ABSTRACT: In this study, we investigated whether multipotent (human-bone-marrow-derived mesenchymal stem cells [hBM-MSCs]) and pluripotent stem cells (murine-induced pluripotent stem cells [iPSCs] and murine embryonic stem cells [ESCs]) respond to nanocomposite fibrous mats of poly(l-lactic acid) (PLLA) loaded with 1 or 8 wt % of calcium-deficient nanohydroxyapatite (d-HAp). Remarkably, the dispersion of different amounts of d-HAp to PLLA produced a set of materials (PLLA/d-HAp) with similar architectures and tunable mechanical properties. After 3 weeks of culture in the absence of soluble osteogenic factors, we observed the expression of osteogenic markers, including the deposition of bone matrix proteins, in multi/pluripotent cells only grown on PLLA/d-HAp nanocomposites, whereas the osteogenic differentiation was absent on stem-cell-neat PLLA cultures. Interestingly, this phenomenon was confined only in hBM-MSCs, murine iPSCs, and ESCs grown on direct contact with the PLLA/d-HAp mats. Altogether, these results indicate that the osteogenic differentiation effect of these electrospun PLLA/d-HAp nanocomposites was independent of the stem cell type and highlight the direct interaction of stem cell-polymeric nanocomposite and the mechanical properties acquired by the PLLA/d-HAp nanocomposites as key steps for the differentiation process.

INTRODUCTION

The nanocomposite technology offers the opportunity to modulate the surface, mechanical, and physicochemical properties of a biopolymeric matrix.\(^1\)\(^-\)\(^5\) Several polymers such as polycaprolactone, poly(l-lactic acid) (PLLA), polyglycolic acid, and poly(lactic-co-glycolic acid, have been successfully combined with calcium phosphates,\(^6\)\(^-\)\(^7\) glass ceramics, metal oxides,\(^8\)\(^-\)\(^10\) or carbon nanotubes,\(^8\)\(^-\)\(^10\) to develop a more bioactive material for biomedical applications and, in turn, to explore their effect on stem cell fate.\(^11\)\(^-\)\(^16\) Among the putative building compounds, calcium phosphates including hydroxyapatite, tricalcium phosphate, and biphasic calcium phosphate, due to their bone bioactive potential (e.g., osteoinductivity, osteoconductivity and osteointegration), are widely employed as bone-grafting materials for promoting bone healing and stem-cell osteogenic differentiation.\(^17\)\(^-\)\(^24\) This emerges by the work of several authors who using fibrous or solid-walled PLLA in combination with hydroxyapatite obtained the osteogenic differentiation of mesenchymal stem cells.\(^19\)\(^,\)\(^21\)\(^,\)\(^23\) Similarly, electrospun PLLA substrates combined with collagen or nanohydroxyapatite have also been considered to assess the osteogenic differentiation of human umbilical cord blood stem cells.\(^20\)\(^,\)\(^27\)
Recently, Bianco et al.28 produced a PLLA-based micro-fibrous nanocomposite loaded with different amounts (1 or 8 wt %) of synthesized Ca-deficient nano-hydroxyapatite (d-HAp) by means of electro-spinning technique generating a set of materials with similar architecture and tunable mechanical properties.28 The d-HAp was used as a nanofiller due to its remarkable closer similarity to the inorganic natural component of bone and teeth.29,30 Moreover, with respect to stoichiometric HA (s-HAp, Ca/P = 1.667), d-HAp (Ca/P molar ratio ranging from 1.5 to 1.667) offers the advantage of higher solubility, enhancing ion-exchange ability and increasing efficiency in inducing the precipitation of bone-like apatite.28,29

Additionally Zhou et al. demonstrated that the presence of d-HAp is critical to proper functioning of PLA-based electropun mats and that its incorporation decreased PLA fiber diameters, accelerated PLA degradation with the consequent buffered pH decrease, and improved the bioactivity and biocompatibility of the scaffold.31

In this study, we explored the response of multipotent or pluripotent stem cells to PLLA fibrous mats containing 1 and 8 wt % d-HAp with respect to neat PLLA in the absence of soluble exogenous osteogenic inducers.

We selected adult human bone-marrow-mesenchymal stem cells (hBM-MSCs) as representative multipotent stem cells and on the basis of their capacity to generate differentiated cells even if they are cultured on biomaterials.32−35 As pluripotent stem cells, we chose murine-induced pluripotent stem cells (iPSCs). These stem cells may be generated in vitro from somatic cells36 and offer the potential advantage to produce patient-specific donor cells for cell replacement or tissue engineering applications.11,37 Finally, we adopted murine embryonic stem cells (ESCs) as natural stem cell control of iPSCs and based on their pluripotency capability.35,37

Our results showed that PLLA/d-HAp nanocomposites have an active role in inducing human multipotent (hBM-MSCs) and murine pluripotent (iPSCs and ESCs) stem cell differentiation toward the osteogenic lineage in the absence of exogenous soluble differentiating agents.

