Hepatocyte growth factor and insulin-like growth factor 1 were used to repair bone-cartilage defects in bone marrow mesenchymal stem cells in a rabbit model of postmenopausal

Jia-Qi Guan1,2, Chuan-Bo Zang3, Jun-Cen Li1,2, Fei-Fan Chen3, Ya-Hui Wang2, Guang-Dong Zhou1,2,4, Yu Liu1,2,4, Yi-Lin Cao1,2,4,*

1 School of Clinical Medical, Shandong Second Medical University, Weifang 261053, China
2 National Tissue Engineering Center of China, Shanghai 261053, China
3 School of Anesthesiology, Shandong Second Medical University, Weifang 216053, China
4 Shanghai Key Laboratory of Tissue Engineering, Department of Plastic and Reconstructive Surgery, Shanghai 9th People’s Hospital, Shanghai Stem Cell Institute, Shanghai 2000336, China
5 Shanghai Jiao Tong University School of Medicine, Shanghai 2000336, China
* Corresponding author: Yi-Lin Cao, yilincao163@163.com

Abstract: In postmenopausal osteoporosis (PMOP), an imbalance exists in the differentiation of bone marrow mesenchymal stem cells (BMSCs), with a decrease in osteogenic differentiation and an increase in adipogenic differentiation. This imbalance leads to bone marrow adiposity, bone loss, bone fragility, and a substantial rise in fracture risk. After a patient experiences an osteochondral defect due to trauma, it struggles to heal naturally, presenting a clinical challenge for treatment. Our study delved into the abnormal differentiation of BMSCs in PMOP by conducting transcriptome sequencing on BMSCs from a PMOP model (PMOP-BMSCs) and a healthy control model (Normal-BMSCs). We identified insulin-like growth factor 1 (IGF-1) and hepatocyte growth factor (HGF) genes as significantly low-expressed protein-coding genes during the osteogenic cartilage differentiation process of PMOP-BMSCs. Due to the downregulation of its expression, it leads to the deletion of the proteins it encodes IGF-1 and HGF. In order to verify the sequencing results, the feasibility of co-culture the above two growth factors with PMOP-BMSCs to repair osteochondral defects was discussed. The findings indicated that the inclusion of elements enhanced the DNA replication activity and extracellular matrix mineralization of PMOP-BMSCs. It also promoted the construction of tissue-engineered bone in vitro and the up-regulation of Runx2, BMP4, OCN, ACAN, collagen type I, II, and Sox9 osteochondral differentiation markers. In the rabbit model of knee osteochondral injury with PMOP, the group treated with both growth factors and PMOP-BMSCs showed superior outcomes in repairing cartilage and subchondral bone defects compared to the other groups. We suggest that the addition of HGF and IGF-1 increases the expression of osteoblast and cartilage-related genes and proteins, promoting the proliferation and differentiation of osteochondrous bone in PMOP-BMSCs. These findings could offer a novel cell therapy strategy for treating postmenopausal osteoporosis, utilizing growth factors.

Keywords: postmenopausal osteoporosis; bone marrow mesenchymal stem cells; transcriptome sequencing; osteochondral defect; tissue engineering

1. Introduction

Postmenopausal osteoporosis is characterized by a lack of estrogen following menopause, leading to lower bone mineral density and mass, degradation of bone microarchitecture, heightened risk of fractures, and an escalating issue of
osteochondral defects resulting from trauma [1]. Treatment of women with PMOP remains challenging [2]. The process of bone repair starts with the differentiation of MSCs into osteoblasts and chondrocytes, followed by the synthesis of extracellular matrix to support continuous calcification and ossification [3–6]. However, postmenopausal osteoporosis patients have an imbalance in the aging and differentiation of bone marrow mesenchymal stem cells due to the loss of their own estrogen, making it difficult for their osteochondral defects to heal on their own [7–10]. Hence, gaining a deeper comprehension of the mechanisms behind the differentiation of BMSCs could hold therapeutic significance for PMOP.

Bone grafting remains the preferred method for treating bone defects; however, it is accompanied by numerous challenges, including a limited pool of donors, inconsistent graft quality, and the risk of infection [11,12]. The advancement of tissue engineering has introduced novel therapeutic approaches for osteochondral defects in postmenopausal osteoporosis. Tissue engineering approaches aim to regenerate tissues through the use of autologous cells with biodegradable scaffolds, which are transplanted into tissue defects in vivo [13]. In the growth factor-regulated microenvironment, implanted cells secrete matrix leading to the gradual formation of new tissues and the subsequent recovery of tissue defects [14,15]. At present, the implantation capacity of MSCs is poor, and frequent inflammation and ischemia limit the homing and survival of MSCs in vivo, leading to suboptimal repair of osteochondral defects [16–19]. Growth factors (GFs) are a class of proteins that guide cell differentiation and gene expression and regulate cell behavior by activating intracellular signaling pathways [20,21]. GFs are key drivers in tissue regeneration. The GF bone morphogenic protein 2 (BMP-2) is the most widely used osteochondral tissue–inducing factor. However, it has limited clinical application because of the serious side effects, such as vertebral osteolysis, that are associated with its high therapeutic dose requirement [22]. Therefore, finding growth factors that can promote the proliferation and differentiation of PMOP-BMSCs can ameliorate the differentiation imbalance of PMOP-BMSCs from the root cause and achieve the purpose of systematically enhancing PMOP osteochondral repair.

It is known that the ovariectomy (OVX)-induced rat model is widely used for postmenopausal osteoporosis studies. Rabbits are extensively utilized as a model for osteoporosis research due to their size, bone remodeling capacity, and delayed epiphyseal plate closure, which make up for some of the limitations of the rat model. Previous models have relied on adjunctive steroid therapy to achieve osteoporosis. Nowadays, a rabbit model induced by bilateral OVX alone demonstrates significant bone loss, and the OVX rabbit model of postmenopausal osteoporosis constructed in this manner is safe and reproducible [23].

The current study aimed to address the aforementioned issues by utilizing transcriptome sequencing technology to identify appropriate research factors. This research seeks to offer a new perspective on growth factor-based cell therapy, investigate the potential for remedying osteochondral defects in the knee joints of PMOP rabbits, and make a valuable contribution to future clinical applications.

Decreased bone density, microarchitecture degradation, and increased fracture risk characterise postmenopausal osteoporosis (PMOP), a complicated medical condition. As a result of oestrogen deficiency, PMOP patients have poor bone repair
processes that rely on the development of mesenchymal stem cells (MSCs), which impedes the body’s natural ability to fix osteochondral lesions. Researchers are looking into tissue engineering methods that use MSCs and growth factors (GFs) within biodegradable scaffolds because traditional bone grafting has its limits. On the other hand, issues with MSC implantation capacity and adverse effects with GFs like BMP-2 mean that repair results aren’t always up to par. Osteochondral healing could be a success story if GFs could boost PMOP-BMSC proliferation and differentiation. Insights into growth factor-based cell treatment and new research factors can be found by transcriptome sequencing in this work. In order to provide a reliable and repeatable platform for studying PMOP therapies, the study will use a rabbit model of ovariectomy-induced postmenopausal osteoporosis. In the end, our research aims to fill a gap in our knowledge about osteochondral healing mechanisms in PMOP and provide useful information for future clinical applications, all with the goal of helping those that now have no treatment options.

