



# The determination of stem cell fate by 3D scaffold structures through the control of cell shape

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## ABSTRACT

Stem cell response to a library of scaffolds with varied 3D structures was investigated. Microarray screening revealed that each type of scaffold structure induced a unique gene expression signature in primary human bone marrow stromal cells (hBMSCs). Hierarchical cluster analysis showed that treatments sorted by scaffold structure and not by polymer chemistry suggesting that scaffold structure was more influential than scaffold composition. Further, the effects of scaffold structure on hBMSC function were mediated by cell shape. Of all the scaffolds tested, only scaffolds with a nanofibrous morphology were able to drive the hBMSCs down an osteogenic lineage in the absence of osteogenic supplements. Nanofiber scaffolds forced the hBMSCs to assume an elongated, highly branched morphology. This same morphology was seen in osteogenic controls where hBMSCs were cultured on flat polymer films in the presence of osteogenic supplements (OS). In contrast, hBMSCs cultured on flat polymer films in the absence of OS assumed a more rounded and less-branched morphology. These results indicate that cells are more sensitive to scaffold structure than previously appreciated and suggest that scaffold efficacy can be optimized by tailoring the scaffold structure to force cells into morphologies that direct them to differentiate down the desired lineage.

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## 1. Introduction

Use of tissue scaffold properties to direct cell function constitutes a basic tenet in the field of tissue engineering [1]. It is known that surface topography from the micro- to the nanoscale can influence cell behavior [2–4]. For instance, 2D topographical cues can induce osteoprogenitor cell [4,5] and mesenchymal stem cell [6] osteogenesis. Since topography has been reported to direct cell function, the hypothesis that 3D scaffold structure can direct cell differentiation has been tested herein. Many protocols for fabricating polymer scaffolds have been developed that yield a wide variety of structures including salt-leaching (SL) [7], gas foaming (GF) [8], phase-separation (GFPS) [9], electrospinning nanofibers (NF) [10,11] and freeform fabrication (FFF) [12,13]. Scaffolds fabricated by these methods have different physical architectures ranging from porous to fibrous, nanofibrous to microfibrillar, and irregular to uniform. However, a systematic study to determine

how these different scaffold structures influence cell function has not been performed.

Herein, gene expression signatures and osteogenic differentiation of primary human bone marrow stromal cells (hBMSCs) were assessed during culture on a library of scaffolds with varied structure and chemistry. hBMSCs are adult stem cells that can be isolated from bone marrow and can differentiate into bone, fat and cartilage [14,15]. Scaffolds with varied structure were fabricated from the same material poly( $\epsilon$ -caprolactone) (PCL) so that effects of scaffold chemistry could be decoupled from effects of scaffold structure. A number of controls were also tested including 2D flat samples (spun-coat films) and scaffolds made from a different polymer [PDLLA, poly(D,L-lactic acid)] to control for 3D structure and chemistry, respectively. hBMSC sensitivity to differences in scaffold structure were assessed and a mechanism for their response based on cell shape is proposed.

## 2. Materials and methods

### 2.1. PCL Salt-leached scaffolds (PCL\_SL)

PCL (relative molecular mass 80,000 g/mol, Sigma) solution (30% mass fraction in chloroform) was mixed with sieved NaCl (250  $\mu$ m–425  $\mu$ m) at a (NaCl):(PCL

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solution) mass ratio of 4:1 to yield a homogenous paste [16]. The paste was pressed into a disk shaped Teflon mold (12 mm diameter, 4 mm thickness). Scaffolds in molds were air dried for 2 h at room temperature, salt-leached 5 d in deionized water, air-dried for 1 d and stored in a desiccator.

## 2.2. PCL Gas-foamed scaffolds (PCL\_GF)

PCL solution (30% by mass in chloroform) was mixed with sieved  $\text{NH}_4\text{HCO}_3$  (250  $\mu\text{m}$ –425  $\mu\text{m}$ ) at a  $(\text{NH}_4\text{HCO}_3)$ :(PCL solution) mass ratio of 2:1 to yield a homogenous paste [8]. The paste was pressed into a disk shaped Teflon mold (12 mm diameter, 4 mm thickness). Scaffolds in molds were air dried for 2 h at room temperature, removed from molds, incubated in warm d-water (40 °C) until no gas bubbles were generated (2 h), further leached in d-water at room temperature for 3 d, air-dried for 1 d and stored in a desiccator.

## 2.3. PCL Gas-foamed phase-separated scaffolds (PCL\_GFPS)

Gas foamed phase separated scaffolds of PCL were fabricated by combining thermally induced phase-separation technique (TIPS) and a gas-foaming technique [9]. PCL solution (30% by mass in 7:3 volume ratio chloroform:n-butanol) was mixed with sieved  $\text{NH}_4\text{HCO}_3$  (250  $\mu\text{m}$ –425  $\mu\text{m}$ ) at a  $(\text{NH}_4\text{HCO}_3)$ :(PCL solution) mass ratio of 2:1 to yield a homogenous paste. The paste was pressed into a disk-shaped Teflon mold (12 mm diameter, 4 mm thickness) and phase separated at –80 °C for 4 h. Next, the scaffolds were immersed in methanol at –20 °C for 24 h for solvent exchange. Scaffolds were gas-foamed by immersion in warm d-water (40 °C) for 2 h (until no bubbles were released), immersed in d-water at room temperature for 3 d, air-dried for 1 d and stored in a desiccator.

## 2.4. PCL “Big” nanofiber scaffolds (PCL\_BNF)

This protocol yields large diameter PCL nanofibers ( $\approx 900$  nm). PCL solution (30% by mass in 9:1 volume ratio chloroform: methanol) was loaded into a syringe and a syringe pump was used to dispense PCL solution (0.5 mL/h) into a home built electrospinning apparatus [11]. Aluminum foil was used as the target, the distance between needle and target was 15 cm, and voltage was 15 kV. The positive lead from the power supply was fixed to the spinneret which was an 18 gauge needle and the ground lead was fixed to the target (aluminum foil). Non-woven PCL nanofiber mats were collected for 1 h onto tissue culture polystyrene (TCPS) disks (12 mm dia., punched from the bottom of 48-well TCPS plates) placed on the aluminum foil target. TCPS disks were lightly sprayed with 70% by mass ethanol before electrospinning to help fibers adhere to the disks. TCPS disks were made by using a hot 12 mm punch to punch disks from the bottom of TCPS 48-well plates.

## 2.5. PCL “Small” nanofiber scaffolds (PCL\_SNF)

This protocol yields small diameter PCL nanofibers ( $\approx 300$  nm) and is the same as for PCL-BNF except for the following changes: PCL solution 10% by mass in 3:1 volume ratio chloroform: methanol, pump rate 2 mL/h and voltage 16.5 kV.

## 2.6. PCL Freeform fabricated scaffolds (PCL\_FFF)

Disc-shaped freeform fabricated scaffolds (5 mm dia., 2 mm height, in 96-well plates) made by precision extrusion deposition [12] were purchased from 3D Biotek and used as received.

## 2.7. 2D Spin-coated PCL films (PCL\_SC)

Thin films of PCL were then prepared by spin-coating PCL solution (0.8 mL, 10% by mass in glacial acetic acid) at 1000 rpm for 30 s over TCPS disks (100 mm dia.). Films were air-dried at room temperature for 2 h and annealed at 60° C for 30 s to adhere the PCL to the TCPS. Films were punched into 12 mm disks using a hot punch and exposed to sterile PBS to remove residual acetic acid.

## 2.8. PDLLA “Big” nanofiber scaffolds (PDLLA\_BNF)

This protocol yields large diameter PDLLA nanofibers ( $\approx 900$  nm) and is the same as for PCL-BNF except for the following changes: PDLLA (relative molecular mass 100,000 g/mol, Surmodics) solution 15% by mass in hexafluoroisopropanol (1,1,1,3,3,3-hexafluoro-2-propanol), pump rate 1.5 mL/h and voltage 15 kV.

## 2.9. 2D Spin-coated PDLLA films (PDLLA\_SC)

Thin films of PDLLA were then prepared by spin-coating PDLLA solution (0.8 mL, 10% by mass in glacial acetic acid) at 1500 rpm for 30 s over TCPS disks (100 mm dia.). Films were air-dried at room temperature for 2 h and annealed at 60° C for 30 s to adhere the PDLLA to the TCPS. Films were punched into 12 mm disks using a hot punch and exposed to sterile PBS to remove residual acetic acid.

## 2.10. Scaffold characterization

PCL\_SL, PCL\_GF, PCL\_GFPS, PCL\_BNF, PCL\_SNF, PCL\_FFF and PDLLA\_BNF scaffolds were examined by scanning electron microscopy (SEM). PCL\_SL, PCL\_GF and PCL\_GFPS scaffolds were frozen in liquid nitrogen and fractured with a scalpel to expose interior. PCL\_BNF, PCL\_SNF, PCL\_FFF and PDLLA\_BNF scaffolds were examined from the top. Scaffolds were sputter-coated with gold for 90 s and imaged (SEM, 15 kV, Hitachi s-4700-II FE-SEM). NF diameter, FFF strut diameter and FFF strut spacing were measured in SEM images using ImageJ software (NIH) (100 measurements of each). Morphology of 2D PCL\_SC and TCPS samples were viewed by transmitted white light phase contrast microscopy. Gravimetry was used to measure total porosity of PCL\_SL, PCL\_GF, PCL\_GFPS and PCL\_FFF scaffolds using an equation, “Total Porosity =  $1 - [(m/d)/v]$ ”, where “m” is mass of the scaffold (g), “d” is PCL density (1.1 g/mL) and “v” is volume of scaffold (mL) (caliper measurements).

## 2.11. Cell culture

PCL\_SL, PCL\_GF, PCL\_GFPS, PCL\_BNF, PCL\_SNF, PCL\_SC, PDLLA\_BNF and PDLLA\_SC specimens were placed in 48-well tissue-culture polystyrene plates for cell culture experiments. Wells without specimens were used as TCPS controls. PCL\_FFF were put into polystyrene 96-well plates (non-tissue culture treated) for cell culture experiments. Specimens were sterilized by ethylene oxide (Anderson Products) and degassed for 2 d under house vacuum.

