells. *Int J Mol Sci* 2018; **19**: 2422.
66 Chen XY, Ren HH, Wang D, *et al*. Isoliquiritigenin induces mitochondrial dysfunction and apoptosis by inhibition mitoNEET in a reactive oxygen species-dependent manner in A375 human melanoma cells. *Oxid Med Cell Longev* 2019; **2019**: 9817576.

doi: 10.3164/jcbn.20-125