2. Materials and methods

2.1. Model of animal

The Animal Care and Experimentation Committee of Weifang Medical College School of Medicine approved all animal procedures. Forty-six female New Zealand large white rabbits (6 months old, weight 3.5 ± 0.2 kg) were provided by Shanghai Jiao Tong University Nongsheng Experimental Practice Field Co., Ltd. and the animals were randomly divided into two groups: (1) Normal control (normal) group (n = 6) and (2) osteoporosis model (PMOP) group (n = 40). The experimental operation was in accordance with medical ethics. After weighing the animals in the osteoporosis group, we proceeded with intramuscular anesthesia injection at a dosage of 0.2 mL/kg. Following this, we prepped the abdominal skin, disinfected it with iodophor, incised through the skin and subcutaneous tissue at the abdominal midline, performed blunt dissection to reach the muscle layer, separated the muscle tissue, incised the peritoneum, located the fallopian tube, traced along it to identify the ovaries, ligated the fallopian tubes, and finally excised the ovaries. The other side of the ovary was removed in the same way to confirm that there were no active bleeding spots, then sutured in layers, closed the abdominal cavity, and sterilized with iodophor. Following the surgery, a daily intramuscular injection of gentamicin at a dose of 20,000 u/kg was administered for 3 days. After 12 weeks, we established an induced postmenopausal osteoporosis model [24] using the method of removing bilateral ovaries.

2.2. Micro-CT scanning analysis

Following the previously described method [25], the femur was removed, and histomorphometry was performed to quantify the bone mass index using Micro-CT. Utilising a Micro-CT μ80 scanner (Scanco Medical, Switzerland), micro-CT analysis was carried out. Scanco Medical from Switzerland utilized evaluation software to analyze data and generate 3D images. The scanning parameters were set as follows: voltage = 70 kV, current = 114 μA, resolution = 1024 × 1024 pixels. Through Micro-
CT analysis, two-dimensional cross-sectional pictures and three-dimensional reconstructed images were produced. Quantitative data were computed for the following parameters: trabecular number (Tb.N), trabecular separation (Tb.Sp), bone surface area to volume ratio (BS/BV), relative bone volume fraction (BV/TV), connectivity density (Conn.D.), and bone mineral density (BMD).

2.3. Assessment of serum E2 concentration

Assessment of serum E2 concentration. Rabbit venous blood was drawn, centrifuged for 3000r/min for 10 min, the supernatant was taken as a serum sample, detected with QuicKey Pro Rabbit E2 (Estradiol) ELISA Kit (E-OSEL-RB0001, Elabscience, Wuhan, China), rabbit E2 antigen was coated on the microplate label, 50µl serum sample and HRP enzyme-labeled anti-E2 monoclonal antibody working solution were added, and incubated at 37 degrees for 60 min. Rinse the plate. Next, 90 microliters of TMB were added, followed by incubation at 37 degrees Celsius for 15 minutes. Then, 50 microliters of stop solution were added, and the OD value was determined at 450 nm using a microplate reader (SpectraMax M5, USA).

2.4. Isolation and culture of bone marrow MSCs

Without altering the culture media to encourage cell adherence to the culture dish, rabbit bone marrow aspirate was collected, and bone marrow MSCs were separated from the bone marrow using the whole bone marrow applanation culture method. After that, bone marrow MSCs were cultivated for five days. In an incubator set at 37 ℃, 5% CO₂, and 95% humidity, isolated bone marrow MSCs were cultivated in Dulbecco’s modified Eagle’s medium (DMEM; Gibco, Grand Island, NY, USA) supplemented with 10% foetal bovine serum (FBS; Hyclone, Logan, UT, USA) and 1% penicillin/streptomycin. Adherent cells were collected, stored, and maintained through continuous cultivation for 48 h.

2.5. BMSC osteogenic induction and Alizarin red S (ARS) staining

BMSCs were seeded in 6-well plates at a cell density of 2 × 10⁴ cells/cm² and incubated until they adhered. BMSCs were induced to differentiate into osteoblasts using an osteoblast differentiation kit (GUXMX-90021, Cyagen, Guangzhou, China). Alizarin red staining was performed at the 7th day of induction. Wash off the residual medium with PBS and fix the cells with 4% paraformaldehyde (PFA) for 30 min and the residual fixative solution with PBS, and add 2 mL of alizarin red staining solution to each well and stain for 10 min at room temperature. After washing off the residual dye, add 2 mL of PBS to observe the osteogenic staining results under the microscope. The transformation of precursor cells or mesenchymal stem cells (MSCs) into the bone-forming osteoblasts is known as osteogenic differentiation. The formation, growth, and repair of bones depend on this mechanism. In order for mesenchymal stem cells (MSCs) to differentiate into mature osteoblasts that can produce bone matrix and mineralization, they go through a number of molecular and cellular changes regulated by distinct signaling pathways and transcription factors. Important indicators of osteogenic differentiation include the presence of calcium phosphate minerals, the expression of particular genes such
runt-related transcription factor 2 (RUNX2), osteocalcin (OCN), and alkaline phosphatase (ALP). Growth factors, cytokines, mechanical stimuli, and certain in vitro culture conditions are among the variables that might promote and regulate osteogenic differentiation. For fields like regenerative medicine, bone tissue engineering, and the creation of treatments for bone-related diseases, an understanding of osteogenic differentiation is crucial.

2.6. BMSC adipogenic induction and oil red O staining

BMSCs were inoculated into 6-well plates at a cell density of 2 × 10^4 cells/cm^2 and cultured until adherence. BMSCs were induced to undergo adipogenic differentiation using an adipogenic differentiation kit (GUXMX-90031, Cyagen, Guangzhou, China). Oil red staining was carried out on the 7th day of induction. Subsequently, the cells were fixed with 4% paraformaldehyde (PFA) for 30 min, followed by washing off the residual fixation solution with PBS. The working solution was prepared by mixing oil red O and distilled water in a 32 ratio, then centrifuged at 250 × g for 4 min. The supernatant was used, and each well was stained with 2 mL of the solution at room temperature for 30 min. After washing off the residual dye, add 2 mL of PBS and observe the lipidogenic staining effect under the microscope.

2.7. BMSC chondrogenesis induction and Alcian Blue staining

BMSCs were inoculated into 6-well plates at a cell density of 2 × 10^4 cells/cm^2 and cultured until adherence. BMSCs were induced to differentiate into cartilage using the Cartilage Differentiation Kit (GUXMX-90041, Cyagen, Guangzhou, China). Alcian blue staining was performed on day 7 after induction. Following a 30-minute fixation process using 4% paraformaldehyde (PFA), the cells were cleaned using PBS to remove any remaining stationary solution. Following a three-minute soak in Alcian acidification solution (Solarbio), the cells were stained for thirty minutes using Alcian staining solution (Solarbio). The outcomes of the dyeing process were examined under a microscope after any leftover dyes were cleaned off.

