**Supporting Information**

# Photo-Crosslinked Scaffold with Kartogenin-Encapsulated Nanoparticles for Cartilage Regeneration

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## Materials and Methods

### Materials

All chemicals unless mentioned were purchased from Sigma-Aldrich. Sodium hyaluronic acid (HA, the molecular weight of 77 kDa) was purchased from Freda Biochem Co., Ltd. (Shandong, China).

### Methods

*Synthesis route of Kartogenin (KGN)*

Compound 1 (**Figure S6**): A mixture of 4-iodoaniline (6.3 g, 28.7 mmol), phenylboronic acid (3.5 g, 28.7 mmol), $K_2CO_3$ (8.12 g, 71.7 mmol), and Pd $(PPh_3)_2Cl_2$ (55 mg, 0.078 mmol) was added to an oven-dried glass vessel, which was then sealed and evacuated and back-filled with Ar. A solvent mixture (DME/$H_2O$, 1/1 [v/v], 100 mL) was added *via* a syringe, followed by the addition of TBAF (10 mL) in a similar manner. The reaction mixture was stirred at 80 °C for 12 h. After cooling, removal of the solvent under vacuum afforded a gray solid, and the obtained crude product was purified by silica gel column chromatography (ethyl acetate/petroleum ether, 1/4–1/2 [v/v]) to afford the title compound 1 as pale brown crystals in an 80 % yield (3.90 g). Mp: 50 °C−51 °C. $^1$H NMR (500 MHz, $CDCl_3$): δ 7.62 (d, J = 7.7 Hz, 2H), 7.53−7.41 (m, 4H), 7.34 (t, J = 7.2 Hz, 1H), 6.79 (d, J = 7.7 Hz, 2H), 3.74 (brs, 2H). $^{13}$C NMR (125.8 MHz, $CDCl_3$): δ 146.0, 141.2, 131.5, 128.7, 128.1, 126.5, 126.3, 115.4.

Compound 2: Isobenzofuran-1,3-dione (0.786 g, 5.3 mmol) was dissolved in 50 mL of HAc at 60 °C, and then a 1,1′-biphenyl-4-amine (1) (1.0 g, 5.9 mmol in 20 mL HAc) solution was added in 30 min. The reaction mixture was stirred at 60°C for 24 h, followed by cooling down at room

temperature, whereupon a pale white suspension was formed. The resulting crude product was filtered, and the residue was recrystallized with EtOH to afford compound 2 (1.36 g, 90 %).

*Chondrogenesis of synovial derived mesenchymal stem cells* [1]*:*

*1) Isolation and Culture of Synovial Mesenchymal Stem Cells:* The study was approved by the ethical committee of Drum Tower Hospital, Medical School of Nanjing University, and informed consent was obtained from all the subjects. Human synovial membrane tissue was harvested from 6 donors (5 women and 1 man; mean age, 57.7 years; age range, 48–65 years) when they underwent total knee arthroplasty. After temporary storage at 4°C, the synovial tissue was rinsed twice with phosphate-buffered saline (PBS), finely minced, and digested with 0.1% collagenase type I (Gibco, USA). After an overnight incubation at 5% $CO_2$ and 37°C in a humidified atmosphere (Thermo Scientific, USA), the cell suspension was filtered through a 70-μm nylon filter to eliminate tissue remnants, and the strained cell suspension was centrifuged at 1500 rpm for 5 min to collect the cells. Then, the cells were resuspended in expansion medium (DMEM/F12, 5% FBS, 100 U/mL penicillin, 100 mg/mL streptomycin; Invitrogen, USA), plated at $5 \times 10^4$ cells/cm in 100$cm^2$ culture dishes, and allowed to attach for 3 days in a 5% $CO_2$ humidified atmosphere at 37°C. Non-adherent cells were removed by changing the culture medium at every 3–4 days. When confluence was achieved at about 7 days of the primary culture, the cells were washed twice with PBS solution, harvested by treatment with trypsin–EDTA (0.25% trypsin, 1 mM EDTA; Hyclone, USA), and re-plated at a 1:2 dilution for the first subculture. Cell passages were continued in the same way with a 1:2 dilution, when the cells reached confluence. Passage 3 (P3) cells were used for the *in vitro* assays.

