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Supplementary Materials

Preparation and biological evaluations of collagen-like hierarchical Ti surface with superior osteogenic capabilities

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Animal study

20 Male adult New Zealand rabbit (weighing 2.2-2.5 kg) were used in this study. The animal experiment was approved by the Animal Ethic Committee of Zhejiang University (Hangzhou, China). Cylinder Ti implants (2.6 mm in external diameter and 6 mm in length) were prepared into MR and NMR20. After general anesthesia with SuMianXin II (The Military Veterinary Institute, Changchun, CHN), the inside area of bilateral knee joint was hair-shaved and disinfected. Two separate medial and distal skin incisions were performed inside of each knee joints. The wide-flat metaphysis of the lower femur and the upper tibia were exposed by blunt dissection technique. Samples were implanted after drilling with gradually increased drills. Ipsilateral femur and tibia were received implants of the same group, and the contralateral received the other. Penicillin (400,000 U/d) was administrated by intramuscular injection in the first three days after surgery. Bone affinity fluorescent dyes were intramuscularly injected to label new bone formation at the bone-implant surface. Oxytetracycline hydrochloride (O, Aladdin), Alizarin Red S (A, sigma) and Calcein blue (C, Aladdin) were given according to Table S1.

Table S1. Dosage regimen for fluorochromes

Times samples collected (w)	day 3	week 1	week 2	week 4	week 8
2	O	C	S (3 days before sacrificed)		
4	O		C	S (3 days before sacrificed)	
8	O		C		S (3 days before sacrificed)

At each predetermined time, 5 animals were sacrificed, and bones blocks containing implant were retrieved by osteotomy. Samples in femur were subjected to hard tissue slices, and those in tibia were subjected to pull-out test. Hard tissue slices were first observed under a confocal laser scanning microscope (IX83-FV3000, Olympus, JPN) for the deposition of newly formed bone stained by bone affinity dyes. Then, hard tissue slices were stained by toluidine blue and observed by a microscope (DM4000, Leica, GER). Bone-implant contact (BIC) was calculated from medium 1/3 of the implant by the percent of the direct bone contact. Universal testing machine (SANS CTT2500, MTS. Co LTD. USA) was used in the pull-out tests. Pull-out speed range was set to 1.0 mm/min, and the maximum load was recorded as the pull-out force.

