Electronic Supplementary Material (ESI) for Journal of Materials Chemistry B.

Supplementary Information

3D Multifunctional Bi-layer Scaffolds to Regulate Stem Cell Behaviors and Promote Osteochondral Regeneration

Po Zhang [a, b,1], Jialin Chen [b, c, d,1], Yuzhi Sun [a, b], Zhicheng Cao [a, b], Yanan Zhang [b], Qingyun Mo [b], Qingqiang Yao [a, d *], Wei Zhang [b, c, d *]

[a] Department of Orthopedic Surgery, Digital Medicine Institute, Nanjing Medical University Nanjing Hospital. No. 68 Changle Road, Nanjing 210006, P.R. China

[b] School of Medicine, Southeast University, 210009, Nanjing, China

[c] Jiangsu Key Laboratory for Biomaterials and Devices, Southeast University, 210096, Nanjing, China

[d] China Orthopedic Regenerative Medicine Group (CORMed), China

[1] These co-authors contributed equally to this work.

*Corresponding Author

E-mail addresses: yaoqingqiang@njmu.edu.cn (Q. Yao), zhang.wei@seu.edu.cn (W. Zhang)

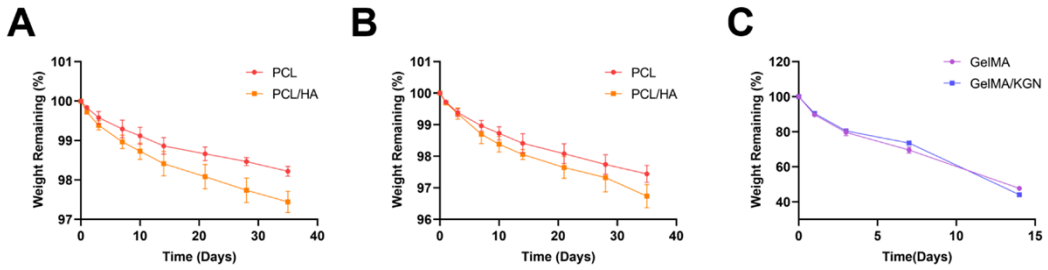

Fig. S1 (A) In vitro degradation curves of PCL and PCL/HA at 37 ℃ in PBS (n=4). (B) In vitro degradation curves of PCL and PCL/HA at 37 ℃ in PBS containing 0.05 % trypsin. (n=4). (C) In vitro degradation curves of GelMA and GelMA/KGN at 37 ℃ in PBS (n=5). Results are shown as mean ± SD.