2.8. Cell proliferation

Bone marrow mesenchymal stem cells were seeded into 96-well plates at a density of 100 μL per well (1 × 10^4 cells) and incubated at 37 ℃ for 1, 3, and 5 days. After incubating the cells at 37 ℃ for 2 h upon reaching the appropriate culture duration, 10 μL of CCK-8 solution (Dojindo, Japan) was added to each well. Subsequently, the absorbance was measured at 450 nm using an enzyme labeling instrument (SpectraMax M5, USA) to determine the level of viable cells.

2.9. RNA sequencing

Total RNA was isolated using the TRIZOL reagent from Life Technologies, USA, following the manufacturer’s protocol. Transcriptome libraries were created using the 101 VAHTS Universal V5 RNA-seq Library Prep kit in accordance with the manufacturer’s instructions. Shanghai Ouyi Biotechnology Co., carried out the transcriptome sequencing and analysis. The Illumina Novaseq 6000 sequencing
technology was utilised to sequence the libraries. R (v3.2.0) was used for PCA analysis of genes (counts) and mapping in order to determine whether the sample contained biological duplicates. DESeq2 was employed for analyzing differentially expressed genes (DEGs), which were defined as genes meeting the criteria of a \( q \)-value < 0.05 and a fold change > 2. R (v3.2.0) was used to carry out a hierarchical clustering analysis of DEGs in order to show the gene expression patterns across samples and groups. Using the R package gradar, radar plots were created for the top 30 genes to show how the expression of up- or down-regulated genes changed. GO [26] (filtering GO entries matching PopHits ≥ 5 across the three classifications; 10 entries in each classification sorted from largest to smallest according to the-log10p-value associated with each entry) and KEGG [27] (filtering Pathway entries matching PopHits ≥ 5, arranged in descending order according to the-log10p-value associated with each entry) to find significantly enriched functional entries, enrichment analysis of differentially expressed genes based on hypergeometric distribution methods was employed. The bars for significantly enriched functional entries were plotted using R (v3.2.0). The results identified 1353 differentially expressed genes, including 669 up-regulated genes and 684 down-regulated genes.

2.10. Edu proliferation assay

Bone marrow mesenchymal stem cells from the third passage were subjected to serum deprivation in DMEM/F12 culture medium without serum and with 0.5% BSA (wt/vol) for 24 h. Bone marrow MSCs were cultured with HGF, IGF-1, or IGF-1+HGF at a dose of 50 ng/mL or untreated (as controls) for 24 h. BMSCs were incubated with the Edu (50 μM; 5-Ethynyl-2′-deoxyuridine; Beyotime, Haimen, Jiangsu, China) work buffer (10%) for 120 min, and Edu staining was performed to observe cell proliferation.

2.11. Preparation of ADM scaffolds

The ADM supplied by Jiangsu You tong Biomedical Technology Co., Ltd. (Nantong, Jiangsu) was sourced from porcine dermis. The dermal tissue was carefully stripped of the overlying soft tissue and the underlying virus was inactivated by UV irradiation. Then, 2 mg/mL trypsin was combined with 4 mg/mL sodium lauryl sulfate to remove cells and cellular antigens from the dermal tissue. Thereafter, the dermis tissue was qualified by a high-speed homogenizer. At pH 4.0–5.5, a 0.2% glutaraldehyde solution was employed to cross-link ADM that had been homogenized, after which the ADM porous scaffolds were obtained through repeated lyophilization and rinsing processes. Extending the observation period to 10–21 days could not be enough to achieve high-quality assessment if the normal duration is 7 days.

The inclusion of undifferentiated controls for staining is essential for the accurate interpretation and validation of staining results in studies involving the repair of bone-cartilage defects in a rabbit model of postmenopausal conditions using hepatocyte growth factor (HGF) and insulin-like growth factor-1 (IGF-1).

Undifferentiated controls in this setting would include keeping a subset of BMSCs in an environment that inhibits their ability to differentiate into
chondrogenic or osteogenic lineages. The staining patterns obtained in the experimental groups receiving HGF and IGF-1 therapy could be compared to these undifferentiated BMSCs (baseline).

The use of homogeneous controls for staining has multiple advantages:

1) Baseline comparison: Non-differentiated cells serve as a reference point for comparing the staining patterns of differentiated cells to. That way, they can tell the difference between background staining and staining that is specific to osteogenic or chondrogenic differentiation.

2) Differentiation validation: Researchers can verify that the staining seen in the experimental groups is really due to osteogenic or chondrogenic differentiation and not just nonspecific staining artefacts by staining both undifferentiated and differentiated BMSCs.

3) Quality assurance: Undifferentiated controls are used as a quality control method to make sure the staining procedures are working properly and that any staining that is detected is specific to the cell type and differentiation state that was planned.

4) Normalization and quantification: Staining data can be normalised by using undifferentiated controls, which allows for quantitative analysis and comparison of different experimental settings.

Therefore, it is crucial to incorporate undifferentiated controls for staining in rabbit models of postmenopausal diseases that involve HGF and IGF-1 treatment for bone-cartilage abnormalities. This will guarantee the validity, specificity, and reproducibility of the staining data.

2.12. Construction of in vitro tissue-engineered bone

Bone marrow MSCs (P3 generation) were starved in serum-free DMEM/F12 medium containing 0.5% BSA (w/v) for 24 h. Subsequently, BMSCs were grouped and cultured for 24 h in 50 ng/mL dose of IGF-1+HGF, HGF, IGF-1 untreated (as control). Afterward, various kinds of BMSCs (5 × 10^7 cells/mL) were seeded onto dermal decellularized matrix (ADM) scaffolds measuring 2 mm in diameter. The cell-scaffold complexes were subsequently transferred into 6-well plates and incubated for 4 h at 37 °C and 5% CO₂. Subsequently, they were transferred to new 6-well plates and cultured for 24 h. After 24 h, osteogenic differentiation was initiated by the addition of an osteogenic induction solution, and the media was replaced every 3 days throughout 8 weeks of in vitro culture.

2.13. Construction of tissue engineering cartilage in vitro

Bone marrow MSCs (P3 generation) were starved in serum-free DMEM/F12 medium containing 0.5% BSA (w/v) for 24 h. Then, bone marrow MSCs were grouped and cultured in 50 ng/mL dose of IGF-1+HGF, HGF, IGF-1 untreated (as control) for 24 h. Subsequently, different types of BMSCs (5 × 10^7 cells/mL) were inoculated into dermal decellularized matrix (ADM) scaffolds with a diameter of 2 mm, and then the cell-scaffold complexes were placed in 6-well plates and cultured for 4 h at 37 °C and 5% CO₂. After 24 h, chondrogenic differentiation was induced by the addition of chondrogenic induction solution, and the medium was changed every
3 days and cultured in vitro for 8 weeks.

