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Supplementary Information

Microporous density-mediated the response of MSCs on 3D trimodal macro/micro/nano-porous scaffolds via fibronectin /integrin and FAK/MAPK signaling pathways

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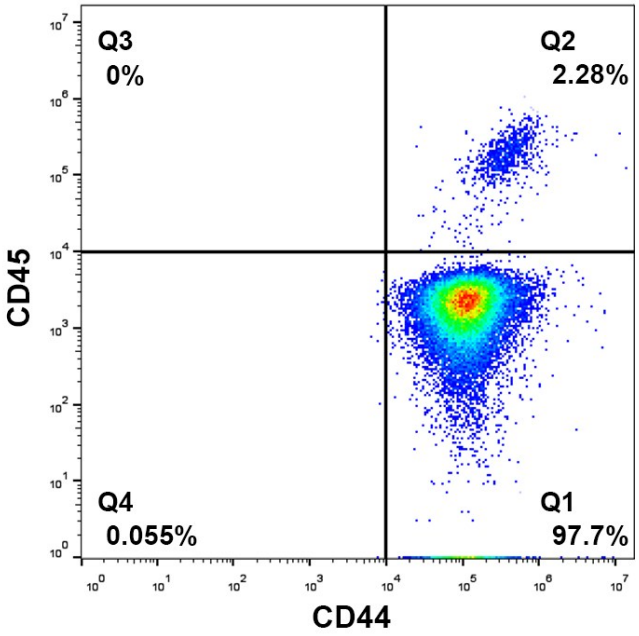

Fig. S1. Flow cytometry analyses of positive (CD44) and negative (CD45) rat bone marrow stromal cells (rBMSCs) surface markers. The results showed that the positive rates were 97.7%, suggesting that the used cells were mainly the MSCs.

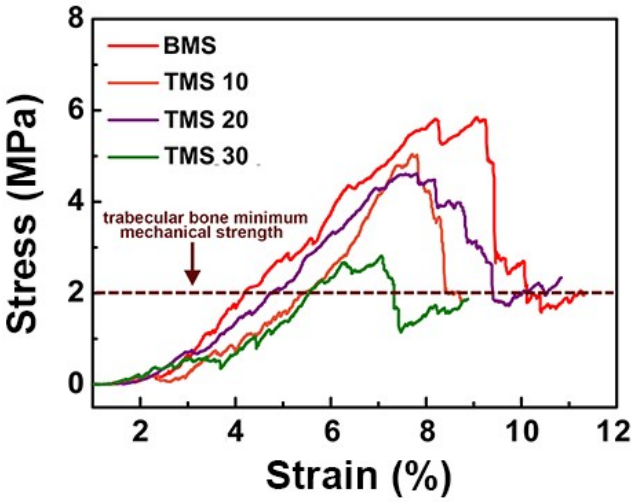

Fig. S2. Typical stress-stain curves of BMS/TMS scaffolds with different microporous densities. The decreasing trend with the increasing content of microporous density could be observed. The minimum mechanical strength of trabecular bone is marked with dashed line for reference.

