Journal of Ethnopharmacology 317 (2023) 116784

Contents lists available at ScienceDirect

Journal of Ethnopharmacology

journal homepage: www.elsevier.com/locate/jethpharm

GuiLu-ErXian Glue extract promotes mesenchymal stem cells (MSC)-Induced chondrogenesis via exosomes release and delays aging in the MSC senescence process

Yong-Hong Yang [a], Che-Sheng Wen [b], Yung-Ling Kuo [c], Su-Ling Fu [a], Tung-Yi Lin [a], Chao-Ming Chen [d,e,f], Po-Kuei Wu [d,e,f], Wei-Ming Chen [d,e], Jir-You Wang [a,d,e,*]

[a] Institute of Traditional Medicine, School of Medicine, National Yang Ming Chiao Tung University, Taipei, Taiwan, ROC
[b] Department of Orthopedics, Cheng-Hsin General Hospital, Taipei, Taiwan, ROC
[c] School of Chinese Medicine for Post Baccalaureate, College of Medicine, I-Shou University, Kaohsiung, Taiwan, ROC
[d] Department of Orthopedics, Taipei Veterans General Hospital, Taipei, Taiwan, ROC
[e] Department of Orthopedics, Therapeutical and Research Center of Musculoskeletal Tumor, Taipei Veterans General Hospital, Taipei, Taiwan, ROC
[f] Institute of Clinical Medicine, School of Medicine, National Yang Ming Chiao Tung University, Taipei, Taiwan, ROC

ARTICLE INFO

Handling Editor: V Kuete

Keywords:
Osteoarthritis
Mesenchymal stem cells
GuiLu-ErXian glue
Exosome
Chondrogenesis
Herbal medicine

ABSTRACT

Ethnopharmacological relevance: The treatment of osteoarthritis (OA) patients is a challenging problem. Mesenchymal stem cells (MSCs) are multipotent cells and play key roles in regenerative medicine for cartilage degeneration. GuiLu-ErXian Glue (GLEXG) is an herbal remedy widely used in traditional Chinese medicine to treat joint pain and disability in elderly OA patients. However, the mechanisms of how GLEXG affects MSCs-induced chondrogensis remains to be elucidated.

Aim of the study: The aim of this study was to investigate the effects of GLEXG on MSC-derived chondrogenesis, both *in vitro* and *in vivo* and its potential mechanisms.

Methods: Using human MSC (hMSCs) as *in vitro* model, the effects of HPLC-profiled GLEXG water extract on chondrogenic differentiation were investigated by 3D spheroid cultures under chondrogenesis-inducing medium (CIM) condition. The chondrogenesis process was evaluated by measuring the sphere sizes, chondrogenesis-related genes expression by reverse transcription real-time PCR that targeted type II/X collagens, SOX9, aggrecan, and protein expression by immunostaining. Anti-TGF-β1 neutralization antibody was used for mechanistic study. Mono-iodoacetate (MIA) induced OA joint was used to evaluate the effects of GLEXG on *in vivo* model. MSCs-derived exosomes were purified for proteomics study and senescence process was evaluated by cumulative population doublings and senescence-associated β-Galactosidase staining.

Results: The results showed that GLEXG enhanced hMSCs chondrogenesis and upregulated RNA expression of type II/X collagen, SOX9 and aggrecan at 0.1 μg/mL, 0.3 μg/mL *in vitro*. *In vivo*, GLEXG at the dose of 0.3 μg intraarticular (i.a.) injection rescued the MIA-induced cartilage defect. Proteomics and ingenuity pathway analysis obtained from MSCs-released exosomes suggested that senescence pathway was less activated in GLEXG group than in vehicle group. Besides, GLEXG was able to increase cumulative population doubling and delayed hMSCs senescence process after four passages in cultures.

Conclusion: we conclude that GLEXG promotes *in vitro* MSC-induced chondrogenesis possibly via exosomes release and delays aging in the MSC senescence process and that treatment with GLEXG (0.3 μg, i.a.) rescued cartilage defects in rat OA knee model.

* Corresponding author. School of Medicine, National Yang Ming Chiao Tung University, No. 155, Sec. II, Linong St, Peitou, 112304, Taipei, Taiwan, ROC.
E-mail addresses: lucas258012@gmail.com (Y.-H. Yang), jasonfennywen@gmail.com (C.-S. Wen), ylkylk@yahoo.com.tw (Y.-L. Kuo), slfu@nycu.edu.tw (S.-L. Fu), biotungyi@gmail.com (T.-Y. Lin), excelnova@gmail.com (C.-M. Chen), drwuvhg@gmail.com (P.-K. Wu), wmchen@vghtpe.gov.tw (W.-M. Chen), yollywang@gmail.com (J.-Y. Wang).

https://doi.org/10.1016/j.jep.2023.116784
Received 28 April 2023; Received in revised form 10 June 2023; Accepted 12 June 2023
Available online 14 June 2023
0378-8741/© 2023 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

List of abbreviation

ACAN	aggrecan
BMP	bone morphogenic protein
CIM	chondrogenic induction medium
Col II	collagen type II
Col X	collagen type X
CPD	cumulative population doublings
FBS	fetal bovine serum
GLEXG	GuiLu-ErXian Glue
hMSCs	human mesenchymal stem cells
MIA	mono-iodoacetate
Micro-CT	micro-computerized tomography
OA	osteoarthritis
PBS	phosphate-buffered saline
RT-PCR	reverse transcription real-time PCR
SA-β-Gal	senescence-associated β-Galactosidase
SOX 9	SRY-box 9
TCM	traditional Chinese medicine
TGF-β	transforming growth factor beta
VEGFA	vascular endothelial growth factor A

1. Introduction

Osteoarthritis (OA) is the most common chronic joint disorder in humans and the main reason for chronic pain and disability worldwide (Katz et al., 2021). The risk factors for OA include age, gender, genetics, and obesity (King et al., 2013; Lanyon et al., 2003; Spector and MacGregor, 2004). Current treatment strategies for OA include non-pharmacological intervention, pharmacological intervention, intraarticular injection, and surgery. While non-pharmacological and pharmacological therapies focus on reducing the pain, and maintaining the function of the joint (Hochberg et al., 2012), the intraarticular injection of corticosteroids, hyaluronic acid, and various blood-derived products is aimed at pain relief and decreasing systemic toxicity (Ayhan et al., 2014). Although surgical intervention plays a vital role in the management OA patients, the long-term outcome of such intervention is far beyond satisfaction.

Mesenchymal stem cells (MSCs) are pluripotent cells that can differentiate into a range of cell types including chondrocytes, osteocytes, and adipose cells (Jiang et al., 2002) and therefore they are good regeneration medicine candidates for the treatment of bone diseases such as osteoporosis and cartilage degeneration. There is consensus that many factors, including receptor ligands, intracellular proteins, and transcription factors, are involved in MSC-induced chondrogenesis. For example, the transforming growth factor β (TGF-β) and bone morphogenic protein (BMP) signaling pathways are essential for expanding chondrocyte populations and inducing chondrocyte differentiation from MSCs (Feng and Derynck, 2005; Tuli et al., 2003). In addition, Wnt- and SRY-box 9 (Sox9)- signaling are also important regulators of chondrogenesis (Zhao et al., 1997) because they affect the expression of some key genes such as Runx2, Col10a1 and VEGFA (J. Liao et al., 2014). Previously, the use of MSC-derived chondrogenesis has been applied clinically for supporting the process of cartilage repair and regeneration within OA joints (Davatchi et al., 2011; Mamidi et al., 2016). Furthermore, some studies have been conducted that have investigated the therapeutic effect of MSCs on OA patients (Lamo-Espinosa et al., 2016; W. S. Lee et al., 2019; Pers et al., 2016). However, findings regarding the effect of Chinese herbal medicines on MSC-derived chondrogenesis are lacking.

Traditional Chinese medicine (TCM) has been practiced in Chinese populations for thousands years to maintain their health and to treat various human disorders. According to "A Comparable Dictionary of Disease Names in Chinese and Western Medicine", OA is an impediment disease, a group of diseases associated with "invasion of wind, cold, and dampness" (Lin, 2004). Recently, some of Chinese herbal remedies, such as Hu-Qian-Wan (Hou et al., 2022), Du-Huo-Ji-Sheng-Tang (Chen et al., 2011) and the Yougui Pill (Zhang et al., 2017) have been shown to improve pain symptoms in animal OA joints. Nonetheless, the effects of Chinese herbs that are commonly used for the treatment of clinical OA patients in relation to MSC-derived chondrogenesis have not been explored.