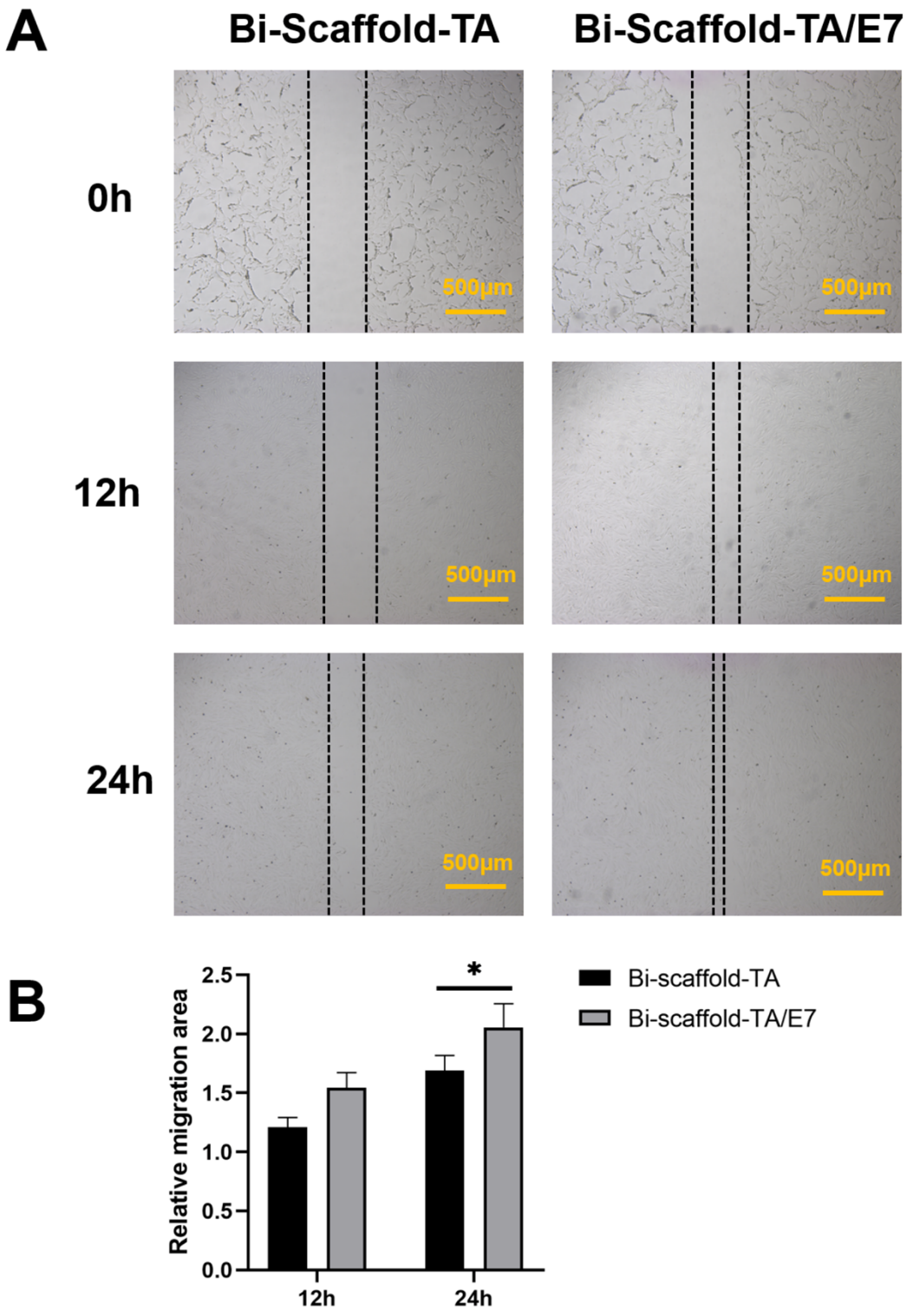

Fig. S2 (A) Scratch assay images of BMSCs migration exposed to the extracts of bi-scaffold-TA or bi-scaffold-TA/E7 at 0 h, 12h, and 24 h. Scale bars = 500 μm. (B) Quantification of the relative migration area. Results are shown as mean ± SD. *p < 0.05. Bi-scaffold: PCL/GelMA

bi-layer scaffold.

