Cell Differentiation

DOI: 10.1002/anie.201405930

Restraint of the Differentiation of Mesenchymal Stem Cells by a Nonfouling Zwitterionic Hydrogel**

*Tao Bai, Fang Sun, Lei Zhang, Andrew Sinclair, Sijun Liu, Jean-Rene Ella-Menye, Ying Zheng, and Shaoyi Jiang**

Abstract: *The success of human mesenchymal stem cell (hMSC) therapies is largely dependent on the ability to maintain the multipotency of cells and control their differentiation. External biochemical and biophysical cues can readily trigger hMSCs to spontaneously differentiate, thus resulting in a rapid decrease in the multipotent cell population and compromising their regenerative capacity. Herein, we demonstrate that nonfouling hydrogels composed of pure poly(carboxybetaine) (PCB) enable hMSCs to retain their stem-cell phenotype and multipotency, independent of differentiation-promoting media, cytoskeletal-manipulation agents, and the stiffness of the hydrogel matrix. Moreover, encapsulated hMSCs can be specifically induced to differentiate down osteogenic or adipogenic pathways by controlling the content of fouling moieties in the PCB hydrogel. This study examines the critical role of nonspecific interactions in stem-cell differentiation and highlights the importance of materials chemistry in maintaining stem-cell multipotency and controlling differentiation.*

The extensive potential applications of human stem cells underscore the significance of elucidating the fundamental mechanism that controls their differentiation.[1] According to previous reports, stem-cell fate can be affected by both biochemical and biophysical signals.[2] Researchers have also begun to realize that the material surrounding a stem cell is highly important in determining its fate.[3] Among current platforms, natural polymers, such as alginate, are commonly chosen owing to their high biocompatibility. Though these natural materials can provide a biocompatible niche, their complex composition makes it difficult to elucidate the biochemical and biophysical aspects of the stem-cell-differentiation mechanism.[3j,4] Artificial materials are usually easier to control,[5] but generally not biocompatible with stem cells, especially for 3D cell encapsulation. High-molecular-weight poly(ethylene glycol) (PEG) is currently the most popular choice among the very limited range of synthetic materials suitable for stem-cell culture.[6] It has also been reported that the fate of stem cells in PEG hydrogels is highly influenced by chemical and physical cues.[3j,6c] However, strictly controlled physical and chemical cues will be very difficult to implement in downstream clinical applications of such systems. The development of a platform that enables hMSCs to maintain their multipotency regardless of biochemical or biophysical influence has been a great challenge.

In contrast to natural polymers and the artificial amphiphilic material PEG, superhydrophilic zwitterionic materials, such as polycarboxybetaine (PCB),[7] are able to strongly resist nonspecific interactions and are highly compatible.[8] It was reported previously that a loosely packed polysulfobetaine coating could support the long-term growth of embryonic stem cells in expansion media, in which cells were able to attach to the surface through nonspecific interactions.[9] However, the influence of nonspecific interactions, and particularly the absence of nonspecific interactions, on the differentiation behavior of stem cells remains unclear. The goal of this study was to demonstrate the differentiation behavior of hMSCs when the degree of nonspecific interactions was altered. This study shows that simple nonspecific interactions between stem cells and biomaterials may be used to control complicated cellular functions, such as stem-cell differentiation. These results not only advance our understanding of the stem-cell-differentiation mechanism, but also lead to simple and controllable biomaterial formulations for the application of stem-cell-based regenerative medicine.

In the present study, the zwitterionic monomer carboxybetaine acrylamide (CBAA) and the cross-linker carboxybetaine dimethacrylate (CBDMA) were used to construct hydrogels designated CBX (Figure 1a). We also replaced the nonfouling zwitterionic cross-linker CBDMA with a hydrophobic cross-linker, ethylene glycol dimethacrylate (EGDMA), to create nonspecific binding sites.[10] These modified hydrogels are denoted CBE (Figure 1a; see Figure S1 in the Supporting Information for the hydrophobicity difference between the selected cross-linkers). The stiffness of hydrogels was varied by altering the cross-linker content. Three different degrees of stiffness were chosen for CBE (low: 2.6 KPa, medium: 27.8 KPa, high: 62.1 KPa) and CBX hydrogels (low: 3.1 KPa, medium: 24.5 KPa, high: 71.2 KPa; see Figure S2; see also Figures S2 and S3 for the swelling ratios and pore-size distributions). We functionalized the integrin-binding molecule cyclic RGD (cRGD) with the matrix components to enable the mediation of cell attach-