Accurate measurement and quantification of different structural factors within tissue samples constitute quantitative morphometric examination of tissue. The quantitative data provided by this analytical method enhances the comprehension of tissue form and composition, supplementing qualitative findings.

Tissue sections are usually prepared first, and then staining procedures are used to bring out more contrast and highlight certain anatomical aspects. These sections are dyed and then inspected under a microscope, with images taken for later examination.

A broad variety of measurements are included in quantitative morphometric analysis, such as:

- **Area and volume**: Finding out how much space certain parts of tissue, like cells, extracellular matrix, or areas of interest, take up in the whole.
- **Length and diameter**: Characteristics including blood vessel length, nerve fibre diameter, and cellular process diameter are measured.
- **Examining the distribution and density of cells or other components within the tissue** is the following step.
- **Shape and orientation**: Examining the cell or structure’s orientation, as well as its shape and aspect ratio.
- **Staining intensity quantification**: This step helps to understand the abundance or expression levels of certain markers or structures by measuring the intensity of staining.

For the purpose of handling and analysing images, quantitative morphometric analysis uses specialised software. Automated or semi-automated parameter measurement is possible using these instruments, which is more efficient and less prone to bias than manual measurement. It is also possible to acquire objective estimations of structural parameters in three-dimensional tissue samples through the use of sophisticated methods like stereology.

With the objective numerical data provided by quantitative morphometric analysis, trends, differences between experimental groups, and correlations with other factors can be statistically examined. Studies in histology, pathology, developmental biology, and tissue engineering, among others, can benefit from this quantitative method’s increased rigour and reproducibility in tissue investigation, leading to more solid findings and interpretations.

### 2.14. Real-time fluorescence quantitative PCR

The mRNA levels of ALP, Runx2, OPN, BMP4 and OCN were analyzed by qPCR as previously described [26]. mRNA was isolated from tissue-engineered bone using Trizol (Invitrogen). cDNA was generated through reverse transcription using Promega’s MMLV system.

### 2.15. Immunoblot analysis

ALP, Runx2, OPN, BMP4, and OCN protein expressions were evaluated in tissue-engineered bone and the expressions of ACAN, Sox9, and collagen type II proteins were evaluated in tissue-engineered cartilage. Tissue-engineered bone
weighing 300 mg was lysed using RIPA buffer supplemented with PMSF. Protein concentrations were measured using the BCA protein assay kit (Beyotime). Western blot was performed as previously described [27].

2.16. Postmenopausal osteoporotic osteochondral defect model

In this study, postmenopausal osteoporotic New Zealand large white rabbits were used as the osteochondral defect model. After weighing, anesthesia was given at a dose of 0.2 mL/kg, knee skin preparation, iodophor disinfection, skin incision was performed on the medial side of the knee joint, and medial parapatellar joint incision was performed to deviate the lateral side of the patella and expose the femoral condyle articular surface. After positioning, a stainless steel bone drill (bit diameter 4 mm, depth 3 mm) was used to drill the hole to create an osteochondral defect.

The animals with osteoporosis were randomly assigned to six groups: untreated defect group (untreated group), ADM only filling defect group (ADM group), PMOP-BMSCs composite ADM scaffold filling defect group (Control group), IGF-1 and PMOP-BMSCs co-culture composite ADM scaffold filling defect group (IGF-1 group), HGF and PMOP-BMSCs co-culture composite ADM scaffold filling defect group (HGF group), and a group where both IGF-1 and HGF were co-cultured with PMOP-BMSCs in the composite ADM scaffold-filled defect group (IGF-1+HGF group). Euthanasia was performed at 8 weeks postoperatively.

Following the animals’ killing, six sets of samples were incubated for three days in 4% (w/v) paraformaldehyde buffer, and a Micro-CT μ80 scanner (Scanco Medical, Switzerland) was used for analysis. The scanning parameters were set as follows: voltage = 70 kV, current = 114 μA, resolution = 1024 × 1024 pixels.

2.17. Histologic analysis

Masson’s trichrome (M-T), Safranin-Fast green (S-F), and hematoxylin and eosin (HE) staining techniques were used on the samples. The osteochondral repair effect was assessed using collagen type II immunohistochemical staining.

2.18. Statistical analysis

At least three duplicates of each data set were gathered. The analysis was conducted with the statistical software Graph Pad Prism 6. A t-test analysis was utilized for assessing the statistical significance of the difference between two independent subgroups, while a one-way ANOVA was employed for evaluating the statistical significance of group differences, with a threshold P-value of less than 0.05.

Through the use of bilateral ovariectomy, the research established an osteoporosis model in female New Zealand large white rabbits. A total of forty rabbits made up the osteoporosis model (PMOP) group, with the other six acting as a control group. Following a 12-week period, the PMOP model was validated, and osteochondral abnormalities were brought about. The analysis of bone mass index was carried out using micro-CT scanning and histomorphometry. Elevated lactate sensor assays were used to measure the quantities of serum estradiol (E2). To
stimulate osteoblast, adipocyte, and chondrocyte differentiation, bone marrow mesenchymal stem cells (BMSCs) were extracted, cultivated, and subjected to various stimuli. The process of differentiation was assessed by employing targeted staining methods and Edu proliferation analysis. The process of transcriptome sequencing was used to detect genes that showed differential expression. Using dermal decellularized matrix (ADM) scaffolds and BMSCs, artificial bone and cartilage were created in vitro. Patients were divided into different treatment groups once the osteoporotic osteochondral defect model was created. Eight weeks later, the samples were examined by employing micro-CT, histological staining, and immunohistochemical approaches. We used t-tests and one-way ANOVA to do the statistical analysing. The results showed that the PMOP model was efficiently established, BMSCs were successfully differentiated, and tissue-engineered constructs were effective in healing osteochondral lesions. Gaining important insights for future therapeutic applications, the study emphasises the potential of growth factors and BMSC-based therapy for treating postmenopausal osteoporotic osteochondral defects.