In our previous study, we demonstrated that a Chinese herbal remedy Du-Huo-Ji-Sheng-Tang and its active component *Ligusticum chuanxiong* were able to inhibit the aging process of human mesenchymal stem cells and promote osteogenic differentiation (J. Y. Wang et al., 2017). Since MSCs are pluripotency, and are important to osteogenesis and chondrogenesis, we speculate that Du-Huo-Ji-Sheng-Tang might also have the potential to promote MSC-induced chondrogenesis if the MSCs are cultured in chondrogenic induction medium (CIM). While using human MSCs (hMSCs) as a screening model, we found that among the five Chinese herbal remedies most commonly used for treating clinical OA patients, a water extract of GuiLu-ErXian Glue (GLEXG), but not Du-Huo-Ji-Sheng-Tang, is able to enhance hMSCs-induced chondrogenesis *in vitro* (supplementary information 1). GLEXG is an herbal remedy widely used in traditional Chinese medicine to nourish blood and qi and to treat kidney deficiency (qi, ying, and yang). GLEXG is composed of deer antlers, turtle shell, Ginseng, and Goji berry. Clinical

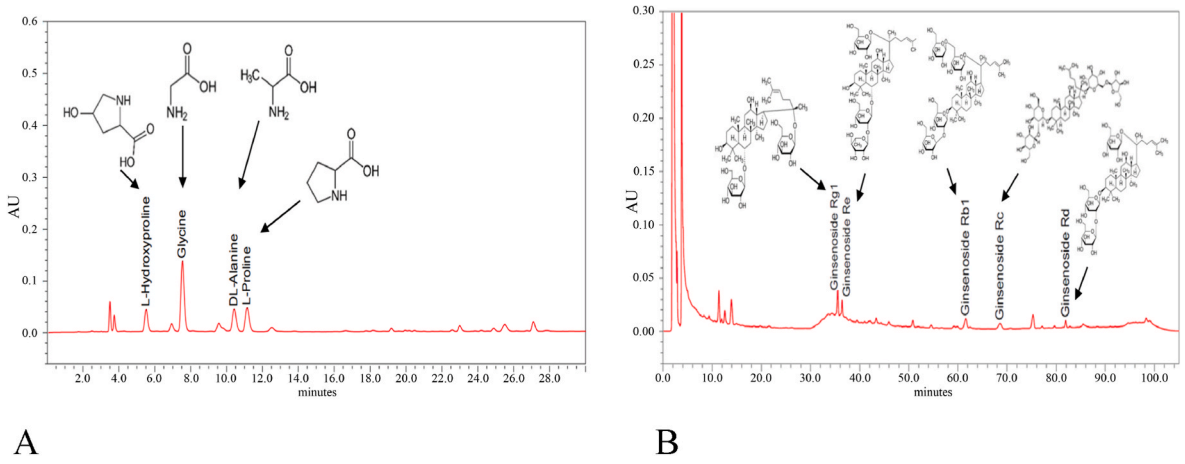


Fig. 1. High performance liquid chromatography analysis on GuiLu-Erxian Glue (GLEXG). Amino acids (A) and ginsenosides (B) profiling in GLEXG was analyzed by HPLC as mentioned in Methods.

observation studies have demonstrated the efficacy of GLEXG regarding joint pain and disability in elderly OA patients (J. A. Liao et al., 2022; Tsai et al., 2014). In addition, experimental evidence has shown that GLEXG not only has potent anti-inflammatory effects in mice (Chou et al., 2018), but also reduces cisplatin-induced damage to BM-MSCs (Ke et al., 2018). However, the mechanisms by which GLEXG enhances hMSCs-induced chondrogenesis remains to be elucidated. Accordingly, the aim of this study was to investigate the effects of GLEXG on hMSC-derived chondrogenesis, both *in vitro* and *in vivo*.

2. Materials and methods

2.1. Cell lines and reagents

The mesenchymal stem cell line (Wharton's jelly-derived mesenchymal stem cells, PCS-500-010) was purchased from American Type Culture Collection Center (ATCC). The surface markers of this cell line were identified by flow cytometry (supplementary information 2). The cells were cultured in complete α-MEM culture medium supplemented with 10% fetal bovine serum (FBS), streptomycin (100 μg/mL) and penicillin (100 units/mL) in a humidified atmosphere containing 5% CO2 at 37 °C. Furthermore, the cells were routine passaged every 3-4 days using 0.25% trypsin-EDTA to keep them in the logarithmic growth phase. The neutralization anti-TGF-β1 antibody was purchased commercially (1D11, Invitrogen, Waltham, USA).

2.2. Herbal remedy preparation

GLEXG is composed of deer antlers 1 gm, turtle shell 0.5 gm, Ginseng Radix et Rhizoma rubra 0.1 gm, and Goji berry (Lycii Fructus) 0.2 gm in a ratio of 10:5:1:2, which was kindly provided by a Good Manufacturing Practice (GMP) company (Chuang Song Zong Pharmaceutical Co., Ltd). The GLEXG water extract was prepared as follows: turtle shell and deer antlers were kept in a slightly boiling state in purified water for 7 days, then Ginseng and Goji berry were added and the decoction is continued for a period of time. After terminating the processes of decoction, the extract was filtered out and concentrated under reduced pressure to obtain the semisolid. The quality control of the GLEXG extract was monitored by high performance liquid chromatography (HPLC) (Fig. 1A and B). The parameters were the following: Waters e2695 Separations Module/Waters 2998 Dual λ Absorbance Detector; Column: Cosmosil 5C18 MS-II, 4.6×250 mm (5 μm); Column Oven Temp: 15 °C; Injecting volume: 20 μL; Flow rate: 1.0 mL/min; Detector: UV 203 nm; Mobile phase: (A) 0.15%H3PO4/H2O, (B) CH3CN. The final preparation was stored at −20 °C until used in the appropriate experiments. To decrease any confounding effect induced by the presence of lipopolysaccharide contamination during the herbal preparation, polymyxin B (10 μg/mL) was used routinely during each experiment *in vitro*.

2.3. Chondrogenic activity assay

To study the effects of herbal remedy on chondrogenesis, $3x10^5$ hMSCs were 3D-cultured using complete growth medium in a low-adherence 15-mL tube. After overnight incubation, the aggregate cells formed spheres without attaching to the tube and this followed by changing to chondrogenic induction medium (CIM) containing serum-free basal medium with 10^{-7} M dexamethasone (Sigma-Aldrich) in insulin transferrin selenium solution (ITS-G; Thermo Fisher Scientific), 40 g/mL L-proline (Sigma-Aldrich), and 50 μg/mL ascorbic acid-2 phosphate (Sigma-Aldrich) (Ciuffreda et al., 2016). The medium was then refreshed every two days with/without (vehicle) co-administration of the water extract (ddH_2O as solvent) of GLEXG in doses ranging of 0-, 0.03–0.1-, and 0.3 μg/mL. This was followed by collection of the chondrogenic sphere at day 14 and 21 for further evaluation. Medium containing exogenous recombinant human TGF-β1 (10 ng/mL) (R&D Systems) was used as a positive control for chondrogenesis. The success of chondrogenesis was validated by immunostaining, RT-qPCR, and Western blot analysis for chondrogenesis-related gene expression as mentioned below.

2.4. Histological analysis and immunohistochemistry staining

The chondrogenic spheres were collected after washing twice with phosphate-buffered saline (PBS) and they were then fixed using 4% paraformaldehyde for 2 h. This was followed by serial dehydration, paraffin wax embedding, and sectioning (4 μm); finally they were mounted on microscope glass slides (MUTO PURE CHEMICALS CO., LTD.). For chondrogenesis evaluation, the specimens were stained with alcian blue (pH 2.5, Scytek), counterstained with hematoxylin (Scytek) and observed under a light microscope (Leica) (Rigueur and Lyons, 2014). For proteins expression in chondrogenic spheres, de-paraffined specimens were stained with primary antibodies against type II collagen (Calbiochem) and type X collagen (Abcam) at 4 °C overnight; this was followed by incubation with biotinylated horse anti-mouse antibody (Vector Laboratories) for 30 min and detection using Vectastain ABC reagent (Vector Laboratories). The spheres also underwent staining with 3,3-diaminobenzidine (DAB) (BioGenex) and counter staining with Mayer's hematoxylin (Sigma-Aldrich).

