Published in final edited form as:

Biomacromolecules. 2010 August 9; 11(8): 1909–1914. doi:10.1021/bm100357t.

Combinatorial Extracellular Matrices for Human Embryonic Stem Cell Differentiation in 3D

Dr. Fan Yang,

Departments of Orthopaedic Surgery and Bioengineering, Stanford University, 300 Pasteur Drive, Stanford, CA, 94304 (USA)

Dr. Seung-Woo Cho,

Department of Biotechnology, Yonsei University, Seoul 120-749 (Korea)

Sun Mi Son,

Department of Chemical Engineering, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Cambridge, MA 02139 (USA)

Dr. Sarah P. Hudson,

Department of Chemical Engineering, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Cambridge, MA 02139 (USA)

Said Bogatyrev,

Department of Chemical Engineering, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Cambridge, MA 02139 (USA)

Lily Keung,

Department of Chemical Engineering, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Cambridge, MA 02139 (USA)

Dr. Daniel S. Kohane,

Department of Anesthesiology, Division of Critical Care Medicine, Children's Hospital, Harvard Medical School, 300 Longwood Ave., Boston, MA 02115 (USA)

Prof. Robert Langer, and

David H. Koch Institute for Integrative Cancer Research, Massachusetts Institute of Technology, 45 Carleton Street, E25-342, Cambridge, MA 02139 (USA), Department of Chemical Engineering, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Cambridge, MA 02139 (USA)

Daniel G. Anderson

David H. Koch Institute for Integrative Cancer Research, Massachusetts Institute of Technology, 45 Carleton Street, E25-342, Cambridge, MA 02139 (USA)

Daniel G. Anderson: dgander@mit.edu

Abstract

Embryonic stem cells (ESCs) are promising cell sources for tissue engineering and regenerative medicine. Scaffolds for ESC-based tissue regeneration should provide not only structural support, but also signals capable of supporting appropriate cell differentiation and tissue development. Extracellular matrix (ECM) is a key component of stem cell niche *in vivo* and can influence stem cell fate via mediating cell attachment and migration, presenting chemical and physical cues, as well as binding soluble factors. Here we investigated the effects of combinatorial extracellular

matrix proteins on controlled human ESC (hESC) differentiation. Varying ECM compositions in 3D markedly affects cell behavior, and optimal compositions of ECM hydrogels are identified which facilitate specific-lineage differentiation of stem cells. To our knowledge, this is the first combinatorial analysis of ECM hydrogels for their effects on hESC differentiation in 3D. The 3D matrices described herein may provide a useful platform for studying the interactive ECM signaling in influencing stem cell differentiation.

Keywords

extracellular matrix; stem cells; combinatorial; three-dimensional; differentiation

1. Introduction

Embryonic stem cells (ESCs) are attractive candidates for tissue regeneration due to their ability to self-renew indefinitely and differentiate into any type of cells in our body. Before ESCs can be widely used for therapeutic purposes, methods must be developed to control their differentiation towards specific lineages. Increasing evidence has shown that the interplay between stem cells and their surrounding microenvironments is critical for regulating stem cell behavior.¹ Stem cells reside in a complex microenvironment *in vivo*, where they constantly interact and respond to multiple types of signals such as soluble and insoluble factors and mechanical forces.² Soluble factors including cytokines and biochemical molecules can induce ESC differentiation toward desired specific lineages.^{3–6} The extracellular matrix (ECM) is an insoluble molecular network consisting of a variety of elements, including interstitial matrix proteins and the basement membrane. Collagens are fibrillar proteins that provide structural support, and are the most abundant proteins in the ECM.⁷ Fibronectin acts as a general cell adhesion molecule and provides binding sites for cell surface receptors and other ECM components.⁸ Laminin provides the scaffolding for basement membranes and can influence cell behavior.⁹ Both fibronectin and laminin bind to collagen and play roles in mediating cell-ECM interactions.