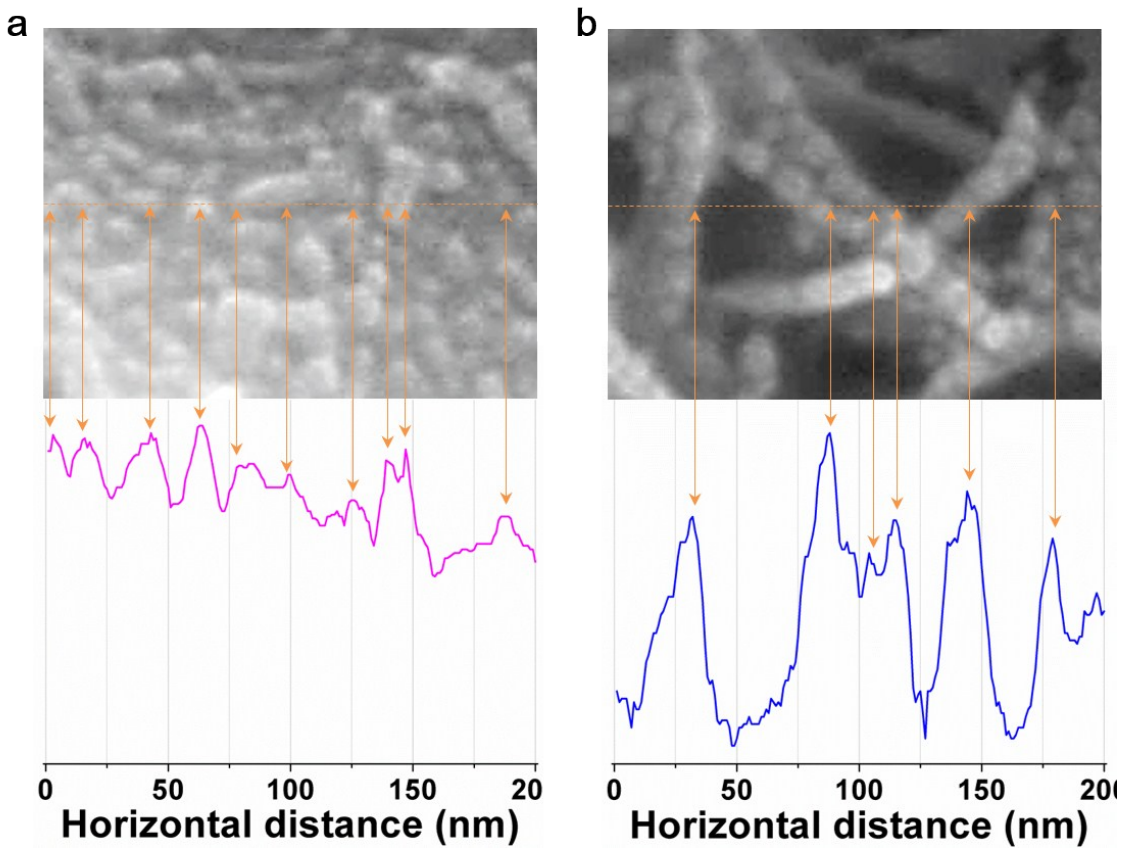

Fig. S1. Sketch showed how the information of the spatial distribution of the nanostructures was analyzed. The horizontal distance between two neighboring points of substrates were measured as peak-to-peak values of the curves obtained from the SEM image at ×100k magnification. (a) MS1, (b) MS20.

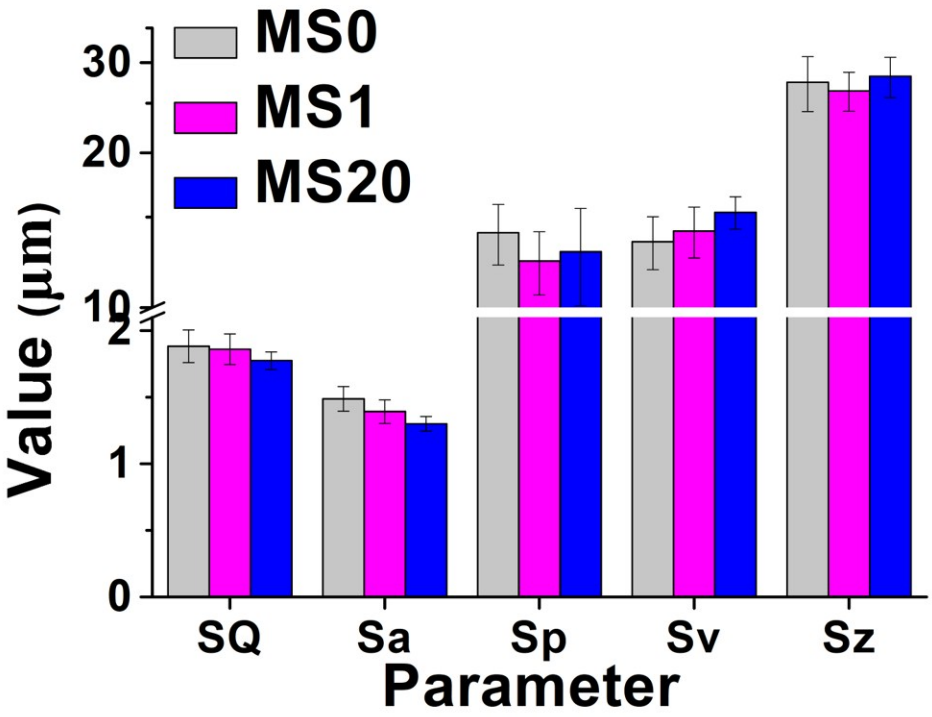

Fig. S2. Roughness analyzed based on the interference microscope.