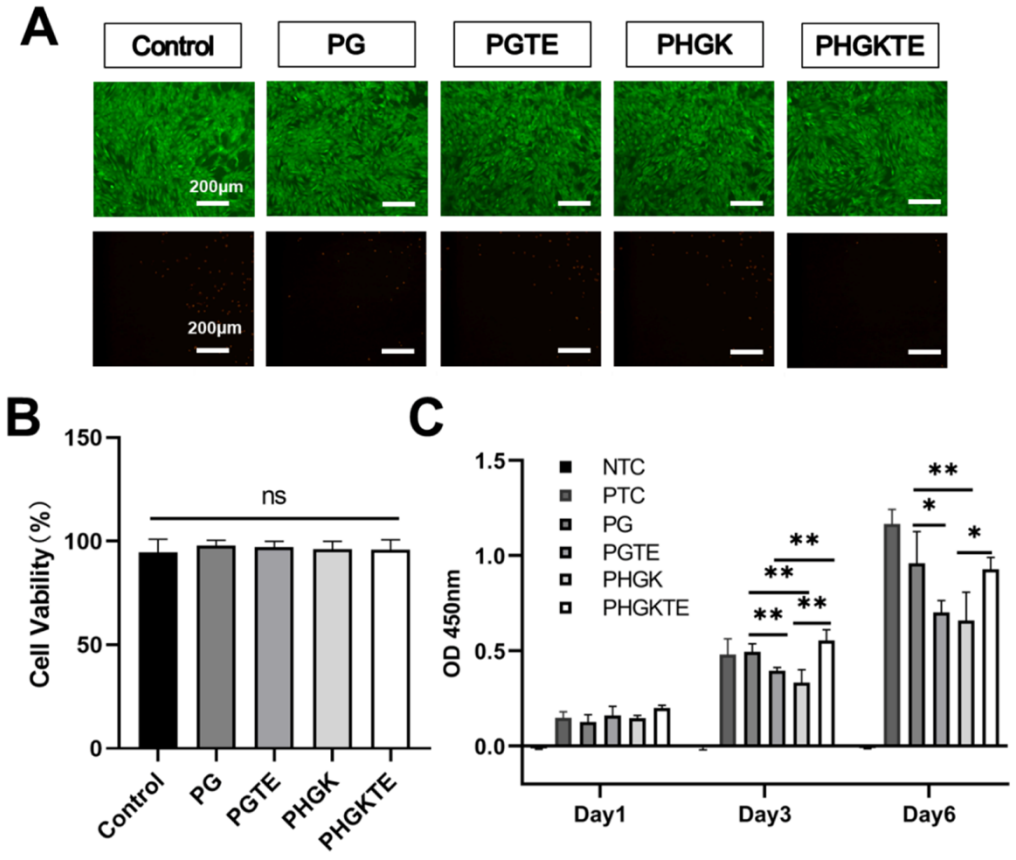

Fig. S3 Cell proliferation in the extracts of the bi-layer scaffolds. (A) The Fluorescence micrographs of BMSCs cultured in the extracts of various bi-layer scaffolds for 3 days were stained with calcein-AM/PI. Calcein AM for live cells (green) and PI for dead cells (red). Scale bars = 500 μm. (B) Quantification of live/dead staining. (C) Cell proliferation in the extracts of different bi-layer scaffolds was measured by CCK-8 on days 1, 3, and 6. Results are shown as mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.001. NTC: Negative control group with DMSO; PTC: Positive control group with normal cell culture medium; PG: PCL/GelMA, PGTE: PCL/GelMA@TA/E7; PHGK: PCL/HA-GelMA/KGN, PHGKTE: PCL/HA-GelMA/KGN@TA/E7.