Previous studies of ECM effects on stem cell differentiation have mainly focused on examining the role of the ECM in regulating mouse ESC differentiation towards lineages such as hepatocytes and cardiomyocytes.^{10,11} However, comparative studies of human ESCs and mouse ESCs have demonstrated major differences in the signaling pathways required to regulate the stem cell fate.¹² Therefore, how ECM regulates human ESC fate remains to be resolved. A recent study has reported that human mesenchymal stem cells in 3D synthetic hydrogels can be induced to differentiate down different pathways by modifying the material surface using 5 different small-molecule functional groups.¹³ This study shows the promise of regulating stem cell fate via matrix cues.

Due to the complexity of cell-ECM and ECM-ECM interactions, optimizing ECM effects on cell behavior remains challenging. To overcome these limitations, combinatorial methods have been developed to screen cell responses to ECM proteins. Flaim and coworkers developed ECM protein microarrays.¹¹ In this study, different combinations of natural ECM proteins such as collagen, laminin and fibronectin were deposited on a hydrogel slide, and their effects on mouse ESC differentiation to cardiac lineage were studied. Several other studies have also developed high-throughput microarray approaches for screening cell material interactions.^{14–18} However, these studies examined cell-material interactions on 2D surfaces, whereas stem cells niche *in vivo* is a 3D environment. In some settings, three dimensionality of the cell microenvironment is important for proper regulation of cell behavior.^{19–22} Furthermore, cell-matrix interactions in 3D matrices can differ from those on 2D substrates.^{21,22} Recently, a combinatorial library of synthetic 3D polymeric

scaffolds was developed and screening with osteoblasts showed that such synthetic libraries may be used for rapidly identifying scaffold formulations that can influence osteoblast adhesion and proliferation.²³

Here we develop a combinatorial array of 3D ECMs for osteogenic and endothelial differentiation of hESC-derived cells (hESdCs). We hypothesize that differentiation of hESdCs in 3D can be modulated by varying the physical structure and the density of cellular binding domains from ECM proteins, and that optimal ECM compositions may exist to facilitate lineage specific differentiation. Therefore, we examined 3D hydrogels based on several key ECM proteins including collagens, fibronectin, and laminin (Fig. 1). We chose collagen type I (Col I) to be the major component of the scaffold as Col I accounts for 90% of bone matrix protein content.⁷ To elucidate the role of ECM cues in controlling the differentiation of hESdCs, Col I hydrogel was interspersed with fibronectin (FN), laminin (LM), and collagen type IV (Col IV) at different concentrations. Specifically, a total of 36 hydrogel compositions were generated in parallel with different amounts of FN (10, 25, 50 $\mu\text{g}/\text{ml}$), LM (10, 50, 100 $\mu\text{g}/\text{ml}$), and Col IV (10, 50, 100 $\mu\text{g}/\text{ml}$) (Fig. 1). Cells were encapsulated in the hydrogels in 3D and cultured in osteogenic differentiation medium or endothelial differentiation medium for 3 weeks before analyses of cellular organization and differentiation.

2. Experimental Section

2.1. Human Embryonic Stem Cell (hESC) Culture

hESC line H9 (WiCell Research Institute, Madison, WI) was grown on inactivated mouse embryonic fibroblasts in hESC growth medium consisting of 80% knockout Dulbecco's Modified Eagle Medium (DMEM, Gibco, Gaithersburg, MD) supplemented with 20% knockout serum, 4 ng/ml basic fibroblast growth factor (bFGF), 1 mM L-glutamine, 0.1 mM 2-mercaptoethanol, and 1% nonessential amino acids (Invitrogen, Carlsbad, CA). For embryoid body (EB) formation, hESC colonies were dissociated into small clumps by incubating at 37°C for 15 min with 2 mg/ml collagenase IV (Gibco). Human embryonic stem cell-derived cells (hESdCs) used in this study were obtained as previously described.²⁴ The hESC clumps were pelleted, resuspended in hESC growth medium without bFGF, and cultured in Petri dishes for 10 days with medium change every other day. The EBs were then transferred to 0.1% (w/v) gelatin-coated plates. Upon 70% confluence, hESC-derived cells (hESdCs) were subcultured in mesenchymal stem cell growth medium consisting of DMEM, 10% fetal bovine serum, 100 mM sodium pyruvate (Gibco), 100 unit penicillin, and 100 $\mu\text{g}/\text{ml}$ streptomycin. Cells were cultured until passage 3 to induce further homogeneous differentiation before use. The hESdCs exhibit a similar morphology to mesenchymal stem cells (MSC) and express MSC surface markers as previously reported.²⁴