Primary human bone marrow stromal cells (hBMSCs, also known as mesenchymal stem cells, Tulane Center for Gene Therapy) were cultured at 37 °C with 5% by volume  $\text{CO}_2$  in  $\alpha$ -minimum essential medium (Invitrogen) supplemented with 16.5% by volume fetal bovine serum (Atlanta Biologicals) and 4 mmol/L L-glutamine [14]. To wet scaffolds fully with culture medium, medium was added to specimens in wells and plates were placed under vacuum for 1 min. The vacuum was released and reapplied two more times. For seeding scaffolds, hBMSCs (80% confluent) were dissociated with 0.25% mass fraction trypsin [containing 1 mmol/L ethylenediaminetetraacetate (EDTA)] and re-suspended in medium. Passage 4 or 5 cells were used for all experiments. Medium was removed from scaffolds and 10,000 hBMSCs in 0.5 mL of medium was added to each well for 48-well plates (PCL\_SL, PCL\_GF, PCL\_GFPS, PCL\_BNF, PCL\_SNF, PCL\_SC, PDLLA\_BNF, PDLLA\_SC, TCPS) or 5000 hBMSCs in 0.2 mL of medium for 96-well plates (PCL\_FFF). Medium was changed twice per week and cells cultured for 4 time points (1 d, 7 d, 21 d, 50 d) as indicated in the figures. In some experiments, cells were cultured in medium containing osteogenic supplements (OS): dexamethasone (10 nmol/L),  $\beta$ -glycerophosphate (20 mmol/L) and ascorbic acid (0.05 mmol/L).

## 2.12. Alizarin red staining

For Alizarin red staining, scaffolds were fixed with 3.7% formaldehyde for 24 h at 37 °C and then stained with Alizarin Red S (10 mg/mL) for 1 h. Scaffolds were washed 5 times with d-water to remove excess stain and air dried. Digital images of stained scaffolds were acquired using a stereomicroscope. All Alizarin red experiments were performed with three specimens ( $n = 3$ ). Two fields of view at both low and high magnification were randomly selected and captured for each specimen and representative images are shown in the figures.

## 2.13. Picogreen DNA assay

The Picogreen DNA assay was used to quantify cell numbers in the scaffolds. Scaffolds were washed with PBS (phosphate-buffered saline) and then incubated with lysis buffer (0.2 mg/mL Proteinase K and 0.02% by mass sodium dodecyl sulfate) for 24 h at 37 °C. After incubation, 100  $\mu\text{L}$  of lysate was transferred to a clean 96-well plate and diluted with 100  $\mu\text{L}$  of Picogreen reagent (Invitrogen, diluted to working concentration as per manufacturer’s protocol). Fluorescence (excitation 485 nm, emission 538 nm) was measured using a plate reader. A DNA standard curve was generated using known DNA concentrations to calibrate readings.

## 2.14. Fluorescence staining of cells

Cells on scaffolds were fixed with 3.7% formaldehyde (mass/volume in PBS buffer) for 15 min, washed in PBS and permeabilized with 0.2% by mass Triton X-100 for 5 min. Samples were rinsed with PBS and stained (1 h) with Alexa Fluor 546-phalloidin (20 nmol/L in PBS) and Sytox green (100 nmol/L in PBS buffer) to stain for F-actin and nuclei, respectively (Invitrogen). Cells were washed with PBS.

## 2.15. Epifluorescence microscopy

For regular epifluorescence microscopy (non-confocal, Fig. 2 and Figs. S2–S4) to assess cell proliferation, specimens were washed with PBS, then water and air-dried before imaging. Two specimens were prepared for each treatment. Two fields of view at both low and high magnification were randomly selected and captured for green (nuclei) and red channels (actin) for each specimen and representative images are shown in the figures.

### 2.16. Confocal microscopy

Confocal microscopy (Zeiss LSM-510) was used to measure cell morphology using samples wet with PBS (not air-dried). High resolution z-stack images were captured with a 40×/0.80 water immersion objective (1 μm z-step size) for nuclei (Sytox green) and actin (Alexa Fluor 546 phalloidin). Two specimens were prepared for each treatment and ten cells were imaged and analyzed for each specimen ( $n = 20$  cells).

Sytox green staining of nuclei was used to make sure that cell morphology was assessed for single cells only (only one nucleus per object). Alexa Fluor 546 phalloidin stained actin images were used to assess cell morphology. Confocal Z-stack projections were constructed (to project entire cell surface into two dimensions) and thresholding was performed to result in binary images. ImageJ was used to measure cell area, aspect ratio and roundness. Cell outlines were created and analyzed for branching. Branching was determined by counting the number of projections from the cell body and the number of branch points originating within that projection with a length greater than 5 μm.

### 2.17. Human genome microarray

Total RNA from hBMSCs in 3D scaffolds and on 2D surfaces at 1 d and 14 d was collected by homogenization of the cell lysate using the QIAshredder followed by purification using the RNeasy Minikit (Qiagen). Four replicates were performed for all treatments wherein a replicate was prepared by pooling cell lysates from 4 samples (expect for PCL\_FFF scaffolds where 8 samples were pooled). Genome-wide transcriptions of hBMSCs were measured by high-throughput screening using the Illumina Human HT-12 v4 Expression BeadChips with 47231 probes for 25130 RefSeq annotated genes (NCBI/NIH) through a contract with Gene Logic (Gaithersburg, MD).

Data analysis was performed using the BRB-Array Tools version 4.1.0 (Biometric Research Branch, National Cancer Institute). The average of the four replicates of TCPS\_1d was used as an *in silico* reference array for median normalization of all of the individual array data. The “significance analysis of microarrays” (SAM) approach [17], with a false discovery rate of 0.05, was chosen to test for significantly changed genes within the osteogenic controls (4 replicates each of TCPS\_1d, TCPS\_14d, TCPS\_OS\_1d and TCPS\_OS\_14d). A total of 9494 genes were identified that were changed significantly between all possible pair-wise comparisons of these treatments. From those 9494 SAM-significant genes, a subset of 831 genes was found to exhibit at least a two-fold change ‘between TCPS\_1d and TCPS\_OS\_1d’ or ‘between TCPS\_14d and TCPS\_OS\_14d’ (this creates “black” regions on the sorted 14d arrays indicating “no change”). Hierarchical cluster analysis (HCA), with centered correlation and average linkage, was performed using all arrays and the 831 two-fold significant genes. A dendrogram of the relationships is shown in Fig. 3a. The  $\log_2$  ratio of the each gene intensity compared to the reference array (see above) was used to show the relative change in gene expression across the dataset. A heat map for the 831 SAM-significant genes is shown in Fig. 3b. Finally, 29 of those genes were found (January 2011) to be in a Gene Ontology [18] cluster for skeletal development (Fig. 3c).

### 2.18. Imaging fluorescent PDLLA\_BNF

In some cases (Fig. 4i–k), PDLLA\_BNF were doped with Rhodamine-123 (5 μg/ml in electrospinning solution, Molecular Probes) so that they could be imaged by fluorescence microscopy. For 3D renderings in Fig. 4i–j, confocal microscopy images (Leica TCS SP5 broadband) were collected with a 63×/0.9 water immersion objective (290 nm Z-step size) for nanofibers (Rhodamine-123) and hBMSC actin (Alexa Fluor 633 phalloidin). For the single Z-slice in Fig. 4k, a confocal microscopy image (Zeiss LSM-510) was collected with a 40×/0.80 water immersion objective for nanofibers (Rhodamine-123) and hBMSC actin (Alexa Fluor 546 phalloidin).

## 3. Results

In order to test if 3D scaffold architecture affects hBMSC behavior, poly(ε-caprolactone) (PCL) scaffolds with different architectures were fabricated by five different techniques (Table 1): salt-leaching (PCL\_SL), gas-foaming (PCL\_GF), gas-foaming phase-separation (PCL\_GFPS), electrospinning nanofibers (PCL\_BNF) and freeform fabrication (PCL\_FFF). Spun-coat films of PCL (PCL\_SC) were used as a flat 2D control for 3Dness. Tissue culture polystyrene (TCPS) was also used as a control flat surface. Using PCL to fabricate these different scaffold architectures enables contributions from material chemistry to be isolated from topographical effects. The specimens presented a wide variety of morphologies as shown by the range of structural parameters including porosities and fiber diameters (Fig. 1; Table 2).

**Table 1**

Scaffold abbreviations.

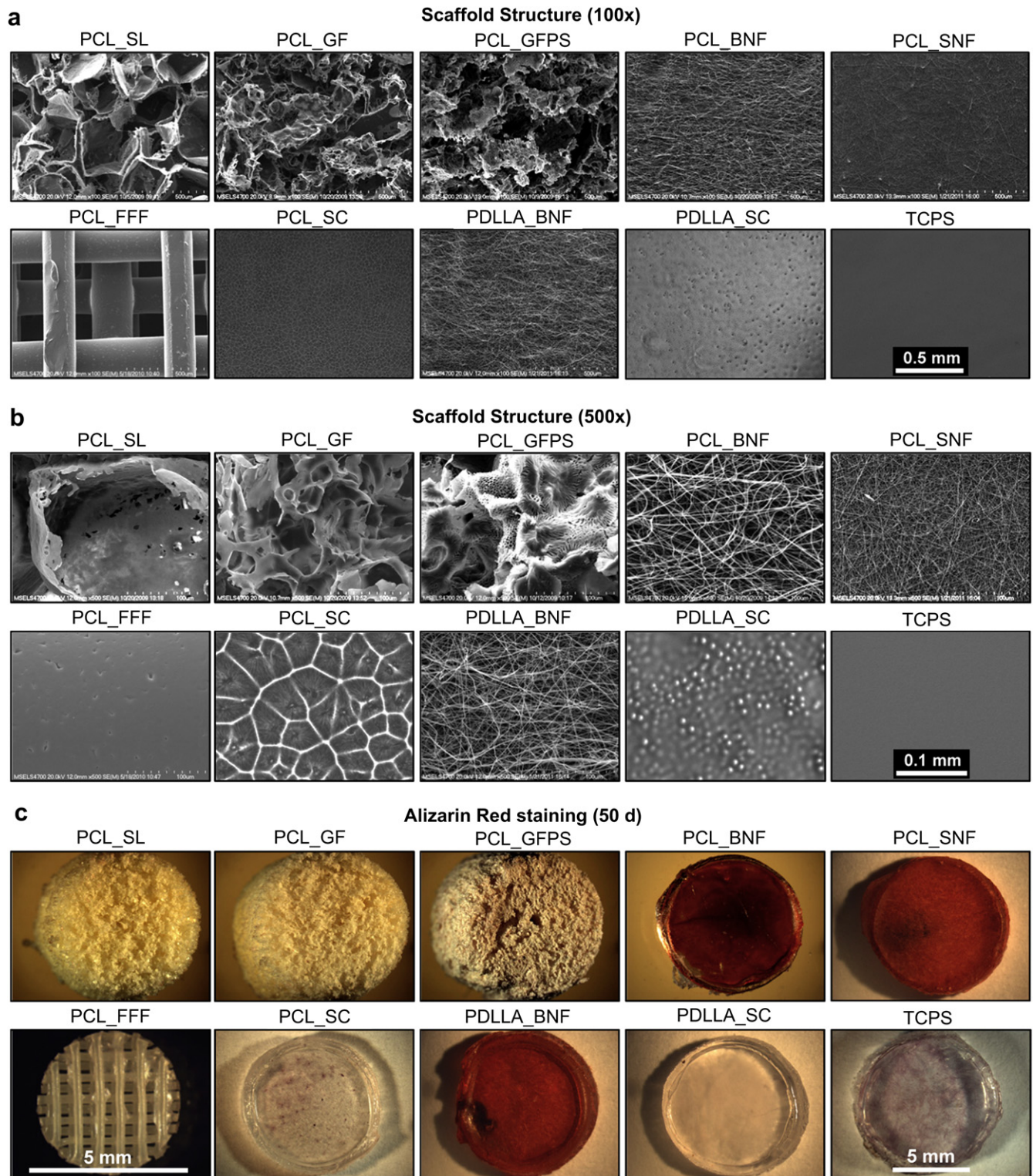
Abbreviation	Definition
PCL_SL	Poly(ε-Caprolactone) Salt-Leached Scaffold
PCL_GF	Poly(ε-Caprolactone) Gas-Foamed Scaffold
PCL_GFPS	Poly(ε-Caprolactone) Gas-Foamed Phase-Separated Scaffolds
PCL_BNF	Poly(ε-Caprolactone) “Big” Nanofiber Scaffolds
PCL_SNF	Poly(ε-Caprolactone) “Small” Nanofiber Scaffolds
PCL_FFF	Poly(ε-Caprolactone) Freeform Fabricated Scaffolds
PCL_SC	Poly(ε-Caprolactone) Spun-Coat Films
PDLLA_BNF	Poly(D,L-Lactic Acid) “Big” Nanofiber Scaffolds
PDLLA_SC	Poly(D,L-Lactic Acid) Spun-Coat Films
TCPS	Tissue Culture Polystyrene
+OS	(+) Osteogenic Supplements

