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Biomaterialized matrix-assisted osteogenic differentiation of human embryonic stem cells

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Abstract

The physical and chemical properties of a matrix play an important role in determining various cellular behaviors, including lineage specificity. We demonstrate that the differentiation commitment of human embryonic stem cells (hESCs), both *in vitro* and *in vivo*, can be solely achieved through synthetic biomaterials. hESCs were cultured using mineralized synthetic matrices mimicking a calcium phosphate (CaP)-rich bone environment differentiated into osteoblasts in the absence of any osteogenic inducing supplements. When implanted *in vivo*, these hESC-laden mineralized matrices contributed to ectopic bone tissue formation. In contrast, cells within the corresponding non-mineralized matrices underwent either osteogenic or adipogenic fate depending upon the local cues present in the microenvironment. To our knowledge, this is the first demonstration where synthetic matrices are shown to induce terminal cell fate specification of hESCs exclusively by biomaterial-based cues both *in vitro* and *in vivo*. Technologies that utilize tissue specific cell-matrix interactions to control stem cell fate could be a powerful tool in regenerative medicine. Such approaches can be used as a tool to advance our basic understanding and assess the translational potential of stem cells.

Keywords

Embryonic Stem Cells; Osteogenic differentiation; Mineralization; Bone mimetic matrices; Biomaterial

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Conflict of interest

Authors declare no conflict of interest.

Introduction

Human embryonic stem cells (hESCs), with unlimited self-renewal potential and the ability to differentiate into all cell types in the human body, hold great promise to treat various diseases and tissue loss.¹ In addition to regenerative therapy, hESC-based tissue models could also be used as a tool to study developmental processes and disease progression, and as drug-screening platforms.^{2, 3} Despite the progress made over recent years, widespread application of hESCs has been impeded by several challenges, of which the main concern is a lack of approaches to direct their differentiation into targeted cells. Advancements over the years have led to the development of a number of strategies ranging from small molecules to mRNAs to genetic manipulations to direct differentiation of hESCs into various phenotypes.⁴⁻⁶ While the importance of soluble factors has been extensively documented, emerging studies show that physical and chemical cues in the matrix could play an equally important role in directing stem cell differentiation and function.⁷⁻¹²

Matrix-assisted differentiation and transplantation of stem cells to treat compromised tissues and organs has enormous potential in wound and fracture healing, tissue regeneration, and restoration of organ functionality.¹³⁻¹⁵ A number of physical and chemical properties of the matrix such as functional groups, hydrophobicity, topography, and rigidity have been identified to influence stem cell differentiation.⁸⁻¹² However, most of these studies utilize matrix cues in conjunction with a medium containing inductive factors to direct differentiation, thus making it difficult to delineate the contributions of individual components.⁸ Furthermore, the extent to which the matrix-based cues can direct differentiation of more naïve stem cells like embryonic stem cells to terminally differentiated cells remains unclear.

In this study, we employ a synthetic matrix recapitulating the calcium phosphate (CaP)-rich bone microenvironment to determine if synthetic matrix-based cues alone are sufficient to direct osteogenic differentiation of hESCs. Previously, we and others have shown that biomaterials containing CaP moieties can promote osteogenic differentiation of osteoprogenitor cells and adult stem cells like mesenchymal stem cells (MSCs).¹⁶⁻²⁵

However, the role of CaP minerals in directing osteogenic commitment of hESCs is not clear. An efficient osteogenic differentiation of hESCs is often achieved through the derivation of mesenchymal progenitor cells and/or culturing them in a medium containing osteogenic inducing components such as β -glycerophosphate, ascorbic acid 2-phosphate, dexamethasone, and/or bone morphogenetic proteins (BMPs).²⁶⁻³² Studies have also utilized the incorporation of bone specific matrix components, such as hydroxyapatite and decellularized bone matrices, in concert with osteogenic-inducing soluble factors to promote the osteogenic differentiation of hESCs and hESC-derived progenitor cells.^{26, 28, 29}

We demonstrate that synthetic matrices containing CaP minerals induce osteogenic differentiation of hESCs in both two-dimensional (2-D) and three-dimensional (3-D) culture conditions in a basal growth medium lacking any osteogenic inducing components. When subcutaneously implanted *in vivo*, the hESC-laden mineralized matrices contributed to ectopic bone formation without teratomas. On the contrary, cells in non-mineralized

matrices underwent either osteogenic or adipogenic fate in response to the local biomaterial-based cues.

Experimental

Materials

Poly(ethylene glycol)-diacrylate (PEGDA) and acryloyl 6-aminocaproic acid (A6ACA) were prepared as previously described.^{9, 33} Briefly, 5.29 mmol of poly(ethylene glycol) (32 g for $M_n = 6$ kDa or 18 g for $M_n = 3.4$ kDa; Sigma Aldrich, catalog numbers: 81260 and 202444, respectively) was dissolved in 300 mL of toluene at 125 °C, followed by refluxing for 4 hours with vigorous stirring. Azeotropic distillation was used to remove the traces of water in the reaction mixture. On cooling this solution to room temperature (~25 °C), 180 mL of anhydrous dichloromethane followed by 1.623 mL (11.64 mmol, 2.2. equivalents) of triethylamine were added with vigorous stirring. The reaction mixture was then transferred to an ice bath to attain 4 °C. To this reaction mixture, 0.942 mL (11.64 mmol, 2.2 equivalents) of acryloyl chloride in 15 mL of anhydrous dichloromethane was added in drop-wise manner for 30 minutes. Following an additional 30 minutes at 4 °C, the reaction mixture was raised to 45 °C and kept overnight. The reaction mixture was filtered at 25 °C through diatomaceous earth to remove quaternary ammonium salt. The filtrate was concentrated using a rotary evaporator and precipitated in excess diethyl ether. Precipitated product was redissolved in dichloromethane and reprecipitated in diethyl ether. The resultant PEGDA was filtered and dried under vacuum at 25 °C for 24 hours. The dried PEGDA was further purified using a Sephadex fine G-25 column (GE Healthcare Life Sciences, catalog number: 17-0032-01) and lyophilized. The PEGDA product was characterized using NMR. ¹H NMR (300 MHz, CDCl₃): 6.39-6.36 ppm (d, 2H), 6.17-6.11 ppm (dd, 2H), 5.93-5.91 ppm (d, 2H), 4.28-4.26 (t, 4H), 3.75-3.74 ppm (m, 8H), 3.62 ppm (broad), 3.48-3.47 ppm (t, 4H)