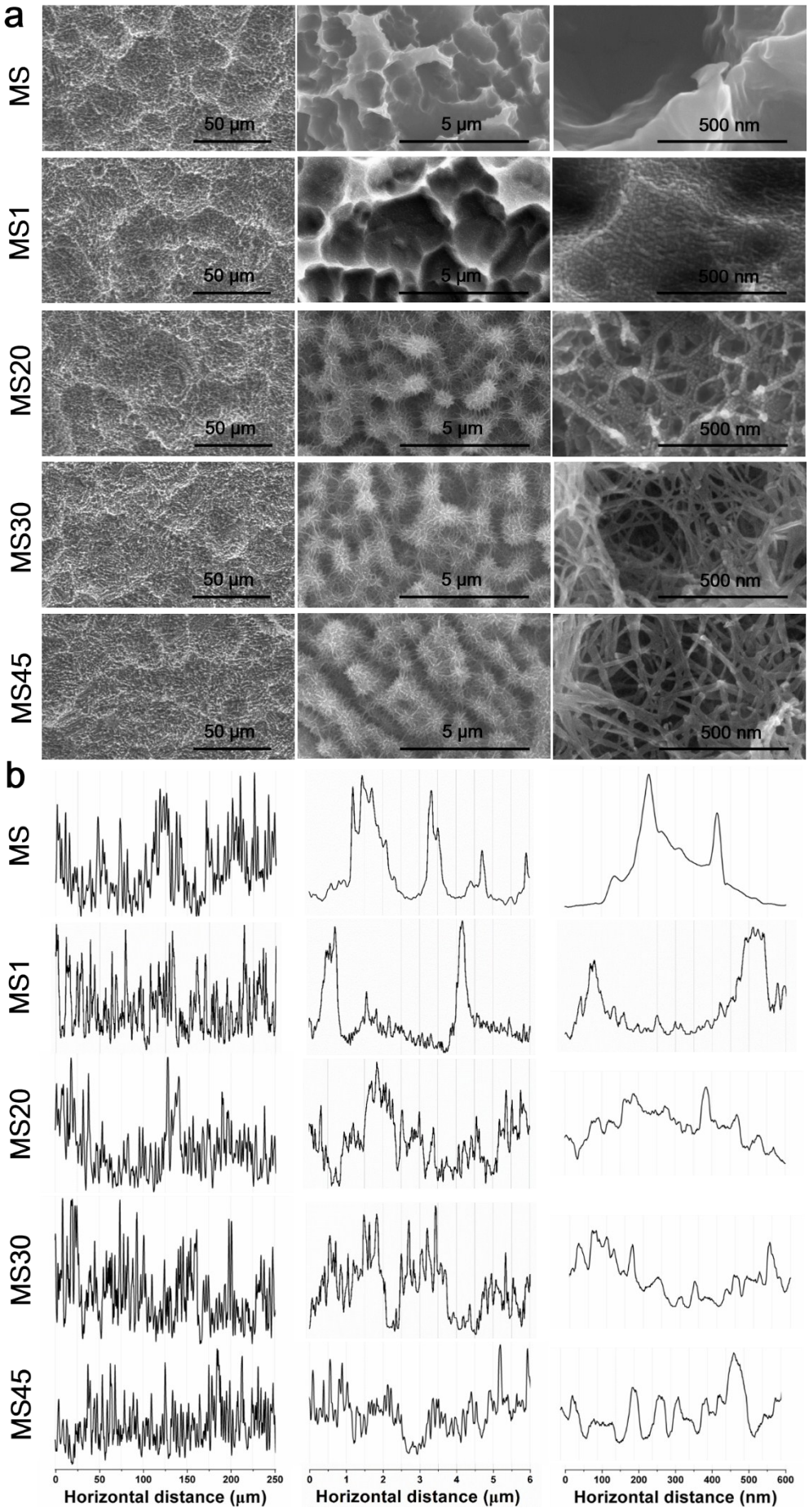
a
MS
MS1
MS20
MS30
MS45
50 μm
5 μm
500 nm
b
0 50 100 150 200 250
Horizontal distance (μm)
0 1 2 3 4 5 6
Horizontal distance (μm)
0 100 200 300 400 500 600
Horizontal distance (nm)