*2) Flow cytometry:* Human MSCs at passage 3 were harvested when reached confluence. One million cells were suspended in 100 µL buffer contained PBS, 2 mM EDTA and 0.5% bovine serum albumin (BSA). Then, 10 µL of fluorescein isothiocyanate (FITC)-coupled antibodies against CD34, CD45, CD11b, CD90, CD105 (MACS, MiltenyiBiotec, Germany) were added. Mouse IgG1 and Mouse IgG2a antibodies (MACS) were used as isotype control. After incubation of 10 minutes in the dark at 4°C, the cells were washed with 2 ml buffer and centrifuged at 300g for 10 minutes. Then, the supernatant was aspirated completely and the cell pellet was resuspended in a 500 µL buffer for analysis by flow cytometry (Becton Dickinson).

*3) Synthesis of Kartogenin:* Compound 1 (Figure S7): A mixture of 4-iodoaniline (6.3 g, 28.7 mmol), phenylboronic acid (3.5 g, 28.7 mmol), $K_2CO_3$ (8.12 g, 71.7 mmol), and Pd $(PPh_3)_2Cl_2$ (55 mg, 0.078 mmol) was added to an oven-dried glass vessel, which was then sealed and evacuated and back-filled with Ar. A solvent mixture (DME/$H_2O$, 1/1 [v/v], 100 mL) was added via a syringe, followed by the addition of TBAF (10 mL) in a similar manner. The reaction mixture was stirred at 80°C for 12 h. After cooling, removal of the solvent under vacuum afforded a gray solid, and the obtained crude product was purified by silica gel column chromatography (ethyl acetate/petroleum ether, 1/4–1/2 [v/v]) to afford the title compound 1 as pale brown crystals in an 80% yield (3.90 g). Mp: 50°C−51°C. $^1$H NMR (500 MHz, $CDCl_3$): δ 7.62 (d, J = 7.7 Hz, 2H), 7.53−7.41 (m, 4H), 7.34 (t, J = 7.2 Hz, 1H), 6.79 (d, J = 7.7 Hz, 2H), 3.74 (brs, 2H). $^{13}$C NMR (125.8 MHz, $CDCl_3$): δ 146.0, 141.2, 131.5, 128.7, 128.1, 126.5, 126.3, 115.4.

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*4) In vitro Chondrogenesis of SMSCs:* Cells ($5 \times 10^5$/mL) were placed into 15mL polypropylene tubes (Fisher) and centrifuged at 1500 rpm for 5 min. The control medium was composed of serum-free high-glucose DMEM supplemented with 100 nM dexamethasone, 50 mg/mL ascorbic acid–2-phosphate, 100 mg/mL sodium pyruvate, and a 1% insulin–transferrin–sodium selenite mix (ITS-mix; Invitrogen). The chondrogenic medium contained the control medium supplemented with KGN, TGF-$\beta$3, or BMP-2. The pellets were divided into 6 groups (group 1-6) according to the different composition of the media. Besides the control group (group 1), 10 μM KGN, 10 ng/mL TGF-$\beta$3 (PeproTech, USA) and 500 ng/mL BMP-2 (PeproTech, USA) were added to the control medium in different combination (group2, KGN; group3, KGN+TGF-$\beta$3; group5, TGF-$\beta$3+BMP-2; group6,TGF-$\beta$3+BMP-2+KGN). We further designed a group in which SMSCs were induced first with TGF-$\beta$3 for 1 week and then with KGN for 2 weeks (group 4). The culture medium was changed every 2 days. After 21 days of culture, portions of the culture pellets were subjected to either RNA isolation or histological analysis.

*5) Evaluation of Chondrogenesis in vitro:* At 14 days after chondrogenic induction, total RNA was extracted from the pellets by using Trizol reagent (Invitrogen) according to the