The lack of osteogenic differentiation of both murine pluripotent and human multipotent stem cells cultured on neat PLLA under the above experimental conditions addresses this result to the new properties acquired by the PLLA/d-HAp nanocomposites.

■ EXPERIMENTAL SECTION

Synthesis of Calcium Deficient Hydroxyapatite Nanopowders (d-HAp). The synthesis of d-HAp was previously reported in detail.28,37 In brief, d-HAp nanopowders were prepared in a double-walled jacket reactor at 40 °C, by precipitation. A stoichiometric volume of Ca(NO3)2·4H2O (99.2%, Sigma-Aldrich) aqueous solution (1 M) was added dropwise to (NH4)2HPO4 (99.2%, Sigma-Aldrich) aqueous solution (1 M) to form CaHPO4·2H2O (99.2%, Sigma-Aldrich) aqueous solution. The pH was continuously monitored and adjusted to 9.0 ± 0.1 by adding NH3·H2O conc. Precipitates were aged in mother liquor for 24 h, washed with NH3·H2O aqueous solution, and vacuum-filtered. Wet as-prepared nanopowders were washed in ethanol and dried in air at room temperature (RT) for 48 h (as-dried nanopowders) to remove absorbed water.

Electrospinning of PLLA and PLLA/d-HAp Suspensions. As-dried d-HAp nanopowder was first suspended in chloroform/12-hydroxystearic acid (HSA, C18H36O3,Sigma-Aldrich) used as surfactant, the concentration being 0.5% w/v, to promote the effective ceramic powder dispersion. The suspension was ultrasonicated for 2 to 3 h. Successively, PLLA (inherent viscosity 0.90 to 1.2 dL/g, Lactel Absorbable Polymers) was added (15% w/v in CHCL3), and the suspension was magnetically stirred for 36 h. Nanocomposites with different contents of d-HAp (1 or 8 wt %) were prepared according to our previous investigations.28

PLLA/d-HAp suspensions were poured in a glass syringe and electropun through a 18G blunt-tip needle in the following conditions: applied voltage 16 kV (Spellman), needle-target distance 10 cm, and feed rate 1 mL/h (KD Scientific). After deposition, the collected membranes were detached from the target by ethanol immersion, dried under vacuum, and stored in a desiccator. As a reference sample, a neat PLLA mat was also produced following the same experimental procedure. The collected mats were designed according to the d-HAp content ratio with respect to PLLA: PLLA (0 wt % d-HAp), PLLA/1d-HAp (1 wt % d-HAp), and PLLA/8d-HAp (8 wt % d-HAp).

Microstructure of PLLA and PLLA/d-HAp Electropun Mats. The microstructure of electropun mats was investigated by scanning electron microscopy (SEM; Leo Supra 35). To evaluate the distribution of the ceramic phase within the polymeric mats, we performed energy-dispersive spectroscopy (EDS) mapping of Ca and P (size 24.3 × 18.2 μm²) (INCA Energy 300, Oxford ELXII detector). The average fiber diameter was determined considering 100 randomly selected fibers from SEM micrographs (ImageJ, NIH). The mean void size was estimated by processing SEM micrographs by means of a custom-made software. The 2-D void size was calculated as the average diameter of equivalent circles having the same area of voids among fibers.28,39

Human-Bone-Marrow Mesenchymal Stem Cells: Isolation and Culture. hBM-MSCs were isolated and cultured as previously described.34,35 In brief, bone marrow cells were taken from the medullary cavities of the femurs of patients undergoing primary total hip replacement. Informed consent was obtained from all donors, and the institutional ethical committee approved the procedures. Mononuclear cells were isolated from bone marrow samples by density gradient on Lympholyte (Cedarlane Laboratories Limited). Human bone mononuclear cells were seeded in 25 cm² tissue-culture flasks at a density of 2.5 × 10⁴ cells/mL and grew in RPMI-1640 (Euroclone) containing 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, and 100 U/mL of penicillin-streptomycin (Euroclone) (control medium). Tissue cultures were incubated at 37 °C in a humidified atmosphere with 5% CO₂. After a 5−7 days of culture, nonadherent cells were discarded, and fresh medium added. After 15 days, fibroblast-like colonies became visible. The medium was changed every 3 days.

Murine Embryonic Stem Cells and Induced Pluripotent Stem Cells Culture. Undifferentiated mouse R1 ESCs and mouse iPSCs (from Stem Cell Research Institute, Leuven, Belgium) were expanded on irradiation-inactivated embryonic fibroblasts (STO cells, Shan Maika, Austin, University of Texas) for 10 days in complete DMEM medium (Invitrogen) containing 20% FBS (Hyclone), 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 5 × 10⁻⁵ M 2-mercaptoethanol, penicillin G (100 U/mL), and streptomycin (10 mg/mL) (all from Invitrogen) and supplemented with leukemia inhibiting factor (LIF, 50U/mL, Chemicon International). This expansion process on LIF maintains the cells in their undifferentiated state.