[*] T. Bai, F. Sun, L. Zhang, A. Sinclair, J. R. Ella-Menye, Prof. S. Jiang
Department of Chemical Engineering, University of Washington
Seattle, WA 98195 (USA)
E-mail: sjiang@uw.edu

S. Liu, Y. Zheng, Prof. S. Jiang
Department of Bioengineering, University of Washington
Seattle, WA 98195 (USA)

[**] This research was funded by the National Science Foundation (DMR 1307375 and CBET-1264477), the Office of Naval Research (N00014-14-1-0090 and N00014-09-1-0137), and the National Institutes of Health (NCI T32 grant no. T32A138312). We thank Dr. Raftery and Dr. Haiwei Gu from the Northwest Metabolomics Research Center (NW-MRC) for assisting with LC–MS assessment.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201405930.

 © 2014 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

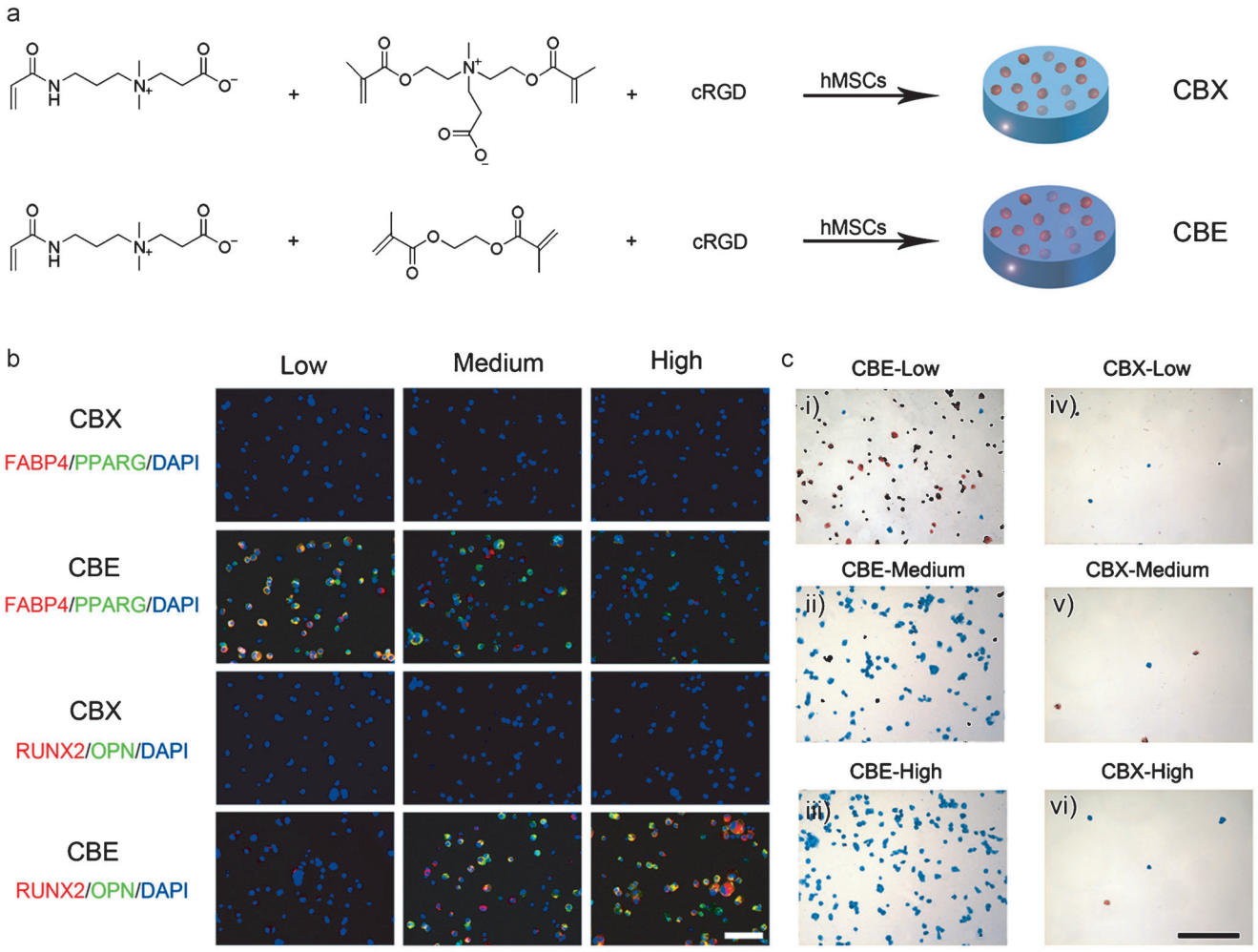

Figure 1. a) Schematic illustration of cRGD conjugation to carboxybetaine monomer or a related cross-linker and cell encapsulation in CBX and CBE hydrogels. b) After culture for 21 days in bipotential differentiation media, cell–hydrogel constructs of CBX and CBE samples were immunostained for adipogenic (PPARG, FABP4) and osteogenic (RUNX2, OPN) biomarkers. Scale bar: 50 μm. c) Representative bright-field images of hMSCs stained with Oil Red O and Fast Blue salt after incubation for 21 days with bipotential differentiation media within i) low-modulus CBE, ii) medium-modulus CBE, iii) high-modulus CBE, iv) low-modulus CBX, v) medium-modulus CBX, and vi) high-modulus CBX. Scale bar: 100 μm.

ment and cellular traction. A scrambled peptide, cyclic RGE (cRGE), was used as a negative-control peptide.

We investigated the nonfouling and integrin-binding properties of peptide-functionalized PCBAA (see Figure S4) and demonstrated the effect of increasing the cross-linker content on the nonfouling properties of CBX and CBE hydrogels by using fluorescently labeled fetal bovine serum (FBS) and cell lysates (CLs). No protein in FBS or CLs was able to adsorb when PCBAA hydrogels were cross-linked with a zwitterionic cross-linker (i.e. CBX hydrogels), regardless of the cross-linker concentration (see Figure S5). In contrast, when PCBAA hydrogels were cross-linked with a hydrophobic cross-linker (i.e. CBE hydrogels), significant protein adsorption from both FBS and CLs was observed, and the protein adsorption increased with elevated hydrophobic-cross-linker content.

Next, hMSCs were encapsulated in CBX and CBE hydrogels and cultured in basal media for 3 days, after which they were transferred to a bipotential differentiation medium (osteogenic and adipogenic) and cultured for 21 days. The interactions between the encapsulated hMSCs and the hydrogel matrix were visualized by focal adhesion staining (see Figure S6). The encapsulated hMSCs showed high viability (see Figures S7 and S8; see also Figure S9 for a summary of their expansion rates). After confirming the biocompatibility of PCB hydrogels, we further explored the differentiation of hMSCs in this environment by using immunohistochemistry (IHC), histological staining, and RNA analysis.