manufacturer's instructions. cDNA was prepared from 1.0 μg of RNA using a PrimeScript RT reagent kit with gDNA Erase (TaKaRa, Japan). Quantitative real-time reverse transcription polymerase chain reaction was performed using the Step-one Plus PCR system (Applied Biosystems, USA), by using SYBR Select Master Mix (Applied Biosystems) and primers prepared according to cartilage-specific genes. The sequences of the primers were as follows: *SOX9* (F: AGCGAACGCACATCAAGAC; R: CTGTAGGCGATCTGTTGGGG),*COL2A1* (F: TGGACGATCAGGCGAAACC; R: GCTGCGGATGCTCTCAATCT), and *COL1A1* (F: GAGGGCCCCAAGACGAAGACATC;R: CAGATCACGTCATCGCACAAC). The expression level was calculated by the $2^{-\Delta\Delta Ct}$ method, and $\beta$-actin was used as the housekeeping gene. At the end of 21 days of culture, the pellets were washed with PBS, fixed in 10% formalin, embedded in paraffin, and sectioned into 5-μm-thick sections. After deparaffinizarion and rehydration, the pellet sections were stained with hematoxylin and eosin. Sections for histochemical studies were fixed, permeabilized, and blocked using an Image-iT-Perm kit (Invitrogen) according to the manufacturer's instructions. Mouse-derived monoclonal antibodies against human collagen type II (1:100 dilution with 3% BSA; Abcam, USA) were applied to the section and incubated at 4°C overnight. On the next day, the sections were washed and a secondary antibody, donkey anti-mouse antibody (1:200 dilution; Invitrogen), was added dropwise followed by 1 h incubationat room temperature. Then, the sections were washed 3 times with PBS and stained with 4′,6-diamidino-2-phenylindole (Invitrogen). The sections were visualized under a fluorescence microscope (Olympus, Japan).

*In vitro release study:* The KGN-loaded PLGA nanoparticles (KGN-NPs) and HA containing KGN-NPs (HA/KGN-NPs) were placed in 1.5 mL phosphatebuffered saline (PBS) solution.

Each tube contained 60 mg nanoparticles. The release studies were performed at 37ºC and 100 rpm in a shaking incubator. For the detection of release kinetics of KGN from KGN-NPs or HA/KGN-NPs, total volume of PBS was collected after centrifugation and replaced by the same volume of PBS at each sampling time. The cumulative release profiles were evaluated *via* HPLC (Agilent pump, controller, autosampler and detector). The samples were analyzed on a Waters C-18 reverse phase column (4.6 × 250 mm, 5 μm) with a mobile phase of acetonitrile/water (v/v, 35/65) plus 0.1% formic acid. The flow rate was set at 1 mL/min and absorbance of CPT was monitored at 274 nm.

*Statistical Analysis:* All the results were reported as mean ± standard deviations. Unpaired *t*-test was used to carry out statistical analysis. $P \leq 0.05$ is considered to be significantly difference. All the values were analyzed by using SPSS software (version 20.0; IBM, America).

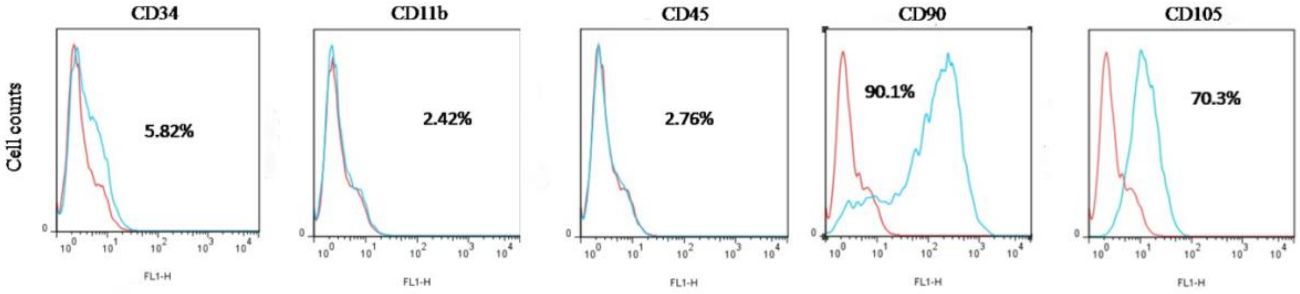


**Figure S1.** Flow cytometric analysis of human MSCs at passage 3 (*n*=3). SMSCs were positive for CD90, CD105 and negative for CD34, CD11b and CD45. CD34, CD11b, CD45,CD90, CD105 expression are shown as the green plot, and the isotype control expression as the red plot.