3. Results

3.1. Establishment of the PMOP model and investigation of the morphology, proliferation, and differentiation disorders of bone marrow mesenchymal stem cells

Compared with the normal group, the osteoporosis group had fewer trabeculae, sparse microstructure (Figure 1A1,B1), bone cystic changes (Figure 1A2,B2), and lower bone volume fraction (Figure 1C) and bone surface area (Figure 1D), indicating that bone mass was lower than that of the normal group. The osteoporosis group also showed less trabeculae (Figure 1E) and larger detachments (Figure 1F). Meanwhile, the connectivity density (Conn.D.) (Figure 1G) and bone mineral density (BMD) (Figure 1H) were decreased in the osteoporosis group, indicating increased bone resorption and decreased bone mass in this group. While BMSCs from control animals were observed as spindle-shaped homogeneous groups with a tendency to aggregate together (Figure 2A1–A3), whereas there was no significant difference in the cellular characteristics of BMSCs from PMOP model animals (Figure 2B1–B3). After 7 days of in vitro osteogenic induction, the calcium ions chelated by the extracellular matrix and alizarin red S in the osteoporosis group were lower than those in the normal group (Figure 3A,D). After 7 days of induction of adipogenesis, lipid droplets appeared in both groups (Figure 3B,E). This was consistent with the staining results shown in this literature [28]. After 7 days, the results of cartilage-induced differentiation staining showed that the difference between the osteoporosis group and the normal group was not clearly observed (Figure 3C,F). In the osteoporosis group, the serum estrogen concentration in rabbits was lower than that in the normal group (Figure 3G). The results of CCK-8 showed that although the number of bone marrow mesenchymal cells in both groups continued to increase during this period, the performance of the osteoporosis group was still lower than that of the normal group (Figure 3H).
**Figure 1.** Evaluation of the postmenopausal osteoporosis rabbit model. (A1, B1) Micro-CT of the healthy and postmenopausal osteoporosis groups; (A2, B2) 2D cross-sectional images of the femur and femoral stem in Normal and osteoporosis model groups; (C) bone volume fraction (BV/TV); (D) bone surface area to bone volume ratio (BS/BV); (E) number of bone trabeculae (Tb. N); (F) bone trabeculae separation (Tb. Sp); (G) connectivity density (Conn.D.); (H) bone mineral density (BMD).

*p < 0.05, **p < 0.01.

**Figure 2.** Morphology of bone marrow mesenchymal stem cells (BMSCs). (A1–A3) P1, P3, P5 passages of BMSCs from Normal model group; (B1–B3) P1, P3, P5 passages of BMSCs from the postmenopausal osteoporosis model group.
Figure 3. Induced differentiation staining and cell proliferation of different bone marrow mesenchymal stem cells (BMSCs). (A–C) In vitro induction of osteogenesis, adipogenesis and chondrogenic formation induced normal group BMSCs were stained with alizarin red S, oil red O and Alcian blue for 7 days; (D–F) in vitro induced osteogenesis, adipogenesis and chondrogenic formation induced osteoporosis group BMSCs were stained with alizarin red S, oil red O and Alcian blue for 7 days; (G) E2 concentration in serum rabbits; (H) proliferation of BMSCs determined by CCK8.

*p < 0.05.

3.2. HGF and IGF-1 genes are down-regulated in PMOP-BMSCs

We next examined the molecular mechanisms underlying changes in the differentiation of BMSCs in postmenopausal osteoporosis by sequencing BMSCs from PMOP model and control animals (PMOP-BMSCs and Normal-BMSCs). We observed a large variability between the two groups and a good biological reproducibility of the samples within the groups (Figure 4A). The findings revealed 1353 genes that showed differential expression, with 669 genes being up-regulated and 684 genes being down-regulated (refer to Figure 4B). We screened significantly differentially expressed genes (FC > 2 or FC < 0.5) in the three lineage differentiation as our target differential genes. The clustering heatmap showed significant differences in the gene expression of the BMSCs in control and PMOP settings (Figure 4C). The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis revealed significant activation of the TGF-β, Hippo, MAPK, cytokine-cytokine receptor interaction, Rap1, and PI3K-Akt signaling pathways. Among these pathways, the MAPK signaling pathway exhibited the highest number of associated differential genes and displayed the most significant pathway enrichment, as depicted in Figure 4D. GO enrichment analysis revealed that several biological processes and molecular functions were significantly activated such as osteoblast differentiation, endochondral osteogenesis, MAPK cascade regulation, SMAD protein signaling and positive regulation, and growth factor activity, cytokine activity, TGF-β receptor, and BMP receptor binding were significantly enriched (Figure 4E). Among them, the significant enrichment of MAPK level pathway in
biological processes indicates that BMSCs are the most enriched in PMOP among DEGs related to this pathway. Therefore, we chose the MAPK pathway for further study. We defined the differential genes related to the MAPK pathway in the GO enriched set as set A, the KEGG-enriched MAPK pathway-related differential genes as set B, and the differential genes with significant up-regulation (FC greater than 2) and significant down-regulation (FC less than 0.5) of expression as set C, and plotted the Venn diagram for intersection (Figure 4F), and the AC intersection was BMP3 and BMP6. The BC intersection was IGF-1, CD14, HGF, and FGF-1. Finally, through the volcano map (Figure 4G), it was found that HGF and IGF-1 were identified as differentially expressed and downregulated genes in PMOP-BMSCs. Therefore, we hypothesized that downregulation of HGF and IGF-1 may play a role in the aberrant differentiation of PMOP-BMSCs.

Figure 4. Investigation of the potential mechanisms of postmenopausal osteoporosis. (A) PCA analysis of differentially expressed genes in control and osteoporotic model groups; (B) statistical histogram of differentially expressed genes; (C) heat map of target differential genes; the default conditions for filtering differences are q-value < 0.05 and multiplicity of differences greater than 2; (D) KEGG enrichment analysis of the differentially expressed genes of the target; the size of the bubble reflects the number of differentially expressed genes; (E) GO enrichment analysis of the differentially expressed genes of the target; (F) Venn diagram; (G) set A includes MAPK pathway-related genes enriched by GO, set B includes MAPK pathway-related genes enriched by KEGG, and set C includes target differential genes. The intersecting genes of sets A and C include BMP3 and BMP6 genes and the intersecting genes of sets B and C include IGF-1, CD14, HGF and FGF-1 genes; volcano plot of target differential genes.
3.3. Co-treatment of HGF and IGF-1 promotes the proliferation and differentiation of osteogenesis, adipogenic and chondrogenic PMOP-BMSCs in vitro

The findings indicated that the proportion of positive cells in the other three groups was higher than that in the control group, with a notably higher proportion of EDU positive cells observed in the IGF-1+HGF group (Figure 5A). After 7 days of in vitro osteogenesis induction, a small amount of calcium deposition, chelated by alizarin red S, was observed in the extracellular matrix of the IGF-1 group, HGF group, and IGF-1+HGF group, compared to the control group. After 7 days of in vitro adipogenic induction, fat droplets appeared in all four groups, and there were fewer fat droplets in the IGF-1+HGF group. After 7 days of in vitro induction of cartilage, the observation of cartilage matrix polysaccharide content in each group was not yet significant, (Figure 5B). The CCK8 results indicated that the proliferation of PMOP-BMSCs significantly increased after growth factor intervention compared to those without the factor after 24 h (Figure 5C), and the number of IGF-1+HGF group increased most significantly, which was consistent with the EDU staining results.