When hBMSCs were cultured on the scaffolds in the absence of osteogenic supplements (OS), the nanofiber scaffolds (PCL\_BNF) induced deposition of a bone-like matrix containing calcium (Fig. 1, Fig. S1). hBMSCs did not produce a calcified matrix on the other scaffold types (PCL\_SL, PCL\_GF, PCL\_GFPS, PCL\_FFF) or the flat controls (PCL\_SC, TCPS) unless OS were added to the culture medium. These results suggest that only the nanofibrous scaffolds can induce hBMSC osteogenesis.

Next, the nanofiber effect was investigated more closely and the effect of nanofiber diameter and chemistry were tested. “Small” diameter 300 nm nanofibers (PCL\_SNF) were fabricated and induced hBMSC osteogenesis in the absence of OS. “Big” 900 nm nanofibers made from another polymer, poly(D,L-lactic acid) (PDLLA\_BNF), induced osteogenesis but control flat PDLLA films (PDLLA\_SC) did not. When control PCL\_BNF were incubated in complete medium without hBMSCs, they did not induce non-specific calcium deposition from medium (Fig. S1). A summary of all experiments is given in Fig. S8. These results indicate that nanofiber scaffolds of different sizes (300 nm and 900 nm) and different chemistries (PCL and PDLLA) induced hBMSC osteogenesis without OS, while flat films of the same polymers required OS to support osteogenic differentiation of hBMSCs.

The effect of scaffold architecture on hBMSC proliferation was tested using Picogreen DNA assay, fluorescence microscopy and stereomicroscopy (Fig. 2; Figs. S2–S5). hBMSCs adhered and proliferated on all specimens tested (PCL\_SL, PCL\_GF, PCL\_GFPS, PCL\_BNF, PCL\_FFF, PCL\_SC, TCPS) both in the presence and absence of osteogenic supplements. For the DNA assay, hBMSC numbers increased the fastest on PCL\_FFF scaffolds (both without and with OS) and the highest amount of DNA extracted from any specimen was for PCL\_FFF in the presence of OS after 21 d culture (Fig. 2). This was observed despite the fact that that PCL\_SL, PCL\_GF, PCL\_GFPS, PCL\_BNF, PCL\_FFF, PCL\_SC and TCPS were seeded with more hBMSCs (10,000) and were cultured in larger wells (48-well) than the PCL\_FFF (PCL\_FFF were seeded with 5000 cells in 96-well plates). The hBMSCs formed a tissue-like matrix at the strut joints on the PCL\_FFF scaffolds by 21 d as observed in fluorescence micrographs and stereomicrographs (both without and with OS) (Fig. 2; Figs. S1, S2 and S4). By 50 d without OS, a tissue-like matrix formed a continuous sheet between some struts on the PCL\_FFF scaffolds (Figs. S1c and S2). These data indicate that hBMSCs adhered and proliferated on all substrates tested (PCL\_SL, PCL\_GF, PCL\_GFPS, PCL\_BNF, PCL\_FFF, PCL\_SC, TCPS) but that PCL\_FFF scaffolds supported the most hBMSC proliferation.

The effect of scaffold architecture on hBMSC gene expression was assessed using microarrays (mRNA). A dendrogram resulting from cluster analysis (Fig. 3a) plus heat maps of the expression level for significantly regulated genes (Fig. 3b) are shown. Heat maps of the significantly regulated genes involved in skeletal



**Fig. 1.** (a,b) Structure of scaffolds is shown by SEM for 3D scaffolds PCL\_SL, PCL\_GF, PCL\_GFPS, PCL\_BNF, PCL\_SNF, PCL\_FFF, PDLLA\_BNF and by phase contrast microscopy for 2D samples PCL\_SC, PDLLA\_SC and TCPS. Scale bar in (a) applies to all images in (a) and scale bar in (b) applies to all images in (b). (c) Stereomicrographs of calcium staining (Alizarin red) for hBMSC osteogenesis on scaffolds in the absence of osteogenic supplements (OS) after 50 d culture. The scale bar in TCPS applies to all panels except PCL\_FFF. Magnification is 18 $\times$  for all images except PCL\_FFF (PCL\_FFF is 32 $\times$ ). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

development are also given (Fig. 3c, Table S1). Cluster analysis indicated that hBMSC gene expression at 14 d on the nanofiber specimens correlated most closely with the osteogenic control treatments. Thus, Node A (TCPS + OS 14d: hBMSCs cultured 14 d in OS on TCPS) and Node B (14d nanofiber specimens: PCL\_BNF,

PCL\_SNF, PDLLA\_BNF) grouped together under Node C. These data indicate that nanofiber scaffolds induced a gene expression signature that was similar to that induced by OS, further supporting the observation that culture of hBMSCs on nanofibrous scaffolds induces hBMSC osteogenesis.

**Table 2**Scaffold structural measurements [mean  $\pm$  S.D. (n)].

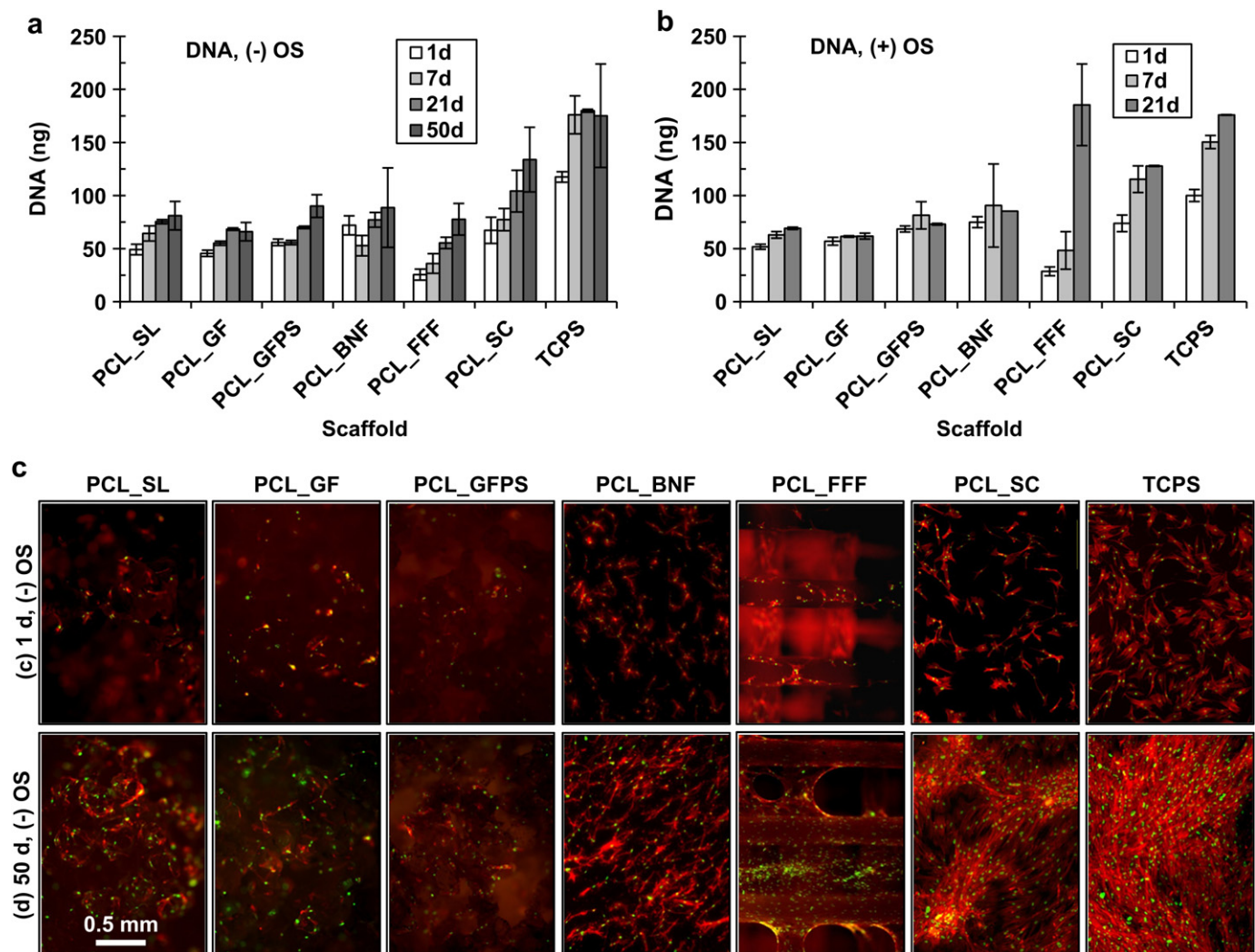
PCL_SL porosity <sup>a</sup> (%)	94.3 $\pm$ 0.7 (10)
PCL_GF porosity <sup>a</sup> (%)	92.6 $\pm$ 0.8 (10)
PCL_GFPS porosity <sup>a</sup> (%)	90.3 $\pm$ 0.7 (10)
PCL_BNF fiber diameter <sup>b</sup> (nm)	910 $\pm$ 526 (100)
PCL_SNF fiber diameter <sup>b</sup> (nm)	326 $\pm$ 128 (70)
PCL_FFF porosity <sup>a</sup> (%)	65.6 $\pm$ 0.9 (10)
PCL_FFF strut diameter <sup>b</sup> ( $\mu$ m)	288 $\pm$ 29 (100)
PCL_FFF strut spacing <sup>b</sup> ( $\mu$ m)	491 $\pm$ 39 (100)
PDLLA_BNF fiber diameter <sup>b</sup> (nm)	942 $\pm$ 234 (70)

<sup>a</sup> Porosity measured by gravimetry.<sup>b</sup> Fiber diameter, strut diameter and strut spacing measured in electron micrographs.