N-acryloyl 6-aminocaproic acid (A6ACA) was prepared as described by Ayala *et al.*⁹ Briefly, 13.11 g (100 mmol) of 6-aminocaproic acid (Sigma Aldrich, catalog number: A2504) and 4.4 g (110 mmol, 1.1 equivalents) of NaOH were dissolved in 80 mL of deionized (DI) water at 4 °C under vigorous stirring. To this, 8.937 mL (110 mmol, 1.1 equivalents) of acryloyl chloride in 15 mL of anhydrous tetrahydrofuran was added dropwise for 30 minutes. The pH of the reaction mixture was maintained between 7.5 and 7.8 until the reaction was complete. The pH of the reaction mixture was then decreased to pH 3 using 6 N HCl at 0 °C, and the solution was extracted using ethyl acetate. The organic layers were collected, combined, and dried over sodium sulfate. The solution was then filtered, concentrated, and precipitated in petroleum ether. The resultant A6ACA was further purified by silica gel (100-200 mesh) column chromatography using 1% methanol in ethyl acetate as the eluent mixture. The purified product was collected and dried overnight prior to use and characterized using NMR. ¹H NMR (300 MHz, CDCl₃): 6.17-6.12 ppm (dd, 1H), 6.07-6.03 ppm (d, 1H), 5.64-5.62 ppm (d, 1H), 3.17-3.14 ppm (t, 2H), 2.08-2.05 ppm (t, 2H), 1.49-1.42 ppm (dd, 4H), 1.25-1.19 ppm (t, 4H)

Synthesis of poly(ethylene glycol) diacrylate-co-acryloyl 6-aminocaproic acid (PEGDA-co-A6ACA) hydrogels—

PEGDA-co-A6ACA hydrogels were synthesized by reacting 1 M A6ACA and 4% PEGDA ($M_n = 6$ kDa) with 0.5% ammonium persulfate (APS) and 0.15% N,N,N',N'-tetramethylethylenediamine (TEMED) in a glass mold with 1 mm spacer for 15 minutes at 25 °C.¹⁶ The resulting hydrogels were incubated in phosphate buffered saline (PBS) overnight with two changes of PBS. The equilibrium-swollen hydrogels were then cut into discs of 1 cm². To create bulk rigidity-matched biomineralized matrices, the above procedure was repeated except 2% PEGDA was used in the precursor solution.¹⁶ The hydrogel discs were biomineralized as described elsewhere.¹⁶ Briefly, hydrogel discs were incubated in DI water for 6 hours and then in modified simulated body fluid (m-SBF) for 6 hours. The hydrogel discs were then rinsed with DI water and incubated in a 40 mM Ca²⁺ and 24 mM HPO⁴³⁻ solution (pH = 5.2) at 25 °C for 45 minutes on a rotating shaker (VWR Mini-shaker) at 200 rpm. The discs were rinsed with DI water, incubated in m-SBF at 37 °C for 48 hours with a daily exchange of m-SBF followed by incubation in PBS for 6 hours. Both non-mineralized and mineralized hydrogels were sterilized through immersion in 70% ethanol (EtOH) for 6 hours, followed by washing in PBS for 4 days with three daily exchanges of PBS prior to cell culture.

Synthesis of PEGDA-co-A6ACA macroporous hydrogels—We used PEGDA-co-A6ACA macroporous hydrogels synthesized by PMMA microsphere templating³⁴ for the 3-D cell culture. The PMMA templates were fabricated using a cylindrical polypropylene mold measuring 8 mm in diameter and filled with 60 mg of PMMA microspheres (Mean diameter = 165 μm, Bangs Laboratories, Catalog number: BB05N). The PMMA bead column was vigorously tapped for 1 minute to facilitate packing of the microspheres. Further, 60 μL of a mixture consisting of 20% acetone and 80% EtOH was gently added into the mold. The mold was incubated at 25 °C for 1 minute to soften the PMMA microspheres allowing them to fuse together, then at 80 °C for 1 hour to evaporate the solvent. A precursor solution of 0.5 M A6ACA and 20% PEGDA ($M_n = 3.4$ kDa) with 0.3% Irgacure was poured and allowed to infiltrate the PMMA-packed mold under vacuum for 5 minutes. The mixture was polymerized using UV light at a wavelength of 365 nm (intensity of 42 W/cm²) for 10 minutes. The PMMA template was dissolved in acetone for 3 days with three daily exchanges of acetone. Upon leaching from the PMMA templates, the macroporous hydrogels were gradually hydrated using acetone–water mixtures with decreasing concentrations of acetone (100 to 0%) for one day. The macroporous hydrogels were washed in PBS for 6 hours and punched into cylindrical constructs measuring 5 mm in diameter by 2 mm in height for 3-D *in vitro* cultures and 7 mm in diameter by 2 mm in height for subcutaneous implantation. Mineralization of macroporous hydrogels was conducted as described earlier.¹⁷ Macroporous hydrogels were incubated in m-SBF for 6 hours, then, in a 40 mM Ca²⁺ and 24 mM HPO⁴³⁻ solution (pH = 5.2) under vacuum for 5 minutes to facilitate the infiltration of solution, and finally moved to a rotating shaker at 25 °C for 30 minutes at a speed of 200 rpm. These treated macroporous hydrogels were rinsed with DI water, incubated in m-SBF at 37 °C for 48 hours with a daily change of m-SBF, and immersed in PBS for 6 hours. The non-mineralized and mineralized macroporous hydrogels were sterilized using 70% EtOH for 6 hours and washed with PBS for 4 days prior to cell culture.

Scanning electron microscopy (SEM) and energy dispersive spectra (EDS)—

To examine the morphology and composition of mineralized matrices, they were rinsed in DI water for 5 minutes, sliced into flat pieces, flash-frozen in liquid nitrogen, and lyophilized overnight. Prior to SEM imaging, the samples were subjected to an Ir coating for 7 seconds using Emitech sputter coater (Emitech, K575X). The SEM images were acquired using Philips XL30 ESEM. The Ca/P atomic ratio was obtained from elemental analysis using Oxford Energy Dispersive Spectra (EDS) with INCA software. The pore diameter of macroporous hydrogels in their dried state was measured from three different SEM images using ImageJ. The pore diameters of non-mineralized and mineralized macroporous hydrogels in the swollen state were measured from three different bright-field images using ImageJ. Roughly 10-15 pores within each image were analyzed to determine the pore diameter. The data are presented as means $\pm$ standard errors.

Cell culture—Human embryonic stem cells (hESCs; HUES9 and H9) were cultured on mitotically inactivated mouse embryonic fibroblasts (MEFs) using Knockout DMEM (Life Technologies, catalog number: 10829-018) supplemented with 10% Knockout Serum Replacement (KSR; Life Technologies, catalog number: 10828028), 10% human plasmonate (Talecris Biotherapeutics), 1% non-essential amino acids, 1% penicillin/streptomycin, 1% Gluta-MAX, and 55 μ M 2-mercaptoethanol as previously described.³⁵ Cells were passaged using Accutase (Millipore) at approximately 80% confluency and supplemented with fresh media containing 30 ng/mL of bFGF (Life Technologies) daily.