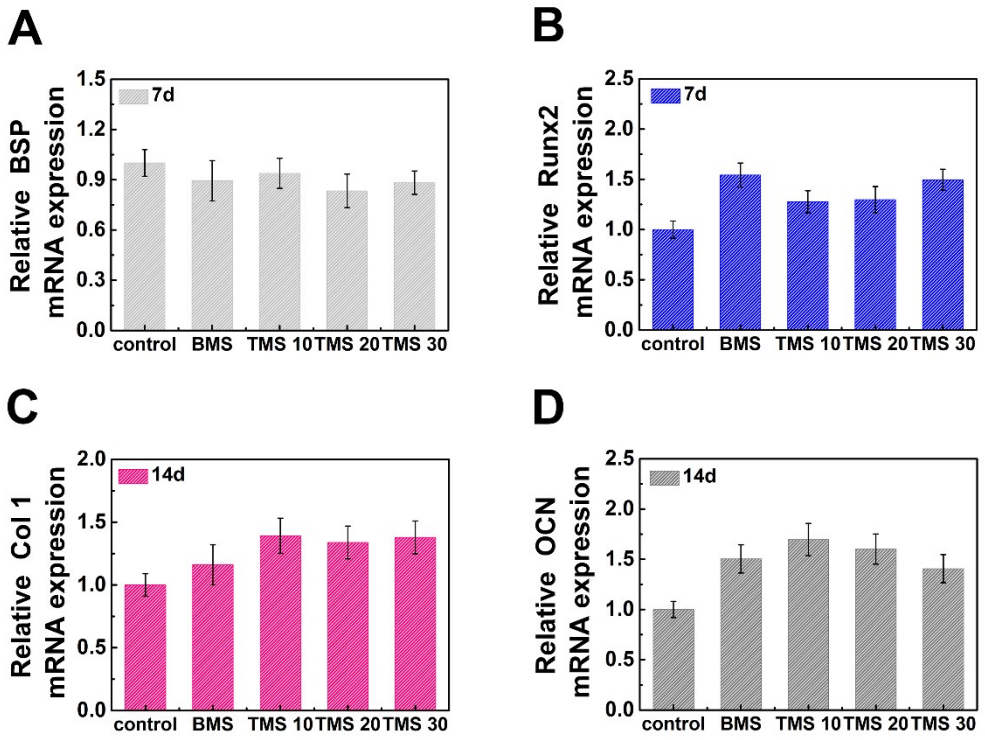

Fig. S3. Effects of dissolved ions on relative mRNA expression of osteogenesis-related gene in rBMSCs on BMS and TMS scaffolds. (A-B) Gene expression of BSP (A) and Runx2 (B) were examined using RT-qPCR after culturing rBMSCs on the leach liquor of the all scaffolds at 7 d (200mg scaffolds/mL α-MEM after soaking for 3 d). (C-D) Gene expression of Col 1 (C) and OCN (D) were measured after 14 d cultured on a series of the leach liquor of scaffolds (200mg scaffolds/mL α-MEM after soaking for 3 d). All the expressions of gene have no significant difference, indicating the ionic extract has no obvious influence on the osteogenic differentiation of rBMSCs. Similar results were obtained from three independent experiments (n=3).

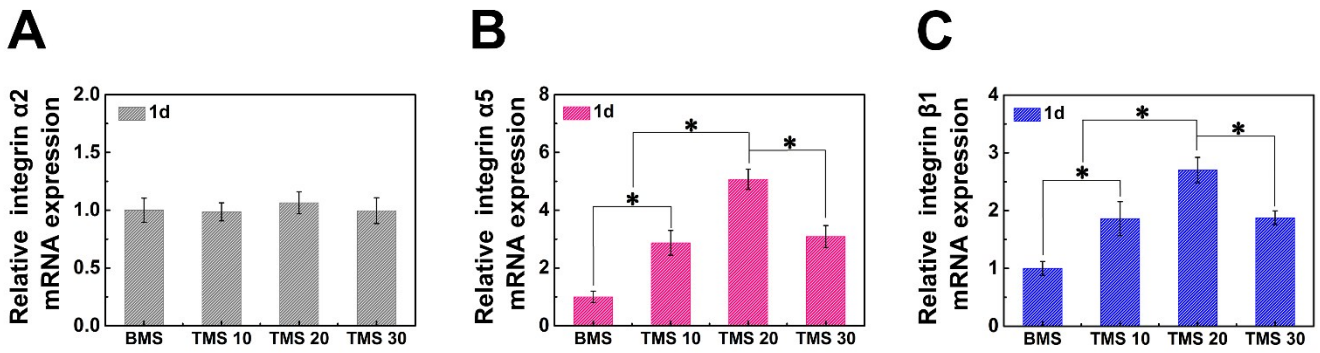

Fig. S4. Effects of micropores density on integrin subunits gene expression on different scaffolds. Gene expression of integrin α2 (A), integrin α5 (B) and integrin β1 (C) were studied via RT-qPCR after culturing 1d, respectively. The results indicated the expression of integrin α2 was no obvious variation on all scaffolds while compared with BMS, the gene expression of integrin α5 and integrin β1 presented prominent promotion on TMSs especially for TMS 20, indicating that the microporous density could validly medicate the expression of integrin α5 and integrin β1 instead of integrin α2. Asterisks indicate significant differences, *$p < 0.05$.