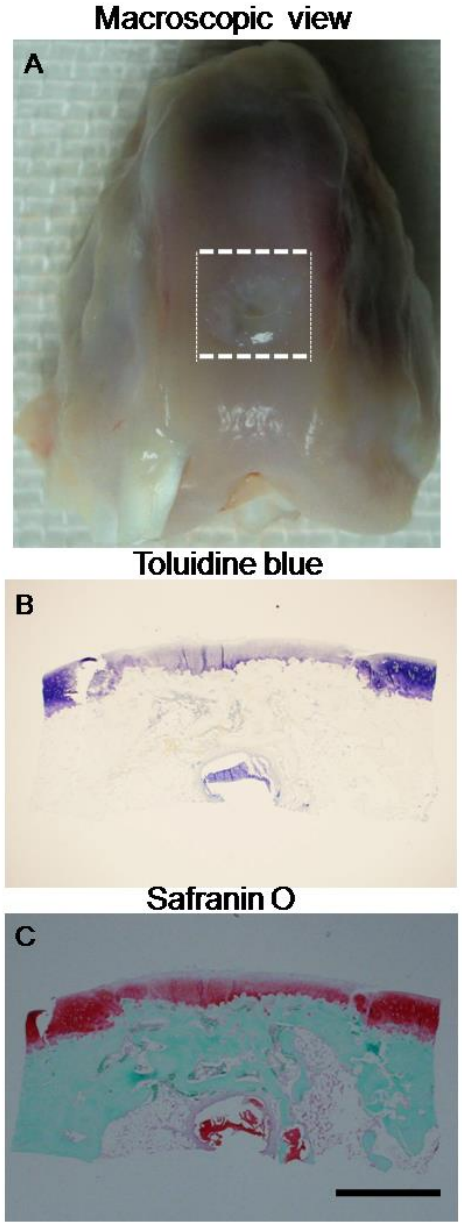


**Figure S2**. *In vivo* cartilage defects repair using photo-crosslinked scaffold and empty PLGA nanoparticles without KGN. A) Macroscopic appearance of the specimens harvested at 12 weeks after operation. B) Toluidine blue staining of the sections showed a less positive result than experimental group. C) Safranin O staining of the sections showed cartilage-like tissue formation in the cartilage defects, but the staining was a little lighter than that of the experimental group. Scale bar: 2mm in (B) and (C).