2.5. Total RNA extraction and reverse transcription quantitative PCR (RT-qPCR)

The total RNA of the chondrogenic spheres were extracted using a GENEzol TriRNA Pure Kit (Geneaid) according to the manufacturer's instructions, followed by using a NanoDropTMLite (Thermo Fisher Scientific) to measure the quality of the RNA extract spectrophotometrically. Complementary DNA (cDNA) was obtained by reverse transcription using an iScript cDNA Synthesis Kit (Bio-Rad). Quantitative RT-PCR was performed on a StepOnePlus™ Real-Time PCR System (Thermo) in duplicate using SYBR® Green qPCR Mastermixes (QIAGEN). The commercially available primers used in this study were for type II collagen: forward 5′- TGGCTGACCTGACCTGATGTCC-3', reverse 5′- GCAGTCTGCCCAGTTCGGTC-3'; for SOX9: forward 5′- CCAAGCGCATTACCCACTTGTG-3′, reverse 5′- CGATTCTCCATCATCCTC-3'; for aggrecan: forward 5′- TGCAAGGAGACAGAGGACAC-3', reverse 5′-ACACAGGTCCCCTTCGTAGCTG-3'; for type X collagen: forward 5′-AGGCCCACTACCCAACACCAAGA-3′, reverse 5′- CGTAGCCTGGTTTTC CTGGTGGTC-3'; and for RNA45SN1: forward 5′- GGAATTGACGGAAGGGCACCA-3′, reverse 5′- CACCACCACCCACGGAATCG-3' (internal control). The relative expression of above genes as mRNA was normalized to the amount of RNA45SN1 in the same RNA extract. All samples were analyzed in duplicate in each experiment and three or four experiments were used for the statistical analysis.

2.6. Effects of neutralization anti-TGF-β1 antibody on hMSCs chondrogenesis

To investigate the mechanism behind GLEXG-induced hMSCs chondrogenesis, neutralization anti-TGF-β1 antibody (1D11, Invitrogen, Waltham, USA) was administrated with CIM. In short, 3 x 10^5 hMSCs were seeded and the GLEXG (0.1 μg/mL)-containing medium was refreshed every two days with or without neutralization anti-TGF-β1 antibody (0.5 μg/mL). This was followed by collection of the chondrogenic spheres at day 14 d for real-time PCR evaluation.

2.6.1. Isolation of extracellular vesicles or exosomes from GLEXG-treated hMSCs

Extracellular vesicles (exosomes) were isolated from the hMSCs culture condition medium. In brief, $5x10^5$ hMSCs with different doses of GLEXG extract (0- to 0.3 μg/mL) were cultured in a 10 cm dish with 6 mL complete culture medium for 2 d and the condition medium was collected by two sequential centrifugations at 300×*g* for 5 min. The

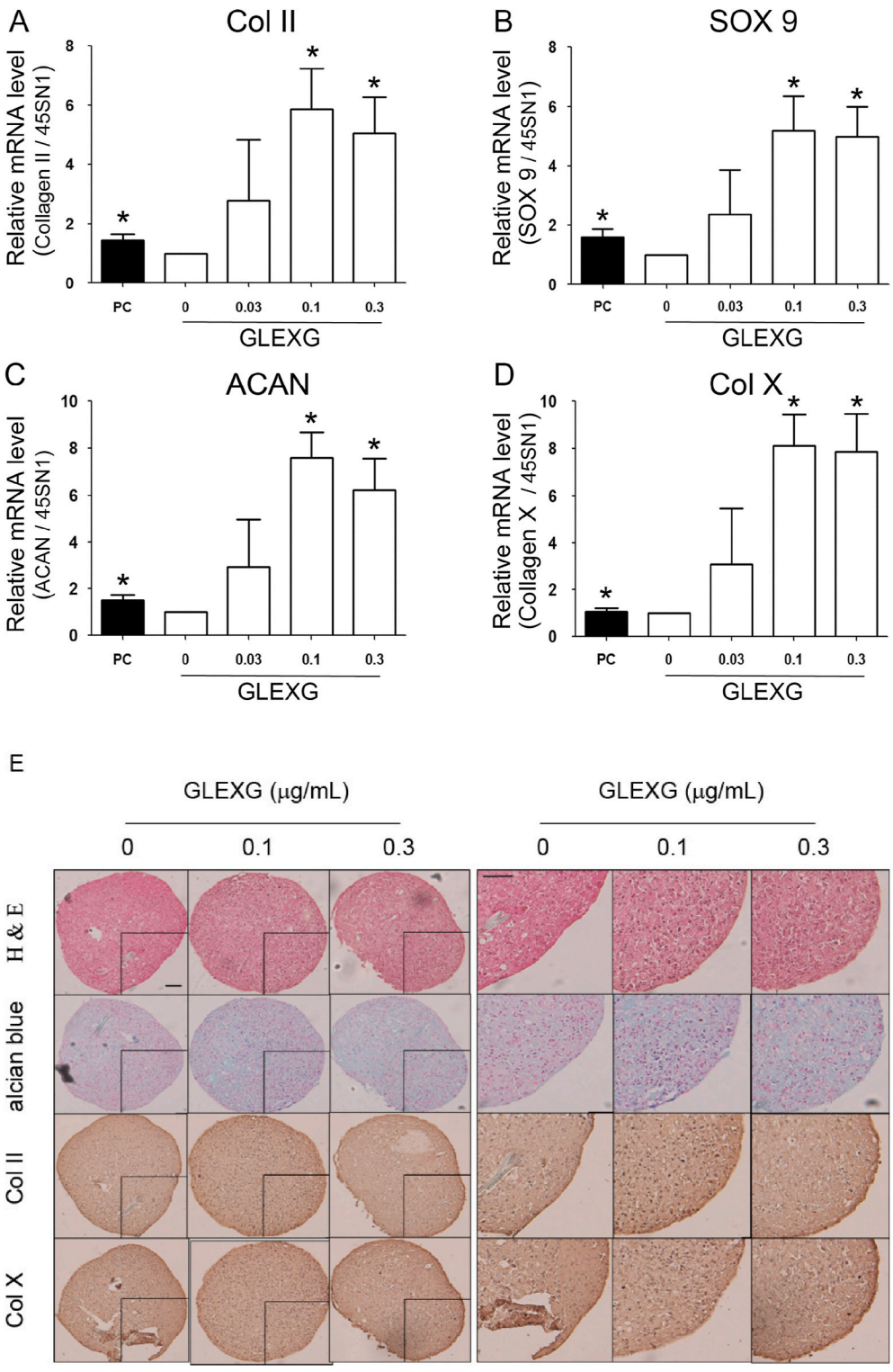


Fig. 2. Effects of GLEXG on hMSCs-derived chondrogenesis *in vitro*. After administration of GLEXG (0-0.3 μg/mL) on hMSCs for 3 weeks, chondro-sphere was measured (supplementary information 3), while chondrogenesis-related gene expression such as Collagen II (A), SOX 9 (B), Aggrecan (ACAN, C), and Collagen X (D) were analyzed by RT-PCR and immunohistochemistry staining (E) at 2 weeks and 3 weeks after treatment, respectively. For proteins expression in chondrogenic spheres, the de-paraffined specimens were stained with primary antibodies against type II collagen and type X collagen. Stained by alcian blue, the blue color indicates the successfulness of cartilage formation containing mucopolysarcharide. Right panel is the manified view of insert in left panel. Bar indicates 50 μm. Asteriks indicate $p < 0.05$ compared to vehicle group.

supernatant was then centrifuged two times at 2000×*g* for 30 min at 4 °C. Finally, the GLEXG-hMSCs-exosomes were pelleted by ultracentrifugation at 100,000×*g* for 90 min at 4 °C. There present was confirmed by the presence of the surface markers CD63 and CD81 (Cell Signaling) using immunofluorescence and Western blotting as well as transmission electron microscopy (Y. Wang et al., 2017). No hMSCs were present in the exosome fraction. The vehicle and GLEXJ-treated exosomes were then ready for proteomics analysis and senescence assay.

2.7. Western blotting

hMSCs derived exosomes were extracted using lysis buffer containing 1% Triton X-100, 10 mM Tris, pH 7.4, 150 mmol/L KCl and protease inhibitor cocktail (CompleteMini). The concentrations of protein were measured using the Bradford protein assay (Pierce) (Bradford, 1976). Protein (1 μg) was separated by 12% SDS-PAGE and, following this, the protein was transferred to a PVDF membrane. The membrane was blocked with 5% bovine serum albumin, which was followed by probing