Cell colonies were lightly trypsinized and plated by 20 μL of hanging drops into bacteriological-grade Petri dishes in culture medium without LIF. The absence of the feeder layer and LIF stimulated the formation of free-floating cell aggregates (embryoid bodies). After 5 days, the embryoid bodies were collected and resuspended in medium containing α-modified Eagle’s medium containing 15% FBS and 100 U/mL of penicillin-streptomycin (Euroclone).

Spacing of PLLA and PLLA/HAp Mats with Stem Cells. Neat PLLA, PLLA/1d-HAp, and PLLA/8d-HAp mats were sterilized through immersion in pure ethanol for 30 min, followed by three rinses in PBS and then deposited in a 24-well plate. We seeded 50 μL of hBM-MSCs suspension (2 × 10⁵ cells), or 5 days embryonic bodies from ESCs and iPSCs (at a density of 2 × 10⁶ cells/mL) were seeded...
dropwise on sterilized electropun mats. We added 500 µL of culture medium to each well. The medium was changed every 3 days. Similar experiments were performed seeding stem cells on tissue culture polystyrene (TCP5) as internal control. Samples were harvested and further analyzed at each time point. All of the experiments were performed five times in triplicates.

**Osteogenic Differentiation of Stem Cells on PLLA and PLLA/d-HAp.** hBM-MSCs and 5 day old embryoid bodies seeded on neat PLLA and PLLA/d-HAp nanocomposite mats were incubated with the appropriate basal growth medium (BM) for 21 days in a humidified incubator at 37 °C and 5% CO2. The medium was changed every 3 days.

As control of osteogenic differentiation, similar experiments were performed maintaining stem cell-mats cultures in osteogenic medium (OM) using MSCs differentiation basal medium-osteogenic supplemented with the SingleQuots containing: dexamethasone, l-glutamine, ascorbate, pen/strep, mesenchymal cell growth supplement (MCGS), and β-glycerophosphate (Lonzza).

In some experiments stem cells were cultured on transwells (Corning), seeding hBM-MSCs on the bottom of the transwell and the PLLA-derivative patch on the transwell insert to avoid a direct interaction of stem cells with the investigated materials. Cell number and the PLLA-derivative patch dimension were according to the other experiments. Cultures were maintained for 21 days only in the presence of basal medium; then, stem cells were investigated for osteogenic differentiation. All of the experiments were performed five times.

**Cells Viability Assay.** Cell viability was evaluated by incubating 2 × 10^6 cells/mL cultured on PLLA, PLLA/1d-HAp and PLLA/8d-HAp at different time points with XTT (2,3-bis[2-methoxy-4-nitro-5-sulphophenyl]-2H-tetrazolium-5-carboxanilide inner salt) (Sigma-Aldrich) according to the manufacturer’s recommendation. The absorbance of the samples was measured using a microtiter plate reader (ELISA reader, GDV) at 450 nm with a reference wavelength at 650 nm.

**Histological Analysis.** Upon culturing, both neat and hybrid mats were fixed with 4% paraformaldehyde, embedded in Tissue-Tek OCT (Sakura), snap frozen, and cut into 15 µm slices. Slices were further processed for DAPI staining and images were captured with fluorescence microscopy (Eclipse-TE2000-S, Nikon) equipped with the F-ViewII FireWire camera (Soft Imaging System, Olympus) and Cell’F View image software (Olympus).

**Alkaline Phosphatase Staining.** Alkaline phosphatase (ALP) staining was used to assess the osteogenic differentiation. Cultures were washed with PBS twice and fixed with 4% paraformaldehyde dissolved in PBS for 10 min at RT. Cultures were washed in H2O and then incubated with 500 µL of FAST BCIP/NBT (Sigma-Aldrich) substrate solution for 2 h at RT. Cells were washed twice with distilled water, and photos were captured with a digital camera (PowerShot G10, Canon).

**Alizarin Red S Staining.** Alizarin red staining was used to assess the osteogenic differentiation. Cultures were washed with PBS twice and fixed with 4% paraformaldehyde dissolved in PBS for 10 min at RT. Cultures were washed in H2O and then incubated with 500 µL of Alizarin red staining (Lonza) solution for 20 min at RT. Cells were washed twice with distilled water, and photos were captured with a digital camera (PowerShot G10, Canon) and bright-field microscopy (Eclipse-TE2000-S, Nikon).

Quantitative analysis of Alizarin Red S staining was performed by determining OD_{560} values of stained cultures subjected to OM treatment and comparing these values to those obtained from control medium cultures. The procedures were performed according to the manufacturer’s instruction. Colorimetric quantification of Alizarin Red extracted solution was measured using a microtiter plate reader (ELISA reader, GDV) at 405 nm wavelength.