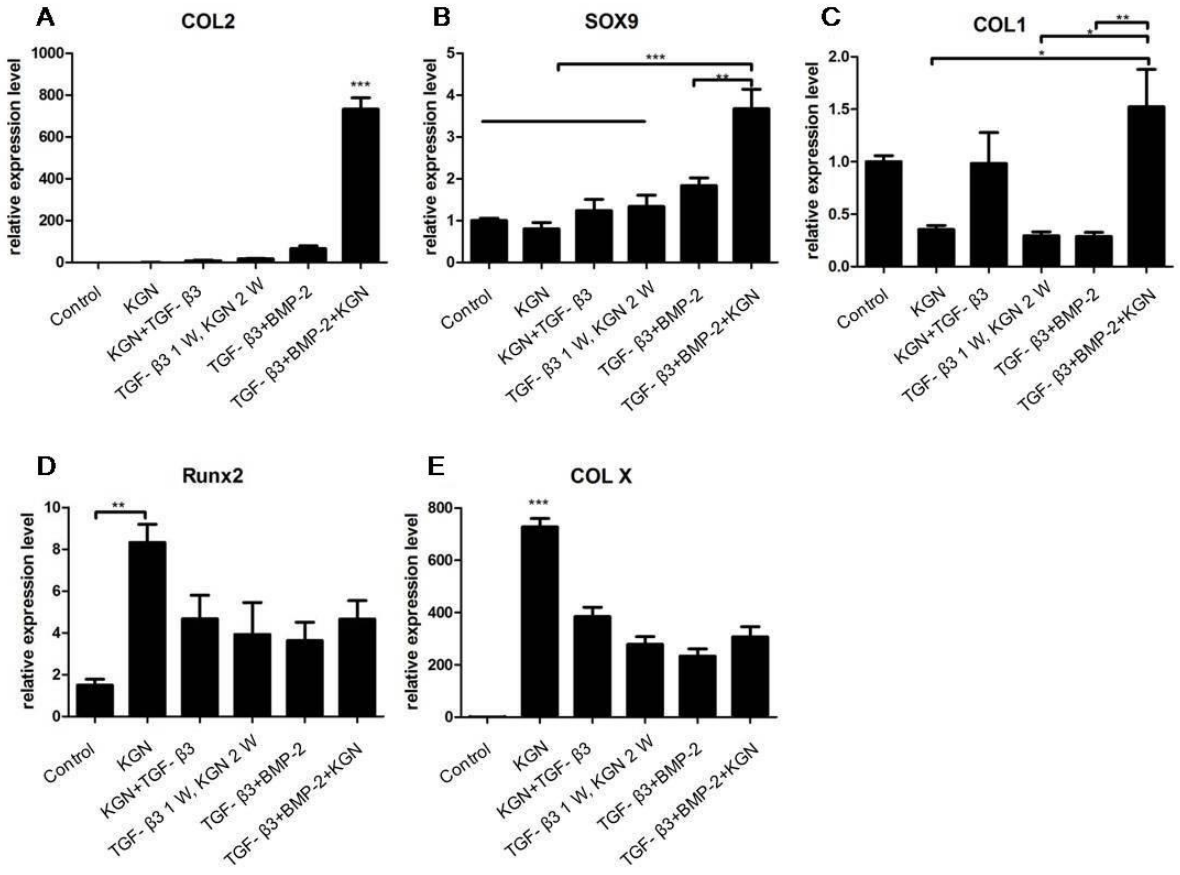


**Figure S3.** Expression levels of Chondrocyte marker genes and hypotrophy-related genes after 21 days of induction. Relative expression levels of collagen type II (A), *SOX9* (B), type I collagen (C), *RUNX2* (D) and type X collagen (E). Results represent the mean ± standard error of the mean (SEM), $n$=3. *, $P$<0.05; **, $P$<0.01; and ***, $P$<0.001.

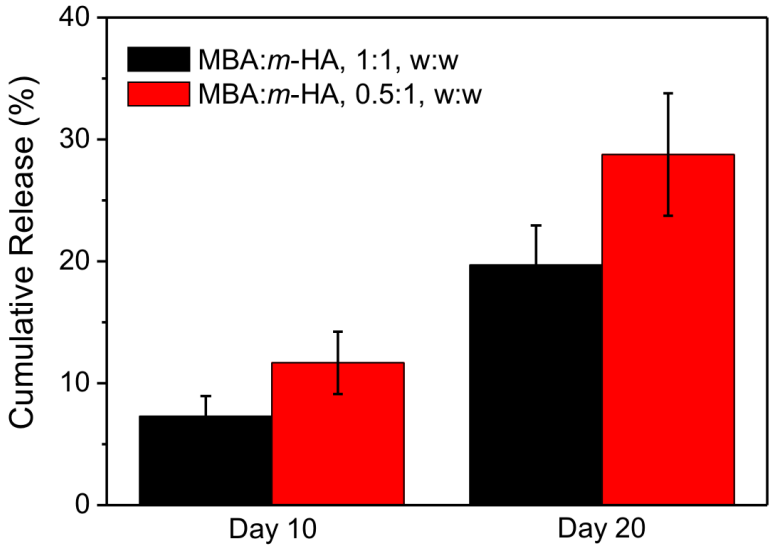


**Figure S4.** *In vitro* release of the KGN from PLGA nanoparticls-encapsulated HA hydrogel made from different ratio of MBA and *m*-HA at 37 $^{o}$C. Results represent the mean ± standard error of the mean (SEM), *n*=3.

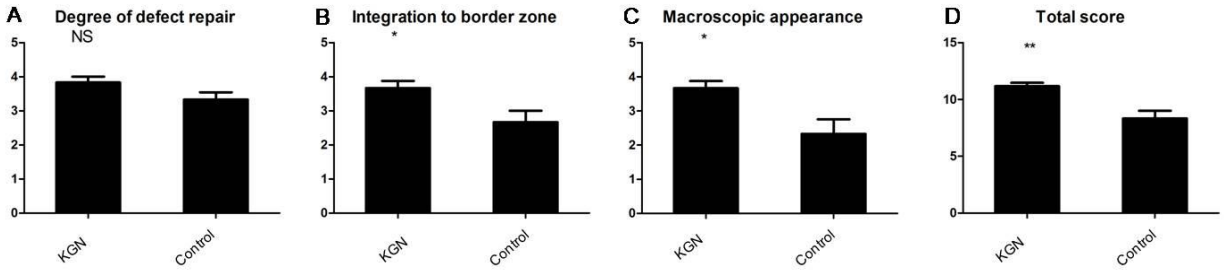


**Figure S5.** The results of macroscopic scoring system for the cartilage defects repair quality evaluation. A) Degree of the defect repair. B) Integration to board zone. C) Macroscopic appearance. D) Total score. Results represent the mean ± standard error of the mean (SEM), $n$=3; *, $P$<0.05; **, $P$<0.01; and ***, $P$<0.001.