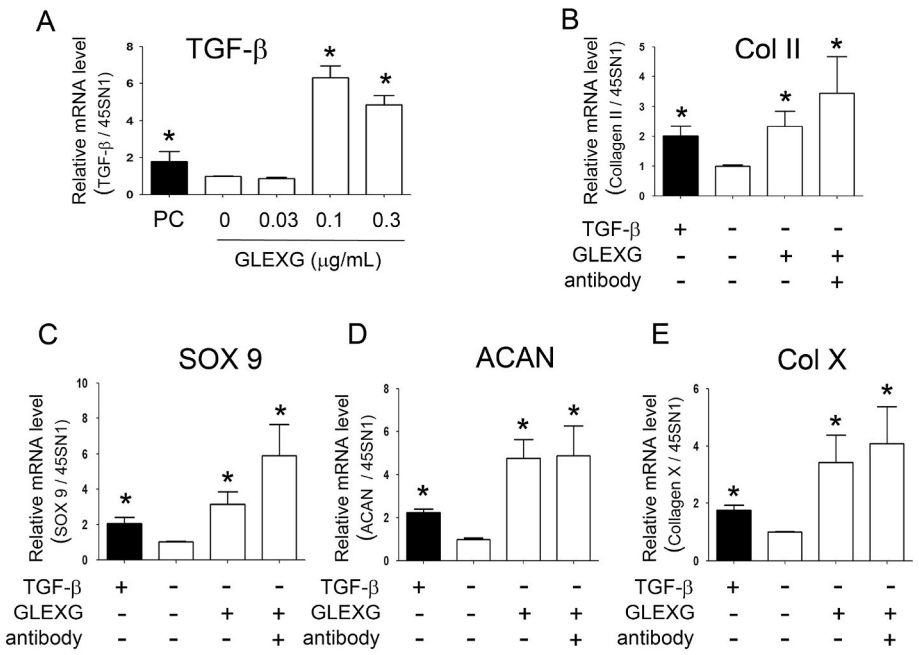


Fig. 3. The role of TGF-β in GLEXG-induced hMSCs-derived chondrogenesis. After administration of GLEXG (0-0.3 μg/mL) on hMSCs for 2 weeks, TGF-β gene expression was evaluated by real-time PCR (A). Besides, 3 x 10^5 hMSCs were seeded and the GLEXG (0.1 μg/mL)-containing medium was refreshed every two days with or without neutralization anti-TGF-β1 antibody (0.5 μg/mL), followed by collection of the chondrogenic sphere at day 14 d for chondrogenesis-related genes such as type II collagen (B), SOX 9 (C), Aggrecan (D), and collagen type X (E) by real-time PCR evaluation. Exogenous administration of TGF-β1 (10 ng/mL) into CIM was used as positive control (PC) for hMSCs-induced chondrogenesis. Asterisks indicate $p<0.05$ compared to vehicle group.

with specific primary antibody against CD63 (GeneTex), CD81 (GeneTex) at 4 °C overnight. Detection was then carried out using conjugated appropriate secondary antibodies and a chemiluminescence assay (Millipore, MA). Membranes were exposed to Analytik Jena™ UVP ChemStudio PLUS (Thermo fisher scientific) to visualize bands.

2.8. Proteomics analysis

After GLEXG-hMSCs-exosomes had been isolated from the hMSC cultures, vehicle-exosomes (0 μg/mL) and GLEXG-exosomes (0.1 μg/mL) were treated with lysis buffer and sent for proteomics analysis. Finally, four paired groups of exosomes (vehicle & GLEXG) were subjected to statistical analysis by unpaired Student *t*-test.

2.9. The hMSCs aging process

Since hMSCs are not transformed cells, they age and become senescent after long term passage. The senescence associated phenotype of hMSCs were evaluated by calculation of the cumulative population doublings (CPD) (K. S. Lee et al., 2013; J. Y. Wang et al., 2017) and the presence of senescence associated (SA) β-galactosidase staining. In summary, hMSCs ($1 \times 10^3/cm^2$) were initially seeded into complete medium and allowed to undergo continuous growth with subculture every week. Cell numbers were measured by the trypan blue exclusion assay over a total of five passages. CPD was measured using the formula $[\log_{10}(N_H) - \log_{10}(N_1)]/\log 10$, where N_H is the harvested cell number and $N_1$is the plated cell number.

2.10. Senescence associated β-galactosidase (SA-β-galactosidase) staining

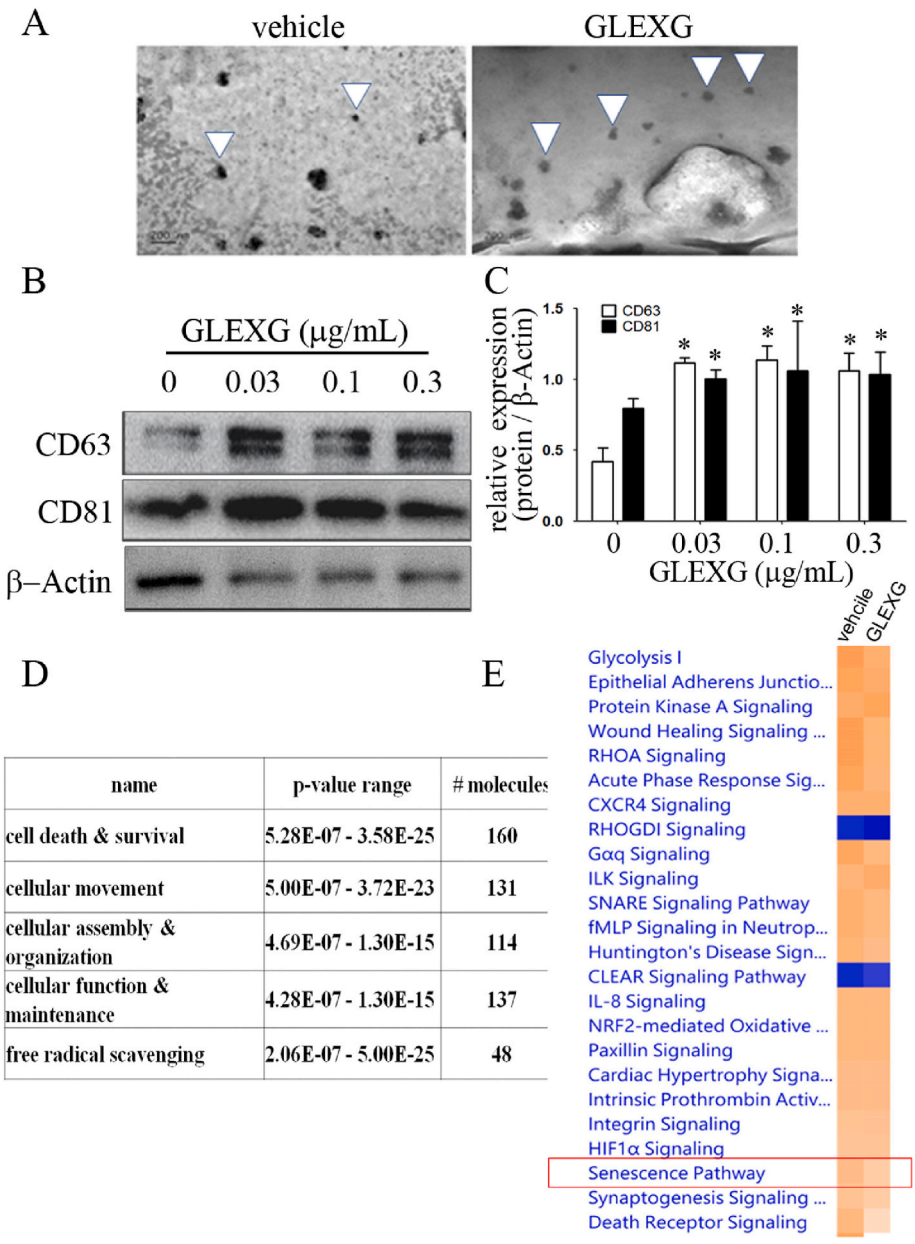
The trend in hMSCs senescence was evaluated by SA-β-galactosidase staining as described previously (J. Y. Wang et al., 2017). In brief, hMSCs after four continuously subcultures, were washed with PBS and fixed using 2% formaldehyde at RT for 5 min. After washing, the cells were incubated at 37 °C with fresh senescence-associated β-Gal (SA-β-Gal) chromogenic substrate solution containing 1 mg/mL 5-bromo-4-chloro-3-indolyl-β-galactoside (X-Gal, Cell Signaling Technology), 40 mM citric acid (pH 6.0), 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM NaCl, and 2 mM $MgCl_2$. The experiments were repeated in triplicate and data are presented as the mean percentage of β-galactosidase (+) cells.

2.11. Animal osteoarthritis (OA) model

A cartilage degeneration animal model was established to evaluate the effects of the GLEXG extracts on MSC chondrogenesis. Study protocols that involved experimental animals were approved by Taipei Veterans General Hospital (IACUC No. 2019-224). Male *Sprague Dawley* rats (280-350g) purchased from LASCO Biotechnology were kept in cages and treated following the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines. Under adequate anesthesia using Zoletil: Rompun = 1:1, the OA rat model was injected mono-iodoacetate (MIA, 1 mg/50 μL) (Sigma–Aldrich, St. Louis, MO, USA) once at the day 0 (Zhang et al., 2017). After 2 weeks induction, micro-CT examination was conducted in each rat as described below. The rats were randomized into four groups, namely, 1) sham OA (normal) group: rats without OA induction; 2) OA group: OA rats with above-mentioned OA induction and treated with GLEXG (0 μg); 3) OA + GLEXG (0.1 μg) group: OA rats treated with GLEXG (0.1 μg); 4) OA + GLEXG (0.3 μg) group: OA rats treated with GLEXG (0.3 μg). GLEXG was administrated every two weeks intra-articularly until sacrifice (supplementary information 4).