2.2. Extracellular Matrix (ECM) Hydrogels

Bovine dermal collagen type I (Col I) hydrogels (1.5 mg/ml) were prepared by diluting the Col I solution (3.0 mg/ml, BD Biosciences, San Jose, CA) with 10 $\times$ phosphate buffered saline (Gibco) (9:1 v/v) and adjusting the pH to 7.4 using NaOH (Sigma, St. Louis, MO). The solution was then added into 48-well plates and incubated at 37°C for 1 h to induce gelation. No cross-linking agent was used. To create a combinatorial collection ECM hydrogels, Col I-based hydrogels (1.5 mg/ml) was interspersed with variable amounts of fibronectin (FN), laminin (LM), or collagen type IV (Col IV). Specifically, FN (1.0 mg/ml, Sigma) stock solution was added to the Col I solution (1.5 mg/ml) to obtain a final FN concentration of 10, 25 or 50 $\mu\text{g}/\text{ml}$. LM (1.0 mg/ml, Sigma) or Col IV (0.7 mg/ml, BD Biosciences) solutions were added to Col I solution to reach a final concentration of 10, 50 or 100 $\mu\text{g}/\text{ml}$, respectively. The solutions were then neutralized to pH 7.4, and incubated in

48-well plates for 1 h at 37°C for gelation. The resulting ECM hydrogel collection has 36 different compositions in total (Fig. 1), and all experiments were done in triplicates.

2.3. Human Embryonic Stem Cell-Derived Cell (hESdC) Differentiation in ECM-Hydrogels

hESdCs were suspended in the Col I solutions with different compositions of ECM proteins (1.0×10^7 cells per 1 ml of each solution) and incubated for 1 h at 37°C to form the gels. All groups were cultured in osteogenic medium as previously defined 26 or endothelial differentiation medium (EGM-2, Lonza, Walkersville, MD). Osteogenic medium consists of high-glucose Dulbecco's modified Eagle's medium (DMEM) (Gibco), 100 nM dexamethasone (Sigma), 50 mg/ml ascorbic acid-2-phosphate (Sigma), 10 mM β -glycerophosphate (Sigma), 10% fetal bovine serum (Gibco), 100 unit/ml penicillin and 100 mg/ml streptomycin (Gibco). All samples were incubated for three weeks before harvest, with medium change three times a week.

2.4. Scanning Electron Microscopy (SEM)

For SEM, samples were first fixed overnight in 2.5% glutaraldehyde at room temperature. Samples were then lyophilized and sputter coated with gold-plutonium alloy (10 nm thickness) under vacuum before SEM analysis. SEM analysis was conducted using a FEI/Philips XL30 FEG ESEM (FEI, Hillsboro, OR).

2.5. Quantitative Real-Time Polymerase Chain Reaction (PCR)

Gene expression of osteogenic or endothelial markers by hESdCs cultured in hydrogels was examined using quantitative real-time PCR ($n=3$ per group). Total RNA was extracted from hydrogel samples as previously described. 25 A reverse transcription reaction was performed with 1 μ g of total RNA using SuperScript^{TM} III reverse transcriptase (Invitrogen). Real-time PCR was performed using a 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). Osteocalcin expression was quantified using SYBR Green detecting reagents, with primer sequence as following: Osteocalcin, Forward 5'-GAC TGT GAC GAG TTG GCT GA-3' and Reverse 5'-CTG GAG AGG AGC AGA ACT GG-3'. β -actin, Forward 5'-TGG CAC CAC ACC TTC TAC AAT GAG C-3' and Reverse 5'-GCA CAG CTT CTC CTT AAT GTC ACG C-3'. Endothelial marker von Willebrand Factor (vWF) expression was quantified using Universe Fast PCR Master Mix (Applied Biosystems) with TaqMan® Gene Expression Assays (Applied Biosystems) for target (vWF: Hs01109438_m1) and endogenous control (glyceraldehyde 3-phosphate dehydrogenase (GAPDH): Hs02758991_g1). All samples were analyzed in triplicates. The expression level of target gene was first normalized to endogenous control (β -actin or GAPDH), 26 and results are presented as relative fold changes in all groups using normalized mRNA level in group 1 as controls.