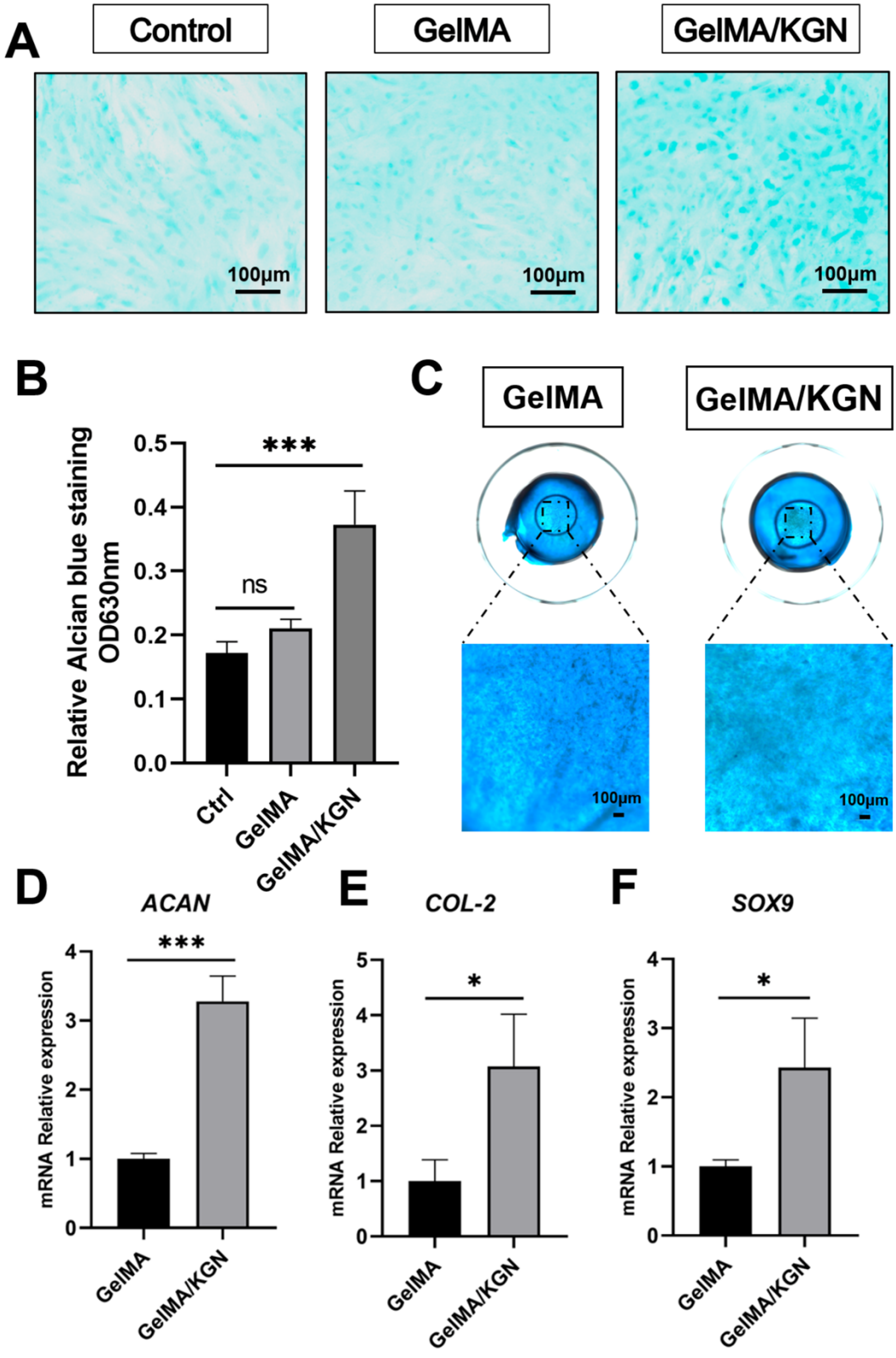

Fig. S4. (A) Alcian blue staining of BMSCs in monolayer cultured in GelMA and GelMA/KGN

extract chondrogenic medium in vitro (n=5). Scale bars = 100 μm. (B) Quantification of Alcian blue staining by measuring the absorbance of the eluent at 630 nm. (C) Alcian blue staining of BMSCs seeded on the GelMA and GelMA/KGN hydrogel cultured in a chondrogenic medium (n=5). Scale bars = 100 μm in high-magnification images (D) to (F) Gene expression (ACAN, COL-2, and SOX9) of BMSC cultured in GelMA and GelMA/KGN extract chondrogenic medium for 7 days by Real-time PCR (n=3.) Results are shown as mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.001.