According to current differentiation theories, the fate of hMSCs can be tuned by adjusting the mechanical properties of their support.[3d] As mentioned above, three different moduli were chosen for both CBE and CBX hydrogels. After culture for 21 days in the bipotential medium, we assessed osteogenesis by staining and visualizing RUNX2 and osteopontin (OPN). Similarly, we assessed adipogenesis with the

© 2014 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

receptor PPARG and fatty acid binding protein 4 (FABP4). Interestingly, both osteogenic and adipogenic biomarkers remained absent from cells grown in all CBX hydrogels (Figure 1 b). These results suggested that neither mechanical-property-associated cell traction nor soluble differentiation factors conclusively drove stem-cell-differentiation choices. In sharp contrast to the lack of differentiation, we observed that in CBX hydrogels, adipogenic biomarkers were highly expressed in cells cultured in soft CBE hydrogels (2.6 KPa) and disappeared quickly when the modulus increased. As compared to the soft CBX constructs, it was apparent that a minimal concentration of hydrophobic components encouraged hMSCs to differentiate to an adipogenic lineage. On the other hand, osteogenic biomarkers were clearly expressed in the stiffer (medium- and high-modulus) CBE hydrogel samples, with increased nonspecific-binding sites resulting in increased expression. This result indicates that an increase in the mechanical strength of the matrix in the presence of nonspecific interactions can help hMSCs shift their differentiation from adipogenesis to osteogenesis.

After visualizing the characteristic surface antigens, we further explored specific chemicals and enzymes secreted within differentiated hMSCs. Adipogenesis in hMSCs can be indicated by the presence of neutral lipids,[11] which can be visualized by staining with Oil Red O. Likewise, osteogenesis can be reflected by alkaline phosphatase (ALP),[11] which can be stained with Fast Blue salt. Stem cells were differentiated actively in CBE hydrogels (Figure 1 c i–iii). Nearly all the differentiated cells committed to adipogenesis in a soft CBE matrix (2.6 KPa) and began to choose the osteogenic pathway as the stiffness was increased. In contrast, nearly all hMSCs in CBX hydrogels remained in an undifferentiated state, with the very few differentiated cells randomly committed (Figure 1 c iv–vi). We also functionalized the CBX hydrogels with different densities of cRGD. However, the varied cRGD density was not able to compromise the viability of encapsulated hMSCs or cause active differentiation of hMSCs (see Figures S10 and S11).