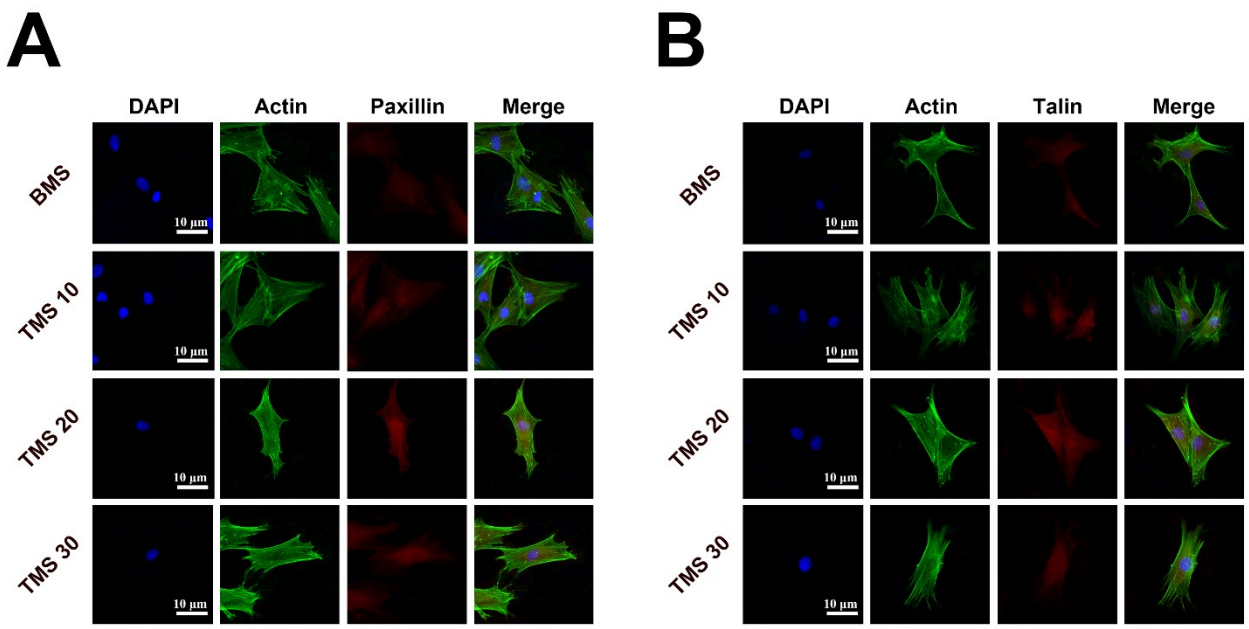

Fig. S5. Immunofluorescent analysis of paxillin (A) and talin (B) in rBMSCs cultured on BMS and TMS scaffolds for 1 d (red: paxillin/talin, green: actin cytoskeleton, blue: cell nuclei, scale bar = 10 μm). The results showed that TMS presented dramatically higher paxillin/talin intensities in unit area than BMS, especially for TMS 20, suggesting TMS 20 was the most beneficial to the FAs formation.

Table S1.

Primers used in real-time PCR.