**Immunofluorescences.** Immunofluorescence experiments were performed as already described. In brief, paraformaldehyde fixed cells were permeabilized, blocked (PBS+10% FBS, 0.1% Triton X-100) for 1 h at RT and incubated when necessary with phalloidin (Alexa-fluor-488 phalloidin, Invitrogen) for 20 min. Then, the cells were further incubated overnight at 4 °C with other primary antibodies (i.e., anti-α-tubulin, Santa Cruz Biotechnology) and antivinculin (clone hVIN-1, Sigma-Aldrich)) for adhesion and morphological analyses, while osteogenic differentiation was marked using antibodies against: osteocalcin (OC) (Santa-Cruz Biotechnology), osteopontin (SPP1) (Sigma-Aldrich), and antitype I collagen (COL1) (Chemicon International). Finally, after staining with Alexa-fluor-594 nm rhodamine, nuclei were counterstained with DAPI (Vector Laboratories).

Images were acquired with fluorescence microscopy (Eclipse-TE2000-S, Nikon) using the F-ViewII Fire Wire camera (Soft Imaging System, Olympus).

**Extraction of the ECM Proteins and ELISA Assay.** To measure the amount of the extracellular matrix proteins after cell cultures, we treated mats of neat PLLA, PLLA/1d-HAp, and PLLA/8d-HAp as previously reported. The protein concentration was evaluated by the BCA protein assay kit (Pierce Biotechnology). After matrix extraction, the mats were incubated, once again, for 24 h at 37 °C with 1 mL of sample buffer. No protein content was detected.

A 96-well plate was coated with 100 µL (20 µg/mL) of the extracted extracellular matrix proteins in 50 mM Na2CO3 pH 9.5 at 4 °C overnight. After washes with PBS containing 0.1% (v/v) Tween-20 and incubation with blocking solution (PBS containing 2% (w/v) BSA) at 22 °C, coated samples were incubated at 22 °C for 2 h with the L. Fisher antibodies against: type-I and -III collagen, decorin, osteonectin, osteocalcin, anti-fibronectin rabbit polyclonal antiserum (http://csdb.nidcr.nih.gov/csdb/antisera.htm, National Institutes of Health, Bethesda, MD), and fibronectin rabbit antihuman polyclonal IgG were used for the determination of human bone proteins. Anti-alkaline-phosphatase, -decorin, -osteonectin, and -fibronectin rabbit antimouse polyclonal antibodies (Santa-Cruz Biotechnology) were used for the quantification of murine bone matrix. Murine type-I collagen and osteopontin were measured using L. Fisher antibodies. Murine type-III collagen and osteocalcin were not evaluated due to the unavailability of the antibodies. After washing, immune-complexes were incubated with 100 µL of HRP-conjugated goat anti-rabbit IgG at 22 °C for 1 h and finally incubated with development solution (phosphate-citrate buffer with o-phenylenediaminedihydrochloride substrate). Reactions were stopped with 100 µL of 0.5 M H2SO4 and the absorbance was measured at 490 nm with a microplate reader (BioRad Laboratories). The amount of extracellular matrix proteins was expressed as pg/(cell × mats) and referred to a calibration curve performed with each purified ECM protein.

**Real-Time RT-PCR.** Total RNA from cells seeded on different mats (1 × 10^6 cells plated on neat PLLA, PLLA/1d-HAp, and PLLA/8d-HAp) after treatment in OM and in control cultures, was isolated using an Rneasy Mini Kit (Qiagen). Reverse transcription was carried out using 1 µg of total RNA in the presence of 200U of Super Script LI Reverse Transcriptase and 10 ng/mL of random hexamers as reverse primers (Invitrogen). Real-time RT-PCR was performed using the primers detailed in Table S1 in the Supporting Information file and the SYBR Green Master Mix (Strategene) in an Mx 3000P (Stratagene), in accordance with the manufacturer’s procedures. The relative quantification of mRNA of each gene was determined by the comparative 2-ΔΔCt method where the target is normalized to the endogenous reference 18S rRNA. The ΔCt was determined by subtracting the Ct of 18S rRNA from the Ct of the target. Reactions were carried out in triplicate.

**Field Emission Scanning Electron Microscopy (FE-SEM).** Cell-matrix interactions were also evaluated by FE-SEM, after 21 days of culture. The cells were rinsed twice with PBS and fixed in 2.5% glutaraldehyde for 30 min at RT. Samples were dehydrated by adding progressively more concentrated ethanol (from 5 to 100% v/v) every 5 min. The final dehydration in pure ethanol was followed by critical point dryer (CPD, Emitech K550). Once dried, the samples were gold sputter-coated before examination by FE-SEM (Supra 5.0 Zeiss), at an accelerating voltage of 5 kV.

**Statistical Analysis.** All data were reported as the mean value ± mean standard error of each group. Data were the mean of five
independent experiments. One-Way ANOVA test with Bonferroni’s multiple comparison test (prism version 4, 2004 edition; GraphPad) was used for statistical analysis, and \( p < 0.05 \) was considered significant.