2.6. Histology

Cell-hydrogel constructs were harvested at 3 weeks after culture. Specimens were fixed in 10% (v/v) buffered formaldehyde, dehydrated with a graded ethanol series, and embedded in paraffin. Specimens were sliced into 4- μ m sections and stained with hematoxylin and eosin (H&E) and Masson's trichrome to detect tissue morphology and collagen deposition.

2.7. Immunofluorescent staining

Tissue sections were immunofluorescently stained using primary antibodies against osteocalcin (Chemicon, Temecula, CA) and collagen type II (DAKO, Carpinteria, CA). Section stained with non-specific primary antibody is used as a negative control. The staining signals were visualized with FITC-conjugated secondary antibodies (Invitrogen).

The stained sections were examined using a fluorescent microscope (Carl Zeiss, Oberkochen, Germany).

2.8. Statistical Analysis

Quantitative data are expressed as mean $\pm$ standard deviation. Statistical analysis was performed by the analysis of variance (ANOVA) using a Bonferroni test. A value of $p < 0.05$ was considered statistically significant.

3. Results and Discussion

3.1. Morphological analyses of ECM hydrogel network

The microscopic structure of ECM hydrogel network was examined by scanning electron microscopy (SEM). Col I matrices alone showed a homogeneous fibrillar network consisting of fibers of approximately 200–300 nm diameter (Fig. 2). Interspersing other ECM components (FN, LM, and Col IV) significantly changed the structures and morphology of the network (Fig. 2). For example, the addition of FN (25 $\mu\text{g/ml}$) (Group 8) increased matrix heterogeneity, with some aggregates and amorphous regions interspersed in and along the collagen fibers. Further addition of LM (50 $\mu\text{g/ml}$) to FN-Col I hydrogel (Group 10) induced a much denser fibrillar network, with thinner fibers and smaller pore size (Fig. 2). Interspersing Col IV (50 $\mu\text{g/ml}$) to FN-Col I network (Group 13) resulted in a fibrillar network structure similar to Col I network alone, with higher heterogeneity in pore size and fiber size (Fig. 2). A previous study has examined the effects of FN and LM on structural property of 3D collagenous network, where it was reported that FN and LM were organized in aggregates, interspersed in collagen gel and distributed along collagen fibers in thin fibrils. 10

3.2. Effects of ECM compositions on osteogenic differentiation

Extracellular matrix (ECM) provides cells with not only structural support but also biochemical and physical cues to regulate cell phenotype. Interspersing ECM proteins into Col I-based hydrogels significantly influenced the osteogenic differentiation of hESdCs (Fig. 3). Quantitative gene expression analysis for osteocalcin, a mature bone marker, showed that low to intermediate concentration of FN (10–25 $\mu\text{g/ml}$) and high concentration of LM (50–100 $\mu\text{g/ml}$) (Groups 3, 4, 10, and 11) significantly promoted ($p < 0.05$) osteogenic differentiation of hESdCs (Fig. 3). Intermediate concentration of FN (25 $\mu\text{g/ml}$) and high concentration of LM (100 $\mu\text{g/ml}$) (Group 11) induced a 53-fold higher expression of osteocalcin compared to control (Group 1). Similar trend was observed with early bone marker such as Cbfa1, where G11 expressed 102% more Cbfa1 than G1, suggesting a synergistic effect between FN and LM on osteogenic differentiation. Interestingly, further increase of FN concentration to 50 $\mu\text{g/ml}$ decreased the osteocalcin expression compared to groups with 25 $\mu\text{g/ml}$ FN, suggesting an optimal FN density exists for promoting osteogenesis.