Fig. S3 A series of titanium surfaces with continuously changed topographies were constructed on Ti surfaces. (a) SEM images showed that as the alkali treating time prolonged, structures on macro- (Left panel) weakened, structures on microscale complexed (Middle panel) and structures on nanoscale (Right panel) were enhanced. (b) Typical curves of macro-, micro- and nanoscale structures were mapped by the grayscale-dependent topography analysis based on the high-resolution SEM images. At macroscale, the amplitude of the curves from the MS0 group to the MS 20, to MS 30 and to MS45 group showed a decline trend with the speed appearing first slow back quick; at microscale, curves of all the three surfaces were with similar amplitude and peak to peak value, but the curves of the multiscale complex surfaces were complicated with more small fluctuations than the MS0 group; at nanoscale, the multiscale complex surfaces were apparently more complex than the MS0 group.

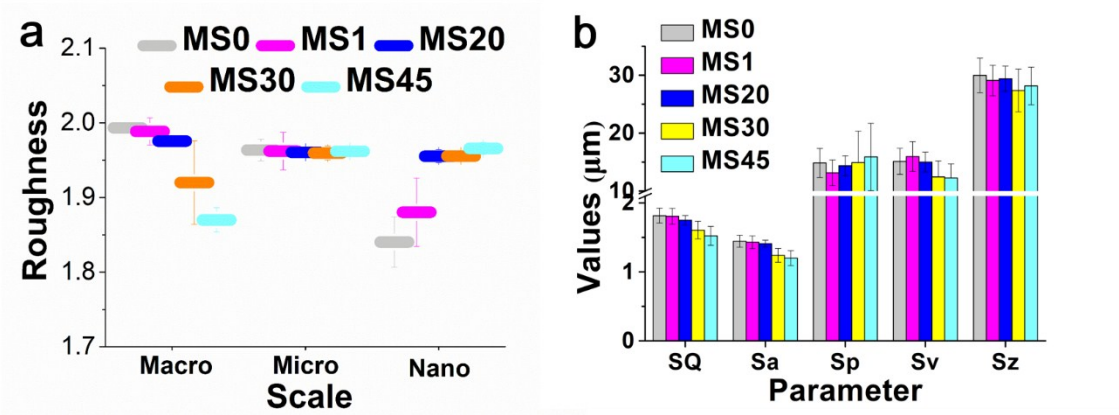

Fig. S4 Roughness based on the grayscale analysis of SEM images could present more precise information than that on the interference microscope. (a) Roughness based on the grayscale analysis of SEM images, (b) Roughness based on the interference microscope.