**RESULTS**

**Microstructure of PLLA and PLLA/d-HAp Electrospun Mats.** Neat PLLA (0 wt % d-HAp) and PLLA loaded with 1 or 8 wt % d-HAp were fabricated as described in the Experimental Section and characterized as reported by Bianco et al.\(^{28}\)

SEM micrographs of electrospun PLLA and PLLA/d-HAp samples showed architecture with randomly oriented fibers (Figure 1A). Nanocomposites loaded with 1 wt % d-HAp (PLLA/1d-HAp) presented the most homogeneous microstructure (Figure 1A; EDS mapping Figure 1A, a,b). The increase in d-HAp to 8 wt % (PLLA/8d-HAp) led to inhomogeneous fiber morphology associated with the presence of microsized agglomerates rich in Ca and P located on or within the polymeric fibers (Figure 1A; EDS mapping Figure 1A, c,d).\(^{28}\) Neat PLLA and PLLA/d-HAp nanocomposite mats were composed of micrometric and submicrometric fibers that delimited 2-D voids sized 8 and 14 \( \mu \text{m} \), respectively. (See Figure 1B for average fibers diameter and 2-D void size.) The bimodal diameter distribution can be associated with a process instability occurring during fiber deposition. Both neat PLLA and PLLA/d-HAp mats showed a peculiar topography characterized by nanopores distributed along the fiber surface as a result of a phase separation in the electrospun that led to polymer-rich regions and solvent-rich regions, the latter being responsible for the formation of nanopores.\(^{28}\)

Dynamic mechanical properties (storage modulus and loss modulus) of the investigated substrates showed a decreasing trend with the increase in the d-HAp content. The storage modulus values \( (E') \) were 90, 30, and 10 MPa, and the loss modulus values \( (E'') \) were 8.7, 2.8, and 1.2 MPa for PLLA, PLLA/1d-HAp, and PLLA/8d-HAp, respectively.\(^{28}\)

**hBM-MSC Interaction with PLLA and PLLA/d-HAp Mats.** First, we evaluated the viability of hBM-MSCs cultured on neat PLLA, PLLA/1d-HAp, and PLLA/8d-HAp mats. Results indicated comparable levels of the mitochondrial dehydrogenase activity on hBM-MSCs cultured in BM on PLLA and TCPS, whereas a slight decrease in the enzyme activity was detected on stem cells seeded on PLLA/d-HAp mats (Figure 2A). All PLLA-derivative mats-stem cell cultures were void of cell debris or necrosis signs during the culture period.

Next, we assessed the interaction of hBM-MSCs with each substrate (neat PLLA, PLLA/1d-HAp, and PLLA/8d-HAp). All 3-D mats provided sufficient space for cell adhesion, distribution, growth, and engraftment into the deeper layers of their microarchitecture (Figure 2B,C).

hBM-MSCs cultured on PLLA/d-HAp and neat PLLA showed a similar cytoskeleton organization with F-actin-containing fibers arranged as straight, cable-like bundles crossing the cytoplasm along the fiber axes. Stem cells exhibited cellular protrusion extending in multiple directions because of vinculin adhesion spots with exposition of a slightly branched shape, perhaps as an effect of multiple PLLA fibers adhesion (Figure 2B, representative images). Moreover, DAPI staining of OCT slices revealed similar cellular ingrowth with a decreasing local cell number from the surface to the bottom and a comparable cell density in all mats (Figure 2C, representative images).

**Osteogenic Differentiation of hBM-MSCs Seeded on PLLA and PLLA/d-HAp Mats.** hBM-MSCs were cultured on those materials for 21 days in BM and analyzed for the osteogenic differentiation to investigate the osteoinductive capability of PLLA and PLLA/1d-HAp and PLLA/8d-HAp. As control, we conducted the same experiments in the presence of OM. The differentiation was evaluated by (i) FE-SEM analysis (Figure 3), (ii) expression of osteogenic genes (Figure 4A), (iii) alkaline phosphatase activity (Figure 4B,C), and (iv)
deposition (Table 1) and organization of osteogenic matrix proteins (Figure S2 on Supporting Information).

FE-SEM analyses performed after 21 days, revealed similar cellular morphology and interaction with PLLA/d-HAp nanocomposite and neat PLLA in both basal and OM (Figure 3).

Stem cells cultured on PLLA/d-HAp mats in BM expressed the highest levels of BGLAP and BMP-2 genes and a comparable level of RUNX-2 gene with respect to stem cells cultured on neat PLLA and TCPS (Figure 4A). As expected under OM condition, stem cells (see Figure S1 in the Supporting Information for cell viability curve) expressed high levels of BGLAP and BMP-2 genes and low level of RUNX-2 gene expression in all of the mats used (PLLA/d-HAp and neat PLLA cultures) (Figure 4A).