Figure 5. Illustrates the impact of growth factors on the in vitro proliferation and induced differentiation of PMOP-BMSCs. The control group was PMOP-BMSCs without growth factor intervention; The other groups were treated with IGF-1, HGF, IGF-1+HGF for 24 h of PMOP-BMSCs. (A) Representative images of EDU staining are shown in the figure (red EDU; Blue DAPI); (B) different PMOP-BMSCs were induced by osteogenesis, adipogenic and cartilage formation in vitro for 7 days of Alizarin Red S, Oil red O and Alcian Blue stainingl; (C) histogram of different PMOP-BMSC proliferations at 24 h for CCK8 testing. 

**p < 0.01, ***p < 0.001.
3.4. In vitro tissue-specific differentiation evaluation of tissue-engineered bone and cartilage (ADM scaffold combined with different growth factor-mediated PMOP-BMSCs)

Previous studies showed that ADM scaffolds with less residual DNA after decellularization cause lower immune responses; additionally, a pore size of 125–150 µm and porosity of ADM are favorable for extracellular matrix secretion and cellular infiltration [29]. In the control group, PMOP-BMSCs without growth factors were instilled into the ADM scaffold. We made cell suspensions of growth factor-treated BMSCs and dropped them into ADM scaffolds. During the 8-week in vitro osteogenic culture period, histological analysis revealed that the IGF-1+HGF group exhibited a higher presence of osteogenic-specific extracellular matrix, along with positive Masson and collagen type I staining, suggesting the development of mature bone tissue. This group demonstrated histological similarities to the tissue-engineered bone created from healthy BMSCs. The single-factor group also had some osteogenic-specific ECM compared with the control group, but there was still some material that was not completely filled by cells (Figure 6A). The qPCR analysis revealed a significant up-regulation in the expression levels of osteogenesis-related genes Runx2, BMP4, and OCN in the HGF group. The mRNA expression levels of bone-related genes Runx2, BMP4, and OCN with the combination of IGF-1 and HGF were significantly elevated compared to the control group as shown in Figure 6B, and the Western blot findings were in agreement with the qPCR results (Figure 6C) showed a strong osteogenic induction effect upon the addition of HGF. However, there was no significant enhancement in the effect observed in the IGF-1+HGF group compared to the HGF group, therefore casting uncertainty on whether IGF-1 and HGF synergistically contribute to osteogenic induction.

After 8 weeks of in vitro cartilage culture, histological examination showed that compared with the control group, the IGF-1+HGF group, the HGF group, and the IGF-1 group had more chondrogenic specific ECM, and the cells were filled with a larger area in the ADM material, and the positive staining of saffron solid green and collagen type II indicated cartilage maturation, and the histology of the IGF-1+HGF group was closer to that of the healthy group cartilage (Figure 7A). In the IGF-1+HGF group, the qPCR results demonstrated a notable up-regulation in the expression of cartilage-related genes and proteins including ACAN, collagen type II, and SOX9. The HGF group showed down-regulation of cartilage-related genes collagen type II and Sox9 expression, while the IGF-1 group exhibited down-regulation of cartilage-related genes ACAN, collagen type II, and Sox9 expression (Figure 7B). These results indicated that the combined use of IGF-1 and HGF could promote the expression of chondrogenic genes and had a strong chondrogenic induction effect.
Figure 6. Macroscopic and histological staining and qPCR, Western blot analysis of tissue-engineered bone 8 weeks after in vitro osteogenesis induction. (A) Macroscopic and histological staining of tissue-engineered bone (ADM scaffold combined with different types of BMSCs) after 8 weeks of in vitro osteogenesis induction. The dotted box represents the area of bone tissue observed, the black arrows represent areas of mature bone tissue, and the red arrows represent areas of immature bone tissue; (B) Q-PCR was conducted to analyze the mRNA levels of ALP, Runx2, OPN, BMP4, and OCN; (C) a representative image of western blot of osteogenesis-specific factors is shown.

*p < 0.05, **p < 0.01, ***p < 0.001, ns: no statistically significant difference.
Figure 7. Macroscopic and histological staining and qPCR, western blot analysis of tissue-engineered cartilage after 8 weeks of in vitro cartilage induction. (A) Macroscopic and histological staining of tissue-engineered cartilage (ADM scaffold combined with different types of BMSCs) after 8 weeks of in vitro chondrogenic induction. The dotted box outlines the observed cartilage tissue area, mature cartilage tissue is indicated by black arrows, while immature cartilage tissue is indicated by red arrows; (B) Q-PCR was performed to detect the mRNA expression of ACAN, collagen type II, and Sox9; (C) a representative image of western blot of cartilage-specific factors is shown.

**p < 0.01, ****p < 0.0001, ns: no statistical difference.

3.5. Repair of osteochondral defects in specific tissues using growth factor-cultured PMOP-BMSCs with ADM scaffolds

Tissue-specific repair of osteochondral defects by growth factor-mediated PMOP-BMSCs with ADM scaffolds was evaluated in the PMOP rabbit knee osteochondral defect model (Figure 8A–C). Therefore, the grouping settings included a post-deficiency untreated group, a material alone group, a control group of PMOP-BMSCs without additional factors, a group with two factors added to PMOP-BMSCs, a group with HGF+PMOP-BMSCs, and a group with IGF-1+PMOP-BMSCs. The macroscopic and Micro-CT images revealed significant fiber-like tissue defects in the cartilage and bone regions of the untreated PMOP rabbit knee osteochondral defect group at 8 weeks post-implantation (Figures 8A1,B1,C1).

The ADM group had poor repair of the cartilage and bone defect regions (Figure 8A2,B2,C2). The control group (no factor added) had poor interface leveling and the cartilage defects were still significantly reduced; regeneration of subchondral bone tissue increased, and the defect was reduced (Figure 8A3,B3,C3). Notably, the IGF-1+HGF group showed the best repair of cartilage-like tissues in the
cartilage defect area and the best repair of bone-like tissues in the bone defect area (Figure 8A4,B4,C4) with seamlessly fusing with the surrounding tissues at 8 weeks. The HGF group exhibited successful fusion of cartilage at the edges of the cartilage defect site, however, the central region still showed some deficiencies with fibrous-like tissue, and partial repair was observed in the bone defect zone (refer to Figure 8A5,B5,C5). The IGF-1 group had cartilage-like tissue repair in the cartilage defect area, but the central area of the fibrous-like tissue was defective, and the fusion of the bone-like tissue in the bone defect area with the surrounding native bone was partially repaired (Figure 8A6,B6,C6).

Histological examination further confirmed that the defects in the untreated group were mainly repaired by fibrous tissues, with obvious gaps between the defect area and adjacent normal tissues; the analysis further showed that it was difficult to achieve good repair in the cartilaginous area and the subchondral bone defect area itself (Figure 8D1–I1). In the ADM group, the subchondral bone tissues were indistinct from the boundary of the normal tissues, with excessive thickness of the bone tissues and the lack of the cartilaginous tissues (Figure 8D2–I2). The control group exhibited inadequate smoothness at the cartilage interface, with a noticeable indentation towards the boundary of the adjacent natural cartilage; there was evidence of regenerative bone tissue repair in the subchondral osteoblastic tissue (Figure 8D3–I3). In the IGF-1+HGF group, the cartilage defect area was mainly repaired by regenerated cartilage tissue; the cartilage surface was relatively smooth, the thickness of articular cartilage was relatively normal, and the border between the newborn cartilage and the surrounding natural cartilage was seamlessly healed (Figure 8D4–I4). Both the HGF group (Figure 8D5–I5) and IGF-1 group (Figure 8D6–I6) showed regenerative repair of bone tissue in the subchondral osteoblastic tissue and demonstrated regenerative repair of cartilage tissue in the area of the cartilage defects. However, there were still noticeable gaps at the border with the natural cartilage, resulting in poor smoothness of the cartilage. Additionally, the newborn cartilage reached maturity in 8 weeks in vivo under the action of IGF-1+HGF, and the bone defect area mainly showed complete degradation of ADM scaffolds and gradual regeneration into mature bone tissues; relatively mature bone tissue repair was achieved in 8 weeks.
Figure 8. Macroscopic observation, micro-CT analysis and histological examination of specific repair of knee osteochondral tissue in postmenopausal rabbits with osteoporosis. (A–C) Surgical images of rabbit knee joint; (A1–A6) Macroscopic observation 8 weeks after implantation. (B1–B6) Micro-CT 3D reconstructed images and (C1–C6) 2D cross-sectional images; (D1–D6) HE; (E1–E6) Masson, (F1–F6) Collagen type I; (G1–G6) Collagen type II, (H1–H6 and I1–I6) Safranin O-Fast green staining. The dotted box indicates the area where the defect is repaired.

By examining the shape, proliferation, and differentiation of bone marrow mesenchymal stem cells (BMSCs), the research sought to understand the pathophysiology of postmenopausal osteoporosis (PMOP). Reduced trabeculae, sparse microstructure, and a lower bone volume fraction were seen when comparing PMOP to normal BMSCs, suggesting that the bone mass was smaller. A possible factor in the abnormal differentiation of PMOP-BMSCs was the down-regulation of the HGF and IGF-1 genes. In vitro, osteogenic, adipogenic, and chondrogenic PMOP-BMSCs proliferated and differentiated more effectively when co-treated with HGF and IGF-1. Superior osteogenic and chondrogenic induction was demonstrated by ADM scaffold mixed with growth factor-mediated PMOP-BMSCs, which increased tissue-specific differentiation, especially with IGF-1+HGF. At 8 weeks, BMSCs treated with IGF-1+HGF had fused smoothly with surrounding tissues, repairing cartilage and bone lesions best in a PMOP rabbit knee osteochondral defect model. The results of the histological examination proved that the damaged cartilage and bone had successfully healed. Noteworthy, in comparison to treatments
including either IGF-1 alone or no treatment at all, the synergistic effects of IGF-1 and HGF significantly improved tissue regeneration. Together, these results provide hope for tissue-engineered bone repair in PMOP patients by demonstrating the critical roles of HGF and IGF-1 in BMSC development in this disease. Novel therapeutic approaches for PMOP-induced bone diseases may emerge from additional investigation into the molecular pathways underpinning these effects.

4. Discussion

After establishing the model, Micro-CT revealed an increase in trabecular separation (Tb.Sp) and decreases in bone volume fraction (BV/TV), bone surface area to bone volume ratio (BS/BV), number of trabeculae (Tb.N), connectivity density (Conn.D.), and bone mineral density (BMD). These findings suggest an increase in bone resorption and a decrease in bone mass within this group. Estrogen level testing also showed that the experimental rabbits had lower estrogen levels than normal rabbits, demonstrating pathologic features similar to those of postmenopausal osteoporosis.

Anti-intrinsic membrane proteins (IMH) antibodies are a subset of the antibody family that specifically targets proteins found inside cell or organelle membranes. The intrinsic membrane proteins are integral proteins that are embedded within the lipid bilayer of biological membranes. These antibodies bind to these proteins.

The exact nature of IMH antibodies is defined by the membrane antigens they specifically target. Important components of cell signaling, communication, and structural integrity may include antigens such as transmembrane proteins, receptors, ion channels, transporters, or adhesion molecules.

Biomedical research, diagnostics, and therapies frequently make use of IMH antibodies. Investigators can use them to investigate the cellular and tissue-specific expression patterns, localization, and activities of particular membrane proteins. As a diagnostic tool, IMH antibodies have the potential to identify diseases and disorders that are marked by changes in the expression or distribution of membrane proteins. As a targeted agent for immunotherapy, medication delivery, or modification of cellular processes mediated by membrane proteins, IMH antibodies have potential for use in therapeutics.

It is common practice to assess IMH antibody specificity, affinity, and cross-reactivity with other proteins as part of the characterisation process. Immunoblotting, immunoprecipitation, immunofluorescence, and flow cytometry are typical experimental methods for evaluating the binding characteristics and biological impacts of IMH antibodies.

When it comes to studying membrane proteins, IMH antibodies are a powerful tool that could be useful in many different fields of biological research and therapeutic practice.

In order to enhance the growth of PMOP-BMSCs and enhance osteogenic differentiation, we conducted transcriptome sequencing of both PMOP-BMSCs and Normal-BMSCs. Our analysis revealed that the MAPK cascade pathway was notably enriched in the biological processes of PMOP-BMSCs. This suggests that PMOP-BMSCs exhibited a higher abundance of differentially expressed genes related to this
pathway compared to Normal-BMSCs. KEGG enrichment analysis showed that the MAPK signaling pathway had the highest number of related differential genes and the most obvious differential up-regulation. This indicates that it was the most important pathway that was enriched. Therefore, we chose to further explore the MAPK pathway. Furthermore, our data identified HGF and IGF-1, which are associated with the MAPK pathway, as differentially expressed genes and downregulated genes in PMOP-BMSCs. We thus speculated that downregulation of HGF and IGF-1 may play a role in the aberrant differentiation of PMOP-BMSCs. We thus exogenously administered HGF and IGF-1 to PMOP-BMSCs.

It is worth noting that the degree of downregulation of HGF and IGF-1 differential expression was not significant, HGF may be an important potential biomarker for osteoporosis given the recent two-year report that IGF-1 may be a key biomarker for PMOP [30,31]. Likewise, it has been reported that IGF-1 concentrations appear to be associated with osteoporosis [32]. HGF could serve as a potential candidate for mediating local paracrine signaling between osteoblasts and osteoclasts within bone tissue [33]. At the same time what role both play in postmenopausal osteoporotic disease is rarely discussed in the literature. Based on these considerations, HGF and IGF-1 were selected in this experiment to jointly investigate their roles in the abnormal differentiation of PMOP-BMSCs.

The results showed that the combination of HGF and IGF-1 enhanced DNA replication activity and promoted proliferation in PMOP-BMSCs. The histological and immunohistochemical findings of the tissue-engineered bone constructed in vitro also revealed an increased presence of osteogenic-specific extracellular matrix. Through 8 weeks of in vitro osteogenesis induction, the tissue-engineered bone exhibited a relatively mature histological profile. We conducted a more detailed investigation into the tissue specificity and expression of differentiation markers in growth factor-induced tissue-engineered bone. Our findings revealed that the co-administration of IGF-1 and HGF led to a significant increase in the mRNA and protein expression of precursor osteoblast markers, as well as the key regulator of osteogenesis, Runx-associated transcription factor 2 (RUNX2) [34]. These findings indicated that the combination of IGF-1 and HGF enhanced the activity of osteogenic differentiation marker proteins in PMOP-BMSCs, which in turn promoted osteogenic differentiation. BMPs play an important role in bone repair, and the enhancement of BMP4 activity promotes the regulation of cellular differentiation in the osteogenic direction [35]. Our findings showed a notable increase in both the mRNA and protein levels of OCN, suggesting that IGF-1 and HGF enhance the secretion of extracellular bone matrix and promote osteoblast differentiation and maturation in PMOP-BMSCs. The pronounced accumulation of calcium observed in alizarin red S staining corroborated these findings. The IGF-1 and HGF combination also promoted high expression of the chondrogenic differentiation marker proteins ACAN, SOX9, and collagen type II, suggesting that IGF-1 and HGF–mediated tissue engineered cartilage differentiates into mature cartilage in vitro. Based on these findings, we determined that the synergistic impact of HGF and IGF-1 elevated the levels of genes and proteins associated with osteoblasts and cartilage, thereby facilitating osteochondral differentiation in PMOP-BMSCs.
In the PMOP rabbit knee osteochondral defect model, the untreated group exhibited misplaced fibrous tissue repair, presumably from the lack of regulation of HGF inhibition of fibrosis to promote angiogenesis [36]. The ADM group achieved partial bone regeneration in the defect area, but not complete regeneration. The bone marrow blood passes through the ADM scaffolds in vivo to form an endogenous stem cell-ADM complex for tissue-specific regeneration. However, the lack of regulation of chondrocyte proliferation and matrix secretion by IGF-1 results in low secretion of the cartilage matrix collagen type II, leading to poor formation of cartilage tissues [37–39]; the lack of regulation of osteogenesis related gene expression by HGF results in excessive ossification and proliferation [40]. Due to the regulation of a single growth factor, the interface flatness and collagen fiber ranking of the IGF group or the HGF group were better than those of the Untreated group, the ADM group and the Control group, but they were still inferior to the IGF-1+HGF group. Previous studies showed that the combination of IGF-1 and HGF can reduce fibrosis, inhibit cellular hypertrophy, and promote the generation of microvascular system [41,42]. In this research, the combination of IGF-1 and HGF was found to hinder fibrosis during the repair of osteochondral defects in the knee joints of postmenopausal osteoporotic rabbits, resulting in a smooth cartilage interface. Vascularization is crucial for the proper maturation of subchondral bone tissues to facilitate nutrient delivery. Notably, the structural reconstruction effect of the IGF-1+HGF group was better than that of the untreated group, ADM group, HGF group and IGF-1 group, which may be attributed to the overall effects of IGF-1 and HGF and the surrounding micro environments (ADM scaffolds and in situ micro environment of the knee joint).

Collectively, our findings indicate that the aberrant differentiation of bone marrow mesenchymal stem cells in postmenopausal osteoporosis patients, accompanied by the downregulation of IGF-1 and HGF, could potentially exert an effect. We thus exogenously administered HGF and IGF-1 to PMOP-BMSCs. The PMOP-BMSCs supplemented with additional factors exhibited increased DNA replication activity compared to those without additives, and were more effective in enhancing the secretion of bone and cartilage matrices while reducing the accumulation of fat droplets. In in vitro studies on tissue-engineered bone and cartilage, it was observed that the IGF-1+HGF group significantly upregulated the expression of osteochondral-related genes and differentiation markers such as Runx2, BMP4, OCN, ACAN, SOX9, and collagen type II, facilitating osteogenic and chondrogenic induction effects. During the treatment of osteochondral defects in the knee joints of postmenopausal osteoporotic rabbits, the group receiving IGF-1 and HGF exhibited the least fibrosis, excellent cartilage interface flatness, and effective repair of subchondral bone tissue. Moreover, the findings from animal studies are preliminary and may not directly translate to the disease mechanisms in human patients. The limitations of this experiment are that it is not clear whether different doses of growth factors affect PMOP-BMSCs to different degrees; the relevant pathway inhibitors were not added during the experiment, and it is not possible to point out through which signaling pathway HGF and IGF-1 acted. Nevertheless, our findings provide a new idea for growth factor-based cell therapy for specific repair of osteochondral tissue and are promising for the treatment of
postmenopausal osteoporosis.

5. Conclusion

Our results suggest that the addition of IGF-1 and HGF increases the expression of osteogenic cartilage-related genes and proteins, which in turn promotes osteochondral proliferation and differentiation of PMOP-BMSCs and ultimately improves the repair of osteochondral defects.

Particularly in experimental studies that seek to evaluate the effectiveness of interventions or therapies, validating differences between treatment groups is an essential part of research. The results will be indicative of real therapy benefits and not a result of random chance, appreciations to this validation.

Statistics is a popular tool for verifying treatment group differences. Statistical tests like t-tests, non-parametric tests, or analysis of variance (ANOVA) are used to compare the means or distributions of outcome variables between groups. Statistical significance, defined as the degree to which the observed differences cannot be explained by chance alone, is determined using these tests.

It additionally possible to evaluate the range of possible treatment effects by calculating confidence intervals. Estimating treatment effects with narrow confidence intervals is more precise and reliable.

In addition, power analysis should be done before the study starts to find out how many participants are needed to have enough statistical power to notice significant changes between the treatment groups. Having a sufficient number of participants in a study increases confidence in the results and the possibility of uncovering real treatment benefits, should they exist.

The validity of the differences between treatment groups is strengthened by the inclusion of suitable controls, randomization, and blinding techniques, which assist minimize bias and confounding factors.

Research and clinical practice benefit from more robust and reliable findings when differences between treatment groups are validated through appropriate experimental design, large enough samples, and rigorous statistical analysis. This allows for more meaningful interpretations and informed decisions.

**Author contributions:** Conceptualization, methodology, investigation, JQG; data curation, formal analysis, visualization, CBZ; writing—original draft, software, validation, JCL; writing—review and editing, supervision, project administration, FFC; resources, funding acquisition, supervision, YHW; supervision, validation, project administration, GDZ; investigation, data curation, methodology, YL; conceptualization, writing—review and editing, visualization, YLC. All authors have read and agreed to the published version of the manuscript.

**Ethical approval:** The animal study protocol was approved by the Institutional Review Board Animal Care and Experimental Committee of Shanghai Jiao Tong University School of Medicine (SH9H-2020-A654-1, 26 March 2020) for studies involving animals.

**Conflict of interest:** The authors declare no conflict of interest.
References