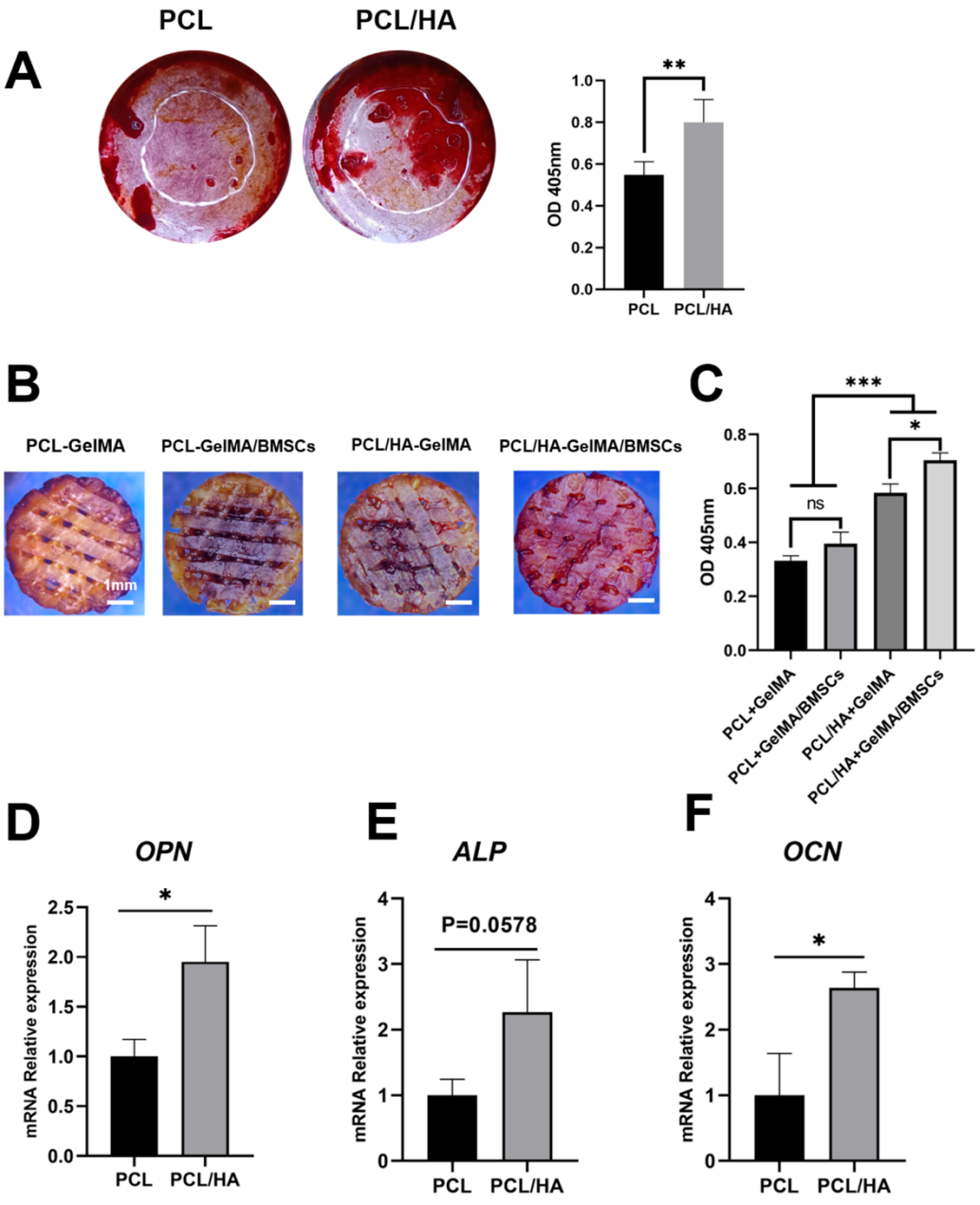

Fig. S5. Evaluation of osteogenic differentiation of the lower PCL-HA layer. (A) The alizarin red staining and quantification of the PCL and PCL/HA scaffolds leaching liquor for detecting calcium deposition on day 14 days of osteogenic induction. (B) The alizarin red staining and quantification of the PCL and PCL/HA scaffolds with or without loading BMSCs-loaded GelMA for detecting calcium deposition on day 14 days of osteogenic induction culture. Scale bars = 100 μm. (D) to (F) Gene expression (OPN, ALP, and OCN) of BMSC cultured in PCL

and PCL/HA extract osteogenic medium for 7 days by Real-time PCR (n=3). Results are shown as mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.001.