Prior to seeding hESCs, the sterilized hydrogel discs, macroporous hydrogels, and cell culture grade coverslips (CS; diameter = 15 mm, Fisherbrand, catalog number: 1254582) were coated with matrigel (BD Biosciences, catalog number: 354277), following the manufacturer's protocol, to promote cell adhesion. Briefly, matrigel solution was thawed overnight at 4 °C and diluted with chilled DMEM solution at 1:86 ratio. All matrices (mineralized and non-mineralized hydrogels and coverslips) were then coated with matrigel solution at 4 °C for overnight followed by incubating at 37 °C for 1 hour. For 2-D cultures, cells were seeded onto the matrices at an initial cell density of 1×10^4 cells/cm² and cultured in a growth medium consisting of high glucose DMEM, 5% fetal bovine serum (FBS; hyclone), 4 mM L-glutamine, and 50 U/mL penicillin/streptomycin at 37 °C and 5% CO₂ with a media change every two days. For 3-D cell culture involving macroporous hydrogels, the hydrogels were partially dried for 1 hour to reach a weight loss of approximately 40% prior to plating hESCs; the weight loss is attributed to the evaporation of water from the macroporous hydrogels. 10 μ L of cell suspension containing 3×10^5 cells (HUES9 or H9) was seeded into the macroporous hydrogels and incubated at 37 °C and 5% CO₂ for 2 hours to allow for cell infiltration, followed by adding 1.5 mL of growth medium.

Calcium and phosphate assays—To determine Ca²⁺ and PO₄³⁻ contents of the mineralized matrices, samples were rinsed with DI water for 5 minutes to remove non-bound CaP moieties followed by lyophilization for 24 hours. The lyophilized samples were homogenized in 0.5 M HCl by vortexing at 25 °C for 3 days. To probe the dissolution of CaP moieties from the mineralized matrices, matrigel-coated and non-coated mineralized matrices were incubated in 1.5 mL of 50 mM Tris-HCl buffer (pH = 7.4) at 37 °C for 7

days; 300 μ L of incubation media was collected and replaced with fresh solution on a daily basis. The measurements of Ca^{2+} and PO_4^{3-} were conducted for matrix homogenates and collected media. For the calcium assay, 20 μ L of sample solution was mixed with 1 mL of assay solution (Pointe Scientific, catalog number: C7503) and its absorbance was measured at 570 nm using a UV-vis spectrophotometer (Beckman Coulter, DU 730). The phosphate assay was conducted by following a method described elsewhere.³⁶ Briefly, 125 μ L of sample solution was mixed with 1 mL of assay solution consisting of 1 part 10 mM ammonium molybdate, 2 parts acetone, and 1 part H_2SO_4 followed by 100 μ L of 1 M citric acid. Absorbance of the solution was measured at 380 nm using a UV-vis spectrophotometer.

Immunofluorescent staining—To evaluate the pluripotency of hESCs prior to culture, the cells were stained for NANOG and OCT4. Osteogenic differentiation of hESCs was examined by staining for osteocalcin (OCN). For immunofluorescent staining, the cells were fixed with 4% paraformaldehyde at 25 °C for 10 minutes and blocked for 30 minutes in a buffer solution containing 3% bovine serum albumin (BSA) and 0.1% Triton™ X-100 at 25 °C. The fixed cells were incubated with primary antibodies against NANOG (1:200; rabbit polyclonal, Santa Cruz Biotechnology, catalog number: sc-33759), OCT4 (1:200; rabbit polyclonal, Santa Cruz Biotechnology, catalog number: sc-9081) or OCN (1:100; mouse monoclonal, Abcam, catalog number: ab13420) in a blocking solution at 4 °C for 16 hours. After washing in PBS, the cells were incubated with secondary antibodies (1:250; goat anti-rabbit Alexa Flour 647, Life Technologies or goat anti-mouse Alexa Flour 568, Life Technologies) for 60 minutes and rinsed with PBS. The nuclei were stained with Hoechst 33342 (2 μ g/mL; Life Technologies, catalog number: H1399) at 25 °C for 5 minutes, mounted onto glass slides with Vectashield, and imaged using a fluorescence microscope (Axio Observer.A1, Carl Zeiss). Images were taken in the linear mode for the same exposure time and the background was uniformly subtracted from all images using ImageJ. For F-actin staining, the fixed cells were incubated with phalloidin (1:250; Alexa Flour 288, Life Technologies) in a blocking buffer solution at 25 °C for 60 minutes. F-actin staining images after 3 days of culturing were used to quantify the circularity of the cells using the equation below.¹⁶ Approximately 30 cells from three different images were used for the calculation.

$$\text{Circularity} = \frac{4\pi A}{p^2} \quad (1)$$

where A and p represent the cellular area and perimeter, respectively.

Quantitative Reverse Transcription Polymerase chain reaction (qRT-PCR)—

Following RNA extraction of homogenized samples in TRIzol, 1 μ g of RNA was reverse-transcribed to cDNA using iScript cDNA synthesis kit (Bio-Rad). Human Osteogenesis PCR array (RT² Profiler™ PCR Array Human Osteogenesis, SA Biosciences, catalog number: PAHS-026) was used to determine the changes in the transcription profiles of the hESCs cultured on various matrices. The heat map was generated to compare relative expressions for each gene with the colors red (high), green (low), and black (medium). The differentiated hESCs were also characterized for a number of target genes such as RUNX2, OCN, SPPI,

and NANOG as a function of culture time via qPCR. Quantitative real-time PCR (qPCR) reactions were performed using SYBR select master mix (Life technologies, catalog number: 4472908) and the ABI Prism 7700 Sequence Detection System (Applied Biosystems). Expression at each time point was normalized to the corresponding housekeeping gene (GAPDH) and to the undifferentiated hESCs, and expressed as fold change thereof. The primers used in qRT-PCR are listed in the Supplementary Table S1.

Live-dead assay—Live-dead assay for 3-D cultures was performed 3 days post cell seeding to examine cell viability (LIVE/DEAD Viability/Cytotoxicity kit, Life technologies, catalog number: L-3224). Cell-laden macroporous hydrogels were cut into thin slices and incubated in the assay solution containing 0.05 v/v% of calcein-AM and 0.2 v/v% of ethidium homodimer-1 in DMEM at 37 °C for 30 minutes. Matrix slices were then rinsed with PBS and imaged using a fluorescence microscope. The percentage of viable cells was determined by counting the live cells, which were stained with green dye, relative to the total number of cells. Images from 3-4 different sections were used to determine the viability. All 3-D matrices supported a similar cell viability of approximately 90%.

DNA assay—DNA assay was performed at 7 and 28 days of culture for HUES9 and H9-laden macroporous hydrogels to determine the cell numbers. Collected samples were frozen, lyophilized, and digested in 1 mL of papain solution consisting of 125 µg/mL of papain in 10 mM L-cysteine, 100 mM phosphate, and 10 mM EDTA at pH = 6.3 for 16 hours at 60 °C. DNA contents were measured by using Quant-iT PicoGreen dsDNA kit (Life technologies, catalog number: P11496) according to the manufacturer's protocol. Briefly, digested samples and PicoGreen reagent were diluted with TE buffer at 1:50 and 1:200 dilutions, respectively. One part each of sample solution and PicoGreen reagent were mixed and their fluorescence was measured at 535 nm using a multimode detector (Beckman Coulter, DTX 880). The DNA contents were normalized to the dry weight of the cell-laden matrix.