We hypothesize that the nonspecific cell–matrix interaction is indispensable for hMSCs to initiate differentiation. When cultured in nonfouling CBX hydrogels, hMSCs are located in an environment in which most nonspecific signals are eliminated (Figure 2). As a consequence, neither physical nor chemical factors can promote these hMSCs to differentiate. In contrast to the CBX hydrogels, the CBE hydrogels can provide nonspecific cues to the anchored hMSCs, thus enabling them to initiate the process of differentiation. Therefore, hMSCs are prone to commit to the osteogenic pathway when cultured in CBE hydrogels with a high modulus and the adipogenic pathway when cultured in CBE hydrogels with a low modulus (Figure 2). This behavior further indicates that the intensity of nonspecific cues provided may alter the differentiation preference of hMSCs. We demonstrated that the degree of nonspecific interactions can conclusively determine the fate of hMSCs (see Figure S12).

We further assessed the expression of multipotent biomarkers, CD166 antigen (encoded by the ALCAM gene) and STRO-1, in hMSCs encapsulated in CBX and CBE hydrogels. Whereas hMSCs cultured in the completely nonfouling CBX hydrogels were positive for stem-cell markers STRO-1 and ALCAM, independent of the matrix modulus, most of the hMSCs in CBE hydrogels were not able to express these stem-cell biomarkers (Figure 3). This result suggests that nonspecific binding triggered differentiation, and hMSCs in CBE hydrogels lost their multipotency when cultured with

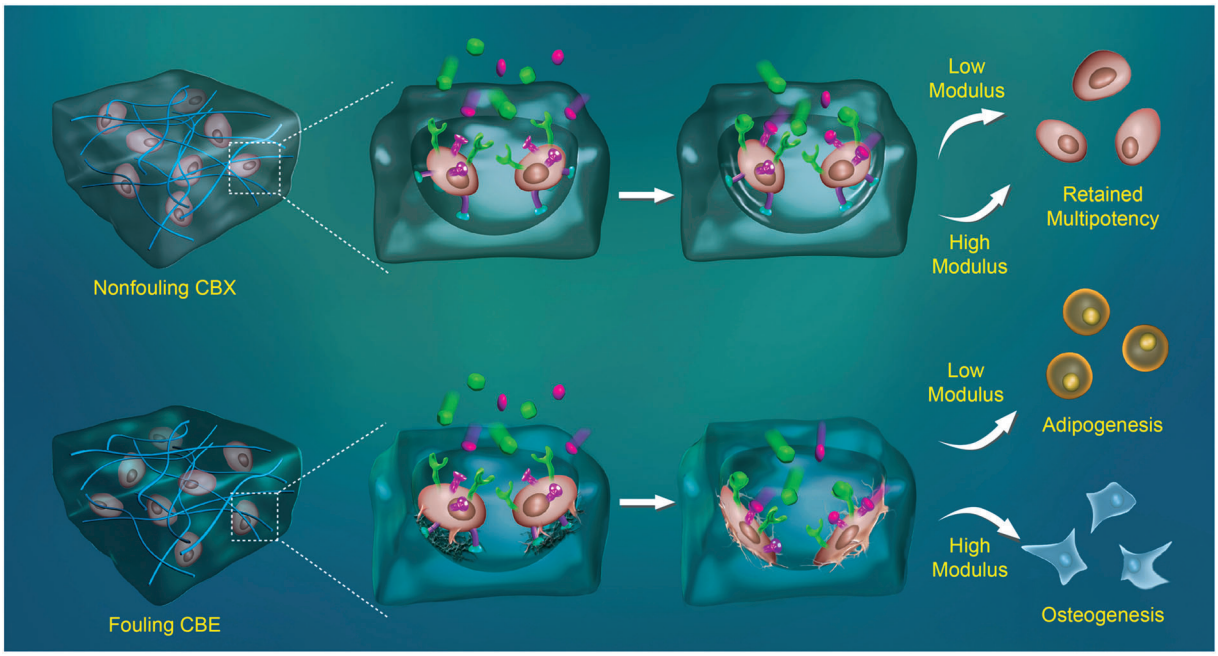

Figure 2. Schematic illustration of the behavior and fate choice of hMSCs in CBX or CBE hydrogels. cRGD moieties are denoted as cyan hemispheres, and differentiation factors are denoted as colored particles.