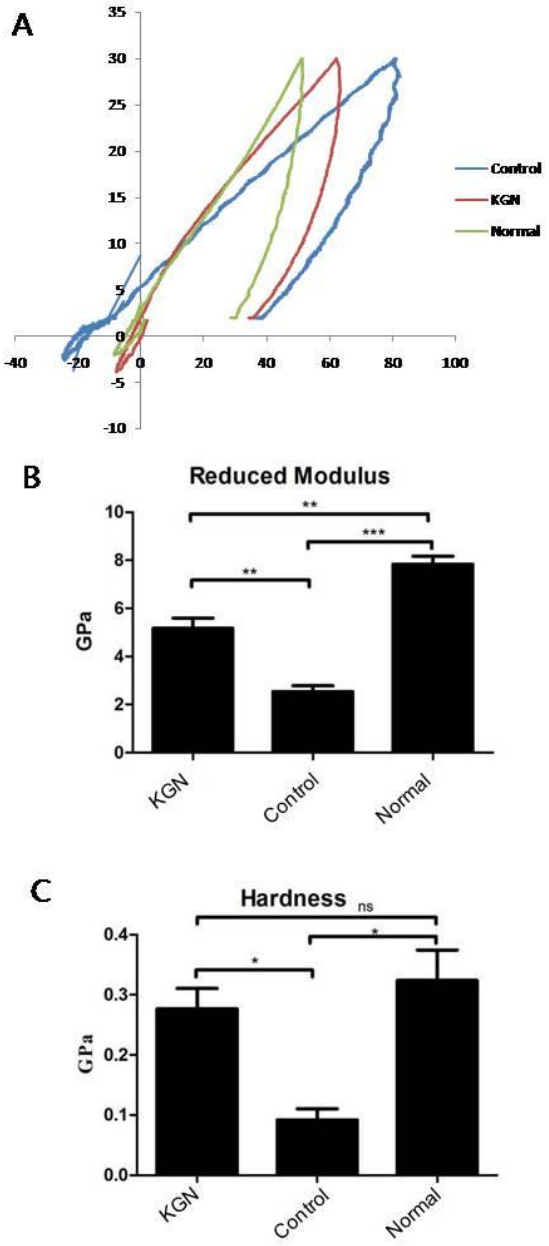


**Figure S6.** Biomechanical tests of repaired cartilage. A) Representative load-displacement curves of different groups were recorded within a test range of 80 nm. B) and C) The biomechanical properties of repaired tissue were calculated according to the biomechanical curves: (B) reduced modulus; (C) hardness. Results represent the mean ± standard error of the mean (SEM), $n$=3; *, $P$<0.05; **, $P$<0.01; and ***, $P$<0.001.

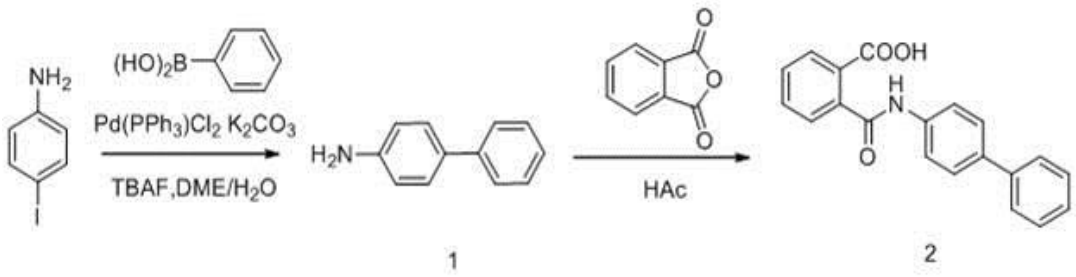


**Figure S7.** Synthesis route of KGN.

**References:**

1. Huang, H.; Zhang, X.; Hu, X.; Shao, Z.; Zhu, J.; Dai, L.; Man, Z.; Yuan, L.; Chen, H.; Zhou, C.; Ao, Y. A Functional Biphasic Biomaterial Homing Mesenchymal Stem Cells for in Vivo Cartilage Regeneration. *Biomaterials* **2014**, 35, 9608-9619.