Subcutaneous implantation—Animal experiments were performed according to the protocols approved by Institutional Animal Care and Use Committee (IACUC) of University of California, San Diego and National Institute of Health (NIH). Macroporous hydrogels (7 mm in diameter and 2 mm in height) were seeded with 1 million hESCs (HUES9 and H9) and incubated in growth media for 6 days *in vitro*. For subcutaneous transplantation of the cell-laden matrices, 3-month-old immunodeficient NOD.CB17-Prkdc^{scid}/J mice were administered with ketamine (100 mg/kg) and xylazine (10 mg/kg), a 1-cm long incision was made in the back of the mice and four subcutaneous pouches (cranial-left and -right and caudal-left and -right) were constructed by blunt dissection using a 1-cm wide spatula. Four of either hESCs-laden non-mineralized or mineralized matrices were subcutaneously transplanted into the end of each pouch (n = 8 per group; left and right sides of cranial and caudal back of mice) and the skin was carefully sutured. After the surgery, all mice were housed in separated cages.

Micro-computed tomography (µCT) of subcutaneously implanted matrices—For µCT analysis, excised implants were fixed in 4% paraformaldehyde at 4 °C for 4 days

and dehydrated in an ethanol–water mixture with a gradual increase in ethanol until equilibrated in pure ethanol. The fixed implants were then dried and subsequently placed in 15 mL centrifuge tubes along with phantom calibration rods prior to measurements. Time-dependent hard tissue formation was evaluated at 4 and 8 weeks of post-implantation. Mineralized matrices prior to implantation were imaged using μ CT to account for the effect of mineralization. Matrices were scanned at 9 μ m/pixel resolution using SkyScan 1076 μ CT scanner (Bruker). Scans were reconstructed via NRecon software (SkyScan) and built into 3-dimensional models using CTAn software (SkyScan) after applying the same threshold values of 90–190 for all groups to selectively account for the minerals, while eliminating the background. Quantification of mineral density was performed as percentage of mineral volume per total volume of the matrix (BV/TV) with CTAn software. Videos for 3-dimensional objects in 360° rotation were constructed using CTVol software (SkyScan). Cross-sectional images in coronal, transverse, and sagittal planes at the center of the implants were analyzed with DataViewer and CTAn softwares.

Histology for 3-D *in vitro* cultured matrices and subcutaneously implanted matrices

—For histological analysis, cell-laden matrices were fixed with 4% paraformaldehyde at 4 °C for 1 day for *in vitro* cultures and 4 days for excised implants. The constructs were processed as reported elsewhere.³⁷ Briefly, the fixed samples were decalcified in 10% ethylenediaminetetraacetic acid (EDTA, Fluka, catalog number: 03677, pH = 7.3) at 4 °C for 3 hours for *in vitro* cultures and 2 days under gentle rotation for excised implants. The decalcified samples were washed gently with PBS.

For paraffin-embedded sections, demineralized samples were gradually dehydrated in an ethanol–water mixture with increasing ethanol concentrations until reaching pure ethanol. Following incubation of samples in CitriSolv (Fisher scientific, catalog number: 22-143975) for 1 hour with two changes, samples were transferred into a mixture of paraffin (Richard-Allan Scientific, catalog number: 8337) containing 5% poly(ethylene-co-vinyl acetate) (PEVA; vinyl acetate, 25 wt%; melt index: 19 g/10 minutes; Sigma Aldrich, catalog number: 437220) and incubated at 70 °C under vacuum for one day with three vent/vacuum cycles. Following embedding in paraffin-PEVA mixture, sample blocks were sliced into 8- μ m-sized sections using a rotary microtome (Leica, RM2255). The sections were briefly hydrated in DI water at 40 °C for 5 minutes and dried overnight at 37 °C. Prior to histological staining, sections were deparaffinized in CitriSolv at 60 °C for 15 minutes with two changes and hydrated in an ethanol-water mixture with decreasing ethanol concentrations. For H&E staining, rehydrated paraffin-embedded sections were incubated in a hematoxylin solution (Ricca Chemical Company, Gill 2, catalog number: 3536-16) for 5 minutes and thoroughly rinsed with DI water. Sections were then stained with Eosin-Y (Richard-Allan Scientific, catalog number: 7111) for 30 seconds, followed by three washes in DI water. The stained sections were visualized using a microscope under bright-field mode.

For immunohistochemical (IHC) staining of osteocalcin (OCN) and human-specific lamin B, rehydrated paraffin-embedded sections were subjected to antigen retrieval by incubating in 10 mM citric acid buffer (EMD Millipore, catalog number: CX 1725, pH = 6.0) at 85 °C for 20 minutes, followed by cooling at 25 °C for 20 minutes. Sections were washed in PBS,

treated with a blocking buffer solution containing 3% BSA and 0.1% Triton^{TM} X-100 at 25 °C for 60 minutes, and incubated overnight with primary antibodies against OCN (1:100; mouse monoclonal, Abcam, catalog number: ab13420) or lamin B (1:100; rabbit polyclonal, Thermo Scientific, catalog number: RB-10569-P0) in a blocking buffer at 4 °C. Following thorough washes in a mixture of PBS and 0.05 v/v% Tween 20 (PBS-Tween 20), sections were immersed in 3% hydrogen peroxide (Acros Organics, catalog number: 41188) for 7 minutes, followed by washing in PBS-Tween 20. Sections were exposed to horseradish peroxidase (HRP)-conjugated secondary antibodies (1:100; goat anti-mouse, Santa Cruz Biotechnology, catalog number: sc-2005 or donkey anti-rabbit, Jackson ImmunoResearch, catalog number: 711-035-152) in a blocking buffer at 25 °C for 60 minutes. After several washes in PBS-Tween 20, sections were developed in 3-3'-diaminobenzidine (DAB) peroxidase substrate (Vector Laboratories, catalog number: SK-4100) for 10 minutes to produce the brown reaction product. Following three times of washing in PBS-Tween 20, sections were imaged using a microscope in color mode under H-filter. For lamin B staining, mouse skin tissue was used as a negative control.

For frozen sections, demineralized samples were incubated in 20% sucrose solution and gradually embedded in OCT at 4 °C for 1 day. OCT-embedded samples were transferred into isopentane, frozen in liquid nitrogen, and sectioned into 12 µm-thick slices using cryostat (Leika CM3050). For Oil Red O staining, OCT-embedded frozen sections were dried in air for 30 minutes, incubated in DI water at 4 °C for 20 minutes to dissolve OCT, and air-dried for 10 minutes. Samples were immersed in propylene glycol (Sigma-Aldrich, catalog number: W294004) at 25 °C for 10 minutes to completely remove residual water. Sections were stained with 0.5% Oil Red O (Sigma-Aldrich, catalog number: O-0625) solution in propylene glycol at 60 °C for 10 minutes. Afterwards, stained sections were thoroughly washed in 85% propylene glycol, briefly rinsed in DI water, and subsequently imaged using a microscope in color mode under H-filter.