Large ALP positive staining areas were visualized in nanocomposite PLLA/d-HAp mats, even in the absence of OM (Figure 4B, line BM). No ALP staining was detected in neat PLLA under the same culture conditions (Figure 4B, line BM). These results were confirmed by an ELISA assay and showed a significant enzyme increase (two- to four-fold) in cells grown in BM on PLLA/d-HAp compared with those cultured on neat PLLA and TCPS (Figure 4C). So far, under OM culture conditions, levels of ALP were highest on all mats and TCPS (Figure 4C).

After 3 weeks under BM conditions, a sustained wide range deposition of bone ECM proteins (type I collagen, osteonectin, decorin, type III collagen, osteopontin, osteocalcin, and fibronecin) was detected in cells cultured on PLLA/d-HAp mats compared with that measured on neat PLLA-cell cultures (Table 1, column BM). These results were further enhanced under OM conditions, where hBM-MSCs cultured on all mats consistently released the analyzed bone ECM proteins (Table 1, column OM).

Additional evidence of the PLLA/d-HAp osteoinductive properties were by osteopontin and type I collagen immunofluorescence analyses. As expected, osteopontin and type I collagen were highly organized within all cell cultures independently of the substrates under OM culture conditions (Figure S2, column OM in the Supporting Information).
Notably, a significant matrix protein organization was detected on PLLA/d-HAp cultures even under basal growth conditions (Figure S2, column BM in the Supporting Information).

**Osteogenic Differentiation of Murine iPSCs and ESCs Seeded on PLLA and PLLA/d-HAp Mats.** As to whether the PLLA/d-HAp had an osteogenic differentiation effect on pluripotent stem cells, we conducted similar experiments using murine iPSCs and ESCs.

First, we investigated the osteogenic differentiation aptitude of murine iPSCs and ESCs. Pluripotent stem cells were cultured as described in the Experimental Section under the Yamanka condition.\(^3\) iPSCs generated were carefully analyzed for their pluripotency and cell biology characteristics.\(^4\) Five day old embryoid bodies (Figure 5A) were isolated, plated on TCPS, and induced to differentiate toward osteogenic lineages (Figure 5B–E). After 21 days under osteogenic induction, iPSCs released OC, expressed osteogenic genes, strongly increased ALP expression, and accumulated Ca\(^{2+}\) within the cells, as shown by Alizarin Red S micrographs and quantification (Figure 5B–E; line iPSCs). Similar findings were observed in cultures of ESCs treated under the same experimental conditions (Figure 5B–E; line ESCs).

Next, we evaluated the iPSCs and ESCs viability on neat PLLA and PLLA/d-HAp mats, in both BM and OM. Results revealed similar levels of mitochondrial dehydrogenase activity in iPSCs and ESCs cultured on all mats and TCPS (Figure 6A).

Then, we explored the osteogenic differentiation potential of iPSCs and ESCs on PLLA-derivative mats under BM and OM. We found expression of osteogenic genes Spp1, Bglap, and Runx-2 (Figure 6B) and a sustained deposition in a wide range of relevant ECM proteins (decorin, fibronectin, osteonectin, osteopontin, type I collagen) in iPSCs cultured on PLLA/d-HAp compared with cells cultured on neat PLLA under BM culture (Table 2, column BM). These results (gene expression and ECM deposition) were comparable to those obtained treating iPSCs with OM (Figure 6B; Table 2, column OM).

ESCs recapitulated the cell behavior of iPSCs to each mat. In basal medium, osteogenic genes and ECM proteins were expressed only by ESCs cultured on PLLA/d-HAp (Figure 6B; Table 3, column BM; Figure S3 on Supporting Information, BM), whereas those osteogenic markers were highly increased under OM conditions in ESCs cultured on all substrates (Figure 6B; Table 3, column OM; Figure S3 on Supporting Information, OM).

The overall data demonstrated that PLLA/d-HAp nano-composite mats supported the osteogenic differentiation of iPSCs and ESCs in vitro in the absence of soluble osteogenic inducers (supplied by appropriate OM). Because similar data were obtained using hBM-MSCs, we assumed that the potential osteoinductive activity of PLLA/d-HAp mats is independent of the stem cell type.

**Effect of the PLLA and PLLA/d-HAp Mats on Stem Cell Differentiation.** Next, we explored how PLLA/d-HAp caused multi/pluripotent stem cell osteogenic differentiation in the absence of soluble osteogenic inducers.
First, we investigated the relevance of the direct interaction of stem cells with PLLA/d-HAp or neat PLLA for the differentiation process. We adopted the transwell culture system to maintain stem cells and materials separated but in the same microenvironment (Figure 7A). After 21 days of culture in BM, stem cells were evaluated for osteogenic differentiation. Alizarin Red S assay showed the absence of calcium deposits in cells cultured on PLLA/d-HAp and neat PLLA (Figure 7B). Slight signals were comparable to that observed on control cultures (stem cells cultured on TCPS). The absence of osteogenic differentiation was confirmed by the lack of ALP staining in all cell cultures (data not shown).