 © 2014 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

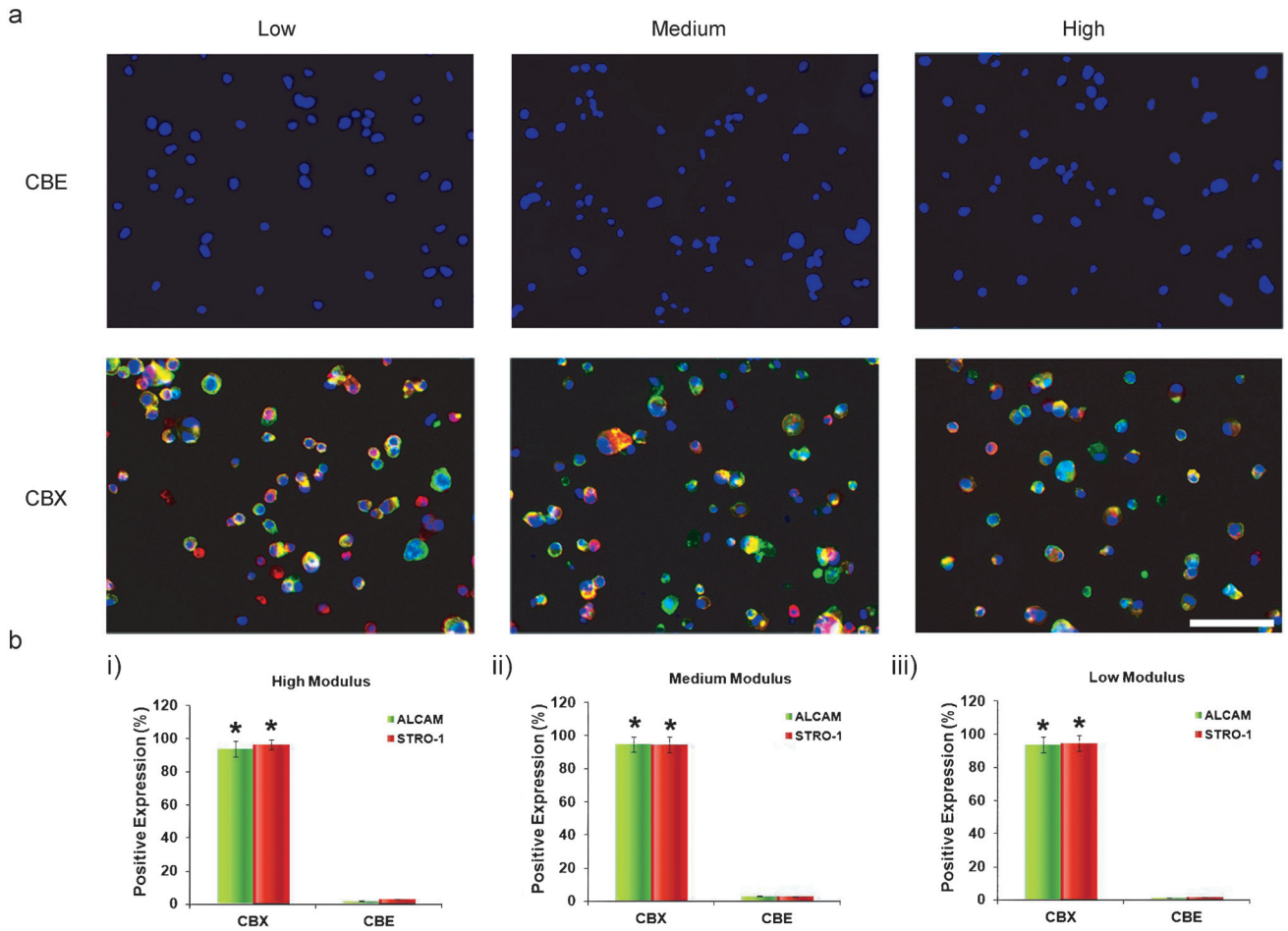

Figure 3. a) Detection of undifferentiated hMSCs by immunofluorescence staining by visualization of the undifferentiated-cell markers ALCAM (green) and STRO-1 (red; nuclei: blue) in CBX hydrogels and CBE hydrogels. Scale bar: 50 μm. b) Percentage of cells expressing either ALCAM or STRO-1 when encapsulated in CBX and CBE hydrogels with different moduli (high: i; medium: ii; low: iii), after incubation for 21 days in bipotential differentiation media. Three sections at different positions of each hydrogel disk were used, and at least five different fields were randomly examined in each section. Asterisks denote statistical significance as compared with CBE hydrogels (**$p < 0.001$, *t*-test). Error bars represent the standard error of the mean from 5 individual experiments.

differentiation media. We also examined RNA expression by quantitative real-time PCR (qRT-PCR) and observed the same phenomenon (see Figure S13).