Gene	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')
BSP	ACAGCTGACGCTGGAAAGTTG	ACCTGCTCATTTTCATCCACTTC
Runx2	CGGCCCTCCCTGAACTCT	TGCCTGCCTGGGATCTGT
Col 1	GGTATGCTTGATCTGTATCTGC	AGTCCAGTTCTTCATTGCATT
OCN	CTGACAAAGCCTTCATGTCCAA	GCGGGCGAGTCTGTTCACTA
Integrin α2	CACAGTTCATTTTTAGGTTACT	CACATTGCCATGCTTGTTAACA
Integrin α5	ACAGTTCGAGCCCATGGCT	CTGAACACATTCTTTATGCTC
Integrin β1	CTACTGGTCCCGACATCATCC	TGACCACAGTTGTCACGGCAC
GAPDH	CCCCCAATGTATCCGTTGTG	TAGCCCAGGATGCCCTTTAGT

Supplementary methods

1. *Flow cytometry*

For flow cytometry analysis, cells were detached with 0.05% trypsin/EDTA and then were washed in PBS. 1×10^5 cells were stained with FITC anti-rat CD44 antibody (BioLegend, Clone OX-49, 203906) and Alexa Fluor® 647 labeled anti-rat CD45 antibody (BioLegend, Clone OX-1, 202211) according to manufacturer indications. All tubes were incubated in the dark for 30 min at 4°C. Then cells were washed with PBS and finally analyzed by using the BD Accuri™ C6 Flow Cytometer System. For each tube, 10000 events were acquired. Flowjo 10 was used for data analysis.

2. *Mechanical strength*

The mechanical strength of the scaffolds ($10\times10\times10$ mm^3) was detected by a universal testing machine (AG-2000A, Shimadzu Autograph, Shimadzu Co., Ltd., Japan) at a loading rate of 1mm/min. Three replicates were carried out for each group, and the results were expressed as means ± standard deviation (means ± SD).

3. *Immunofluorescent staining*

Immunostaining of talin was measured after 14 days of culture at a density of 5×10^4 cells/scaffold ($10\times10\times2$ mm^3). After being fixed with 2.5% glutaraldehyde for 15 min, the cells were permeabilized with 0.1% Triton X-100 solution and blocked with 5% bovine serum albumin (BSA) for 1 h. Then paxillin were stained with mouse-anti-talin 1/2 IgG (Abcam, HK, ab11188) at 4°C overnight, followed by incubation with Alexa Fluor® 647 labeled goat-anti-mouse IgG (Abcam, HK, ab150115) for 2 h at room temperature. For the immunostaining of paxillin were performed by using anti-paxillin antibody and Alexa Fluor® 647 labeled goat-anti-rabbit IgG (Abcam, HK, ab32084 and ab150079). Cell cytoskeletons were identified by Alexa-Fluor® 488 phalloidin (Sigma, StLouis, USA) and nuclei were stained by DAPI (Sigma, St Louis, USA) according to the manufacturer's protocols. Confocal laser scanning microscope (CLSM, A1, Nikon, Japan) was used to examine cell cytoskeleton arrangement, talin and paxillin distribution on the surfaces

of all scaffolds.

4. *Quantitative real time PCR analysis*

Osteogenic gene expression was quantitatively analyzed using real-time quantitative reserve transcription-polymerase chain reaction (RT-qPCR) system (Bio-Rad, Hercules, CA, USA). Cells were seeded at a density of 5×10^4 cells/well and osteogenic inductive medium was applied. After 1d, 7d, and 14d of culture, total RNA was lysed using Trizol Reagent (Takara, Tokyo, Japan). First strand complementary DNA (cDNA) was synthesized using Prime Script RT reagent kit (Takara, Tokyo, Japan) following manufacturer's instructions. RT-qPCR was carried out in 20 μL of reaction volume which contained 10 μL of SYBR Premix Ex Taq™, 0.4 μL each of 50 μM forward and reverse primers, 1 μL of cDNA template diluted 1:5, and 8.2 μL of RNase free water. The integrin α2/α5/β1, runt-related transcription factor 2 (Runx2), bone sialoprotein (BSP), collagen 1 (Col 1), osteocalcin (OCN) and the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were evaluated. Cultures without scaffolds were set as blank control and all experiments were performed in triplicate to obtain the average data.