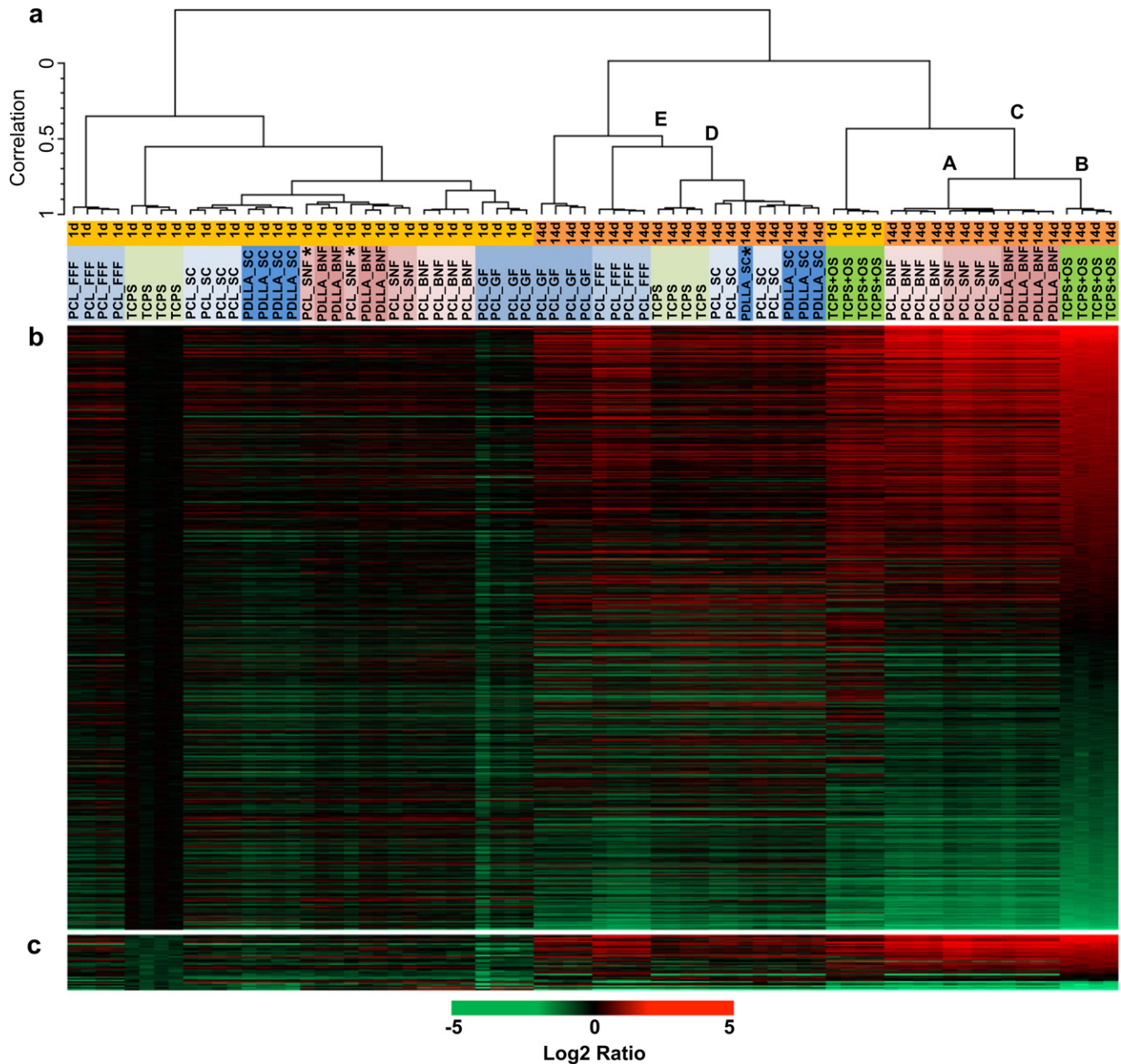
It is also astonishing that for the different treatments, the 4 replicates group together almost exclusively (Fig. 3a). Indeed, only three treatments (marked with an asterisk in Fig. 3) would need to be relocated to give perfect segregation by treatment in the dendrogram. Also, all 14 d flat specimens (TCPS, PCL\_SC, PDLLA\_SC) group together under node D, indicating that the hBMSCs can sense the 2-dimensionality of these substrates. For these experiments, specimen architecture was more important than surface chemistry,

since PCL\_SC grouped with the other 2D specimens (PDLLA\_SC, TCPS) instead of with other PCL specimens. Likewise, PDLLA\_SC grouped with the other 2D specimens (PCL\_SC, TCPS) instead of with the other PDLLA specimen (PDLLA\_BNF). These results indicate that hBMSCs are exquisitely sensitive to scaffold structure resulting in a unique gene expression profile for each type of scaffold tested. The results also suggest that scaffold structure has a bigger influence on stem cell behavior than does polymer chemistry.

The ability of nanofibers to drive osteogenic differentiation of hBMSCs was investigated further to understand the mechanism. Fluorescence micrographs suggested that cell shape could be driving osteogenesis on nanofiber scaffolds. hBMSCs assumed a spindly, highly branched morphology on PCL\_BNF which was very different than the well spread morphology observed for flat films (PCL\_SC) (Figs. S2–S4). Since cell morphology is tightly linked to cell function [19,20], this possibility was investigated further using high resolution confocal microscopy to quantify and compare cell morphology on PCL nanofibers (PCL\_BNF) and films (PCL\_SC) after 1 d culture (Fig. 4, Fig. S7). hBMSCs cultured on PCL\_BNF had smaller cell area, higher aspect ratio, lower roundness and more branching than did hBMSCs on PCL\_SC. Strikingly, hBMSCs cultured on PCL\_SC in the presence of OS also took on a spindly, branched



**Fig. 2.** (a,b) hBMSC cell numbers measured by Picogreen DNA assay after culture (a) without and (b) with OS. Error bars are S.D. ( $n = 4$ ). (c) Fluorescence micrographs ( $40\times$ ) of hBMSCs cultured on scaffolds without OS for 1 d or 50 d. Nuclei are green (Sytox green) and actin is red (AlexaFluor 546 phalloidin). Scale bar applies to all images in (c). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)



**Fig. 3.** Gene expression profiles of hBMSCs on scaffolds and control surfaces at 1d and 14d revealed by microarrays. (a) Dendrogram from hierarchical cluster analysis organizing experiments by similarity. Only three treatments (marked with an asterisk) would need to be relocated to give perfect segregation by treatment in the dendrogram. (b) Heat map of relative gene expression for 831 genes identified to be significantly regulated in osteogenic controls (at least a two-fold change 'between TCPS 1d and TCPS\_OS 1d' or 'between TCPS 14d and TCPS\_OS 14d'). Data are organized from highest up-regulation to lowest down-regulation for TCPS\_OS 14d. (c) From the 831 genes shown in (b), a subset of 29 genes that are involved in skeletal system development was identified by Gene Ontology. Color scale at bottom of figure applies to (b,c) and is  $\log_2$  of the ratio of fluorescence intensity to the mean value for the four TCPS 1d controls.

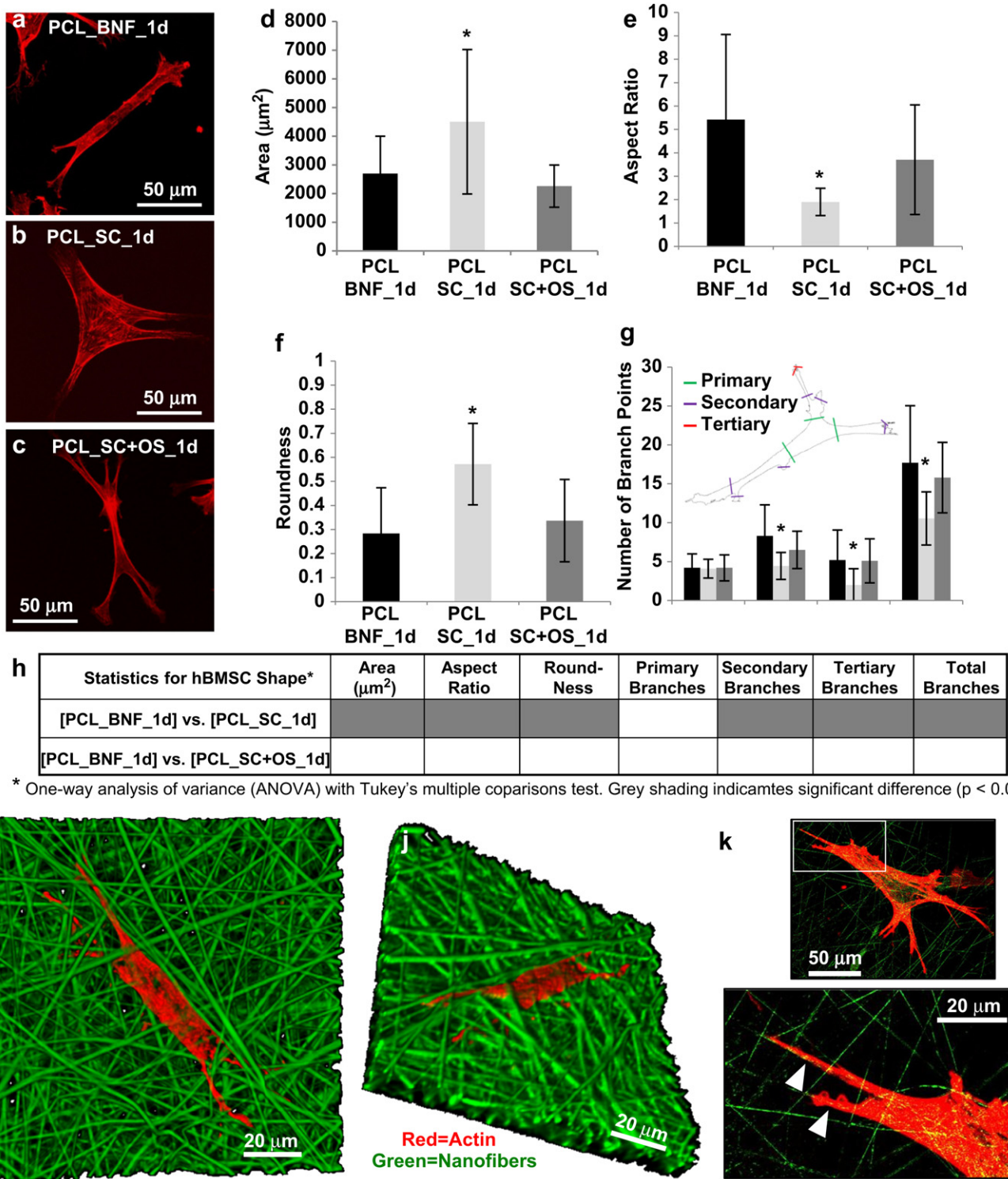
morphology that was similar to hBMSCs on PCL\_BNF (Fig. 4). Thus, elongated, highly branched hBMSC morphologies induced osteogenesis regardless of whether morphological changes were driven by scaffold architecture (nanofiber scaffolds) or by biochemical supplements (OS).

Confocal 3D renderings of hBMSCs on fluorescent nanofiber scaffolds show that the hBMSCs penetrate into the nanofiber scaffolds (Fig. 4i–j). In addition, the hBMSCs extended projections that follow along the individual nanofibers resulting in the elongated, branched hBMSC morphology (Fig. 4k). The hBMSCs cannot attach to the aqueous medium in the voids between the nanofibers and have no choice but to adhere to the nanofibers, which forces them

into and elongated and highly branched morphology. Taken together, these results suggest that the intrinsic structure of the nanofiber scaffolds forces the hBMSCs into an osteogenic morphology that drives their osteogenic differentiation.

#### 4. Discussion

The cluster analysis dendrogram (Fig. 3) is powerful because it provides an unbiased approach of sorting scaffold treatments according to gene expression patterns. The analysis considers all genes and does not focus on pre-selected markers. In addition, this approach provides a unique way to analyze cell–material interactions



**Fig. 4.** (a–c) hBMSC morphology (400×) after 1 d culture for (a) PCL\_BNF, (b) PCL\_SC and (c) PCL\_SC + OS. Actin is red (AlexaFluor 546 phalloidin) and projections of confocal z-stacks are shown. (d–g) Cell morphology using quantified for (d) area, (e) aspect ratio, (f) roundness and (g) number of primary, secondary, tertiary and total branch points for hBMSCs cultured 1 d on PCL\_BNF, PCL\_SC and PCL\_SC + OS. Confocal z-stack projections of Alexa Fluor 546 phalloidin-stained cells were used for analysis. Error bars are S.D. and 20 cells were analyzed for each treatment ( $n = 20$ ). Inset in (h) defines analysis. Asterisk in (d–g) indicates significantly different from PCL\_BNF [ $p < 0.05$ , one-way analysis of variance (ANOVA) with Tukey's test for multiple comparisons]. (h) Statistical analysis for hBMSC morphology. (i–k) Confocal imaging of hBMSCs (red, actin, Alexa Fluor 633 phalloidin) cultured on PDLLA\_BNF (green, Rhodamine 123 doping) for 24 h (i) 3D view of a confocal z-stack (630×). (j) 3D view of the Z-stack shown in (i) but rotated in 3D space to show how hBMSCs have migrated down into the nanofibers (630×). (k) One Z-slice (400×) shows how hBMSCs extend projections that follow along the nanofibers (white arrowheads). The area outlined by the white box in the top image is enlarged in the bottom image. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

where the sorting of the treatments provides insight into which material properties are influencing cell fate. In Fig. 3a, the different scaffold treatments sort by scaffold structure over scaffold composition, suggesting that scaffold structure has a greater ability to control cell fate than does composition. Combining the microarray cluster analysis with the scaffold library approach, where many types of scaffold architectures are systematically compared side-by-side, robustly demonstrates that cells are more sensitive to scaffold structure than previously appreciated.

Although the advantages of nanofiber scaffolds for tissue engineering are well-documented and their ability to promote osteogenesis has been demonstrated [21–23], the mechanism for these effects has not been defined. The current scaffold library results further emphasize the ability of nanofibers to induce osteogenesis by systematically comparing multiple scaffold architectures side by side. A nanofibrous topography was key since only the nanofiber scaffolds, of all the scaffold morphologies that were tested (SL, GF, GFPS, NF, FFF), induced osteogenic differentiation. In addition, the current results provide a mechanistic basis for the effects of nanofibers. Nanofibers provided fibrous adhesion sites for the hBMSCs that caused the hBMSCs to take on an elongated, highly branched morphology which drove them down and osteogenic lineage (Fig. 4). The current experiments suggest that the osteoinductive effects of nanofibers come from their ability to force cells into an osteogenic morphology.

Decellularized extracellular matrices (dECM) have been very successful as tissue engineering scaffolds and collagen nanofibers are the dominant structure of dECM [24–27]. Synthetic polymer nanofiber scaffolds such as PCL\_BNF mimic the structure of the collagen nanofibers present in dECM [11]. The current results suggest that the dECM scaffolds may be effective because their nanofibrous structure drives cells into morphologies that enhance their differentiation. It will be interesting to see if this holds true in future work.

It is well-established that cell morphology and cell function are strongly linked [19,20] and that this principle applies to hBMSC osteogenesis [28–30]. McBeath et al. demonstrated that modulation of hBMSC shape with surface patterning could be used to switch hBMSC differentiation between osteogenic or adipogenic [28]. In addition, Rodriguez et al. observed that osteogenic supplements caused changes in hBMSC morphology [29]. In vivo, hBMSCs progress to osteoblasts and then to mature osteocytes. While osteoblasts are cuboidal, osteocytes are highly branched, residing in lacunae and radiating multiple extensions down canaliculi [31]. Thus, nanofiber scaffolds may force the hBMSCs into a highly branched “osteocyte-like” morphology, which pushes the hBMSCs toward an osteogenic lineage.

The data suggest that scaffolds should be designed to drive cells into morphologies that will induce the desired cell function (proliferation, migration, differentiation, etc.). Models for cell spreading could be adapted to predict cell shape on different 3D structures. Alternatively, more extensive libraries of 3D structures could be probed for their effect on cell shape and function to create a catalog of scaffold structures versus cell shape/behavior. Coupling scaffold structure to cell shape could yield new targets for scaffold design that could enhance the inherent regenerative capabilities of scaffolds.

## 5. Conclusions

A systematic comparison of multiple scaffold types highlights the unexpected sensitivity that cells have for scaffold structure. hBMSCs took on a unique gene expression profile on each type of scaffold structure tested demonstrating that hBMSCs are highly responsive to scaffold architecture. In addition, the effect of scaffold structure on

stem cell behavior was mediated by cell shape. Nanofibers scaffolds induced osteogenic differentiation of hBMSCs by forcing the cells into an elongated, highly-branched, osteogenic morphology. These results advance our understanding of how scaffold architecture can control cell fates. The data suggest a new paradigm for improving 3D tissue scaffold efficacy where the structure of the scaffold can be designed to drive cells into morphologies that direct their differentiation down a desired lineage.

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## Appendix. Supplementary material

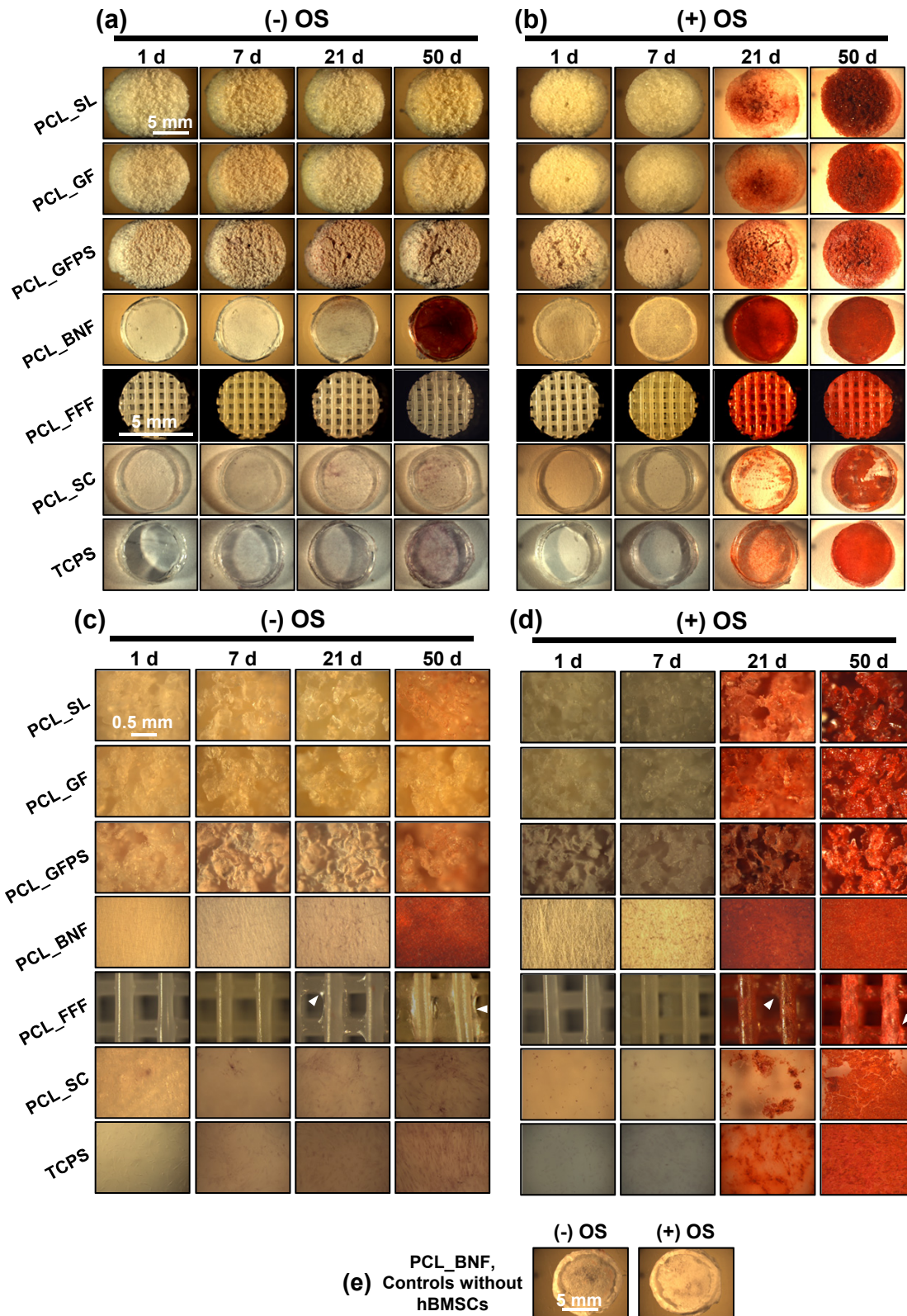
Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.biomaterials.2011.08.054.

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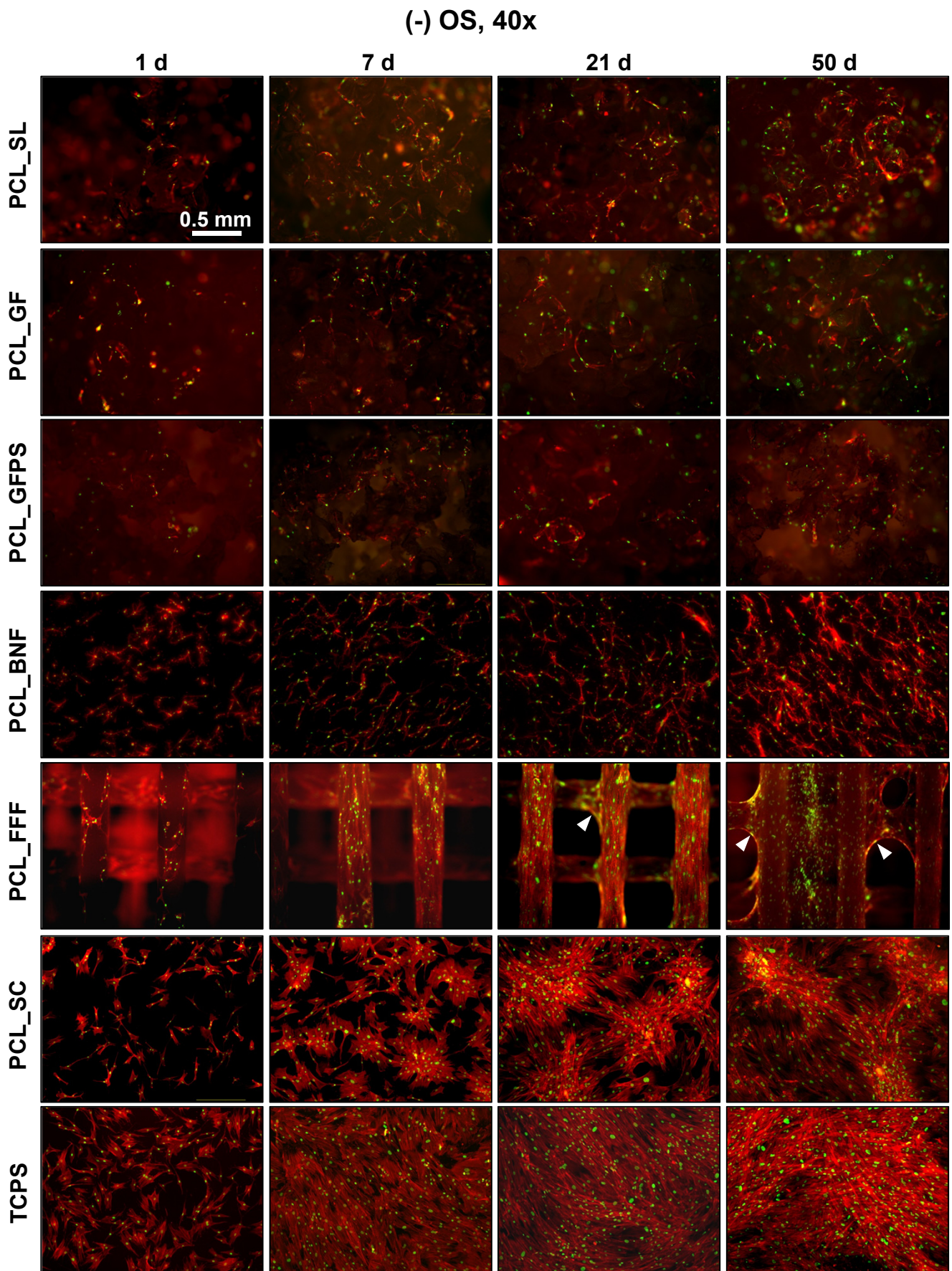
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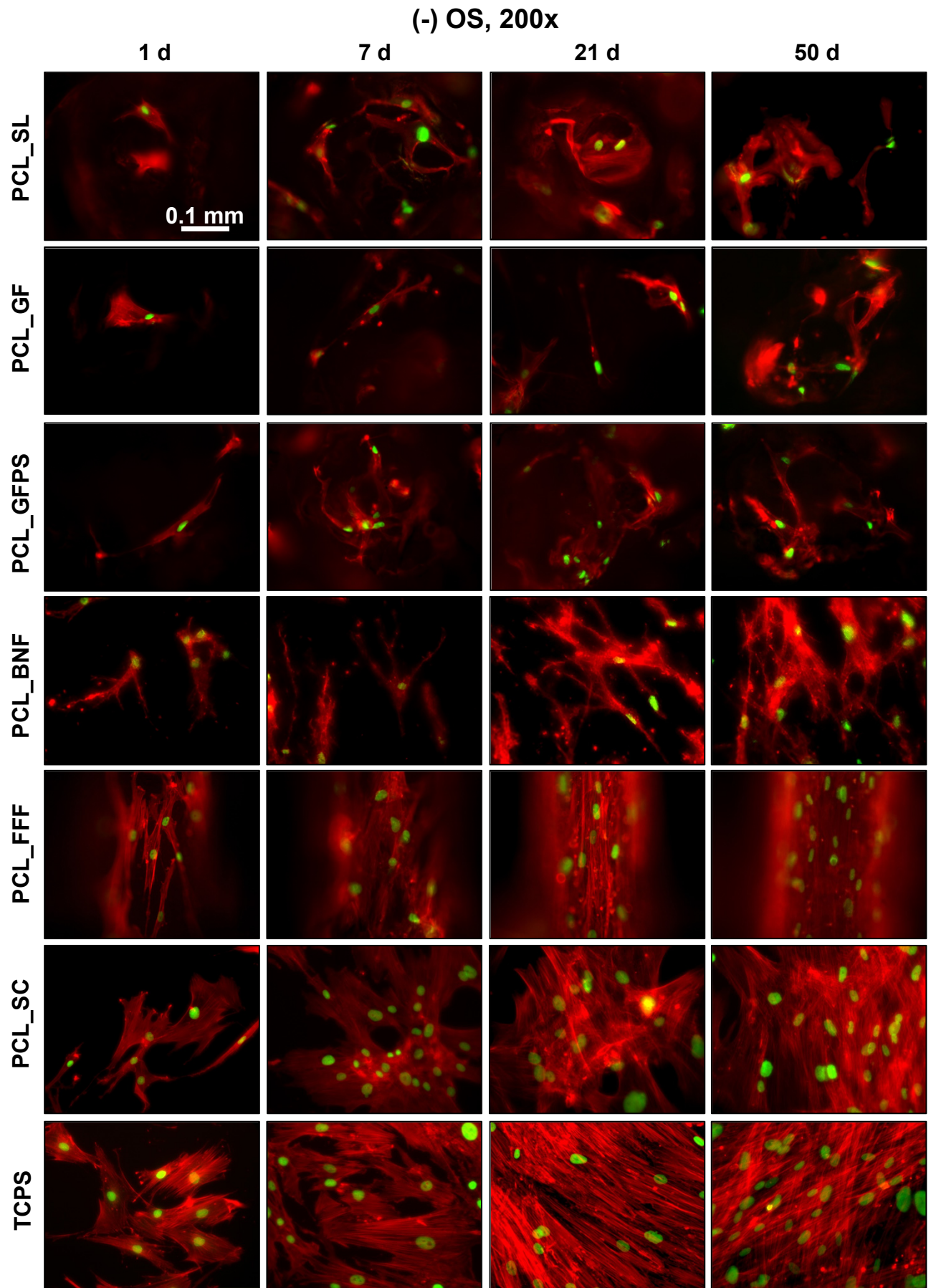
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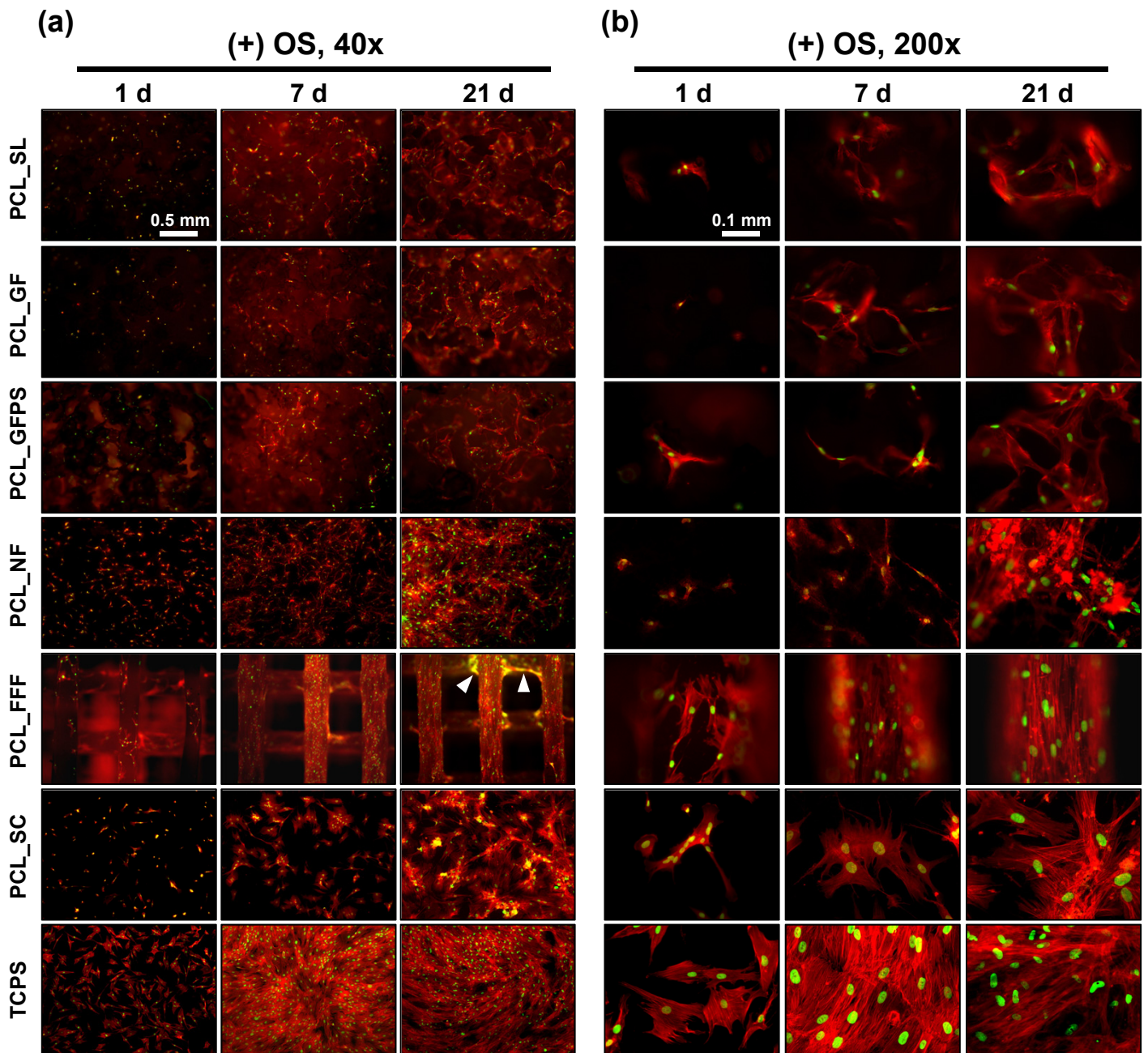
**Fig. S1.** Stereomicrographs of calcium staining (Alizarin red) for hBMSC osteogenesis. (a,c) hBMSCs cultured without osteogenic supplements (OS). (b,d) hBMSCs cultured with OS. (a,b) All images are 18x except for PCL\_FFF images which are 32x. Scale bar in upper left of panel (a) applies to all images in panels (a,b) except PCL-FFF. The scale bar in the left PCL-FFF image in panel (a) applies to all PCL-FFF images panels (a,b). (c,d) All images are 128x and scale bar in upper left of panel (c) applies to all images in panels (c,d). (e) Control PCL\_BNF scaffolds cultured without hBMSCs do not mineralize. PCL\_BNF scaffolds were incubated in hBMSC medium without hBMSCs (both without and with OS) for 50 d (medium changed twice weekly) and stained with Alizarin red. Images were collected on a stereomicroscope (18x) and scale bar applies to both images. Arrowheads in (c,d) PCL\_FFF images indicate tissue-like matrix formation.



**Fig. S2.** Fluorescence micrographs (40x) of hBMSCs cultured on scaffolds without OS. Nuclei are green (Sytox green) and actin is red (Alexa Fluor 546 phalloidin). Scale bar applies to all images. Arrowheads in PCL\_FFF indicate tissue-like matrix.

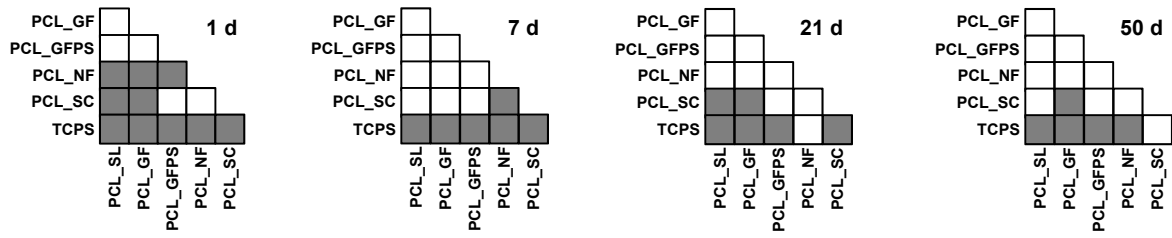


**Fig. S3.** Fluorescence micrographs (200x) of hBMSCs cultured on scaffolds without OS. Nuclei are green (Sytox green) and actin is red (Alexa Fluor 546 phalloidin). Scale bar applies to all images.

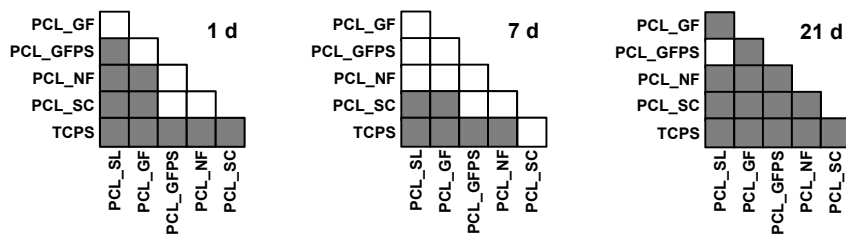


**Fig. S4.** Fluorescence micrographs of hBMSCs cultured on scaffolds with OS. Nuclei are green (Sytox green) and actin is red (Alexa Fluor 546 phalloidin). (a) 40x images. Scale bar in upper left of panel (a) applies to all images in panel (a). Arrowhead in PCL\_FFF indicates tissue like matrix formation. (b) 200x images. Scale bar in upper left of panel (b) applies to all images in panel (b). Data for “50 d” images are omitted since cell layers became too dense to stain efficiently.

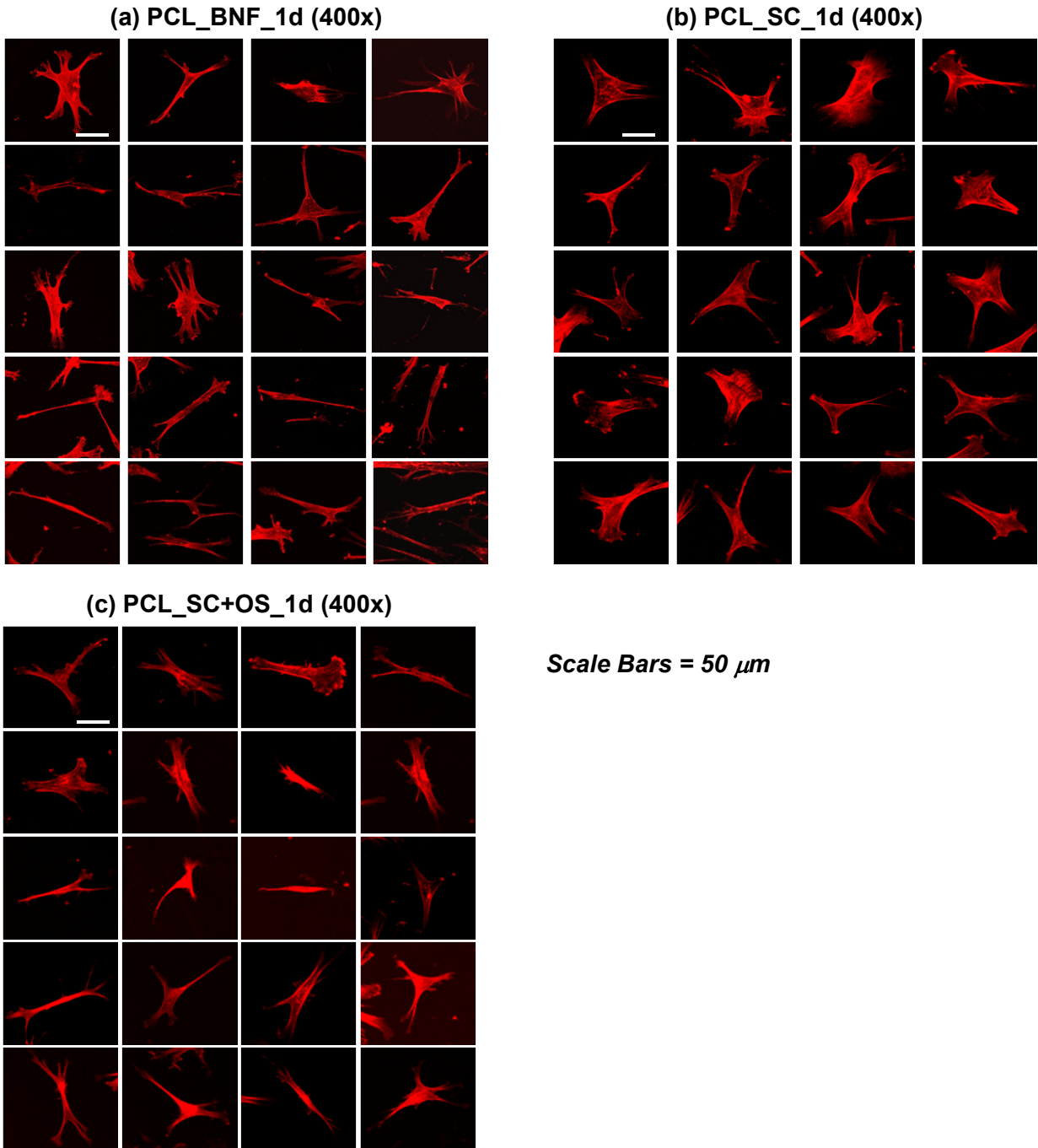
(-) OS



(+) OS



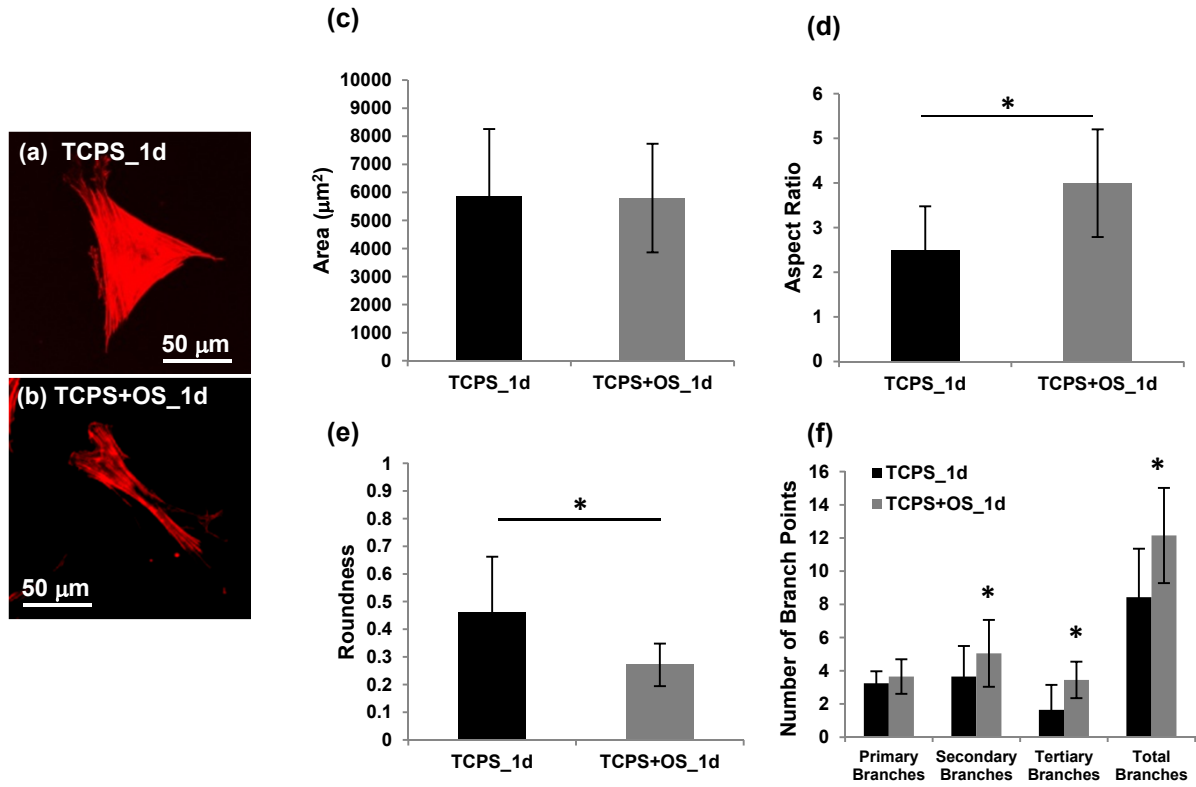
**Fig. S5.** Statistical analysis of Picogreen DNA data (shown in Fig. 2) using 1-way ANOVA with Tukey's test for multiple comparisons. Grey squares represent significant differences ( $p < 0.05$ ).



(d) Cell Shape Measurements [mean (S.D.)]

Culture Condition	Area ( $\mu\text{m}^2$ )	Perimeter ( $\mu\text{m}$ )	Aspect Ratio	Roundness	Primary Branches	Secondary Branches	Tertiary Branches	Total Branches
PCL_BNF_1d	2697 (1303)	1250 (503)	5.42 (3.65)	0.283 (0.190)	4.2 (1.8)	8.3 (4.0)	5.2 (3.9)	17.7 (7.3)
PCL_SC_1d	4507 (2518)	1760 (662)	1.90 (0.59)	0.572 (0.169)	4.1 (1.2)	4.4 (1.7)	2.0 (2.1)	10.6 (3.4)
PCL_SC+OS_1d	2261 (734)	1633 (493)	3.71 (2.34)	0.337 (0.171)	4.2 (1.7)	6.5 (2.4)	5.1 (2.8)	15.8 (4.5)

**Fig. S6.** (a-c) Image library used for cell shape measurements shown in Fig. 4. Each image is a confocal z-stack projection of actin staining (Alexa Fluor 546 phalloidin) for hBMSCs cultured 1 d for (a) PCL\_BNF, (b) PCL\_SC and (c) PCL\_SC+OS. (d) Cell shape measurements used for plots shown in Fig. 4. Twenty cells were analyzed for all measurements ( $n = 20$ ).



**Fig. S7.** (a,b) hBMSC morphology (400x) on TCPS and TCPS+OS after 1 d culture. Actin is red (Alexa Fluor 546 phalloidin). (c-f) Cell morphology quantified for (c) area, (d) aspect ratio, (e) roundness and (f) number of primary, secondary, tertiary and total branch points for hBMSCs cultured 1 d on TCPS or TCPS+OS. Confocal z-stack projections of actin staining (Alexa Fluor 546 phalloidin) were used for analysis. Error bars are S.D. and 20 cells were analyzed for each treatment (n = 20). (g) Statistical analysis for one way ANOVA with Tukey's test for multiple comparisons. Grey shading indicates significant differences (p < 0.05).



**Fig. S8. Summary of Results**

Scaffolds					Results			
Abbreviation	Specimen	Scaffold Morphology	Scaffold Morphological Parameters	Osteogenic Supplements	Osteogenesis (Alizarin Red)	Proliferation (Picogreen DNA)*	Microarray Profile Groups Under "Node C"	Elongated, Highly Branched hBMSC Morphology (Confocal, Quantitative)
PCL_SL	Poly( $\epsilon$ -Caprolactone) Salt-Leached Scaffold	Macroporous	94% Porosity	(-)	(-)	(+)	untested	untested
PCL_GF	Poly( $\epsilon$ -Caprolactone) Gas-Foamed Scaffold	Macroporous	93% Porosity	(-)	(-)	(+)	(-)	untested
PCL_GFPS	Poly( $\epsilon$ -Caprolactone) Gas-Foamed Phase-Separated Scaffolds	Macroporous	90% Porosity	(-)	(-)	(+)	untested	untested
PCL_BNF	Poly( $\epsilon$ -Caprolactone) "Big" Nanofiber Scaffolds	Nanofibrous	910 nm Fiber Diameter	(-)	50 d	(+)	(+)	(+)
PCL_SNF	Poly( $\epsilon$ -Caprolactone) "Small" Nanofiber Scaffolds	Nanofibrous	326 nm Fiber Diameter	(-)	50 d	untested	(+)	untested
PCL_FFF	Poly( $\epsilon$ -Caprolactone) Freeform Fabricated Scaffolds	Microfibrous	66% Porosity; 288 $\mu$ m Strut Dia.; 491 $\mu$ m Strut Spacing	(-)	(-)	(+++)	(-)	untested
PCL_SC	Poly( $\epsilon$ -Caprolactone) Spun-Coat Films	Flat	not applicable	(-)	(-)	(+)	(-)	(-)
PDLLA_BNF	Poly(D,L-Lactic Acid) "Big" Nanofiber Scaffolds	Nanofibrous	942 nm Fiber Diameter	(-)	50 d	untested	(+)	untested
PDLLA_SC	Poly(D,L-Lactic Acid) Spun-Coat Films	Flat	not applicable	(-)	(-)	untested	(-)	untested
TCPS	Tissue Culture Polystyrene	Flat	not applicable	(-)	(-)	(+)	(-)	(-)
PCL_SL	Poly( $\epsilon$ -Caprolactone) Salt-Leached Scaffold	Macroporous	94% Porosity	(+)	21 d	(+)	untested	untested
PCL_GF	Poly( $\epsilon$ -Caprolactone) Gas-Foamed Scaffold	Macroporous	93% Porosity	(+)	21 d	(+)	untested	untested
PCL_GFPS	Poly( $\epsilon$ -Caprolactone) Gas-Foamed Phase-Separated Scaffolds	Macroporous	90% Porosity	(+)	21 d	(+)	untested	untested
PCL_BNF	Poly( $\epsilon$ -Caprolactone) "Big" Nanofiber Scaffolds	Nanofibrous	910 nm Fiber Diameter	(+)	21 d	(+)	untested	untested
PCL_SNF	Poly( $\epsilon$ -Caprolactone) "Small" Nanofiber Scaffolds	Nanofibrous	326 nm Fiber Diameter	(+)	untested	untested	untested	untested
PCL_FFF	Poly( $\epsilon$ -Caprolactone) Freeform Fabricated Scaffolds	Microfibrous	66% Porosity; 288 $\mu$ m Strut Dia.; 491 $\mu$ m Strut Spacing	(+)	21 d	(+++++)	untested	untested
PCL_SC	Poly( $\epsilon$ -Caprolactone) Spun-Coat Films	Flat	not applicable	(+)	21 d	(+)	untested	(+)
PDLLA_BNF	Poly(D,L-Lactic Acid) "Big" Nanofiber Scaffolds	Nanofibrous	942 nm Fiber Diameter	(+)	untested	untested	untested	untested
PDLLA_SC	Poly(D,L-Lactic Acid) Spun-Coat Films	Flat	not applicable	(+)	untested	untested	untested	untested
TCPS	Tissue Culture Polystyrene	Flat	not applicable	(+)	21 d	(+)	(+)	(+)

\*One plus sign is greater than 1-fold increase , three plus signs is greater than 3-fold increase and 6 plus signs is greater than 6-fold increase.

**Table S1. List of 29 SAM-Significant Genes Expressed in Skeletal System Development<sup>a</sup>**

Illumina Probe <sup>b</sup>	Log <sub>2</sub> Ratio <sup>c</sup>	Gene Name
ILMN_1677636	4.95	cartilage oligomeric matrix protein
ILMN_1789507	4.45	collagen, type XI, alpha 1
ILMN_2071809	4.16	matrix Gla protein
ILMN_1716246	3.88	frizzled-related protein
ILMN_1651958	3.76	matrix Gla protein
ILMN_1772910	3.63	growth arrest-specific 1
ILMN_1676663	3.14	tumor necrosis factor receptor superfamily, member 11b
ILMN_2062701	2.98	growth arrest-specific 1
ILMN_2413956	2.90	insulin-like growth factor 2 (somatomedin A); insulin; INS-IGF2 readthrough transcript
ILMN_2086470	2.87	platelet-derived growth factor receptor, alpha polypeptide
ILMN_1681949	2.53	platelet-derived growth factor receptor, alpha polypeptide
ILMN_1707077	2.44	sortilin 1
ILMN_2392803	2.38	collagen, type XI, alpha 1
ILMN_2305407	2.25	zinc finger and BTB domain containing 16
ILMN_1733756	2.18	collagen, type XII, alpha 1
ILMN_1801205	2.02	glycoprotein (transmembrane) nmb
ILMN_1699867	1.96	insulin-like growth factor 2 (somatomedin A); insulin; INS-IGF2 readthrough transcript
ILMN_1790338	1.95	paired related homeobox 2
ILMN_1758164	1.85	stanniocalcin 1
ILMN_2402817	1.83	zinc finger and BTB domain containing 16
ILMN_1665033	1.60	natriuretic peptide receptor C/guanylate cyclase C (atrionatriuretic peptide receptor C)
ILMN_1747650	1.51	bone morphogenetic protein 6
ILMN_2407389	1.46	glycoprotein (transmembrane) nmb
ILMN_1672908	1.32	twist homolog 1 (Drosophila)
ILMN_1672776	1.17	collagen, type X, alpha 1
ILMN_1665865	1.17	insulin-like growth factor binding protein 4
ILMN_1721626	0.89	AT rich interactive domain 5B (MRF1-like)
ILMN_1736178	0.53	AE binding protein 1
ILMN_1759598	0.49	distal-less homeobox 5
ILMN_1682775	0.35	endothelin 1
ILMN_1783182	0.32	fibrillin 1
ILMN_1765578	0.10	TCDD-inducible poly(ADP-ribose) polymerase
ILMN_1651354	-1.15	secreted phosphoprotein 1
ILMN_2129927	-1.27	exostoses (multiple) 1
ILMN_1746085	-1.58	insulin-like growth factor binding protein 3
ILMN_1785699	-1.58	parathyroid hormone-like hormone
ILMN_2396875	-1.59	insulin-like growth factor binding protein 3
ILMN_2314169	-1.92	parathyroid hormone-like hormone

<sup>a</sup> These are the same data as shown in Fig. 3c and are sorted in the same order: highest to lowest gene expression levels for TCPS\_OS 14d as compared to TCPS 1d.

<sup>b</sup> There are 38 Illumina probes represented in this table and they correspond to 29 unique Gene Ontology terms.

<sup>c</sup> Values are log<sub>2</sub> of the ratio of fluorescence intensity for 'the average of the four TCPS\_OS 14d specimens' to 'the mean value for the four TCPS 1d controls'.