It is believed that the differentiation behavior of hMSCs is directly dependent on the cytoskeletal contractility.[7] Therefore, we further evaluated the effect of several pharmacological agents known to modulate the cytoskeleton on the differentiation behavior of encapsulated hMSCs in CBX and CBE hydrogels. The percentage of hMSCs that were differentiated into adipocytes or osteoblasts in control culture media without the addition of pharmacological agents is shown in Figure 4a,i and Figure 4b,i. It is known that nocodazole can interfere with the polymerization of microtubules, thus increasing cell contractility.[7b] This agent indeed reversed the fate choice of hMSCs in a CBE hydrogel with a low modulus and gave a high rate of osteogenesis in all cases (Figure 4a ii). Three other agents able to directly inhibit the contractility of the cytoskeleton—blebbistatin, which inhibits myosin II, cytochalasin D, which inhibits F-actin polymerization, and Y-27632, which inhibits ROCK—resulted in a significant decrease in osteogenesis with a corresponding increase in adipogenesis in all of the CBE hydrogels (Figure 4a iii,v). It is also known that α5β1-integrin can mediate coupling of the cytoskeleton to the extracellular matrix and enable mechanical signal transduction.[7b] We therefore examined cells encapsulated in cRGD-modified CBE hydrogels in the presence of function-blocking antibodies against α5β1-integrin. We found that most hMSCs committed to adipogenesis in all CBE hydrogels in the presence of these antibodies, and the influence of stiffness on differentiation was removed (Figure 4a vi).

We also tested the influence of these pharmacological agents on the differentiation of hMSCs in CBX hydrogels. As presented in Figure 4b, unlike in CBE hydrogels, the hMSCs in CBX hydrogels of varying rigidity exhibited a very low rate (< 5%) of differentiation, and no significant difference was observed among CBX hydrogels treated with different pharmacological agents. These results further demonstrate that actomyosin contractility is not able to influence the fate choice of hMSCs in the absence of nonspecific interactions,