Our results also indicate that multiple ECM interactions can play a role in regulating stem cell differentiation. FN alone (Group 1, 8, and 15) did not affect the osteogenic differentiation process (Fig. 3). However, in the presence of LM and Col IV, FN significantly enhanced osteogenic differentiation of hESdCs within a specific concentration range (Fig. 3). LM alone (Groups 22, 26, and 30) showed no or moderate effects on osteogenesis, but increased osteocalcin expression 53-fold with FN (25 $\mu\text{g/ml}$). Col IV alone did not increase osteocalcin expression (Groups 34–36) (Fig. 3), but increased osteocalcin expression 16-fold in the presence of FN (Group 21) and LM (Group 25) ($p < 0.05$). Our results confirm that ECM compositions interact in a complex manner and cellular response to individual ECM components cannot necessarily be used to predict the cellular response.

Specific lineage differentiation of stem cells may also be modulated by the mechanical property of the matrix on which the cells are grown. 27 By tuning the elastic modulus of Col I-coated polyacrylamide hydrogel, mesenchymal stem cells were reported to differentiate towards various pathways including neurogenic, myogenic, or osteogenic lineages. 14 In our study, the matrix is a Col I-based hydrogel and a previous report has shown that interspersing Col I hydrogel with other ECM components such as FN or LM does not significantly alter the mechanical property of the network 10 within the concentrations examined here. Therefore, we believe the altered stem cell responses to various ECM groups observed in our study were likely not caused by the mechanical property of the matrices. There are several other factors that may be responsible for the observed cellular response including matrix ligand presentation, surface topography of the fibers, network pore size etc. For example, encapsulated stem cells may respond to the matrix ligands through focal adhesion complexes, which lead to corresponding cytoskeleton reorganization. Focal adhesion kinase signaling has been reported to play an important role in regulating ECM-induced osteogenic differentiation of human mesenchymal stem cells,²⁸ and may influence the hESdC osteogenesis in a similar manner. Furthermore, varying ECM compositions led to an obvious change in the network density, which affects the transport of soluble factors that are involved in cell differentiation.

Previous work has examined the effects of various ECM compositions on stem cell adhesion and differentiation and showed that ECM proteins such as FN and LM may promote mesenchymal stem cell (MSC) adhesion and differentiation towards bone pathway. 29–31 While the effects of individual ECM proteins have been extensively explored, the influence of combinatorial ECM proteins on stem cell differentiation in 3D remain unclear, and optimal concentration is yet to be determined. Furthermore, most previous work examined cell-material interactions on 2D surfaces, while cell-matrix interactions in 3D matrices can differ significantly from those on 2D substrates.^{21–22} The findings from this study will provide valuable guidance for designing next generation of 3D synthetic stem cell niche to mimic the ECM microenvironment and promote specific stem cell differentiation.

3.3. Effects of ECM compositions on tissue formation

While the gene expression assay directly quantifies the effects of ECM matrices on osteogenic differentiation, histology provides information on the effects of ECM compositions on tissue formation. Our results demonstrated that varying ECM compositions in 3D markedly affected cellular organization and tissue formation. Histological analysis (hematoxylin and eosin (H&E) and Masson's trichrome staining; Fig. 4) revealed that interspersing Col I gel with singular additional ECM component (FN, LM, or Col IV) did not increase the cellularity compared to the control (Group 1). In contrast, interspersing Col I gel with two additional ECM components markedly promoted cellular organization and tissue formation. In general, groups with high level of osteocalcin expression (Groups 3, 4, 10, 11, 14, 18, 21, and 25) demonstrated denser structure, significantly higher cellularity, and stronger collagen matrix deposition (Fig. 4). In one ECM condition (Group 11), hESdCs produced an evenly distributed matrix (Fig. 5A, B). Homogeneous and intense staining of collagen type II and mature bone marker osteocalcin was detected throughout the construct, suggesting these cells underwent endochondral ossification (Fig. 5C, D). While the processes of osteogenesis and matrix formation are correlated and exhibited similar trend, we also noticed some variance in the fold of change. For example, the matrix formation in group 13 is much higher than group 3 and comparable to group 14, yet the osteocalcin expression in group 13 is not as significantly upregulated. Such difference may be due to the multi-factorial changes in the ECM matrices induced by varying ECM compositions and concentrations in 3D. Our scanning electron microscopy data (Fig. 2) showed that varying ECM compositions led to an obvious change in the network density and pore size, which

directly affects the transport of soluble factors that are involved in cell differentiation. Meanwhile, varying ECM compositions may also change cellular remodeling dynamics and matrix metalloproteinase (MMP) activity, which will directly influence the matrix formation. The extracellular matrix also serves as a depot for growth factors, from which they can be released into the surrounding microenvironment and regulate cell phenotype and tissue formation.^{32, 33} Varying ECM compositions may also influence the ECM-growth factor interactions, which would in turn influence stem cell fate via autocrine or paracrine cell signaling. All these factors, in part or together, likely contribute to the observed changes in stem cell differentiation and tissue formation.

3.4. Effects of ECM compositions on endothelial differentiation

Bone is a highly vascularized tissue and an ideal scaffold for bone tissue engineering should promote both bone tissue formation and angiogenesis. To identify an optimal ECM-based scaffold for angiogenesis induction, hESdCs were encapsulated in the 3D-hydrogels and cultured in endothelial differentiation medium for 3 weeks. Endothelial differentiation was evaluated by quantitative gene expression analysis of von Willebrand Factor (vWF), a mature endothelial marker. These results show that intermediate concentration of FN (25 $\mu\text{g}/\text{ml}$) induced ~7-fold higher vWF expression (Fig. 6), while a further increase of FN concentration (50 $\mu\text{g}/\text{ml}$) reduced vWF expression back to the baseline level. In the presence of an intermediate concentration of FN (25 $\mu\text{g}/\text{ml}$), LM and Col IV significantly enhanced vWF expression (group 10, 11, and 14). In contrast, LM and Col IV markedly inhibited vWF expression in the presence of high concentration FN (50 $\mu\text{g}/\text{ml}$) (group 18 and 21). These results further highlight the importance of ECM interactions in influencing stem cell differentiation. By modulating the density of binding signals and matrix physical structures, we can direct stem cells to differentiate preferentially towards specific lineages. Two hydrogel compositions (Groups 10 and 11) demonstrated relatively high efficacy in inducing both osteogenic and endothelial differentiation (Fig. 3 and Fig. 6), and may hold potential for applications in vascularized bone tissue engineering.

4. Conclusion

In conclusion, we have demonstrated that a combinatorial analysis of ECM gels can facilitate the identification of optimal ECM matrices for hESdC differentiation in 3D. To our knowledge, this is the first combinatorial analysis of ECM hydrogels for their effects on hESC differentiation in 3D. This approach may be useful for the study of other clinically relevant human stem cell types, or be combined with various soluble factors to study stem cell differentiation in the context of interacting ECM and growth factor signaling. Successful tissue regeneration strategies rely on optimized stem cell niche design and extensive efforts are being dedicated in developing biomaterials to direct stem cell fate.³⁴ Advances in understanding cell-extracellular matrix interactions and signaling would provide valuable insight in guiding the design of biomimetic materials to regulate stem cell fate for various tissue regeneration applications.

Acknowledgments

This work was supported by National Institute of Health (NIH) grants (DE016516 and EB000244) and Juvenile Diabetes Research Foundation (JDRF) grant (17-2007-1063). Fan Yang would also like to thank NIH for postdoctoral fellowship support.

References

1. Scadden DT. *Nature*. 2006; 441:1075–1079. [PubMed: 16810242]

2. Metallo CM, Mohr JC, Detzel CJ, Pablo JJ, Wie BJ, Palecek SP. *Biotechnol Prog.* 2007; 23:18–23. [PubMed: 17269664]
3. D'Amour KA, Agulnick AD, Eliazar S, Kelly OG, Kroon E, Baetge EE. Efficient differentiation of human embryonic stem cells to definitive endoderm. *Nat Biotechnol.* 2005; 23:1534–1541. [PubMed: 16258519]
4. D'Amour KA, Bang AG, Eliazar S, Kelly OG, Agulnick AD, Smart NG, Moorman MA, Kroon E, Carpenter MK, Baetge EE. *Nat Biotechnol.* 2006; 24:1392–1401. [PubMed: 17053790]
5. Darabi R, Gehlbach K, Bachoo RM, Kamath S, Osawa M, Kamm KE, Kyba M, Perlingeiro RC. Functional skeletal muscle regeneration from differentiating embryonic stem cells. *Nat Med.* 2008; 14:134–143. [PubMed: 18204461]
6. Yang L, Soonpaa MH, Adler ED, Roepke TK, Kattman SJ, Kennedy M, Henckaerts E, Bonham K, Abbott GW, Linden RM, Field LJ, Keller GM. *Nature.* 2008; 453:524–528. [PubMed: 18432194]
7. Kern B, Shen J, Starbuck M, Karsenty G. *J Biol Chem.* 2001; 276:7101–7107. [PubMed: 11106645]
8. Pankov R, Yamada KM. Fibronectin at a glance. *J Cell Sci.* 2002; 115:3861–3863. [PubMed: 12244123]
9. Timpl R, Rohde H, Robey PG, Rennard SI, Foidart JM, Martin GR. *J Biol Chem.* 1979; 254:9933–9937. [PubMed: 114518]
10. Battista S, Guarnieri D, Borselli C, Zeppetelli S, Borzacchiello A, Mayol L, Gerbasio D, Keene DR, Ambrosio L, Netti PA. *Biomaterials.* 2005; 26:6194–6207. [PubMed: 15921736]
11. Flaim CJ, Chien S, Bhatia SN. *Nature Methods.* 2005; 2:119–125. [PubMed: 15782209]
12. Wei CL, Miura T, Robson P, Lim SK, Xu XQ, Lee MY, Gupta S, Stanton L, Luo YQ, Schmitt J, Thies S, Wang W, Khrebtukova I, Zhou DX, Liu ET, Ruan YJ, Rao M, Lim B. *Stem Cells.* 2005; 23:166–185. [PubMed: 15671141]
13. Benoit DS, Schwartz MP, Durney AR, Anseth KS. *Nat Mater.* 2008; 7:816–823. [PubMed: 18724374]
14. MacBeath G, Schreiber SL. Printing proteins as microarrays for high-throughput function determination. *Science.* 2000; 289:1760–1763. [PubMed: 10976071]
15. Anderson DG, Levenberg S, Langer R. *Nature Biotechnology.* 2004; 22:863–866.
16. Anderson DG, Putnam D, Lavik EB, Mahmood TA, Langer R. *Biomaterials.* 2005; 26:4892–4897. [PubMed: 15763269]
17. Simon CG Jr, Eidelman N, Kennedy SB, Sehgal AI, Khatri CA, Washburn NR. *Biomaterials.* 2005; 26:6906–6915. [PubMed: 15939467]
18. Nakajima M, Ishimuro T, Kato K, Ko IK, Hirata I, Arima Y, Iwata H. *Biomaterials.* 2007; 28:1048–1060. [PubMed: 17081602]
19. Cukierman E, Pankov RD, Stevens R, Yamada KM. *Science.* 2001; 294:1708–1712. [PubMed: 11721053]
20. Grinnell F. *Trends Cell Biol.* 2003; 13:264–269. [PubMed: 12742170]
21. Chun TH, Hotary KB, Sabeh F, Saltiel AR, Allen ED, Weiss SJ. *Cell.* 2006; 125:577–591. [PubMed: 16678100]
22. Hotary KB, Allen ED, Brooks PC, Datta NS, Long MW, Weiss SJ. *Cell.* 2003; 114:33–45. [PubMed: 12859896]
23. Yang Y, Bolikal D, Becker M, Kohn J, Zeiger D, Simon C. *Adv Mater.* 2008; 20:2037–2043.
24. Hwang NS, Varghes S, Zhang Z, Elisseeff J. *Tissue Eng.* 2006; 12:2695–2706. [PubMed: 16995803]
25. Yang F, Williams CG, Wang DA, Lee H, Manson PN, Elisseeff J. *Biomaterials.* 2005; 26:5991–5998. [PubMed: 15878198]
26. Mori R, Wang Q, Danenberg KD, Pinski JK, Danenberg PV. *The Prostate.* 2008; 68:1555–1560. [PubMed: 18651557]
27. Engler AJ, Sen S, Sweeney HL, Discher DE. *Cell.* 2006; 126:677–689. [PubMed: 16923388]
28. Salaszyk RM, Klees RF, Williams WA, Boskey A, Plopper GE. *Exp Cell Res.* 2007; 313:22–37. [PubMed: 17081517]
29. Gronthos S, Simmons PJ, Graves SE, Robey PG. *Bone.* 2001; 28:174–181. [PubMed: 11182375]

30. Keselowsky BG, Collard DM, Garcia AJ. PNAS. 2005; 102:5953–5957. [PubMed: 15827122]
31. Hidalgo-Bastida LA, Cartmell SH. Tissue Engineering Part B. 2010 Apr 7. Epub ahead of print.
32. Discher DE, Mooney DJ, Zandstra PW. Science. 2009; 324:1673–1677. [PubMed: 19556500]
33. Uebersax L, Merkle HP, Meinel L. Tissue Engineering Part B. 2009; 15:263–289.
34. Designing materials to direct stem-cell fate. Lutolf MP, Gilbert PM, Blau HM. Nature. 2009; 462:433–441. [PubMed: 19940913]

Figure 1. Schematic illustration of compositions and layout of the combinatorial ECM hydrogels. Stem cell-seeded ECM mixtures were deposited into 48-well plates, and cultured in either osteogenic differentiation medium or endothelial differentiation medium. All experiments were done in triplicate. Col I: collagen I; FN: Fibronectin; LM: Laminin; Col IV: collagen IV.

Figure 2.

SEM micrographs of collagen network interspersed with various ECM components. (A, E) Col I (1.5 mg/ml), (B, F) Col I + FN (25 µg/ml), (C, G) Col I + FN (25 µg/ml) + LM (50 µg/ml), (D, H) Col I + FN (25 µg/ml) + Col IV (50 µg/ml). Scale bars: A–C, 2 µm; D, 5 µm.

Figure 3. Quantitative gene expression of osteocalcin, a mature bone marker, by the encapsulated hESdCs after 3 weeks of culture in ECM gels under osteogenic conditions. All experiments were done in triplicates and results were presented as relative fold changes in all groups using normalized mRNA level in group 1 as controls (*; $p < 0.05$).

Figure 4. Histology of hESdC-ECM gels 3 weeks after culture. (A) H&E staining showed varying ECM components significantly influenced cellular organization and tissue development. (B) Masson's trichrome staining demonstrated intense collagen deposition (blue) in some groups. Histology (H&E; Masson's Trichrome; magnification $\times 4$). Scale bars: A, B, 500 μm .

Figure 5. Histology and immunofluorescent staining of leading hESdC-hydrogel group with the highest osteocalcin expression [Group 11: Col I + FN (25 μ g/ml) + LM (100 μ g/ml)]. Tissue morphology and matrix production was shown by (A) H&E staining, (B) Masson's trichrome staining, and immunofluorescent staining for (C) collagen type II, (D) osteocalcin and (E) negative control.

Figure 6. Quantitative gene expression of a mature endothelial marker von Willebrand Factor (vWF) by the encapsulated hESdCs after 3 weeks of culture in ECM gels with endothelial differentiation medium. All experiments were done in triplicates and results were presented as relative fold changes in all groups using normalized mRNA level in group 1 as controls (*; $p < 0.05$).