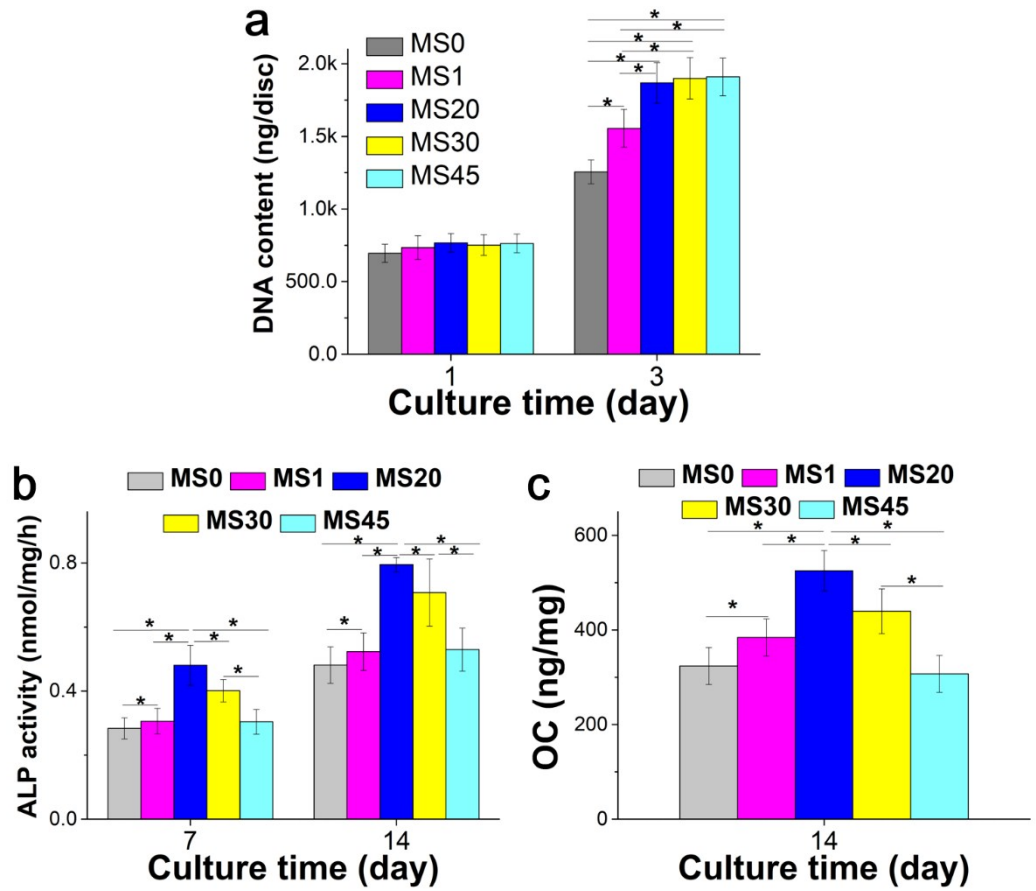

Fig. S5 Among the titanium surfaces with continuously changed topographies, theability of cell adhesion, proliferation ncreased fast from the MS0 to the MS20, the increased slowly from the MS20 to the MS45, while osteogenetic differentiation was increased fast from the MS0 to the MS20 then decreased rapidly. (a) cell adhesion and proliferation, (b) ALP activity, (c) OC procudtion.

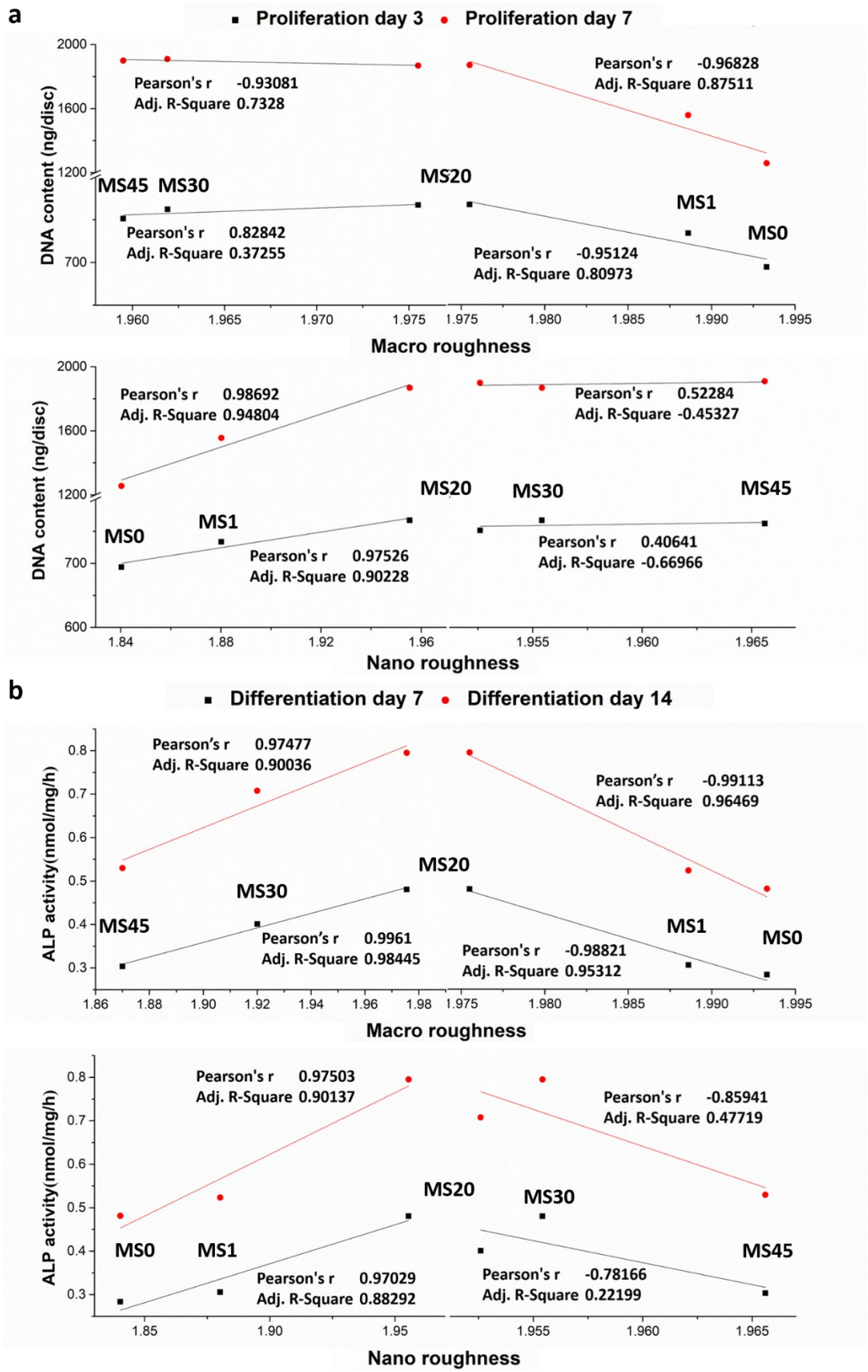

Fig. S6. Correlation analysis indicated that, macro roughness had strong negative correlation with cell proliferation while nano roughness had strong positive correlation,

the macro roughness had strong positive correlation with cell differentiation. (a) Correlation analysis of surface roughness and cell proliferation, (b) Correlation analysis of surface roughness and cell differentiaton.

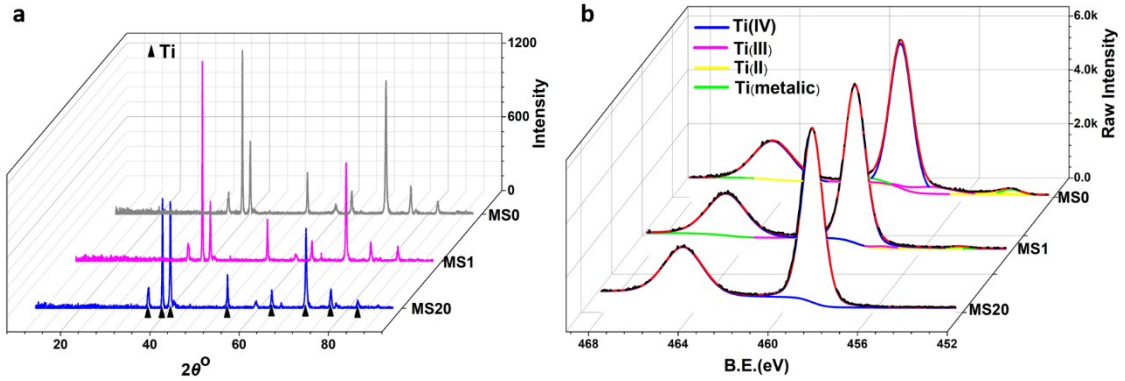

Fig. S7. XPS and XRD analysis. (a) XPS spectra showed that the oxide films of the three groups were mainly amorphous titanium dioxide, with the detectable Ti metallic signals slightly decreased from the MS0 group to the MS1 group and MS20 group. (b) XRD analysis shows that all the three groups had identical x-ray diffraction patterns. No crystalline phases were detected in any of the three groups.

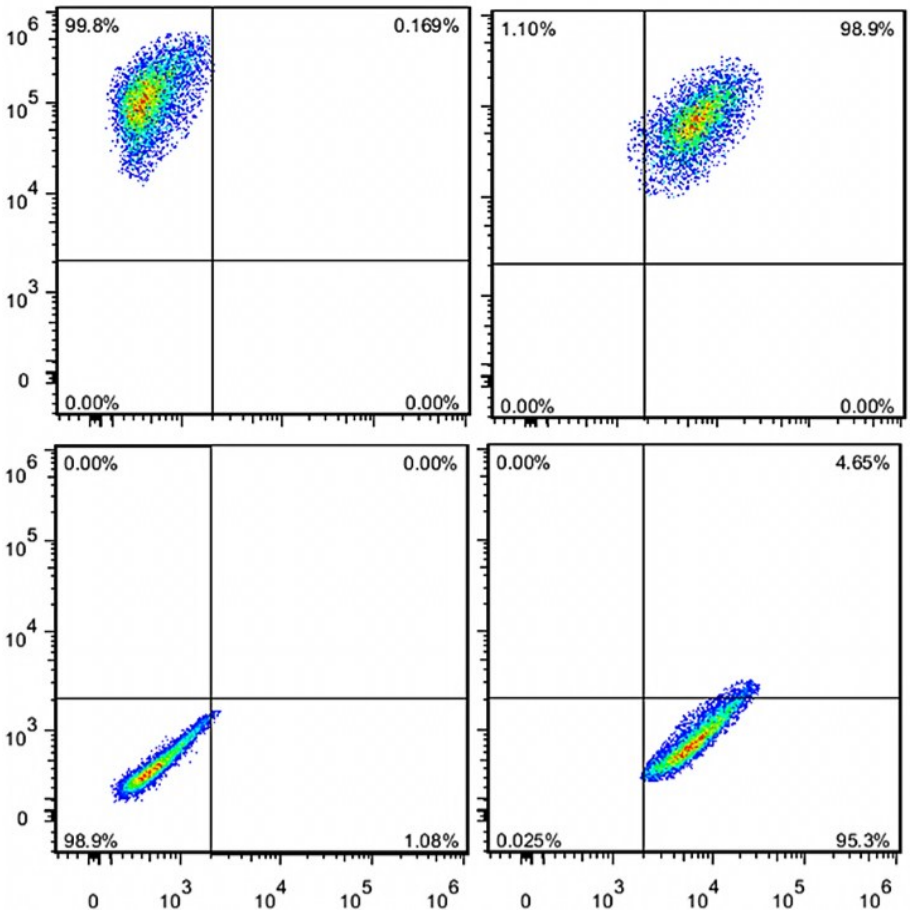

Fig. S8 BMMs were identified by flow cytometry. Flow cytometry data show that cells cultivated in this study were F4/80^{high} CD11b^{high} innate macrophages (the lower left: blank, the upper left: CD11b positive, the lower right: F4/80 positive, the upper right: CD11b+F4/80 positive).

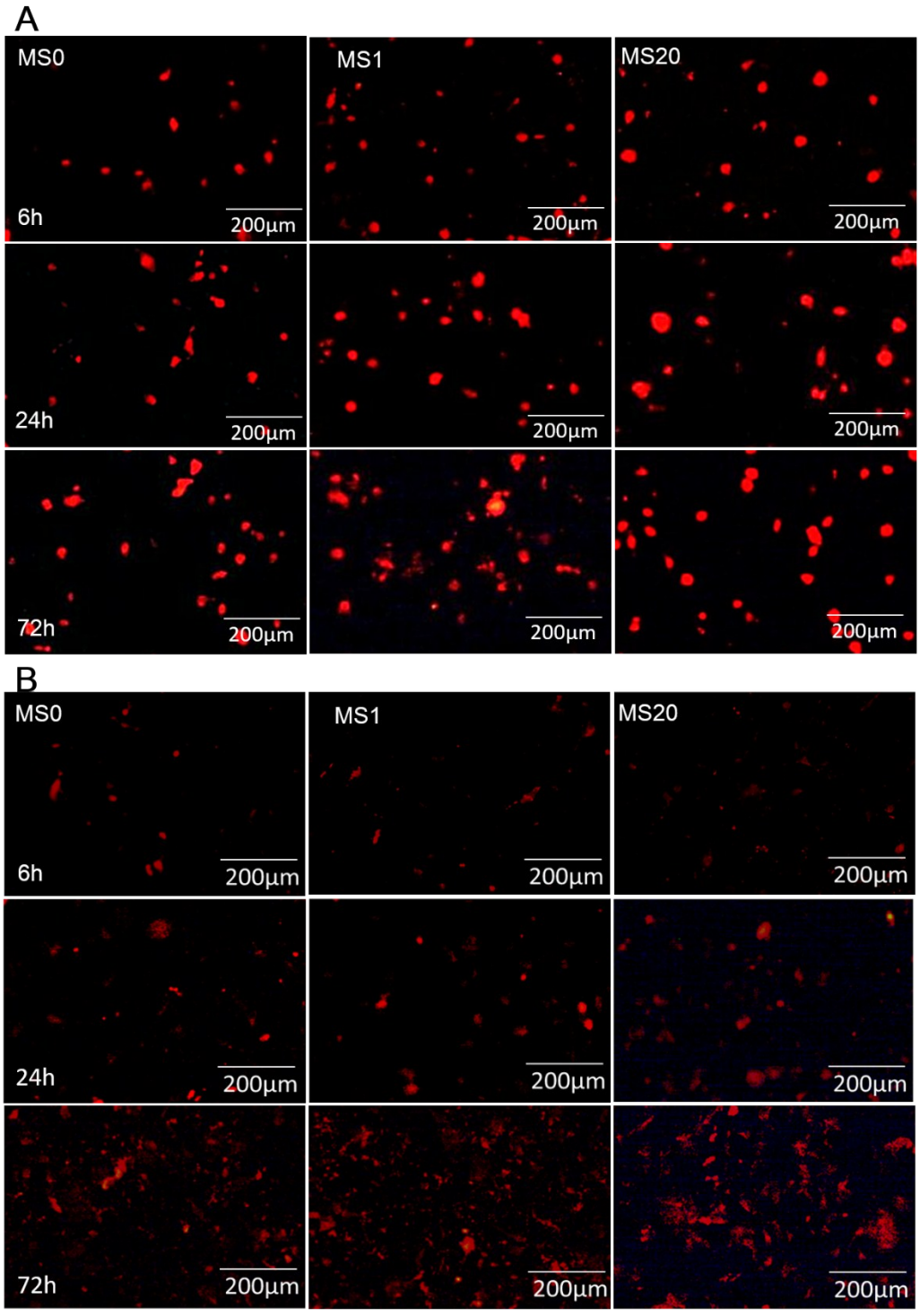

Fig. S9. Vinculin fluorescent staining showed that more vinculin was expressed by the MS20 group. (a) BMMs, (b) MC3T3-E1.