2.12. Micro-CT imaging and histological analysis for the OA joints

At day 14 and 42, each rat was anesthetized, and micro-CT scans were taken using a U-CTHR system (MILabs, Netherlands). The CT image was reconstructed using ITK-snaps software (Yushkevich et al., 2006), and were then analyzed for the presence of bone remodeling, fracture callus and bony lesions. At day 42, well-anesthetized animals were sacrificed and the joints were harvested for further histopathology, gross morphology and immunohistology assessment. Briefly, the joints were fixed in 4% paraformaldehyde for a day before being decalcified in Surgipaph Decalcifier II (LEICA) for 6 days at 37 °C. Next, the samples were processed for pathology examination and stained with Hematoxylin and eosin stain and Safranin-O/Fast green stain. The pink region of rat cartilage as identified by Safranin-O staining was quantified as the area of positive remained chondrocytes.



name	p-value range	# molecules
cell death & survival	5.28E-07 - 3.58E-25	160
cellular movement	5.00E-07 - 3.72E-23	131
cellular assembly & organization	4.69E-07 - 1.30E-15	114
cellular function & maintenance	4.28E-07 - 1.30E-15	137
free radical scavenging	2.06E-07 - 5.00E-25	48

Fig. 4. Effects of GLEXG on hMSCs-secreted exosomes. After GLEXG treatment in serum-free culture medium on hMSCs for 2 d, extracellular vesicles (exosomes) were isolated from culture condition medium as described in Methods. Electron microscopic examination (A) and Western blot for CD63 and CD81 expression (B, C) were performed to validate the presence of exosomes. The differential expressed peptides in GLEXG-treated exosomes obtained from proteomics were subjected to ingenuity pathway analysis (D, E). Asterisks indicate $p<0.05$ compared to vehicle group.

2.13. Statistics

Data are expressed as mean ± standard deviation (SD). Independent t tests or Mann-Whitney U tests were performed for comparison of data from two independent samples. More than two groups (dose-dependent effects) were compared by one-way ANOVA with Dunnet's post hoc test. More than two variables (time effects and dose effects) were analyzed by two-way ANOVA. A p value < 0.05 was considered statistically significant.

3. Results

3.1. GLEXG extract enhances hMSCs-induced chondrogenesis in vitro

To investigate the effect of GLEXG on hMSCs-induced chondrogenesis, hMSCs were cultured in chondrogenic induction medium (CIM) with or without GLEXG. Chondrogenic mRNA markers were measured at day 14 after induction, while spheroid size and expression of extracellular matrix markers were evaluated at day 21. The results showed that there was no significantly increased sphere size when GLEXG-treated and the vehicle group were compared (supplementary information 3). However, GLEXG (at 0.1 μg/mL, 0.3 μg/mL) did enhance chondrogenesis *in vitro*, including an increase in chondrogenic markers, for example an upregulation of the RNA expressionof type II collagen (Fig. 2A), SOX9 (Fig. 2B), Aggrecan (Fig. 2C), and collagen type X (Fig. 2D) as measured by RT-qPCT and of collagen II and X protein expression as assessed by alcian blue and immunohistochemical staining (Fig. 2E).

3.2. The role of TGF-β1 in GLEXG-hMSCs-chondrogenesis

TGF-β is a key factor in hMSCs-induced chondrogenesis and was used by us as a positive control. Using CIM (without TGF-β1) as induction medium, we found that GLEXG was able to enhance hMSC chondrogenesis, which suggests that GLEXG might possess activity similar to TGF-β1. Our results showed that GLEXG increased not only various chondrogenic markers (Fig. 2A-D), but also the mRNA expression of TGF-β1 in the 0.1 μg/mL and 0.3 μg/mL groups (Fig. 3A). However, after

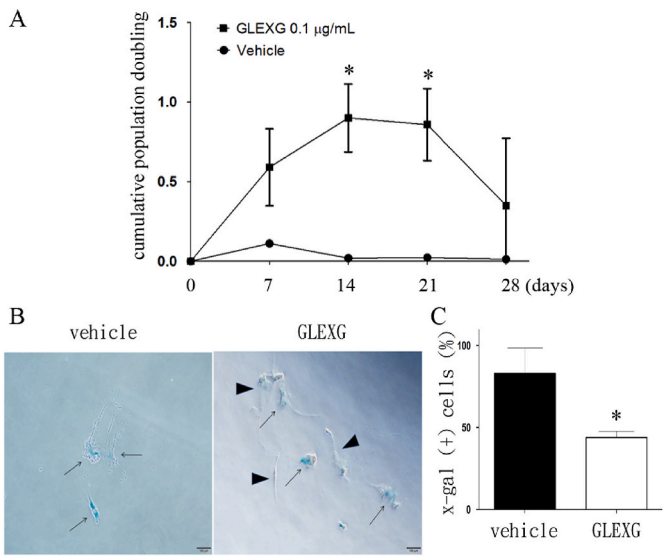


Fig. 5. Effects of GLEXG on the senescence process of human mesenchymal stem cells (hMSCs). The senescence associated phenotypes were evaluated by cumulative population doubling (A) and β-galactosidase staining (B). The experiments were repeated in triplicate and data were presented as the mean percentage of β-galactosidase (+) cells (C). Arrows indicate β-galactosidase staining (+) cells, while arrow heads indicate live hMSCs. *, $p<0.05$ by two way ANOVA (A) and unpaired student *t*-test (C).

pretreatment with neutralization anti-TGF-β1 antibody in the culture medium, there was no significant change in the mRNA expression levels of type II collagen (Fig. 3B), SOX9 (Fig. 3C), aggrecan (Fig. 3D), and collagen type X (Fig. 3E) compared to the GLEXG-treated group. These results suggest that production of endogenous TGF-β is not the main mechanism by which GLEXG-induced hMSCs chondrogenesis occurs.

3.3. Exosomes released from hMSCs

There is consensus that hMSCs excretes exosomes into their culture medium. After GLEXG treatment in serum-free culture medium for 2 d, we found that there was an increase in hMSCs-derived exosomes in the GLEXG-treated group compared to the vehicle group (Fig. 4 A). Western blot analysis showed that CD63 and CD81 protein expression levels were higher in GLEXG-treated group than in the vehicle group (Fig. 4B and C).

3.3.1. Proteomics analysis of the GLEXG-hMSCs-exosomes

To elucidate the differences in peptide expression between GLEXG-treated and vehicle-treated hMSCs-derived exosomes, proteomic analysis was performed on these two groups of exosomes. Ingenuity pathway analysis of the peptides from the proteomics analysis of the GLEXG-hMSCs-exosomes showed that the cell death and survival pathway (Fig. 4D) was the top pathway present in the GLEXG-treated hMSCs exosomes, while the senescence pathway showed reduced activation in the GLEXG-treated group compared to the vehicle-treated group (Fig. 4E).

3.4. GLEXG decreases hMSCs senescence in vitro

Since senescence is a typical feature of transformed hMSCs and because this process results in a loss of cells from the population, we next explored the effects of GLEXG on hMSCs senescence. The results showed that GLEXG was able to increase cumulative population doubling (Fig. 5A) and decrease the level of senescence of hMSCs after four passages in cultures (Fig. 5B and C).

3.5. Effects of GLEXG on an in vivo OA model

A rat OA model was established by injecting mono-iodoacetate (MIA) into intra-articular space of rats for 14 days, which was followed by micro-CT analysis to detect bony lesions, fracture callus, and bone remodeling. After intra-articular treatment with GLEXG (0, 0.1, or 0.3 μg every two weeks, well-anesthetized animals were sacrificed at day 42 and their joints were harvested for pathology examination and safranin O staining (Fig. 6A). The results showed that treatment with GLEXG (0.3 μg, i.a.) rescued cartilage defects in this OA knee model (Fig. 6B).

4. Discussion

There is consensus that MSCs play an important role in processes involved in regenerating bone clinically; these process include MSC-derived osteogenesis and chondrogenesis. In the present study we have demonstated that GLEXG is able to promote MSC-derived chondrogenesis, both *in vitro* and *in vivo*, via MSC-secreted exosomes. A reduction in the senescence processese within MSCs is postulated here to be part of the many mechanisms that occur during GLEXG-induced MSC-derived chondrogenesis.

Among many compound remedies commonly used to treat OA patients clinically, Du-Huo-Ji-Sheng-Tang has been shown to promote hMSC-induced osteogenic differentiation (J. Y. Wang et al., 2017) and has the potential to decrease cartilage degradation in an OA rabbit model (Chen et al., 2011). However, our screening results show that GLEXG, but not Du-Huo-Ji-Sheng-Tang, is able to induce MSC-derived chondrogenesis *in vitro*. GLEXG is composed of deer antlers 1 gm, turtle shell 0.5 gm, Ginseng 0.1 gm, and Goji berry 0.2 gm. Recent investigations have shown that anlter extract is able to enhance proliferation and differentiation activity in chondrocytes (Yao et al., 2019), which provides suporting evidence for GLEXG having the potential to promote hMSC-derived chondrogenesis. Using MSCs as an *in vitro* model, *Dioscorea opposita* Thunb (Yang et al., 2020), the Epimedium genus (Wang et al., 2018), and *Arctium lappa* L. (Wu et al., 2020) have also been demonstrated to promote hMSCs-induced chondrogenesis.

TGF-β plays an important role in hMSCs-induced chondrogenesis. However, clinical application of TGF-β in chondrogenesis is still controversial. For examples, TGF-β induces cell hypertrophy in terminal chondrogenic differentiation (Mardani et al., 2013) and evokes synovial membrane inflammation and osteophyte formation (Pelttari et al., 2006). Using CIM without TGF-β1 as induction medium, we found that GLEXG enhanced hMSCs-induced chondrogenesis. We postulate that GLEXG possesses an activity similar to TGF-β. However, after pretreatment with neutralization anti-TGF-β1 antibody in the culture medium, there was no significant changes in the mRNA expression levels of type II/X collagen, SOX9, and aggrecan compared to the GLEXG-treated group. These results suggest that production of endogenous TGF-β contributes little to GLEXG-induced hMSCs chondrogenesis. The exact mechanism by which GLEXG promotes hMSCs chondrogenesis remains to be further elucidated, which is now on going.

Although MSCs are pluripotent cells that can be differentiated into osteocytes and chondrocytes, there are still arguments that they are good therapeutic candidates for the treatment of many bone diseases. The major debate is the uncertainty associated with the tumorigenic potential of hMSCs when treating human diseases. In many countries, including Taiwan, the use of hMSC-based cell therapy in clinical applications is limited by government regulations. Therefore, hMSC-secreted exsomes have potential in regeneration bone diseases. There is consensus that exosomes, a subtype of extracellular vesicles that are released from MSCs, play an important role in intercellular communication and have the potential to be used in regeneration medicine, including cardiac repair (Han et al., 2019), neurodegeneration treatment (Masoudi Asil, Ahlawat, Guillama Barroso and Narayan, 2020), and wound repair (Hu et al., 2016). Furthermore, MSC-secreted

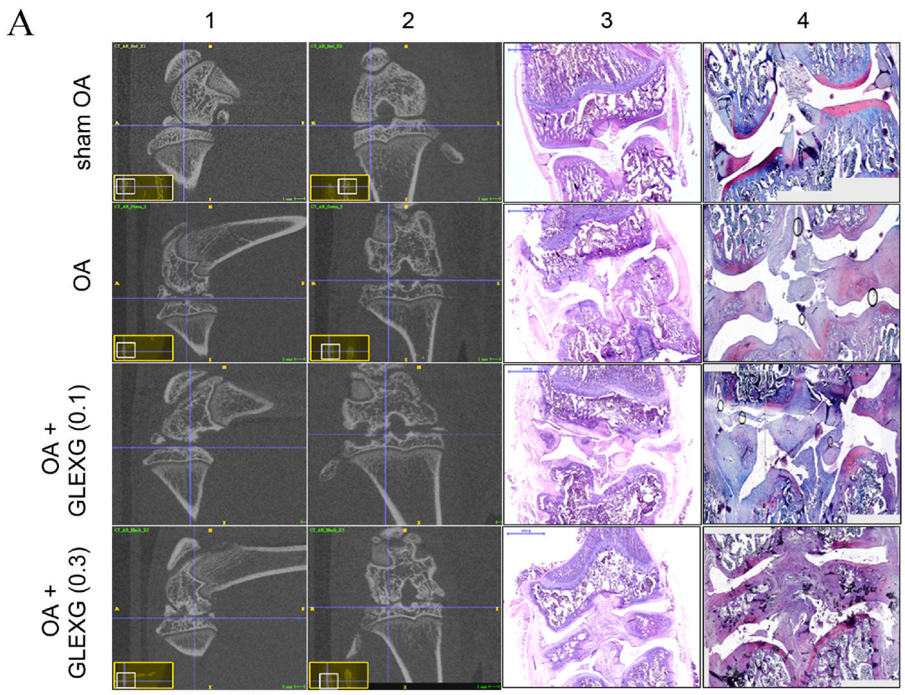
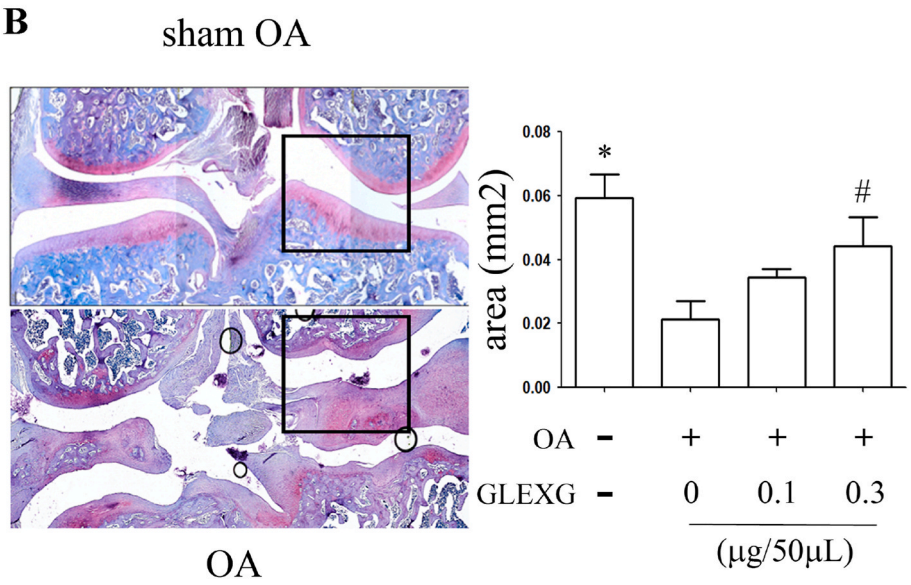


Fig. 6. Effects of GLEXG on *in vivo* osteoarthritis (OA) model. Rat OA model was established by injecting mono-iodoacetate (MIA) into intra-articular space as described in Methods. After intra-articular treatment of GLEXG (0-, 0.1-, 0.3 μg) in SD rats, each rat was anesthetized, and micro-CT scans were taken at day 14 and 42, using a U-CTHR. The CT images (A) including sagittal view (1) and coronal view (2) were reconstructed as described in Methods. At day 42, anesthetized animals were sacrificed and the joints were harvested for further histopathology (H & E staining, 3) and Safranin-O/Fast green staining (4). The pink region of rat cartilage in Safranin-O staining (B, left insert) was quantified and analyzed. *, $p < 0.05$, Mann-Whitney U test. #, $p < 0.05$, one way ANOVA with Dunnet post hoc test. Bar indicates 2000 μM.



exosomes also have the potential to treat many bone/joint diseases (Meng et al., 2018; Zhai et al., 2020). Recent studies have demonstrated that low-intensity pulsed ultrasound (LIPUS)-mediated BMSC-derived exosomes promote an increase in chondrocyte proliferation and the synthesis of extracellular matrix while at the same time suppressing interleukin-1β-induced activation of the nuclear factor kappa B pathway (Q. Liao et al., 2021). Furthermore, kartogenin-treated MSC-derived exosomes have been shown to enhance cartilage regeneration (Shao et al., 2021; Xu et al., 2021). Our findings that GLEXG promotes MSC-derived chondrogenesis via MSC-secreted exosomes are consistent with above-mentioned studies.

It is well known that aging process occurs when MSCs are cultured for a long time. A previous study has suggested that Chinese herbal remedies possess activities that can slow down the aging process of MSCs (J. Y. Wang et al., 2017). Furthermore, it has been shown that Guilu Erxian decoction is able to attenuate cisplatin-induced damage to BM-MSCs (Ke et al., 2018). Using proteomics and ingenuity pathway analysis of hMSC-secreted exosomes, we found that the senescence pathway showed lower activatioin in GLEXG-treated exosomes compared to the vehicle-treated group. Ginseng is one of the components of GLEXG. A recent study has demonstrated that Rg3 in Ginseng improves the proliferation and differentiation activity and prevents the

senescence process of human mesenchymal stem cells via enhancing capabilities of mitochondrial functions through a Ca2+-dependent pathway (Hong et al., 2020). In addition, Ginsenoside Rg1 has been shown to improve differentiation by inhibiting senescence of human bone marrow mesenchymal stem cell (Wang et al., 2020). The fact that Ginsenoside Rg1 can also be detected during the HPLC profiling of GLEXG (referred to Fig. 1b) provides evidence to support our findings.

In summary, we conclude that GLEXG promotes *in vitro* MSC-induced chondrogenesis possibly via exosomes release and delays aging in the MSC senescence process and that treatment with GLEXG (0.3 μg, i.a.) rescued cartilage defects in rat OA knee model. These results suggest that the application of hMSCs-secreted exosomes to the treatment of OA patients has potential in the future.

Ethics approval and consent to participate

Study protocols involving experimental rats followed ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines and were approved by the Institutional Animal Committee of and Taipei Veterans General Hospital (IACUC No. 2019-224).

Funding

This work was supported by grants from Cheng-Hsin project (CHGH109-(N)21) and Taipei Veterans General Hospital (V110D56-001). Besides is kindly supported by Sophie Chang and Morris Chang.

CRediT authorship contribution statement

Yong-Hong Yang: wrote the manuscript, Writing – original draft, performed the experiments and data analysis, Data curation, Formal analysis. **Che-Sheng Wen:** formed the idea and, Funding acquisition. **Yung-Ling Kuo:** provided knowledge of traditional Chinese medicine and the materials of GLEXG. **Su-Ling Fu:** provided academic comments and suggestion during the manuscript drafting, Writing – original draft. **Tung-Yi Lin:** provided academic comments and suggestion during the manuscript drafting, Writing – original draft. **Chao-Ming Chen:** provided clinical experience and comments, provided experimental facilities, provided experimental facilities. **Po-Kuei Wu:** provided clinical experience and comments, provided experimental facilities. **Wei-Ming Chen:** provided experimental facilities. **Jir-You Wang:** formed the idea and, Funding acquisition, wrote the manuscript, Writing – original draft, performed the experiments and data analysis, Data curation, Formal analysis.

Declaration of competing interest

We declare that we have no conflicts of interest, including financial and non-financial interests.

Data availability

Data will be made available on request.

Acknowledgements

We were in debt to Chou, SS for his technique supports. This work was kindly financially supported by Sophie Chang and Morris Chang.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jep.2023.116784.

References

Ayhan, E., Kesmezacar, H., Akgun, I., 2014. Intraarticular injections (corticosteroid, hyaluronic acid, platelet rich plasma) for the knee osteoarthritis. World J. Orthoped. 5 (3), 351–361. https://doi.org/10.5312/wjo.v5.i3.351.

Bradford, L.W., 1976. Problems of ethics and behavior in the forensic sciences. J. Forensic Sci. 21 (4), 763–768. Retrieved from. https://www.ncbi.nlm.nih.gov/pubmed/972302.

Chen, C.W., Sun, J., Li, Y.M., Shen, P.A., Chen, Y.Q., 2011. Action mechanisms of du-huo-ji-sheng-tang on cartilage degradation in a rabbit model of osteoarthritis. Evid Based Complement Alternat Med, 571479. https://doi.org/10.1093/ecam/neq002, 2011.

Chou, Y.J., Chuu, J.J., Peng, Y.J., Cheng, Y.H., Chang, C.H., Chang, C.M., Liu, H.W., 2018. The potent anti-inflammatory effect of Guilu Erxian Glue extracts remedy joint pain and ameliorate the progression of osteoarthritis in mice. J. Orthop. Surg. Res. 13 (1), 259. https://doi.org/10.1186/s13018-018-0967-y.

Ciuffreda, M.C., Malpasso, G., Musaro, P., Turco, V., Gnecchi, M., 2016. Protocols for in vitro differentiation of human mesenchymal stem cells into osteogenic, chondrogenic and adipogenic lineages. Methods Mol. Biol. 1416, 149–158. https://doi.org/10.1007/978-1-4939-3584-0_8.

Davatchi, F., Abdollahi, B.S., Mohyeddin, M., Shahram, F., Nikbin, B., 2011. Mesenchymal stem cell therapy for knee osteoarthritis. Preliminary report of four patients. Int J Rheum Dis 14 (2), 211–215. https://doi.org/10.1111/j.1756-185X.2011.01599.x.

Feng, X.H., Derynck, R., 2005. Specificity and versatility in tgf-beta signaling through Smads. Annu. Rev. Cell Dev. Biol. 21, 659–693. https://doi.org/10.1146/annurev.cellbio.21.022404.142018.

Han, C., Zhou, J., Liang, C., Liu, B., Pan, X., Zhang, Y., Li, Y., 2019. Human umbilical cord mesenchymal stem cell derived exosomes encapsulated in functional peptide hydrogels promote cardiac repair. Biomater. Sci. 7 (7), 2920–2933. https://doi.org/10.1039/c9bm00101h.

Hochberg, M.C., Altman, R.D., April, K.T., Benkhalti, M., Guyatt, G., McGowan, J., American College of, R, 2012. American College of Rheumatology 2012 recommendations for the use of nonpharmacologic and pharmacologic therapies in osteoarthritis of the hand, hip, and knee. Arthritis Care Res. 64 (4), 465–474. https://doi.org/10.1002/acr.21596.

Hong, T., Kim, M.Y., Da Ly, D., Park, S.J., Eom, Y.W., Park, K.S., Baik, S.K., 2020. Ca(2+)-activated mitochondrial biogenesis and functions improve stem cell fate in Rg3-treated human mesenchymal stem cells. Stem Cell Res. Ther. 11 (1), 467. https://doi.org/10.1186/s13287-020-01974-3.

Hou, P.W., Liu, S.C., Tsay, G.J., Tang, C.H., Chang, H.H., 2022. The traditional Chinese medicine "Hu-Qian-Wan" attenuates osteoarthritis-induced signs and symptoms in an experimental rat model of knee osteoarthritis. Evid Based Complement Alternat Med 2022, 5367494. https://doi.org/10.1155/2022/5367494.

Hu, L., Wang, J., Zhou, X., Xiong, Z., Zhao, J., Yu, R., Chen, L., 2016. Exosomes derived from human adipose mensenchymal stem cells accelerates cutaneous wound healing via optimizing the characteristics of fibroblasts. Sci. Rep. 6, 32993 https://doi.org/10.1038/srep32993.

Jiang, Y., Jahagirdar, B.N., Reinhardt, R.L., Schwartz, R.E., Keene, C.D., Ortiz-Gonzalez, X.R., Verfaillie, C.M., 2002. Pluripotency of mesenchymal stem cells derived from adult marrow. Nature 418 (6893), 41–49. https://doi.org/10.1038/nature00870.

Katz, J.N., Arant, K.R., Loeser, R.F., 2021. Diagnosis and treatment of hip and knee osteoarthritis: a review. JAMA 325 (6), 568–578. https://doi.org/10.1001/jama.2020.22171.

Ke, B., Shi, L., Xu, Z., Wu, G., Gong, Y., Zhu, L., Wang, X., 2018. Flavored Guilu Erxian decoction inhibits the injury of human bone marrow mesenchymal stem cells induced by cisplatin. Cell Mol Biol (Noisy-le-grand) 64 (6), 58–64. Retrieved from. https://www.ncbi.nlm.nih.gov/pubmed/29808802.

King, L.K., March, L., Anandacoomarasamy, A., 2013. Obesity & osteoarthritis. Indian J. Med. Res. 138 (2), 185–193. Retrieved from. https://www.ncbi.nlm.nih.gov/pubmed/24056594.

Lamo-Espinosa, J.M., Mora, G., Blanco, J.F., Granero-Molto, F., Nunez-Cordoba, J.M., Sanchez-Echenique, C., Prosper, F., 2016. Intra-articular injection of two different doses of autologous bone marrow mesenchymal stem cells versus hyaluronic acid in the treatment of knee osteoarthritis: multicenter randomized controlled clinical trial (phase I/II). J. Transl. Med. 14 (1), 246. https://doi.org/10.1186/s12967-016-0998-2.

Lanyon, P., Muir, K., Doherty, S., Doherty, M., 2003. Age and sex differences in hip joint space among asymptomatic subjects without structural change: implications for epidemiologic studies. Arthritis Rheum. 48 (4), 1041–1046. https://doi.org/10.1002/art.10886.

Lee, K.S., Cha, S.H., Kang, H.W., Song, J.Y., Lee, K.W., Ko, K.B., Lee, H.T., 2013. Effects of serial passage on the characteristics and chondrogenic differentiation of canine umbilical cord matrix derived mesenchymal stem cells. Asian-Australas. J. Anim. Sci. 26 (4), 588–595. https://doi.org/10.5713/ajas.2012.12488.

Lee, W.S., Kim, H.J., Kim, K.I., Kim, G.B., Jin, W., 2019. Intra-articular injection of autologous adipose tissue-derived mesenchymal stem cells for the treatment of knee osteoarthritis: a phase IIb, randomized, placebo-controlled clinical trial. Stem Cells Transl Med 8 (6), 504–511. https://doi.org/10.1002/sctm.18-0122.

Liao, J., Hu, N., Zhou, N., Lin, L., Zhao, C., Yi, S., Huang, W., 2014. Sox9 potentiates BMP2-induced chondrogenic differentiation and inhibits BMP2-induced osteogenic differentiation. PLoS One 9 (2), e89025. https://doi.org/10.1371/journal.pone.0089025.