Remarkably, these experiments also indicated that levels of Ca$^{2+}$ released by the PLLA/d-HAp nanocomposites in the culture medium were below the level required for the osteogenic induction.

Then, we investigated the significance of the dynamic mechanical properties of neat PLLA and PLLA/d-HAp with the expression of osteogenic markers. As reported in Section 3.1, the dispersion of increased amounts of d-HAp to PLLA modified the dynamic mechanical properties of the polymer matrix, the values of the storage modulus and loss modulus, according to the following trend: neat PLLA > PLLA/1d-HAp > PLLA/8d-HAp.28

Table 1. ECM Protein Deposition by hBM-MSCs Cultured on PLLA-Derivative Mats

<table>
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<th>markers</th>
<th>BM</th>
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<td>18 ± 1</td>
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<td>37 ± 2$^b$</td>
<td>64 ± 3$^b$</td>
<td>38 ± 2$^b$</td>
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<td>6 ± 2$^b$</td>
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<td>18 ± 2$^b$</td>
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<tr>
<td>osteonectin</td>
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</tbody>
</table>

$^a$ELISA quantification of total bone ECM protein released by hBM-MSCs seeded on neat and nanocomposite PLLA mats cultured for 21 days in the absence (BM)/presence (OM) of osteogenic medium. Only PLLA/d-HAp cultured in the absence of soluble osteogenic agents induces hBM-MSCs to release high levels of decorin, fibronectin, osteocalcin, osteonectin, osteopontin, type I collagen, and type III collagen. High levels of ECM proteins were still detected in the presence of osteogenic medium in all mats. $^b$p < 0.05 versus PLLA BM.
Here we reported the correlation of the deposition levels of each ECM protein (selected as representative differentiation markers) released by hBM-MSCs, murine iPSCs, and ESCs with the storage modulus ($E'$) of the substrate (selected as a representative mechanical property) on which stem cells were plated (Figure S4 in the Supporting Information). We observed
that the level of ECM proteins was highest in multipotent and pluripotent stem cells cultured on mat with the lowest $E$ (PLLA/8d-HAp), sharply lower in mat with intermediate $E$ (PLLA/1d-HAp) and lowest in mat with the highest $E$ (PLLA), thus validating the osteogenic potential of the mats containing d-HAp (PLLA/d-HAp) compared with neat PLLA (Figure S4 in the Supporting Information).

## DISCUSSION

In this work we demonstrated the potential of PLLA/d-HAp nanocomposite fibrous mats with respect to neat PLLA to drive human multipotent (hBM-MSCs) and murine pluripotent (iPSCs and ESCs) stem cells toward osteogenic lineage in the absence of exogenous soluble differentiating agents.

Despite the fact human BM-MSCs, murine iPSCs and ESCs showed comparable cell adhesion and morphology either on neat PLLA or on PLLA/d-HAp platforms, we found the expression of osteogenic markers only in stem cells cultured on PLLA/d-HAp mats: (i) levels of RUNX-2 gene; (ii) up-regulation of BGLAP and BMP-2 gene expression; (iii) high expression of ALP activity; (iv) a significant deposition of ECM proteins (osteonectin, osteocalcin, osteopontin, decorin, type I collagen and type III collagen); and (v) high cellular organization of type I collagen and osteopontin.

Notably, under these experimental conditions (BM) the osteogenic differentiation process was recapitulated by stem cells cultured on PLLA containing either 1 or 8 wt % d-HAp, thereby suggesting that even d-HAp as low as 1 wt % was sufficient to activate the molecular signals for the onset of differentiation mechanisms. Conversely, when we conducted the same experiments on a transwell system, to maintain stem cells on the bottom of the transwell and materials on the transwell insert to avoid a direct interaction but together in the same milieu, the osteogenic induction was lost in all cultures.

**Table 2. ECM Protein Deposition by Murine iPSCs Cultured on PLLA-Derivative Mats**

<table>
<thead>
<tr>
<th>markers</th>
<th>PLLA</th>
<th>PLLA/1d-HAp</th>
<th>PLLA/8d-HAp</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BM</td>
<td>OM</td>
<td>BM</td>
</tr>
<tr>
<td>decorin</td>
<td>1.56 ± 0.08</td>
<td>4.5 ± 1.0b</td>
<td>2.33 ± 0.03b</td>
</tr>
<tr>
<td>fibronectin</td>
<td>1.30 ± 0.07</td>
<td>3 ± 1.0b</td>
<td>1.7 ± 1.0b</td>
</tr>
<tr>
<td>osteonectin</td>
<td>0.49 ± 0.03</td>
<td>2.3 ± 1.0b</td>
<td>0.90 ± 0.08b</td>
</tr>
<tr>
<td>osteopontin</td>
<td>1.7 ± 0.1</td>
<td>3.6 ± 1.0b</td>
<td>2.4 ± 0.2b</td>
</tr>
<tr>
<td>type I collagen</td>
<td>5.4 ± 1.1</td>
<td>11.7 ± 2.3b</td>
<td>7.4 ± 1.8b</td>
</tr>
</tbody>
</table>