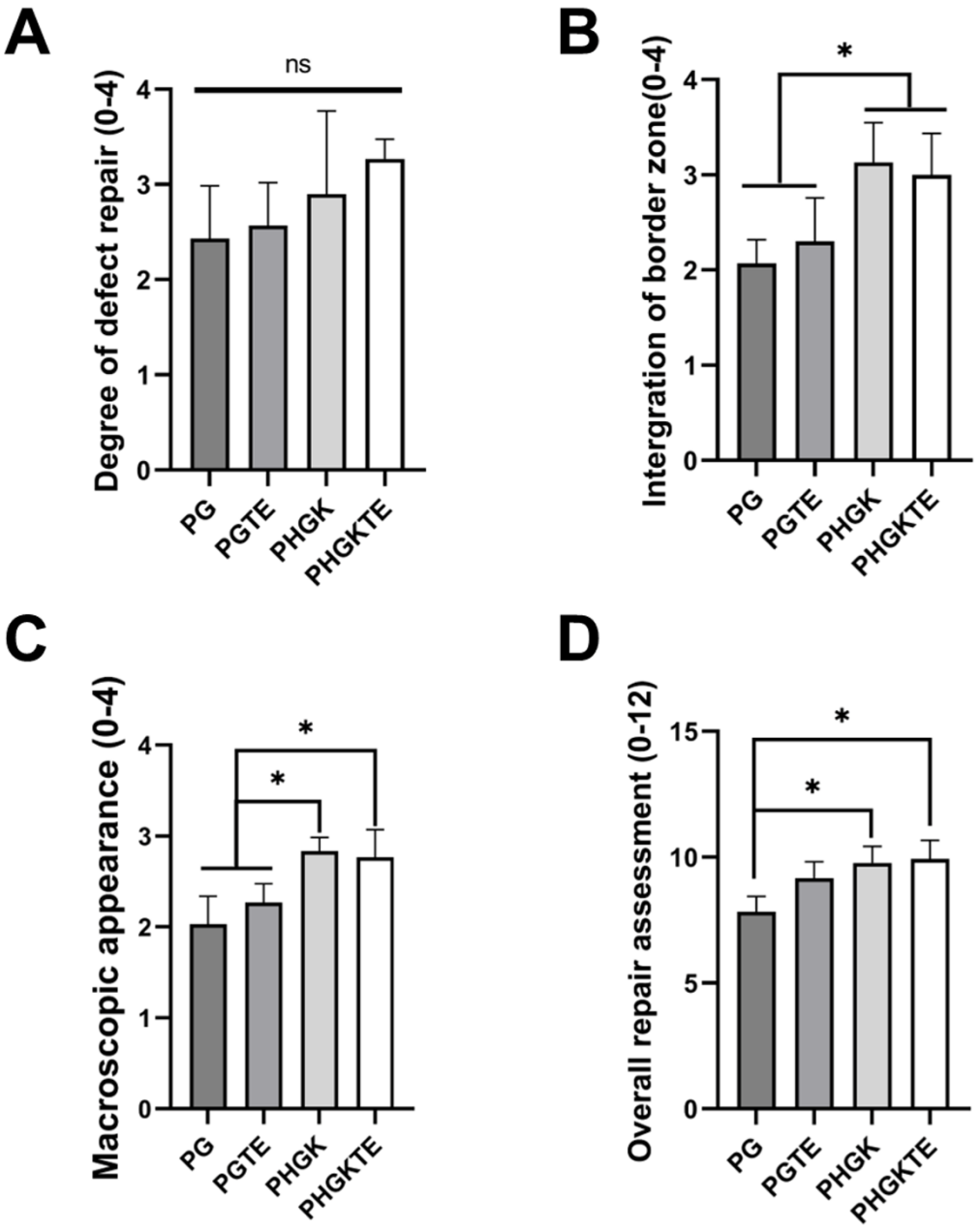

Fig. S6 Macroscopic evaluation of the various bi-layer scaffold into osteochondral defect at 12 weeks after implantation. Quantitative sub-scores of the different groups according to the ICRS scoring system included Degree of defect repair (A), Integration of border zone (B),

Macroscopic appearance (C), and Overall repair assessment (D). Results are shown as mean ± SD. *P < 0.05.

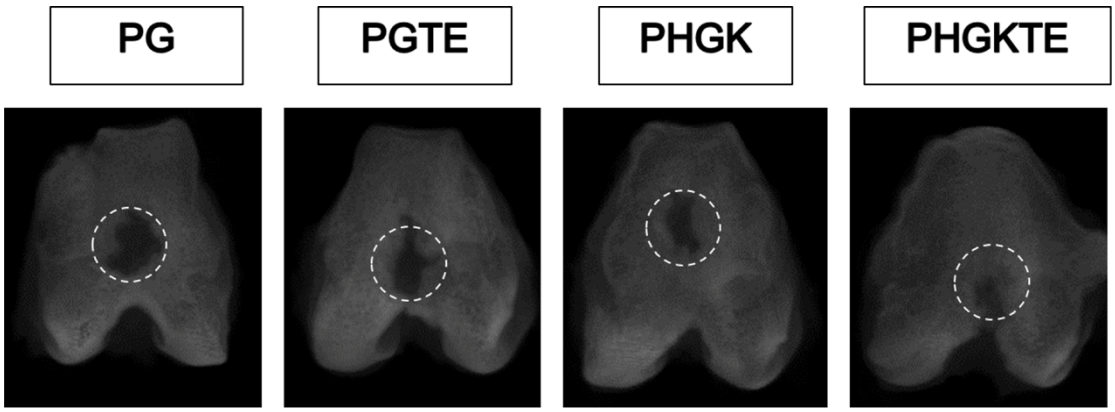

Fig. S7 Three-dimensional reconstruction images from the micro-CT data of the PG, PGTE, PHGK, and PHGKTE. The white dotted circles represent the original defect regions.