 © 2014 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

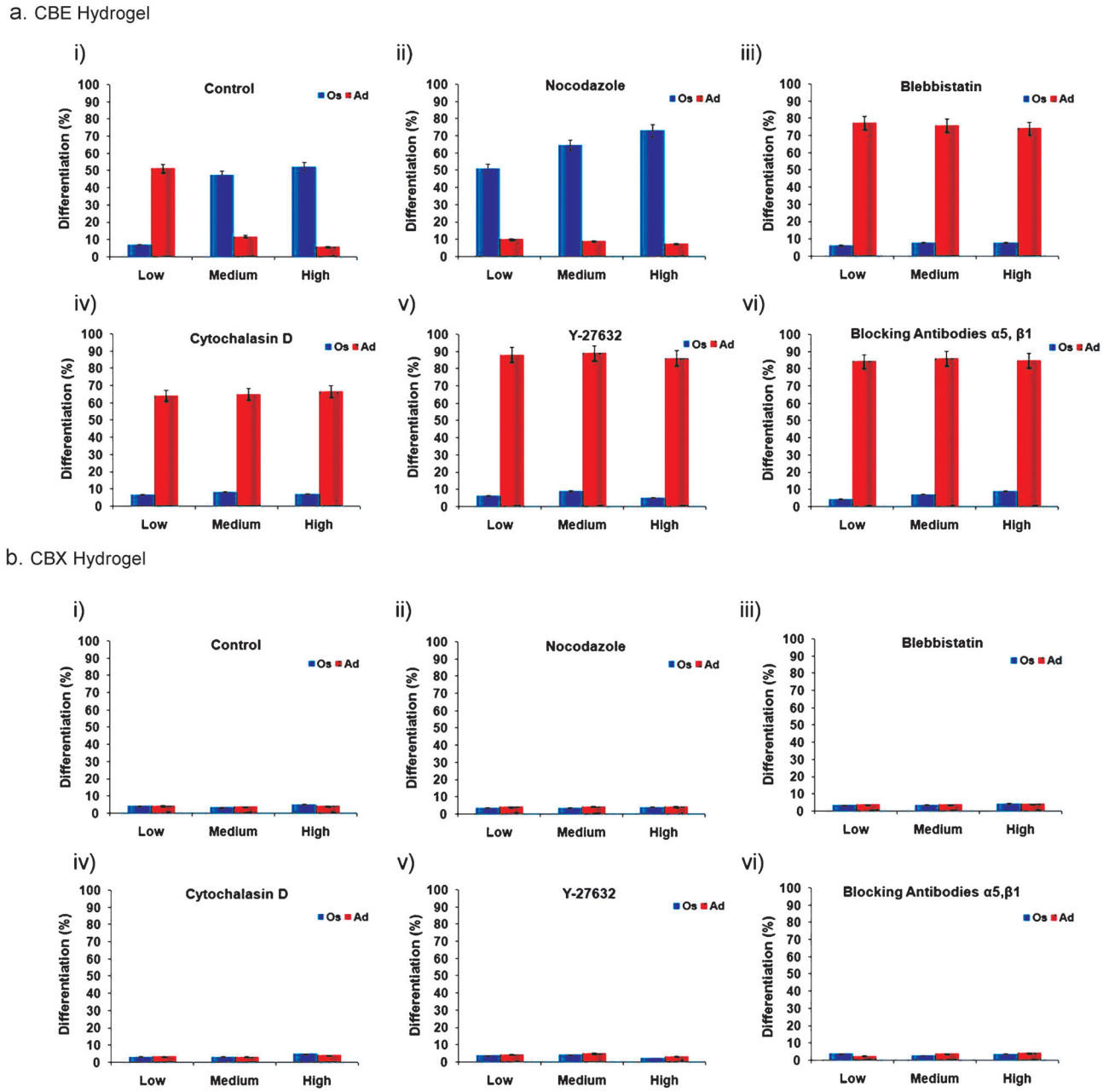

Figure 4. a i, b i) Percentage of cells in CBE or CBX hydrogels differentiating into adipocytes (stained by Oil red O) or osteoblasts (stained by Fast Blue salt) in normal bipotential differentiation media. a ii–vi, b ii–vi) Percentage of cells in CBE or CBX hydrogels differentiating into adipocytes or osteoblasts in the presence of cytoskeleton disruptors and integrin-blocking antibodies.

and that the nonspecific interactions between the extracellular matrix and cells are indispensable for hMSCs to initiate the differentiation process.

In summary, as evidenced by IHC, histological staining, and RNA analysis, we have shown that the absence of nonspecific interactions prevents hMSC differentiation in 3D cultures. Stem cells did not undergo differentiation in non-fouling hydrogels, regardless of material stiffness, even after long-term culture in differentiation media or in the presence of cytoskeletal manipulation agents. Furthermore, we found that a fouling background could mediate hMSC differentiation in 3D platforms. The degree of nonspecific interactions could define the differentiated lineage of hMSCs, independent of the stiffness of the substrate or matrix. Although stem cells were investigated in this study, the zwitterionic hydrogels reported can also be applied in the form of bulk hydrogels or nanogels to the encapsulation of other types of cells, proteins, polysaccharides, nucleic acids, nanomaterials, and small molecules.

Received: June 5, 2014
Revised: August 19, 2014
Published online: September 22, 2014

 © 2014 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

Keywords: gels · nonspecific interactions · stem-cell differentiation · zwitterions · zwitterionic hydrogels

[1] a) Y. H. Jiang, B. N. Jahagirdar, R. L. Reinhardt, R. E. Schwartz, C. D. Keene, X. R. Ortiz-Gonzalez, M. Reyes, T. Lenvik, T. Lund, M. Blackstad, J. B. Du, S. Aldrich, A. Lisberg, W. C. Low, D. A. Largaespada, C. M. Verfaillie, *Nature* **2002**, *418*, 41 – 49; b) C. Nombela-Arrieta, J. Ritz, L. E. Silberstein, *Nat. Rev. Mol. Cell Biol.* **2011**, *12*, 126 – 131.

[2] D. E. Discher, D. J. Mooney, P. W. Zandstra, *Science* **2009**, *324*, 1673 – 1677.