Statistical analysis—Statistical analyses were carried out using Graphpad Prism 5. Two-tailed Student's t-test was used to compare two groups. One-way analysis of variance (ANOVA) with the Tukey-Kramer post-hoc test was used to compare multiple groups in the same time point and two-way ANOVA with the Bonferroni post-hoc test was used to compare multiple groups across different time points, and p-values were obtained from each test.

Results and discussion

Development and characterization of mineralized matrices

Synthetic matrices containing CaP minerals (termed as mineralized matrices) were developed by mineralizing poly(ethylene glycol)-diacrylate hydrogels containing acryloyl-6-aminocaproic acid (A6ACA) moieties (PEGDA-co-A6ACA) as described elsewhere.³⁸ We used mineralized hydrogels for 2-D cultures and mineralized macroporous hydrogels for 3-D cultures. Macroporous hydrogels with interconnected pores were fabricated by poly(methyl methacrylate) templating.³⁴ The matrices were characterized for the presence of mineral content, morphology, calcium (Ca²⁺) and phosphate (PO⁴³⁻) contents, and dissolution of CaP minerals into Ca²⁺ and PO⁴³⁻ (Figs. 1, S1a-b, S2a-c).

As evident from the scanning electron microscopy (SEM) images, the matrix-bound CaP minerals showed a plate-like morphology (Fig. 1a,d). The presence of CaP minerals was further confirmed by elemental analysis (Fig. 1a,d) which was also used to measure Ca^{2+} and PO_4^{3-} contents on mineralized matrices (Fig. 1b-c,e-f). Elemental analysis revealed a Ca/P ratio of 1.40 and 1.57 for mineralized hydrogels and mineralized macroporous hydrogels, respectively. Similar to prior studies, CaP components of the mineralized matrices dissociate into Ca^{2+} and PO_4^{3-} when incubated in a medium lacking these ions (Figs. S1a-b, S2b-c).³⁹ Since the matrices were coated with matrigel to enhance the adhesion of hESCs, we compared the dissolution profiles between matrigel-coated and non-coated mineralized hydrogels and observed a similar dissolution profile (Fig. S1a-b). This suggests that the matrigel coating did not have any effect on CaP dissolution, which is in good agreement with a previous work.⁴⁰ The dissociation kinetics of the CaP minerals in conjunction with our previous report, including XRD analyses of these biomineralized matrices suggest that the CaP minerals exhibit a semicrystalline structure.^{41, 42} The pore size of the macroporous hydrogels was determined from the SEM and bright field images (Figs. 1d, S2a). The macroporous hydrogels exhibited a pore diameter of $77.7 \pm 6.7 \mu\text{m}$ in their dried state as determined from the SEM images. This corresponded to a pore diameter of $123.6 \pm 4.9 \mu\text{m}$ and $118.2 \pm 3.6 \mu\text{m}$ (estimated from the bright field images) for the non-mineralized and mineralized macroporous hydrogels, respectively, in their swollen state. This is in agreement with our previous report, which shows no significant reduction in pore size due to mineralization.¹⁷

Mineralized matrices induce osteogenic differentiation of hESCs in 2-D cultures

The effect of mineralized matrices on hESCs (HUES9) was examined and compared against that of corresponding non-mineralized matrices and cell culture grade coverslips. All the cultures were carried out in basal growth medium in the absence of any osteogenic inducing soluble factors. Pluripotent state of the hESCs prior to their culture on various matrices was characterized by their colony formation and staining for NANOG and OCT4 (Fig. S3). The hESCs adhered onto non-mineralized (NM) and mineralized (M) hydrogels and coverslips (CS) and were grown to confluence within 7 days of culture (Fig. S4a). As evident from F-actin staining at 3 days of post-plating, the cells adhered onto all surfaces with similar circularity (Fig. S4b). Analysis of gene expression profile of HUES9 cells demonstrated considerably higher osteogenic gene expression on mineralized matrices compared to non-mineralized matrices and coverslips after 28 days of culture (Fig. 2a). The gene expression profile was further confirmed by quantitative PCR (qPCR) analyses, for markers that are characteristic of cells undergoing osteogenesis, as a function of culture time. The qPCR analyses showed that HUES9 cells cultured on mineralized matrices consistently exhibited an upregulation of osteogenic markers such as RUNX2, OCN, and SPP1 (Fig. 2b). The mineralized matrix-assisted osteogenic differentiation was also evident in protein levels. Cells cultured on mineralized matrices stained positive for OCN, an osteoblast specific marker, with the intensity of OCN staining increasing with culture time (Figs. 2c, S5). No such OCN presence was observed on other substrates, suggesting that the mineral environment provided by the biomineralized matrix is sufficient to direct osteogenic differentiation of hESCs. Supplementary Figure S6 shows the F-actin staining of the cells on all substrates as a function of culture time. Concomitant with differentiation, the hESCs

showed downregulation of pluripotent marker (NANOG) (Fig. S1c). Downregulation of NANOG was observed on all substrates, indicating that none of the culture conditions under study are conducive for supporting pluripotency of HUES9 cells. This is in agreement with previous studies, which showed that the self-renewal of hESCs requires a combination of matrix-based cues and soluble factors such as basic fibroblast growth factor (bFGF).³⁵ While hESCs cultured on all substrates (non-mineralized and mineralized matrices and coverslips) showed downregulation of NANOG, only the cells cultured on mineralized matrices were found to undergo efficient osteogenic differentiation. This finding implies that although the hESCs cultured *in vitro* were poised to differentiate through a loss of pluripotent phenotype, their efficient differentiation into osteoblasts required a bone-specific mineral environment.

Mineralized matrices promote osteogenic differentiation of hESCs in 3-D cultures

Having established the potential of mineralized matrices to induce osteogenic differentiation of HUES9 cells in 2-D cultures, we subsequently evaluated the ability of mineralized matrices to direct osteogenic differentiation of hESCs in a 3-D environment. Mineralized macroporous hydrogel-assisted osteogenic differentiation was compared against that of non-mineralized macroporous hydrogels cultured under identical conditions. We have used two different cell lines, HUES9 and H9. Following 3 days after cell seeding, most of the cells were found to be viable in all matrices (Fig. S2d). DNA quantification as a function of post cell seeding suggested no significant change in cell numbers between the mineralized and non-mineralized matrices (Fig. S2e-f). Similar to the 2-D culture, hESCs on mineralized matrices showed distinct upregulation of osteogenic markers (RUNX2, OCN, and SPP1) (Fig. 3a-b). Immunohistochemical staining for OCN corroborated the findings from the gene expression (Fig. 3c-d). The cells cultured on all the matrices showed downregulation of pluripotent gene (NANOG) akin to 2-D culture systems (Fig. 3e-f). Despite the geometrical differences between the 2-D hydrogel assisted culture and 3-D macroporous hydrogel assisted culture, hESCs exposed to the mineralized matrices underwent osteogenic differentiation, thus underscoring the instructive cues that the mineralized environment provides to direct osteogenic commitment of hESCs.

In addition to the physical cues such as topographical cues, the mineralized matrices containing CaP minerals could contribute to osteogenesis of stem cells through biochemical signaling triggered by the local Ca^{2+} and PO_4^{3-} ions.⁴³⁻⁴⁵ As made evident by the dissolution studies, the CaP minerals readily dissociate into Ca^{2+} and PO_4^{3-} resulting in an increase in their local concentrations. The dissociation and precipitation of the CaP moieties responding to their local concentrations play not only a key role in the binding and release of various osteoinductive growth factors like BMPs,⁴⁶⁻⁴⁸ but will also regulate various signaling cascades involving Ca^{2+} and PO_4^{3-} ions. Studies have shown that the influx of Ca^{2+} ions through L-type calcium channels promotes osteogenic differentiation of MSCs.⁴⁹ Recently, we have shown the role of PO_4^{3-} -ATP-Adenosine signaling cascade in CaP-bearing matrix-mediated osteogenic differentiation of hMSCs.⁴²

Biomaterialized matrices support osteogenic differentiation of hESCs *in vivo*

To determine the potential of mineralized matrices to support the osteogenic differentiation of hESCs *in vivo*, hESC-laden matrices (hESC-laden mineralized and non-mineralized macroporous hydrogels) were subcutaneously implanted into 3-month-old NOD/SCID mice. Subcutaneous implantation is often used to assess the potential contribution of stem cells and scaffolds to *in vivo* bone tissue formation and to determine the pluripotency of hESCs.^{26, 28} The implants were retrieved after 4 and 8 weeks and analyzed for bone tissue formation. Gross images of the excised implants indicated hard tissue formation in all implants with significant differences (Fig. 4a-b). 3-D micro-computed tomography (μ CT) image analyses concurred with the above observations wherein cell-laden mineralized matrices exhibited homogeneous and dense calcification at 4 and 8 weeks post-implantation (Fig. 4c-d, Supplementary Videos S1-2). Unlike cell-laden mineralized matrices, their non-mineralized counterparts showed non-uniform calcification concentrated along the periphery of the implants. The μ CT analyses of mineralized matrices prior to implantation indicated that the implants themselves were not radio-opaque and thus, were not detectable in μ CT (Fig. S7). 3-D μ CT models with 360° views and 2-D cross-sectional images confirmed uniform mineral formation throughout the porous network by hESCs coupled with mineralized matrices and non-uniform mineralization for their non-mineralized counterparts (Fig. S8a-b, Supplementary Videos S1-2). Furthermore, the hESC-laden mineralized matrices were found to have higher mineral densities (BV/TV) compared to their non-mineralized counterparts (Fig. 4c-d, bar graphs).

To verify whether the hard tissue formation observed by 3-D μ CT analyses is indeed due to bone tissue formation, histological examinations were conducted. H&E staining of the cell-laden mineralized matrices demonstrated progressive accumulation of dense extracellular matrix (ECM) that resembles bone tissue at 4 and 8 weeks post-implantation (Fig. 5a-b, right panels). High magnification images demonstrated that a majority of cells were populated along the periphery of newly assembled ECM for cell-laden mineralized matrices at 8 weeks post implantation. The bone tissue formation was further confirmed by OCN staining, where the hard tissue layer within the cell-laden mineralized matrices was stained positive for OCN at 4 and 8 weeks post implantation, revealing *in vivo* bone tissue formation (Fig. 5c-d, right panels). In stark contrast, hESCs-laden non-mineralized counterparts mainly displayed a mixture of fibrous and fat tissues with a thin layer of bone tissue in the mineralized peripheral region of the implant (Fig. 5a-b, left panels, Fig. S9a). Intense Oil Red O staining for lipid molecules further confirmed the presence of fat cells in non-mineralized matrices (Fig. 5e-f, left panels). Amongst the two cell lines, HUES9-laden non-mineralized matrices were found to have higher Oil Red O staining compared to H9-laden matrices. This difference in adipogenic differentiation could be attributed to innate variations in different hESC lines with HUES9 cells retaining a higher adipogenic potential. This is consistent with previous studies that demonstrated cell line-dependent differentiation of pluripotent stem cells.⁵⁰ The human origin of the cells within the cell-laden implants after 8 weeks of transplantation was confirmed by human-specific lamin B staining, which reveals the survival and contribution of transplanted hESCs towards tissue formation *in vivo* in all matrices (Fig. S9b); however, the involvement of infiltrated host cells could not be ruled out. Previously, we have shown the infiltration of host cells into mineralized

macroporous hydrogels and their contribution to ectopic bone tissue in rats, but no such infiltration into the non-mineralized counterparts was observed.¹⁷ Although a long-term investigation is warranted, the *in vivo* findings demonstrate survival and continuous differentiation of transplanted cells responding to the local cues without teratoma formation. Previous studies involving hESCs have shown that they form teratomas by 7 weeks when subcutaneously implanted with biomaterials.²⁶ In this study, the lack of teratoma formation after 8 weeks of implantation suggests that cells lost their pluripotency within the synthetic matrices.

A study by Both *et al.* has reported a lack of *in vivo* bone formation from hESCs transplanted using porous biphasic calcium phosphate ceramics, even with preconditioning of cells in osteogenic-inducing medium for one week prior to their implantation.⁵¹ The observed differences could be attributed to the cell lines and/or the matrix used. The dissolution kinetics of CaP minerals have been thought to play a key role in the ability of CaP-based matrices to direct the osteogenic commitment of stem and progenitor cells and assist in bone tissue formation.^{52, 53} A recent study by Yuan *et al.* has shown that subtle differences in the crystalline phase of CaP ceramics could endow them with different levels of osteoinductive function *in vivo*.⁵⁴

Conclusion

In summary, the results described in this article demonstrate that synthetic matrices, recapitulating a bone-like mineral rich environment, can direct the osteogenic commitment of pluripotent hESCs both *in vitro* and *in vivo*. Altogether, our results show for the first time that the differentiation of human embryonic stem cells into terminal phenotypes can be solely induced by synthetic matrices both *in vitro* and *in vivo*. Such synthetic matrix-mediated stem cell commitment will not only accelerate the translational potential of hESCs, but also provide a platform to elucidate the underlying molecular mechanisms that regulate pluripotent stem cell commitment and the role of the extracellular matrix in this process.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Notes and references

1. Odorico JS, Kaufman DS, Thomson JA. Stem cells. 2001; 19:193–204. [PubMed: 11359944]
2. Murry CE, Keller G. Cell. 2008; 132:661–680. [PubMed: 18295582]
3. Wobus AM, Boheler KR. Physiological reviews. 2005; 85:635–678. [PubMed: 15788707]
4. Borowiak M, Maehr R, Chen S, Chen AE, Tang W, Fox JL, Schreiber SL, Melton DA. Cell stem cell. 2009; 4:348–358. [PubMed: 19341624]
5. Zhu S, Wurdak H, Wang J, Lyssiotis CA, Peters EC, Cho CY, Wu X, Schultz PG. Cell stem cell. 2009; 4:416–426. [PubMed: 19427291]

6. Pang ZP, Yang N, Vierbuchen T, Ostermeier A, Fuentes DR, Yang TQ, Citri A, Sebastiano V, Marro S, Sudhof TC, Wernig M. *Nature*. 2011; 476:220–223. [PubMed: 21617644]
7. Marklein RA, Burdick JA. *Advanced materials*. 2010; 22:175–189. [PubMed: 20217683]
8. Benoit DS, Schwartz MP, Durney AR, Anseth KS. *Nature materials*. 2008; 7:816–823.
9. Ayala R, Zhang C, Yang D, Hwang Y, Aung A, Shroff SS, Arce FT, Lal R, Arya G, Varghese S. *Biomaterials*. 2011; 32:3700–3711. [PubMed: 21396708]
10. Dalby MJ, Gadegaard N, Tare R, Andar A, Riehle MO, Herzyk P, Wilkinson CD, Oreffo RO. *Nature materials*. 2007; 6:997–1003.
11. Engler AJ, Sen S, Sweeney HL, Discher DE. *Cell*. 2006; 126:677–689. [PubMed: 16923388]
12. Huebsch N, Arany PR, Mao AS, Shvartsman D, Ali OA, Bencherif SA, Rivera-Feliciano J, Mooney DJ. *Nature materials*. 2010; 9:518–526.
13. Lutolf MP, Gilbert PM, Blau HM. *Nature*. 2009; 462:433–441. [PubMed: 19940913]
14. Lutolf MP, Hubbell JA. *Nat Biotechnol*. 2005; 23:47–55. [PubMed: 15637621]
15. Hwang Y, Phadke A, Varghese S. *Regenerative medicine*. 2011; 6:505–524. [PubMed: 21749208]
16. Phadke A, Shih YRV, Varghese S. *Macromolecular bioscience*. 2012; 12:1022–1032. [PubMed: 22760917]
17. Phadke A, Hwang Y, Kim S. Hee, Kim S. Hyun, Yamaguchi T, Masuda K, Varghese S. *Eur Cell Mater*. 2013; 25:114–129. [PubMed: 23329467]
18. Yuan H, Fernandes H, Habibovic P, de Boer J, Barradas AM, de Ruiter A, Walsh WR, van Blitterswijk CA, de Bruijn JD. *Proceedings of the National Academy of Sciences*. 2010; 107:13614–13619.
19. Song G, Habibovic P, Bao C, Hu J, van Blitterswijk CA, Yuan H, Chen W, Xu HH. *Biomaterials*. 2013; 34:2167–2176. [PubMed: 23298780]
20. Roberts SJ, Geris L, Kerckhofs G, Desmet E, Schrooten J, Luyten FP. *Biomaterials*. 2011; 32:4393–4405. [PubMed: 21421268]
21. Choi S, Murphy WL. *Biotechnology journal*. 2013; 8:496–501. [PubMed: 23420758]
22. Chou YF, Huang W, Dunn JC, Miller TA, Wu BM. *Biomaterials*. 2005; 26:285–295. [PubMed: 15262470]
23. Vaquette C, Ivanovski S, Hamlet SM, Hutmacher DW. *Biomaterials*. 2013; 34:5538–5551. [PubMed: 23623428]
24. Müller P, Bulnheim U, Diener A, Lüthen F, Teller M, Klinkenberg ED, Neumann HG, Nebe B, Liebold A, Steinhoff G. *Journal of cellular and molecular medicine*. 2008; 12:281–291. [PubMed: 18366455]
25. Cameron K, Travers P, Chander C, Buckland T, Campion C, Noble B. *Journal of Biomedical Materials Research Part A*. 2013; 101:13–22. [PubMed: 22733430]
26. Marolt D, Campos IM, Bhumiratana S, Koren A, Petridis P, Zhang G, Spitalnik PF, Grayson WL, Vunjak-Novakovic G. *Proceedings of the National Academy of Sciences*. 2012; 109:8705–8709.
27. Levi B, Hyun JS, Montoro DT, Lo DD, Chan CK, Hu S, Sun N, Lee M, Grova M, Connolly AJ. *Proceedings of the National Academy of Sciences*. 2012; 109:20379–20384.
28. Hwang NS, Varghese S, Lee HJ, Zhang Z, Elisseeff J. *Tissue Engineering Part A*. 2013; 19:1723–1732. [PubMed: 23510052]
29. de Peppo GM, Marcos-Campos I, Kahler DJ, Alsalman D, Shang L, Vunjak-Novakovic G, Marolt D. *Proceedings of the National Academy of Sciences*. 2013; 110:8680–8685.
30. Sottile V, Thomson A, McWhir J. *Cloning & Stem Cells*. 2003; 5:149–155. [PubMed: 12930627]
31. Bielby RC, Boccaccini AR, Polak JM, Buttery LD. *Tissue engineering*. 2004; 10:1518–1525. [PubMed: 15588411]
32. Smith LA, Liu X, Hu J, Ma PX. *Biomaterials*. 2010; 31:5526–5535. [PubMed: 20430439]
33. Hwang Y, Zhang C, Varghese S. *Journal of Materials Chemistry*. 2010; 20:345–351.
34. Bryant SJ, Cuy JL, Hauch KD, Ratner BD. *Biomaterials*. 2007; 28:2978–2986. [PubMed: 17397918]
35. Chang CW, Hwang Y, Brafman D, Hagan T, Phung C, Varghese S. *Biomaterials*. 2013; 34:912–921. [PubMed: 23131532]

36. Heinonen JK, Lahti RJ. *Anal Biochem.* 1981; 113:313–317. [PubMed: 6116463]
37. Lim JI, Lee Y-K. *Micron.* 2009; 40:756–760. [PubMed: 19473850]
38. Phadke A, Zhang C, Hwang Y, Vecchio K, Varghese S. *Biomacromolecules.* 2010; 11:2060–2068. [PubMed: 20690714]
39. Phadke A, Shih YR, Varghese S. *Macromolecular bioscience.* 2012; 12:1022–1032. [PubMed: 22760917]
40. Maxian SH, Di Stefano T, Melican MC, Tiku ML, Zawadsky JP. *J Biomed Mater Res.* 1998; 40:171–179. [PubMed: 9549611]
41. Phadke A, Zhang C, Hwang Y, Vecchio K, Varghese S. *Biomacromolecules.* 2010; 11:2060–2068. [PubMed: 20690714]
42. Shih Y-RV, Hwang Y, Phadke A, Kang H, Hwang NS, Caro EJ, Nguyen S, Siu M, Theodorakis EA, Gianneschi NC. *Proceedings of the National Academy of Sciences.* 2014; 111:990–995.
43. Ducheyne P, Radin S, King L. *Journal of biomedical materials research.* 1993; 27:25–34. [PubMed: 8380596]
44. Radin SR, Ducheyne P. *J Biomed Mater Res.* 1993; 27:35–45. [PubMed: 8380597]
45. Barradas AM, Monticone V, Hulsman M, Danoux C, Fernandes H, Tahmasebi Birgani Z, Barrere-de Groot F, Yuan H, Reinders M, Habibovic P, van Blitterswijk C, de Boer J. *Integrative biology : quantitative biosciences from nano to macro.* 2013; 5:920–931. [PubMed: 23752904]
46. Autefage H, Briand-Mesange F, Cazalbou S, Drouet C, Fourmy D, Goncalves S, Salles JP, Combes C, Swider P, Rey C. *Journal of biomedical materials research. Part B, Applied biomaterials.* 2009; 91:706–715.
47. Suarez-Gonzalez D, Barnhart K, Migneco F, Flanagan C, Hollister SJ, Murphy WL. *Biomaterials.* 2012; 33:713–721. [PubMed: 22014948]
48. Lee JS, Suarez-Gonzalez D, Murphy WL. *Adv Mater.* 2011; 23:4279–4284. [PubMed: 22039597]
49. Wen L, Wang Y, Wang H, Kong L, Zhang L, Chen X, Ding Y. *Biochemical and biophysical research communications.* 2012; 424:439–445. [PubMed: 22771798]
50. Allegrucci C, Young LE. *Hum Reprod Update.* 2007; 13:103–120. [PubMed: 16936306]
51. Both SK, van Apeldoorn AA, Jukes JM, Englund MC, Hyllner J, van Blitterswijk CA, de Boer J. *J Tissue Eng Regen Med.* 2011; 5:180–190. [PubMed: 20718035]
52. LeGeros RZ. *Chemical reviews.* 2008; 108:4742–4753. [PubMed: 19006399]
53. Osathanon T, Linnes ML, Rajachar RM, Ratner BD, Somerman MJ, Giachelli CM. *Biomaterials.* 2008; 29:4091–4099. [PubMed: 18640716]
54. Yuan H, Fernandes H, Habibovic P, de Boer J, Barradas AM, de Ruiter A, Walsh WR, van Blitterswijk CA, de Bruijn JD. *Proceedings of the National Academy of Sciences of the United States of America.* 2010; 107:13614–13619. [PubMed: 20643969]

Figure 1.

Characterization of mineralized matrices. (a) SEM images and corresponding EDS of non-mineralized (NM) and mineralized (M) hydrogels. Scale bars represent 2 μm . Inset shows high magnification image. Scale bar represents 500 nm. (b) Ca^{2+} and (c) PO_4^{3-} amounts of NM and M hydrogels. (d) SEM images and corresponding EDS of NM and M macroporous hydrogels. Scale bars represent 100 μm . Inset shows high magnification image. Scale bar indicates 1 μm . (e) Ca^{2+} and (f) PO_4^{3-} contents of NM and M macroporous hydrogels. Data are presented as mean $\pm$ standard errors ($n = 3$). Two-tailed Student's t-test was used to compare two groups. Asterisks indicate statistical significances according to p-values (***: $p < 0.001$).

Figure 2. Mineralized matrix-assisted osteogenic differentiation of HUES9 cells in 2-D culture. (a) Transcription profile of HUES9 cells cultured for 28 days in growth medium on non-mineralized matrices (NM), mineralized matrices (M), and coverslips (CS). Relative expressions: red (high), black (medium), green (low). (b) RUNX2, OCN, and SPP1 gene expressions as a function of culture time for HUES9 cells on NM and M matrices and CS in growth medium. (c) Immunofluorescent staining for OCN (green), F-actin (red), and nucleus (Hoechst; blue) of HUES9 after 28 days of culture on NM and M matrices and CS. Scale bars represent 100 μ m. Data are presented as mean $\pm$ standard errors ($n = 3$). Multiple groups in the same time point were compared by one-way ANOVA with Tukey-Kramer post-hoc test. Asterisks indicate statistical significances according to p-values (*: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$).

Figure 3.

Mineralized matrix-assisted osteogenic differentiation of hESCs in 3-D culture. RUNX2, OCN, and SPP1 gene expressions for (a) HUES9 and (b) H9 after 14 and 28 days of culture in growth medium on non-mineralized (NM) and mineralized (M) matrices.

Immunohistochemical staining of OCN for (c) HUES9 and (d) H9 after 14 and 28 days of culture on NM and M matrices. Scale bars represent 100 μ m. NANOG gene expression for (e) HUES9 and (f) H9 after 14 and 28 days of culture on NM and M matrices. Data are shown as mean $\pm$ standard errors ($n = 3$). Two-tailed Student's t-test was used to compare two groups in the same time point. Two-way ANOVA with Bonferroni post-hoc test was used to compare multiple groups in different time points. Asterisks represent statistical significances with each p-value (*: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$).

Figure 4.

In vivo function of hESC-laden mineralized and non-mineralized matrices. Gross images of (a) HUES9-laden and (b) H9-laden non-mineralized (NM) and mineralized (M) matrices after 4 and 8 weeks of implantation. 3-D μ CT images of (c) HUES9-laden and (d) H9-laden NM and M matrices at 4 and 8 weeks post implantation and corresponding bone mineral densities (BV/TV) as a function of post-implantation time. Scale bars indicate 2 mm. Data are displayed as mean $\pm$ standard errors (n = 3). Comparisons of two groups in the same time point were made using two-tailed Student's t-test. Asterisks indicate statistical significances according to p-values (*: p < 0.05; **: p < 0.01).

Figure 5.

Biomaterial-dependent *in vivo* tissue formation of transplanted hESC-laden matrices. H&E staining of (a) HUES9-laden and (b) H9-laden non-mineralized (NM) and mineralized (M) matrices after 4 and 8 weeks of implantation. Scale bars represent 500 μm . High magnification images from the center of NM and M matrices with HUES9 and H9 cells at 8 weeks post-implantation are also provided. Yellow arrows: Bone-like matrix formation. Red arrows: Fat and fibrous tissues. Scale bars indicate 50 μm . Immunohistochemical staining of OCN at the center of NM and M matrices containing (c) HUES9 and (d) H9 cells following 4 and 8 weeks of transplantation. Scale bars indicate 100 μm . Oil Red O staining of NM and M matrices containing (e) HUES9 and (f) H9 cells at 8 weeks post-implantation. Scale bars represent 100 μm .