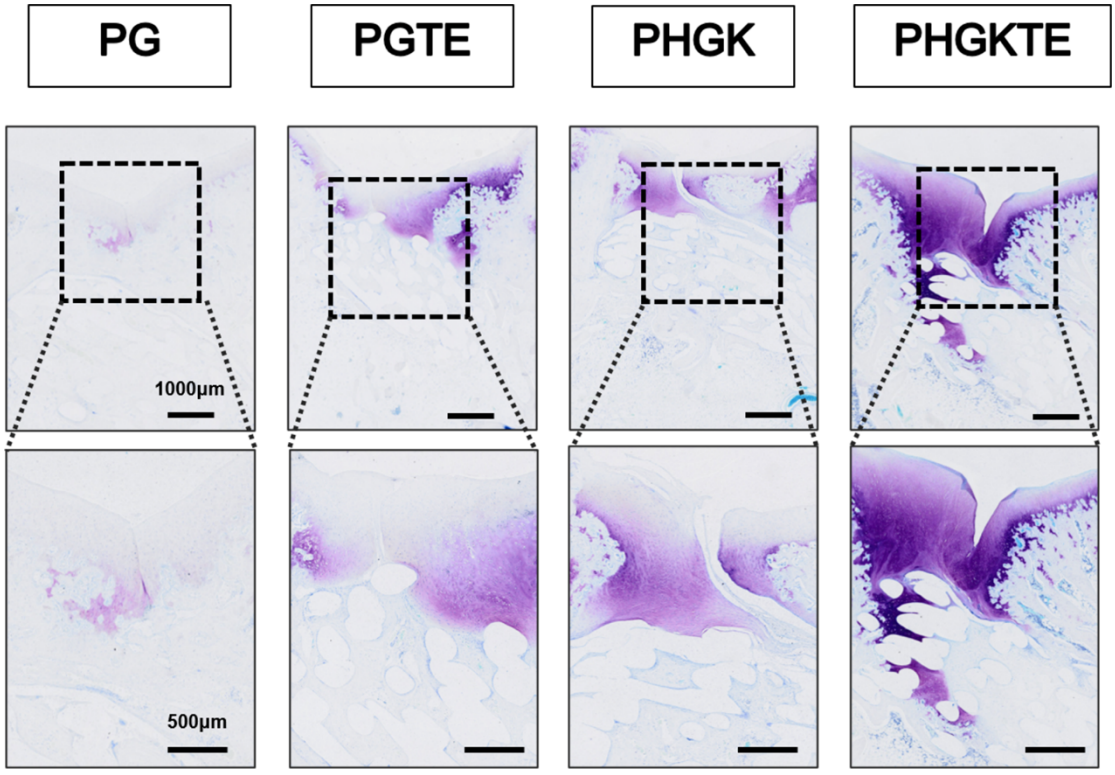

Fig. S8 Toluidine blue staining of the regenerative osteochondral tissue at 12 weeks post-surgery. The lower panels represent higher magnification images of the corresponding black

boxes in the upper panel. Scale bars = 1000μm in upper row and Scale bars = 500μm in lower row.

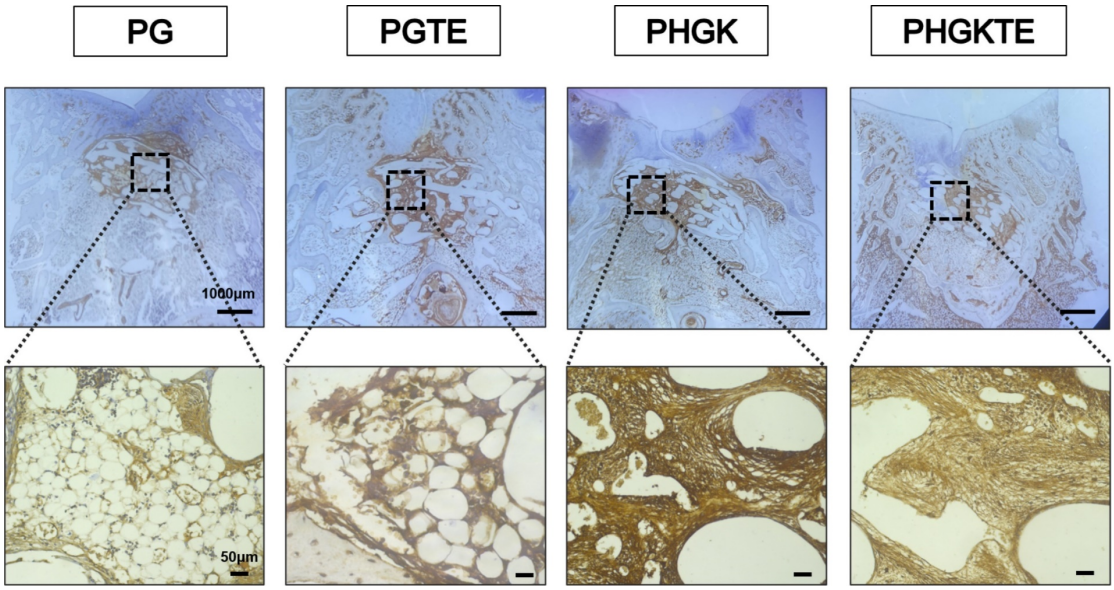

Fig. S9 Immunohistochemical staining of Runx-2 of the regenerative osteochondral tissue at 12 weeks post-surgery. Scale bars = 1000μm in upper row and Scale bars = 50μm in lower row.

Table S1 Detail of primer

Primers	Primer sequence
GAPDH	Forward: GCAAGTTCAACGGCACAG
	Reverse: CGCCAGTAGACTCCACGAC
OPN	Forward: CTCCATTGACTCGAACGACTC
	Reverse: CAGGTCTGCGAAACTTCTTAGAT
ALP	Forward: ACGTGGCTAAGAATGTCATC
	Reverse: CTGGTAGGCGATGTCCTTA
OCN	Forward: CACAAAGAAGCCGTACTCTGT