**Table 3. ECM Protein Deposition by Murine ESCs Cultured on PLLA-Derivative Mats**

<table>
<thead>
<tr>
<th>markers</th>
<th>PLLA</th>
<th>PLLA/1d-HAp</th>
<th>PLLA/8d-HAp</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BM</td>
<td>OM</td>
<td>BM</td>
</tr>
<tr>
<td>decorin</td>
<td>1.90 ± 0.07</td>
<td>4.9 ± 1.0b</td>
<td>2.9 ± 0.8b</td>
</tr>
<tr>
<td>fibronectin</td>
<td>1.20 ± 0.04</td>
<td>3.9 ± 0.8b</td>
<td>1.50 ± 0.06b</td>
</tr>
<tr>
<td>osteonectin</td>
<td>0.42 ± 0.01</td>
<td>2.1 ± 1.0b</td>
<td>0.70 ± 0.01b</td>
</tr>
<tr>
<td>osteopontin</td>
<td>0.98 ± 0.02</td>
<td>1.99 ± 0.11b</td>
<td>1.06 ± 0.07b</td>
</tr>
<tr>
<td>type I collagen</td>
<td>5.9 ± 1.7</td>
<td>9.9 ± 2.5b</td>
<td>6.8 ± 2.0b</td>
</tr>
</tbody>
</table>

**Figure 7.** Absence of osteogenic differentiation in cultures of stem cells and PLLA or PLLA/d-HAp in a transwell system. Absence of Alizarin Red S positive staining in PLLA/1d-HAp and PLLA/8d-HAp indicates the absence of osteogenic stem cell differentiation. No signal was observed on PLLA and TPSC cultures.
is likely that the biological responsiveness to be effective requires the direct interaction between the seeded stem cells and the PLLA/d-HAp substrates. In fact, these experiments might exclude the putative involvement of Ca ions released in the culture medium by PLLA/d-HAp as osteogenic differentiation agent and highlight the mechanical properties acquired by the neat PLLA when combined with d-HAp as requested players for the activation of the differentiative process.

It has been shown that the dispersion of increased amount of d-HAp to PLLA generated a PLLA/d-HAp nanocomposites with decreased mechanical properties (neat PLLA > PLLA/1d-HAp > PLLA/8d-HAp). This trend was inversely recapitulated by the expression levels of osteogenic markers (e.g., deposition levels of the ECM proteins released by hBM-MSCs, murine iPSCs and ESCs cultured on PLLA/d-HAp) that increased with the d-HAp nanofiller amount and with the consequent storage modulus decrement.

These observations are in agreement with several reports describing the effect of chemico-physical, topographical, and mechanical properties of biomaterials on the induction of stem cell differentiation as well as on the preservation of their stem cell status. Indeed, evidence that stem cells are able to convert mechanical cues on biochemical signals and in turn modulate their fate is a relatively recent acquisition. Currently, the molecular basis of this phenomenon (mechanotransduction) is not fully understood. However, the overall reports converge on similar conclusions. They propose that interactions of cell cytoskeleton with a substrate generate forces that are transferred to the nucleus through the actin-intermediate filament system to modulate the gene expression.

Altogether, our results support these concerns because they indicate an involvement of the mechanical features of PLLA/d-HAp in the mechanisms that induced osteogenic differentiation of human multipotent and murine pluripotent stem cells.

CONCLUSIONS

Here we showed that the interaction of human multipotent and murine pluripotent stem cells with PLLA/d-HAp mats in the absence of soluble osteogenic factors resulted in their osteogenic differentiation. Because of the absence of osteogenic induction in both multipotent and pluripotent stem cells cultured on neat PLLA, we addressed the biological response to the dispersion of different amounts of d-HAp into PLLA that produced a set of materials with similar architecture and tunable mechanical properties.

Compared with other papers analyzing the differentiation of iPSCs seeded on different materials, our results represent the first evidence showing the osteogenic differentiation of iPSCs on PLLA/d-HAp in the absence of osteogenic factors.

Despite future experiments needed to draw conclusions for biomedical applications, given the tremendous impact of iPSCs for the generation of patient-specific stem cells, our results could give a relevant contribution for novel regenerative medicine-based applications.

ASSOCIATED CONTENT

Supporting Information

Additional added data show: (i) viability of hBM-MSCs of seeded on neat PLLA and PLLA/d-HAp in OM by XTT assay; (ii) expression of osteogenic markers by hBM-MSCs cultured on neat PLLA and PLLA/d-HAp; (iii) expression of osteogenic markers by iPSCs and ESCs cultured on neat PLLA and PLLA/d-HAp; and (iv) correlation of ECM protein vs storage modulus in stem-cell-material culture.

This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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REFERENCES