Liao, J.A., Yeh, Y.C., Chang, Z.Y., 2022. The efficacy and safety of traditional Chinese medicine Guilu Erxian Jiao in the treatment of knee osteoarthritis: a systematic

review and meta-analysis. Compl. Ther. Clin. Pract. 46, 101515 https://doi.org/10.1016/j.ctcp.2021.101515.
Liao, Q., Li, B.J., Li, Y., Xiao, Y., Zeng, H., Liu, J.M., Liu, G., 2021. Low-intensity pulsed ultrasound promotes osteoarthritic cartilage regeneration by BMSC-derived exosomes via modulating the NF-kappaB signaling pathway. Int. Immunopharm. 97, 107824 https://doi.org/10.1016/j.intimp.2021.107824.
Lin, J.-G. (Ed.), 2004. A Comparable Dictionary of Disease Names in Chinese and Western Medicine. National Research Institute of Chinese Medicine.
Mamidi, M.K., Das, A.K., Zakaria, Z., Bhonde, R., 2016. Mesenchymal stromal cells for cartilage repair in osteoarthritis. Osteoarthritis Cartilage 24 (8), 1307–1316. https://doi.org/10.1016/j.joca.2016.03.003.
Mardani, M., Kabiri, A., Esfandiari, E., Esmaeili, A., Pourazar, A., Ansar, M., Hashemibeni, B., 2013. The effect of platelet rich plasma on chondrogenic differentiation of human adipose derived stem cells in transwell culture. Iran J Basic Med Sci 16 (11), 1163–1169. Retrieved from. https://www.ncbi.nlm.nih.gov/pubmed/24494069.
Masoudi Asil, S., Ahlawat, J., Guillama Barroso, G., Narayan, M., 2020. Nanomaterial based drug delivery systems for the treatment of neurodegenerative diseases. Biomater. Sci. 8 (15), 4109–4128. https://doi.org/10.1039/d0bm00809e.
Meng, F., Li, Z., Zhang, Z., Yang, Z., Kang, Y., Zhao, X., Liao, W., 2018. MicroRNA-193b-3p regulates chondrogenesis and chondrocyte metabolism by targeting HDAC3. Theranostics 8 (10), 2862–2883. https://doi.org/10.7150/thno.23547.
Pelttari, K., Winter, A., Steck, E., Goetzke, K., Hennig, T., Ochs, B.G., Richter, W., 2006. Premature induction of hypertrophy during in vitro chondrogenesis of human mesenchymal stem cells correlates with calcification and vascular invasion after ectopic transplantation in SCID mice. Arthritis Rheum. 54 (10), 3254–3266. https://doi.org/10.1002/art.22136.
Pers, Y.M., Rackwitz, L., Ferreira, R., Pullig, O., Delfour, C., Barry, F., Consortium, A., 2016. Adipose mesenchymal stromal cell-based therapy for severe osteoarthritis of the knee: a phase I dose-escalation trial. Stem Cells Transl Med 5 (7), 847–856. https://doi.org/10.5966/sctm.2015-0245.
Rigueur, D., Lyons, K.M., 2014. Whole-mount skeletal staining. Methods Mol. Biol. 1130, 113–121. https://doi.org/10.1007/978-1-62703-989-5_9.
Shao, J., Zhu, J., Chen, Y., Fu, Q., Li, L., Ding, Z., Zhou, Y., 2021. Exosomes from kartogenin-pretreated infrapatellar fat pad mesenchymal stem cells enhance chondrocyte anabolism and articular cartilage regeneration. Stem Cell. Int. 2021, 6624874 https://doi.org/10.1155/2021/6624874.
Spector, T.D., MacGregor, A.J., 2004. Risk factors for osteoarthritis: genetics. Osteoarthritis Cartilage 12 (Suppl. A), S39–S44. https://doi.org/10.1016/j.joca.2003.09.005.
Tsai, C.C., Chou, Y.Y., Chen, Y.M., Tang, Y.J., Ho, H.C., Chen, D.Y., 2014. Effect of the herbal drug guilu erxian jiao on muscle strength, articular pain, and disability in elderly men with knee osteoarthritis. Evid Based Complement Alternat Med, 297458. https://doi.org/10.1155/2014/297458, 2014.
Tuli, R., Tuli, S., Nandi, S., Huang, X., Manner, P.A., Hozack, W.J., Tuan, R.S., 2003. Transforming growth factor-beta-mediated chondrogenesis of human mesenchymal progenitor cells involves N-cadherin and mitogen-activated protein kinase and Wnt signaling cross-talk. J. Biol. Chem. 278 (42), 41227–41236. https://doi.org/10.1074/jbc.M305312200.
Wang, J.Y., Chen, W.M., Wen, C.S., Hung, S.C., Chen, P.W., Chiu, J.H., 2017a. Du-Huo-Ji-Sheng-Tang and its active component Ligusticum chuanxiong promote osteogenic differentiation and decrease the aging process of human mesenchymal stem cells. J. Ethnopharmacol. 198, 64–72. https://doi.org/10.1016/j.jep.2016.12.011.
Wang, Y., Yu, D., Liu, Z., Zhou, F., Dai, J., Wu, B., Liu, H., 2017b. Exosomes from embryonic mesenchymal stem cells alleviate osteoarthritis through balancing synthesis and degradation of cartilage extracellular matrix. Stem Cell Res. Ther. 8 (1), 189. https://doi.org/10.1186/s13287-017-0632-0.
Wang, Z., Jiang, R., Wang, L., Chen, X., Xiang, Y., Chen, L., Wang, Y., 2020. Ginsenoside Rg1 improves differentiation by inhibiting senescence of human bone marrow mesenchymal stem cell via GSK-3beta and beta-catenin. Stem Cell. Int. 2020, 2365814 https://doi.org/10.1155/2020/2365814.
Wang, Z., Li, K., Sun, H., Wang, J., Fu, Z., Liu, M., 2018. Icariin promotes stable chondrogenic differentiation of bone marrow mesenchymal stem cells in self-assembling peptide nanofiber hydrogel scaffolds. Mol. Med. Rep. 17 (6), 8237–8243. https://doi.org/10.3892/mmr.2018.8913.
Wu, K.C., Weng, H.K., Hsu, Y.S., Huang, P.J., Wang, Y.K., 2020. Aqueous extract of Arctium lappa L. root (burdock) enhances chondrogenesis in human bone marrow-derived mesenchymal stem cells. BMC Complement Med Ther 20 (1), 364. https://doi.org/10.1186/s12906-020-03158-1.
Xu, X., Liang, Y., Li, X., Ouyang, K., Wang, M., Cao, T., Duan, L., 2021. Exosome-mediated delivery of kartogenin for chondrogenesis of synovial fluid-derived mesenchymal stem cells and cartilage regeneration. Biomaterials 269, 120539. https://doi.org/10.1016/j.biomaterials.2020.120539.
Yang, R., Zhang, R., Deng, H., Wang, G., Zhao, J., Yang, H., Zhou, J., 2020. Yam-containing serum promotes proliferation and chondrogenic differentiation of rabbit bone marrow mesenchymal stem cells and synthesis of glycosaminoglycan. Pharmacology 105 (7–8), 377–385. https://doi.org/10.1159/000503932.
Yao, B., Zhang, M., Leng, X., Zhao, D., 2019. Proteomic analysis of the effects of antler extract on chondrocyte proliferation, differentiation and apoptosis. Mol. Biol. Rep. 46 (2), 1635–1648. https://doi.org/10.1007/s11033-019-04612-1.
Yushkevich, P.A., Piven, J., Hazlett, H.C., Smith, R.G., Ho, S., Gee, J.C., Gerig, G., 2006. User-guided 3D active contour segmentation of anatomical structures: significantly improved efficiency and reliability. Neuroimage 31 (3), 1116–1128. https://doi.org/10.1016/j.neuroimage.2006.01.015.
Zhai, M., Zhu, Y., Yang, M., Mao, C., 2020. Human mesenchymal stem cell derived exosomes enhance cell-free bone regeneration by altering their miRNAs profiles. Adv. Sci. 7 (19), 2001334 https://doi.org/10.1002/advs.202001334.
Zhang, L., Wang, P.E., Ying, J., Jin, X., Luo, C., Xu, T., Jin, H., 2017. Yougui pills attenuate cartilage degeneration via activation of TGF-beta/smad signaling in chondrocyte of osteoarthritic mouse model. Front. Pharmacol. 8, 611. https://doi.org/10.3389/fphar.2017.00611.
Zhao, Q., Eberspaecher, H., Lefebvre, V., De Crombrugghe, B., 1997. Parallel expression of Sox9 and Col2a1 in cells undergoing chondrogenesis. Dev. Dynam. 209 (4), 377–386. https://doi.org/10.1002/(SICI)1097-0177(199708)209:4<377::AID-AJA5>3.0.CO;2-F.