	Reverse: GGGGCTGGATAAGCATCCC
ACAN	Forward: CTGCAGACCAGGAGGTATGTGA
	Reverse: GTTGGGGCGCCAGTTCTCAAAT
COL-2	Forward: GTCTGTGACACTGGGACTGT
	Reverse: TCTCCGAAGGGGATCTCAGG
SOX9	Forward: GGCGGAGGAAGTCGGTGAAGAA
	Reverse: GCTCAT GCCGGAGGAGGAGTGT

Table S2 ICRS macroscopic evaluation of cartilage repair*

Cartilage repair assessment ICRS	**Points**
1. Degree of defect repair	
In level with surrounding cartilage	4
75% repair of defect depth	3
50% repair of defect depth	2
25% repair of defect depth	1
0% repair of defect depth	0
2. Integration into the border zone	
Complete integration with surrounding cartilage	4
Demarcating border <1 mm	3
3/4th of graft integrated, 1/4th with a notable border >1 mm width	2
1/2 of graft integrated with surrounding cartilage, 1/2 with a notable border >1	1

mm	
From no contact to 1/4th of graft integrated with surrounding cartilage	0
3. Macroscopic appearance	
Intact smooth surface	4
Fibrillated surface	3
Small, scattered fissures or cracks	2
Several, small or few but large fissures	1
Total degeneration of the grafted area	0
4. Overall repair assessment	
Grade I: normal	12
Grade II: nearly normal	11-8
Grade III: abnormal	7-4
Grade IV: severely abnormal	3-1

*This is a modified version of the system described by Borne et al. (M.P. van den Borne, N.J. Raijmakers, et al. International Cartilage Repair, International Cartilage Repair Society (ICRS) and Oswestry macroscopic cartilage evaluation scores validated for use in Autologous Chondrocyte Implantation (ACI) and microfracture, Osteoarthritis Cartilage, 2007, 15(12):1397-402).

Table S3 Histological scoring system for rabbit osteochondral defects*

Histological scoring system for an evaluation of rabbit osteochondral defects		Score
(a)	**Overall defect evaluation (Throughout the entire defect depth)**	
1	**Percent filling with newly formed tissue**	
	100%	3
	>50%	2
	<50%	1
	0%	0
2	**Percent degradation of the implant**	
	100%	3
	>50%	2
	<50%	1
	0%	0
(b)	**Subchondral bone evaluation (within the bottom 2mm of the defect)**	
3	**Percent filling with newly formed tissue**	
	100%	3
	>50%	2
	<50%	1
	0%	0
4	**Subchondral bone morphology**	
	Normal, trabecular bone	4
	Trabecular bone, both some compact bone	3

	Compact bone	2
	Compact bone and fibrous tissue	1
	Only fibrous tissue or no tissue	0
5	**The extent of new tissue bonding with adjacent bone**	
	Complete on both edges	3
	Complete on one edge	2
	Partial on both edges	1
	Without continuity on either edge	0
(c)	**Cartilage evaluation (within the upper 1mm of the defect)**	
6	**Morphology of newly formed surface tissue**	
	Exclusively articular cartilage	4
	Mainly hyaline cartilage	3
	Fibrocartilage (spherical morphology observed with ≥75% of cells)	2
	Only fibrous tissue (spherical morphology observed with < 75% of cells)	1
	No tissue	0
7	**The thickness of newly formed cartilage**	
	Similar to the surrounding cartilage	3
	Greater than the surrounding cartilage	2
	Less than the surrounding cartilage	1
	No cartilage	0
8	**Joint surface regularity**	

	Smooth, intact surface	3
	Surface fissures (<25% of new surface thickness)	2
	Deep fissures (≥25% of new surface thickness)	1
	Complete disruption of the new surface	0
9	**Chondrocyte clustering**	
	None at all	3
	<25% chondrocytes	2
	25-100% chondrocytes	1
	No chondrocytes present (no cartilage)	0
10	**Chondrocyte and GAG content of new cartilage**	
	Normal cellularity with normal Safranin O staining	3
	Normal cellularity with moderate Safranin O staining	2
	Fewer cells with poor Safranin O staining	1
	Few cells with no or little Safranin O staining or no cartilage	0
11	**Chondrocyte and GAG content of adjacent cartilage**	
	Normal cellularity with normal Safranin O staining	3
	Normal cellularity with moderate Safranin O staining	2
	Fewer cells with poor Safranin O staining	1
	Few cells with no or little Safranin O staining or no cartilage	0

*This is a modified version of the system described by Jiang et al. (Y. Jiang, L. Chen, et al. Incorporation of bioactive polyvinylpyrrolidone-iodine within bilayered collagen scaffolds enhances the differentiation and subchondral osteogenesis of mesenchymal stem cells, Acta

Biomater 2013, 9(9):8089-98)