[3] a) S. Zhu, W. Wei, S. Ding, *Annu. Rev. Biomed. Eng.* **2011**, *13*, 73 – 90; b) S. Yao, S. Chen, J. Clark, E. Hao, G. M. Beattie, A. Hayek, S. Ding, *Proc. Natl. Acad. Sci. USA* **2006**, *103*, 6907 – 6912; c) Y. Sun, C. S. Chen, J. Fu, *Annu. Rev. Biophys.* **2012**, *41*, 519 – 542; d) F. Guilak, D. M. Cohen, B. T. Estes, J. M. Gimble, W. Liedtke, C. S. Chen, *Cell Stem Cell* **2009**, *5*, 17 – 26; e) A. Higuchi, Q.-D. Ling, Y. Chang, S.-T. Hsu, A. Umezawa, *Chem. Rev.* **2013**, *113*, 3297 – 3328; f) A. J. Engler, S. Sen, H. L. Sweeney, D. E. Discher, *Cell* **2006**, *126*, 677 – 689; g) B. Trappmann, J. E. Gautrot, J. T. Connelly, D. G. T. Strange, Y. Li, M. L. Oyen, M. A. C. Stuart, H. Boehm, B. Li, V. Vogel, J. P. Spatz, F. M. Watt, W. T. S. Huck, *Nat. Mater.* **2012**, *11*, 642 – 649; h) K. A. Kilian, B. Bugarija, B. T. Lahn, M. Mrksich, *Proc. Natl. Acad. Sci. USA* **2010**, *107*, 4872 – 4877; i) R. McBeath, D. M. Pirone, C. M. Nelson, K. Bhadriraju, C. S. Chen, *Dev. Cell* **2004**, *6*, 483 – 495; j) N. Huebsch, P. R. Arany, A. S. Mao, D. Shvartsman, O. A. Ali, S. A. Bencherif, J. Rivera-Feliciano, D. J. Mooney, *Nat. Mater.* **2010**, *9*, 518 – 526; k) Y. Han, T. Bai, W. Liu, *Sci. Rep.* **2014**, *4*, 5815.

[4] S. Khetan, M. Guvendiren, W. R. Legant, D. M. Cohen, C. S. Chen, J. A. Burdick, *Nat. Mater.* **2013**, *12*, 458 – 465.

[5] Y. Mei, K. Saha, S. R. Bogatyrev, J. Yang, A. L. Hook, Z. I. Kalcioglu, S.-W. Cho, M. Mitalipova, N. Pyzocha, F. Rojas, K. J. Van Vliet, M. C. Davies, M. R. Alexander, R. Langer, R. Jaenisch, D. G. Anderson, *Nat. Mater.* **2010**, *9*, 768 – 778.

[6] a) D. S. W. Benoit, M. P. Schwartz, A. R. Durney, K. S. Anseth, *Nat. Mater.* **2008**, *7*, 816 – 823; b) A. Singh, J. Zhan, Z. Ye, J. H. Elisseeff, *Adv. Funct. Mater.* **2013**, *23*, 575 – 582; c) K. A. Kilian, M. Mrksich, *Angew. Chem. Int. Ed.* **2012**, *51*, 4891 – 4895; *Angew. Chem.* **2012**, *124*, 4975 – 4979; d) A. M. Kloxin, A. M. Kasko, C. N. Salinas, K. S. Anseth, *Science* **2009**, *324*, 59 – 63.

[7] a) Z. Cao, S. Jiang, *Nano Today* **2012**, *7*, 404 – 413; b) A. J. Keefe, S. Jiang, *Nat. Chem.* **2012**, *4*, 60 – 64.

[8] a) L. Zhang, Z. Cao, T. Bai, L. Carr, J.-R. Ella-Menye, C. Irvin, B. D. Ratner, S. Jiang, *Nat. Biotechnol.* **2013**, *31*, 553 – 556; b) T. Bai, S. Liu, F. Sun, A. Sinclair, L. Zhang, Q. Shao, S. Jiang, *Biomaterials* **2014**, *35*, 3926 – 3933; c) S. Jiang, Z. Cao, *Adv. Mater.* **2010**, *22*, 920 – 932.

[9] L. G. Villa-Diaz, H. Nandivada, J. Ding, N. C. Nogueira-de-Souza, P. H. Krebsbach, K. S. O'Shea, J. Lahann, G. D. Smith, *Nat. Biotechnol.* **2010**, *28*, 581 – 583.

[10] R. S. Kane, P. Deschatelets, G. M. Whitesides, *Langmuir* **2003**, *19*, 2388 – 2391.

[11] M. F. Pittenger, A. M. Mackay, S. C. Beck, R. K. Jaiswal, R. Douglas, J. D. Mosca, M. A. Moorman, D. W. Simonetti, S. Craig, D. R. Marshak, *Science* **1999**, *284*, 143 – 147.

 © 2014 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim