University of Pennsylvania
ScholarlyCommons

Departmental Papers (BE) Department of Bioengineering

June 2007

Hyaluronic acid hydrogel for controlled self-renewal and differentiation of human embryonic stem cells

Sharon Gerecht
Harvard–Massachusetts Institute of Technology

Jason A. Burdick
University of Pennsylvania, burdick2@seas.upenn.edu

Lino S. Ferreira
University of Coimbra

Seth A. Townsend
Massachusetts Institute of Technology

Robert Langer
Harvard–Massachusetts Institute of Technology

See next page for additional authors

Follow this and additional works at: http://repository.upenn.edu/be_papers

Recommended Citation

Gerecht, S., Burdick, J. A., Ferreira, L. S., Townsend, S. A., Langer, R., & Vunjak-Novakovic, G. (2007). Hyaluronic acid hydrogel for controlled self-renewal and differentiation of human embryonic stem cells. Retrieved from http://repository.upenn.edu/be_papers/94

© 2007 by The National Academy of Sciences of the USA. Reprinted from *Proceedings of the National Academy of Sciences in the United Stated*, Volume 104, Issue 27, July 2007, pages 11298-11303.
Publisher URL: http://dx.doi.org/10.1073/pnas.0703723104

This paper is posted at ScholarlyCommons. http://repository.upenn.edu/be_papers/94
For more information, please contact libraryrepository@pobox.upenn.edu.

Hyaluronic acid hydrogel for controlled self-renewal and differentiation of human embryonic stem cells

Abstract

Control of self-renewal and differentiation of human ES cells (hESCs) remains a challenge. This is largely due to the use of culture systems that involve poorly defined animal products and do not mimic the normal developmental milieu. Routine protocols involve the propagation of hESCs on mouse fibroblast or human feeder layers, enzymatic cell removal, and spontaneous differentiation in cultures of embryoid bodies, and each of these steps involves significant variability of culture conditions. We report that a completely synthetic hydrogel matrix can support (i) long-term self-renewal of hESCs in the presence of conditioned medium from mouse embryonic fibroblast feeder layers, and (ii) direct cell differentiation. Hyaluronic acid (HA) hydrogels were selected because of the role of HA in early development and feeder layer cultures of hESCs and the controllability of hydrogel architecture, mechanics, and degradation. When encapsulated in 3D HA hydrogels (but not within other hydrogels or in monolayer cultures on HA), hESCs maintained their undifferentiated state, preserved their normal karyotype, and maintained their full differentiation capacity as indicated by embryoid body formation. Differentiation could be induced within the same hydrogel by simply altering soluble factors. We therefore propose that HA hydrogels, with their developmentally relevant composition and tunable physical properties, provide a unique microenvironment for the selfrenewal and differentiation of hESCs.

Keywords

scaffolds, three-dimensional cultures, vasculogenesis

Comments

© 2007 by The National Academy of Sciences of the USA. Reprinted from *Proceedings of the National Academy of Sciences in the United Stated*, Volume 104, Issue 27, July 2007, pages 11298-11303.
Publisher URL: http://dx.doi.org/10.1073/pnas.0703723104

Author(s)

Sharon Gerecht, Jason A. Burdick, Lino S. Ferreira, Seth A. Townsend, Robert Langer, and Gordana Vunjak-Novakovic

This journal article is available at ScholarlyCommons: http://repository.upenn.edu/be_papers/94

Hyaluronic acid hydrogel for controlled self-renewal and differentiation of human embryonic stem cells

Sharon Gerecht*, Jason A. Burdick[†], Lino S. Ferreira[‡§¶], Seth A. Townsend[‡], Robert Langer*[‡‖], and Gordana Vunjak-Novakovic**[††]

*Harvard–Massachusetts Institute of Technology Division of Health Sciences and Technology and [‡]Departments of Chemical Engineering and Bioengineering, Massachusetts Institute of Technology, Cambridge, MA 02139; [§]Center of Neurosciences and Cell Biology, University of Coimbra, 3004-517 Coimbra, Portugal; [¶]Biocant Centro de Inovação em Biotecnologia, 3060-197 Cantanhede, Portugal; [†]Department of Bioengineering, University of Pennsylvania, Philadelphia, PA 19104; and **Department of Biomedical Engineering, Columbia University, New York, NY 10027

Contributed by Robert Langer, April 26, 2007 (sent for review February 7, 2007)

Control of self-renewal and differentiation of human ES cells (hESCs) remains a challenge. This is largely due to the use of culture systems that involve poorly defined animal products and do not mimic the normal developmental milieu. Routine protocols involve the propagation of hESCs on mouse fibroblast or human feeder layers, enzymatic cell removal, and spontaneous differentiation in cultures of embryoid bodies, and each of these steps involves significant variability of culture conditions. We report that a completely synthetic hydrogel matrix can support (*i*) long-term self-renewal of hESCs in the presence of conditioned medium from mouse embryonic fibroblast feeder layers, and (*ii*) direct cell differentiation. Hyaluronic acid (HA) hydrogels were selected because of the role of HA in early development and feeder layer cultures of hESCs and the controllability of hydrogel architecture, mechanics, and degradation. When encapsulated in 3D HA hydrogels (but not within other hydrogels or in monolayer cultures on HA), hESCs maintained their undifferentiated state, preserved their normal karyotype, and maintained their full differentiation capacity as indicated by embryoid body formation. Differentiation could be induced within the same hydrogel by simply altering soluble factors. We therefore propose that HA hydrogels, with their developmentally relevant composition and tunable physical properties, provide a unique microenvironment for the self-renewal and differentiation of hESCs.

scaffolds | three-dimensional cultures | vasculogenesis

Undifferentiated human ES cells (hESCs), derived from the inner cell mass of the developing blastocyte, are routinely cultured on mouse embryonic fibroblast feeder layers (MEFs) or on surfaces coated with Matrigel (an animal basement membrane preparation extracted from Engelbreth–Holm–Swarm mouse sarcoma), laminin, fibronectin, and human serum (1–4) in MEF-conditioned medium. To induce hESC differentiation, cells are enzymatically removed from monolayers and recultured in the form of embryoid bodies (EBs) on a stromal layer (5) or on extracellular matrix (6, 7). In contrast, during early development, hESCs reside and differentiate within a single 3D environmental milieu. The standard hESC culture protocols are thus limited by the need for cell transfer between the two different and completely separate culture systems for cell renewal and differentiation, which causes significant variability of culture conditions. We investigated the possibility of designing a single culture system that would mimic the early developmental milieu and allow the cells to switch between differentiation states within the same culture setting.

During embryogenesis, inner cell mass cells are embedded in a 3D matrix, which regulates both their self-renewal and differentiation (8, 9). To establish a single, controllable 3D culture system in which hESCs can be maintained as undifferentiated cells and differentiate in response to specific cues, we explored encapsulation of hESC lines in hydrogel scaffolds (that we selected to mimic the developmental milieu) composed of a biologically recognized molecule (that we identified studying the MEF cultures of hESCs). Hydrogels not only have a high water content to promote cell viability, but they are structurally and mechanically similar to the native extracellular matrix of many tissues (10). In combination with recently developed chemically defined media (11), these scaffolds could provide a defined system for hESC culture that does not incorporate any animal components.

Several studies have explored the culture of hESCs in defined 3D settings by using a variety of natural and synthetic scaffolds for cell growth (12), differentiation (13), or lineage guidance (14–18). There has been a considerable effort to replace more biological but less controllable native materials with synthetic materials. The synthetic scaffolding materials explored thus far have not been designed by using developmentally relevant molecules and, in the best case, supported only a short-term self-renewal of hESCs (12). We hypothesized that hyaluronic acid (HA), a nonsulfated linear polysaccharide of (1-β-4)D-glucuronic acid and (1-β-3)*N*-acetyl-D-glucosamine, would support hESC growth *in vitro*, because it coregulates gene expression, signaling, proliferation, motility, adhesion, metastasis, and morphogenesis of hESCs *in vivo* (19). In humans, the HA content is greatest in undifferentiated cells and during early embryogenesis and then decreases at the onset of differentiation (20), where it has a crucial role in regulation of the angiogenic process (21–23). Despite its known role in embryogenesis (19, 20), HA has not been used for the cultivation of hESCs.

We suggest that HA-based hydrogels can maintain the undifferentiated state of hESCs in the presence of conditioned medium from MEFs until soluble factors are introduced to direct cell differentiation. We found that hESCs have active HA binding sites and receptors that are involved in feeder layers and showed that hESCs are able to internalize and process HA. We report that the cultivation of hESCs in HA hydrogels maintained the state of cell self-renewal and enabled EB formation from released cells, whereas the introduction of angiogenic factors readily induced cell sprouting and elongation, indicating a switch to vascular differentiation.

Results and Discussion

HA Is Involved in the Maintenance of Undifferentiated hESCs. We first investigated whether HA plays a role in conventional cultures of

Author contributions: S.G., J.A.B., S.A.T., R.L., and G.V.-N. designed research; S.G., L.S.F., and S.A.T. performed research; L.S.F. contributed new reagents/analytic tools; S.G., J.A.B., S.A.T., and G.V.-N. analyzed data; S.G., J.A.B., R.L., and G.V.-N. wrote the paper; and J.A.B. and L.S.F. designed hydrogels.

The authors declare no conflict of interest.

Abbreviations: EB, embryoid body; HA, hyaluronic acid; FL-HA, fluorescein-labeled HA; MEF, mouse embryonic fibroblast feeder layers; XTT, 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide.

‖To whom correspondence may be addressed. E-mail: rlanger@mit.edu.

[††]To whom correspondence may be addressed at: Department of Biomedical Engineering, Columbia University, William Black Research Building 1605–1611, 650 West 168th Street, MC 104B, New York, NY 10032. E-mail: gv2131@columbia.edu.

This article contains supporting information online at www.pnas.org/cgi/content/full/0703723104/DC1.

© 2007 by The National Academy of Sciences of the USA

undifferentiated hESCs. We observed that MEFs, which form feeder layers for hESC cultivation, produce 8-fold higher levels of HA (840 ng/ml) compared with initial levels in the growth media (105 ng/ml) and that abundant HA binding sites are located intracellularly in undifferentiated hESCs (Fig. 1*A*). These findings are consistent with previous evidence that HA is localized intracellularly, in endosomes and perinuclear tubular vesicles, rough endoplasmic reticulum, nuclei, and nucleoli (24–26). We therefore investigated whether the success of MEF feeder layers for the cultivation of hESCs might be related to their ability to secrete HA.

During development, cellular interactions with HA are mediated by CD44 and CD168. CD44 is a mediator for HA-induced cell proliferation and survival pathways (19) and is present in human cumulus cells, oocytes, early embryos, and prehatched blastocysts (27). CD44 also is involved in the initial binding of HA to the cell surface before its internalization and degradation by acid hydrolysis. CD168 is involved in HA-induced cell locomotion (28) and early embryos (29). During *in vitro* culture, undifferentiated hESCs expressed high levels of CD44 and CD168 (Fig. 1*B*). In fact, hESC colonies cultured on MEFs could be easily visualized by staining for CD44 (Fig. 1*Ci*) or CD168 (Fig. 1*Cii*). Confocal analysis suggests that CD44 is expressed intracellularly (Fig. 1*Ciii*) and CD168 is expressed either on the membrane or intracellularly in undifferentiated cells (Fig. 1*Civ*).

The addition of human fluorescein-labeled HA (FL-HA) to the culture of hESCs on MEFs resulted in the localization of HA receptors to the cell membranes, first at the edges of cell colonies and then at their centers (Fig. 2*A*). FL-HA was observed to be internalized (Fig. 2*Bi*) and localized within the cells (Fig. 2 *Bii* and *Biii*). No internalization of FL-HA could be observed once anti-CD44 was added to the cultures of hESCs, indicating receptor-mediated internalization of HA by hESCs. To examine whether blocking HA internalization effects self-renewal, hESCs were passaged and seeded on MEFs with and without the addition of a mixture of anti-CD44 (clones A3D8 and P3H9) and anti-CD168. After 24 h, colony formation could be observed in both culture conditions. However, fewer colonies (32.5 ± 3.41 vs. 59.2 ± 8.35 per well) and a higher differentiation rate (43.66 ± 0.046% vs. 12.75 ± 0.033%) (Fig. 2*C*) were observed in the cultures supplemented with antibodies. After 48 h, the antibody-containing cultures still had much lower colony numbers (37.75 ± 7.0 vs. 101.5 ± 9.2) and higher differentiation rates (34.5 ± 0.047% vs. 5.93 ± 0.005%) (Fig. 2*C*) than control cultures. This result further suggests that HA receptors, CD44, and CD168 are involved in the self-renewal of hESCs. Immunofluorescence of hESC colonies cultured on MEFs revealed that densely packed colonies expressed human hyaluronidase Hyal 1 and 2 (Fig. 2*D*). RT-PCR analysis corroborated that hESCs express high levels of Hyal 2, one of the isoforms of human hyaluronidase (Fig. 2*E*). It was previously suggested that HA originates from the pericellular material that is degraded intracellularly (30, 31). Our data suggest that hESCs are able to uptake and degrade HA and thereby remodel HA gels, a feature necessary for cell survival and migration.

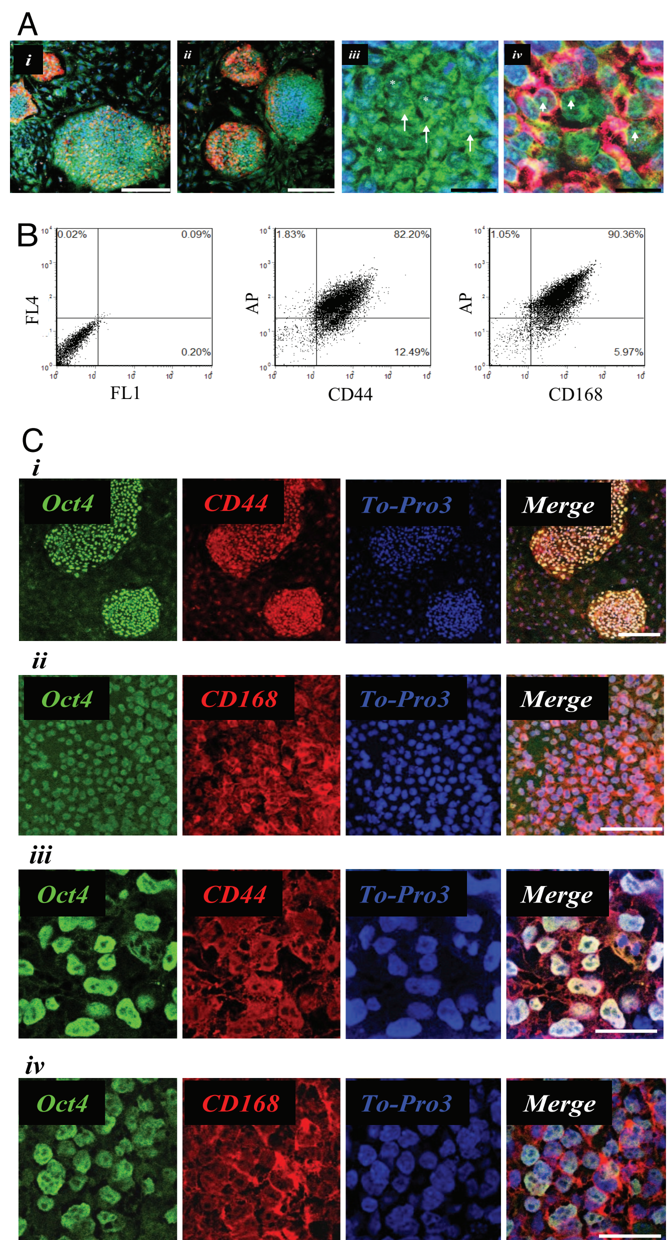

Fig. 1. HA plays a role during hESC culture on MEFs. (*A*) Staining of hESCs (H1 line) grown on MEFs for HA binding site (green), undifferentiated membrane marker TRA-1–81 (red), and nuclei (blue): Intracellular localization of HA (*Ai* and *Aii*), including perinuclear areas (arrows) (*Aiii*) and nuclei (*), and nucleoli (arrowheads) (*Aiv*). (*B*) FACS analysis revealed that compared with isotype control (*Left*), the majority of undifferentiated hESCs were found to express HA receptors CD44 (82%) (*Center*) and CD168 (90%) (*Right*). (*Ci* and *Cii*) By using immunofluorescence staining, undifferentiated hESC colonies were easily detected with undifferentiated cell markers Oct4 (green) and CD44 or CD168 (red), respectively (nuclei: blue). (*Ciii* and *Civ*) Higher magnification suggests intracellular expression of CD44 and either membrane or intracellular expression of CD168. (Scale bars: *Ai*, *Aii*, *Ci*, and *Cii*, 100 μm; *Aiii*, *Ciii*, and *Civ*, 25 μm; *Aiv*, 10 μm.)

HA Hydrogels Provide a Biocompatible Environment for hESC Culture. Rather than adding soluble HA to the culture or modifying biomaterial surfaces with this molecule, we chose to more directly mimic the native environment and encapsulate hESCs in hydrogels fabricated entirely of HA. To accomplish this, HA was modified with photoreactive groups (32) and colonies of hESCs were suspended in a solution of the modified HA and photoinitiator in PBS. This process has been used previously to entrap a variety of mammalian cells (33). In our previous studies, hydrogels comprised of 2 wt% of a 50-kDa macromer supported the highest viability of differentiated mammalian cells (32, 33) and thus were used in these studies. One advantage of HA hydrogels is that the chemistry of the network is easily controlled through reaction conditions and is uniform between the various batches (32), which is difficult or impossible to achieve with naturally derived matrices such as Matrigel. Additionally, the monomer is obtained microbially and does not introduce animal components.

Because hESCs are particularly susceptible to harmful culture conditions (34), it was important to assess any toxicity of the methacrylated HA macromer. Human ESCs were propagated in monolayers with a range of concentrations of the HA macromer (0,

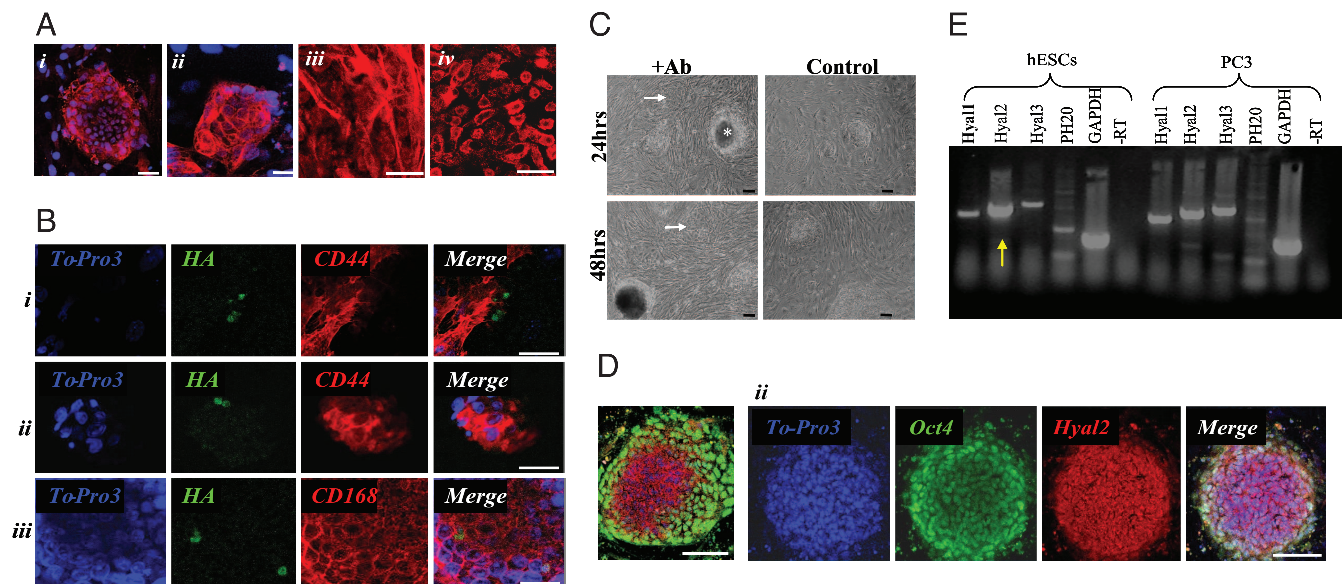

Fig. 2. HA interaction with hESCs. (*A*) Localization of HA receptors in response to addition of human FL-HA to the growth medium of hESCs (H9 line) cultured on MEFs. (*Ai* and *Aii*) Confocal analysis suggests relocalization of HA receptors in cell membranes of both CD44 (*Ai*) and CD168 (shown in red, nuclei shown in blue) (*Aii*). (*Aiii* and *Aiv*) Higher magnification of CD168 localization is shown with (*Aiii*) and without (*Aiv*) the addition of human HA. (*B*) HA uptake by hESC (H9 line) colonies. Undifferentiated hESCs (H1 line) grown on MEFs were incubated overnight with fluorescein-HA and further stained for CD44 and CD168. (*Bi*) Edge of colony suggests internalizing FL-HA via CD44. (*Bii* and *Biii*) Intracellular localization of FL-HA. (*C*) Seeding of hESCs (H9 line) in the presence of anti-CD44 and CD168 resulted in less and differentiated colonies (both at the edge and center of the colonies, as indicated by arrows and asterisks, respectively), whereas control cultures contain expanding undifferentiated colonies. (*D*) Human ESC (H13 line) colonies grown on MEFs positive for Oct4 (green) express Hyal 1 (*Di*) or Hyal 2 (*Dii*) (red; nuclei are shown in blue) mainly in densely packed areas of the colonies. (*E*) RT-PCR analysis revealed high expression levels of a hyaluronidase isomer, Hyal 2, in undifferentiated hESCs (H9 line). PC3 line served as positive control. (Scale bars: 100 μm.)

10, and 50 μl/ml culture medium). Human ESCs formed colonies of proliferating cells at all culture conditions (Fig. 3 *Ai–Aiii*). Comparison of the metabolic activity rates revealed slight toxic effects only at the macromer concentration of 50 μl/ml (Fig. 3*Aiv*), a level corresponding to completely nonpolymerized HA and therefore much higher than that seen by the encapsulated cells. The rate of cell proliferation at a macromer concentration of 10 μl/ml, a level corresponding to a HA hydrogel that was polymerized to 80% incorporation of the macromer, was indistinguishable from that in control medium (Fig. 3 *Aiv* and *Av*). Radical polymerization of loosely cross-linked HA hydrogels occurs at high conversion rates and the release of unreacted macromer is only minimal, thus minimizing any toxicity that may result from the presence of free HA macromer.

Because formation of HA gels involves exposure to low levels of UV light, we explored potential DNA damage to the hESCs during this process. A recent study demonstrated that hESCs express low levels of p53 (compared with mESCs) and that long-term exposure (5 h) to UV light resulted in accumulation of p53 in the cell nuclei (35). Accumulation of p53 could be observed 12 h after exposure to UV (35). We therefore explored whether 10 min of exposure to UV light results in accumulation of p53 in hESCs. We found that p53 accumulated in cells exposed to UV light for 5 h, whereas only background levels of p53 expression were detected in both unexposed cells and those exposed to 10 min of UV light (Fig. 3*B*). This result suggests that photopolymerization of hESCs in HA macromer does not directly damage their DNA.

HA Hydrogels Maintain hESCs in Their Undifferentiated State of Self-Renewal. For encapsulation, hESCs were suspended in a solution of HA macromer and photoinitiator and photopolymerized into a hydrogel network, and constructs were placed within conditioned medium supplemented with basic FGF. Human ESCs encapsulated in the HA hydrogels were uniformly distributed throughout the gel (Fig. 3*C*), forming cell colonies with a range of sizes (Fig. 3*D*). The cells retained metabolic activity (Fig. 3*E*) and early doubling times of ≈36 h, comparable with those in 2D culture systems (34) for up to 5 days, as determined by a 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) assay (Fig. 3*F*). As the cells remodeled the hydrogel, colonies near the surface were released, making it difficult to accurately quantify cell proliferation. We documented the maintenance of hESC viability in HA hydrogels through several markers. The human Ki-67 protein, which is associated with cell proliferation, was expressed by the majority of encapsulated hESCs after 20 days of culture (58 ± 5%) [supporting information (SI) Fig. 6*A*]. Only occasional apoptotic events could be observed by using a Tunnel assay (14 ± 3%). These results correlate to a recent study that showed that >50% of the cell nuclei within hESC colonies grown on MEFs are in a proliferating phase (36). Only infrequent expression of caspase-3, a marker activated in cells undergoing apoptosis, was found within HA-hESC constructs (3 ± 8%) after 20 days of culture. When detected, caspase-3 appeared in a whole colony rather than in single cells within different colonies (SI Fig. 6 *B* and *C*) and only in cultures older than 15 days. Therefore, under the conditions studied, diffusion of nutrients and oxygen to the cells through the 2 wt% HA hydrogel appeared to be rapid enough to support normal cell growth rates. In addition, the cells maintained their typical undifferentiated morphology of colonies within the HA networks (Fig. 3*G*) after 20 days in culture. High cell concentrations, in the range of 5–10 × 10^6 cells per milliliter of the precursor solution, were essential for high viability and sustained cell growth. At hESC concentrations greater than 10 × 10^6 cells per milliliter, large clumps of cells formed that underwent rapid apoptosis, whereas cell concentrations lower than 5 × 10^6 cells per milliliter could not support colony formation within the networks (data not shown). The same phenomenon of concentration dependence of hESC colony formation was observed in 2D monolayers (37, 38).

To determine whether the hESC-HA interactions, and not only the 3D morphology via encapsulation in hydrogel, are critical for

Fig. 3. HA hydrogels support the maintenance of viable hESCs in their undifferentiated state. (*Ai–Aiv*) Undifferentiated hESCs (H9 line) were passaged and recultured on feeder layers for 4 days in culture medium containing: no macromer (*Ai*), 10 μl/ml macromer (corresponding to the hydrogel containing 80% nonpolymerized monomer) (*Aii*), or 50 μl/ml macromer (corresponding to completely nonpolymerized monomer) (*Aiii*). Toxic effects were detected only at the macromer concentration of 50 μl/ml. (*Aiv* and *Av*) XTT assay (*Aiv*) and cell count (*Av*) revealed no negative effect of macromer on cell viability at a concentration of 10 μl/ml and a slight decrease in hESC viability at a macromer concentration of 50 μl/ml. Results are presented as means ± SD (*, $P < 0.05$). (*B*) Low basal levels of p53 are expressed by hESCs (H13 line) 12 h postincubation after 10 minutes of UV exposure, whereas accumulation of p53 in hESCs was observed 12 h postincubation after 5 h UV exposure. (*C* and *D*) Light microscopy revealed uniform distribution of hESC colonies in HA gels (*C*) with a range of colony sizes (*D*). (*E*) Incubation with XTT revealed orange dye in metabolically active hESCs (H13 line) encapsulated in HA hydrogels. (*F*) XTT assay shows comparable growth rates of hESCs encapsulated in HA hydrogels and on Matrigel. (*G*) A representative histological section of hESC-HA constructs (H9 line) cultured for 20 days demonstrates uniform morphology (H&E stain) of undifferentiated colony within 3D networks (folding of the hydrogel is indicated by an asterisk). (*H*) Encapsulation of hESCs (H13 line) in HA hydrogels was compared with dextran hydrogels after 15 days of culture. Light microscope images of both cultures at low and high magnifications and histological sections (H&E stain) demonstrate EB formation in dextran hydrogels vs. colony arrangements of undifferentiated hESCs in HA hydrogels. (Scale bars: *A*, *C–F*, and *H*, 100 μm; *B* and *G*, 25 μm.)

controlled hESC differentiation, hESCs were also encapsulated in networks formed from a different polysaccharide, dextran, using the exact same methodology of photopolymerization. In contrast to the maintenance of undifferentiated hESC colonies in the HA system, dextran hydrogels induced hESC differentiation and the formation of EBs (Fig. 3*H*). These results are consistent with data published for other hydrogel systems that also supported hESC differentiation (14, 18, 39). Therefore, HA hydrogels act as a unique microenvironment for the propagation of hESCs, likely due to the regulatory role of HA in the maintenance of hESCs in their undifferentiated state, *in vitro* as well as *in vivo*.

Human ESCs Released from HA Hydrogels Are Viable and Undifferentiated, and Have Preserved Genetic Integrity. To enable the use of HA hydrogels for research and cellular therapy, the cells need to be released from the hydrogel with preservation of high viability. This can be achieved by treatment with hyaluronidase (32). First, hESCs were examined for their viability during a long-term treatment (i.e., 24 h). Human ESC colonies incubated with growth medium containing 100–2,000 units/ml hyaluronidase preserved their normal morphology with no apparent loss of viability (Fig. 4 *A–D*). Incubation of HA-hESC constructs in growth medium containing 2,000 units/ml hyaluronidase resulted in complete degradation of the hydrogel (Fig. 4 *E* and *F*). We also found that the viability of hESCs incubated with 2,000 units/ml hyaluronidase for 24 h was comparable with that measured for incubation with 1 mg/ml collagenase IV for 30 min (76.5 ± 8% vs. 70 ± 4.5%, respectively). In contrast, hyaluronidase concentrations of <1,000 units/ml resulted in only partial degradation of HA hydrogels over a 24-h period and were associated with low efficiency of hESC retrieval. Importantly, the release of hESCs from the HA hydrogels was associated with the full preservation of cell viability and undifferentiated state. Colonies released from the hydrogels readily adhered to MEFs (Fig. 4*G*) with high adherence efficiency (79 ± 2.5% after 48 h) and proliferated at rates normally seen in standard monolayer cultures (Fig. 4*H*). Furthermore, FACS analysis of released hESCs showed high levels of expression of stem cell markers SSEA4 and alkaline phosphatase (Fig. 4*I*).

The proposed system for hESC culture in an HA hydrogel involves the exposure of hESCs to low-intensity UV light (i.e., ≈10 mW/cm^2 for 10 min) and treatment with hyaluronidase (i.e., 2,000 units/ml for 24 h). Because these factors could potentially affect the genetic integrity of hESCs, karyotype analysis was performed on (*i*) undifferentiated hESCs cultured on MEFs (H9 line p22 and H13 line p25); (*ii*) undifferentiated hESCs cultured on MEFs (H9 line p22 and H13 line p25), all of which were exposed to UV light for 10 min; (*iii*) undifferentiated hESCs cultured on MEFs (H9 line p22 and H13 line p25) treated with hyaluronidase (2,000 units/ml) for 24 h; (*iv*) undifferentiated hESCs (H9 line p38) encapsulated in HA gels for 5 days followed by their release and reculture on MEFs for an additional three passages; and (*v*) H13 p33 encapsulated in HA for 2 weeks and recultured on MEF for two passages. Human ESCs were found to have normal karyotype with no clonal aberration (SI Fig. 7). Hence, the application of UV light, encapsulation, and

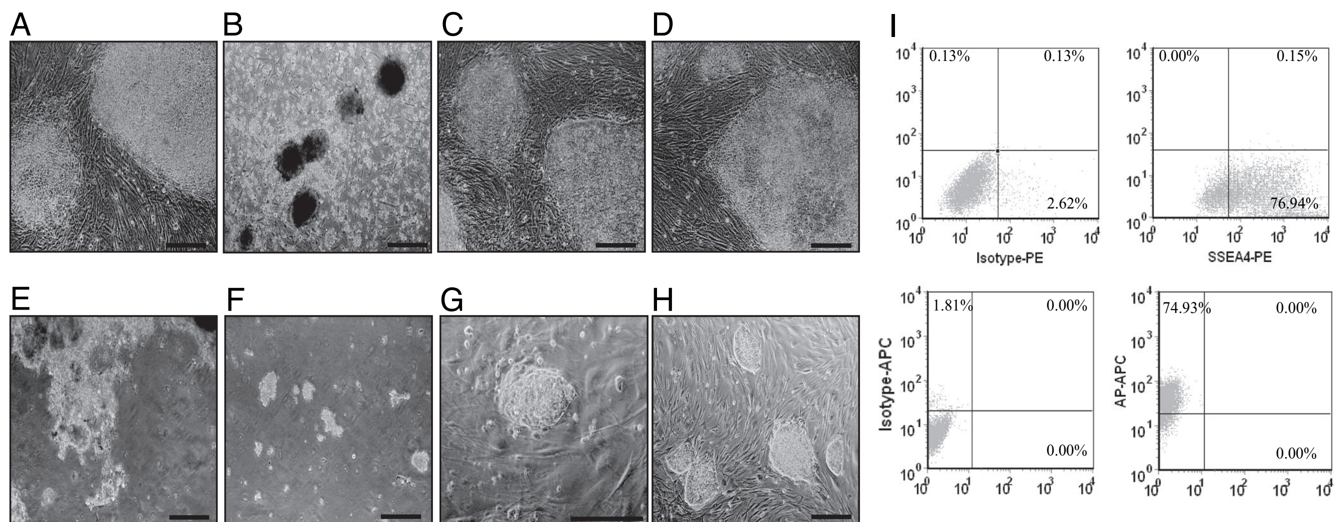

Fig. 4. Cell release from hydrogels and cell karyotyping. (*A–D*) hESCs (H13 line) grown on MEFs were incubated for 24 h in growth medium (*A*), 1% collagenase solution in growth medium (*B*), 1,000 units/ml hyaluronidase solution in growth medium (*C*), and 2,000 units/ml hyaluronidase solution in growth medium (*D*). To release hESCs from HA hydrogel, constructs were incubated with 2,000 units/ml hyaluronidase in growth medium. (*E*) After 18 h, small particles of hydrogels remained that trapped hESCs. (*F*) After 24 h, hESCs colonies were completely released from the hydrogel. (*G* and *H*) hESCs (H9 line) released from the hydrogel after 30 days of encapsulation and cultured on MEFs formed small colonies of undifferentiated cells after 24 h (*G*) and were propagated on MEFs for three passages (*H*). (*I*) FACS analyses of released cells after 20 days of HA culture revealed high levels of SSEA4 and alkaline phosphatase. (Scale bars: 100 μm.)

hyaluronidase at the levels necessary for our work appear to be safe for hESCs.

Human ESCs Encapsulated in HA Hydrogel Maintain Their Capacity for Differentiation. An important advantage of the HA hydrogel system is that the hESCs can be first maintained in their undifferentiated state and then exposed to differentiation factors within the same system or released to be studied with other strategies, *in vitro* or *in vivo*. To illustrate this feature, we compared (*i*) spontaneous differentiation of hESCs via EB formation for hESCs released from hydrogel and (*ii*) induction of vasculogenic sprouting of HA-encapsulated hESCs. Cells that were cultured in HA hydrogels for 30 days, released with hyaluronidase, and subsequently cultured in suspension were found to form EBs containing cell types representative of all three germ layers (SI Fig. 8). HA was observed to play a role in the regulation of angiogenesis and vascular endothelial cell function. In particular, low-molecular-weight degradation products (3–10 disaccharide units) stimulated endothelial cell proliferation, migration, and sprouting (22). Generation of "angiogenic" HA from the naturally occurring HA is mediated by the endoglycosidase hyaluronidase, by processes that are associated with tissue damage, inflammatory disease, and certain types of tumors (21). We therefore explored HA hydrogel culture systems for vascular differentiation. Human ESCs were encapsulated in HA hydrogels and cultured in MEF conditioned medium for 1 week, after which the medium was replaced by angiogenic differentiation medium containing VEGF. Cell sprouting and elongation was observed after 48 h for hESC colonies treated with VEGF (Fig. 5 *A* and *B*). After 1 week of differentiation, staining with specific vascular markers revealed that most sprouting cells were positive for smooth muscle actin (Fig. 5*C*), whereas few were positive for CD34 (Fig. 5*D*).

Materials and Methods

hESCs. Multiple lines of hESCs were studied: H9, H13, and, in several studies, H1 (WiCell Research Institute, Madison, WI).

hESC Culture on MEFs. hESCs were grown on inactivated MEFs in growth medium consisting of 80% knockout DMEM, supplemented with 20% knockout serum replacement, 4 ng/ml basic FGF, 1 mM L-glutamine, 0.1 mM 2-mercaptoethanol, 1% nonessential amino acid stock (Invitrogen). Human ESCs were passaged every 4–6 days with 1 mg/ml type IV collagenase (Invitrogen).

hESC Encapsulation and Release. Methacrylated HA was synthesized as described (32) (*SI Materials and Methods*). It was dissolved at a concentration of 2 wt% in PBS containing 0.05 wt% 2-methyl-1-[4-(hydroxyethoxy)phenyl]-2-methyl-1-propanone (Irgacure 2959), and hESCs were added [(0.5–1) $\times$ 10^7 cells per milliliter of precursor solution]. The mixture was pipetted into a sterile mold

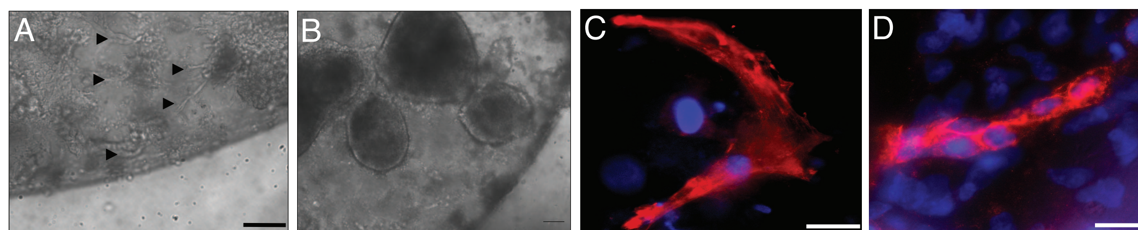

Fig. 5. Differentiation. H9 line cells were cultured in conditioned medium for 1 week followed by the replacement of medium containing VEGF. (*A* and *B*) Cell sprouting was observed after 48 h in gels transferred to medium containing VEGF (arrows) (*A*) compared with gels continuously cultured in conditioned medium (*B*). (*C* and *D*) After 1 week of differentiation, sprouting elongating cells were mainly positive for vascular α-smooth muscle actin (*C*), whereas some were positive for early stage endothelial marker (*D*). CD34 (*in situ* 3D staining of gels). (Scale bars: *A* and *B*, 100 μm; *C* and *D*, 25 μm.)

(50-μl volume per well, to obtain discs with diameters of 3 mm and thicknesses of 2 mm) and photopolymerized [$\approx$10 mW/cm^2 UV light (BlakRay) for 10 min]. The acrylated dextran macromer was prepared as described (18) (*SI Materials and Methods*), and hESCs were encapsulated within the dextran by using the same procedures as for HA hydrogels. Cell–gel constructs were cultivated in MEF conditioned medium as previously described (2). For differentiation, gels were cultured with endothelial growth medium (Cambrex) supplemented with 100 ng/ml VEGF (R & D Systems).

To release encapsulated hESCs, HA constructs were incubated for 24 h in hESC growth medium containing 100, 500, 1,000, or 2,000 units/ml hyaluronidase (Sigma). The percentage of viable hESCs incubated with 2,000 units/ml HAase for 24 h or 1 mg/ml collagenase IV for 30 min was examined by trypan blue. For reculture, cells were collected, centrifuged, washed three times with PBS to remove any hydrogel residues, resuspended in growth medium, and cultured on MEF-coated dishes according to standard methods (35, 36). For adherence studies, released hESC colonies were seeded on four-well plates coated with MEFs and the medium was not changed for 48 h. To estimate the adherence percentage, we collected the media from each well and counted the colonies in the supernatant and those that attached to the MEFs. We excluded single cells from the counts. Results are presented for $n = 3$. For EB formation, released hESCs were recultivated in nonadherent Petri dishes with EB medium (13).

Presence of HA in Medium. MEF-conditioned medium was prepared as described (2) and compared with hESC growth medium with respect to the levels of HA by using an HA test kit (Corgenic).

FACS Analysis. hESCs were removed from MEFs or released from hydrogels and analyzed by means of flow cytometry (*SI Materials and Methods*).

Proliferation Assay. Cell proliferation was detected either by daily cell count or the XTT kit (Sigma) according to the manufacturer's instructions (*SI Materials and Methods*).

Immunohistochemistry. EBs and HA constructs were either embedded in histo-gel or directly fixed in 10% neutral-buffered formalin (Sigma) overnight, dehydrated in graded alcohols (70–100%), embedded in paraffin, sectioned to 4 μm, and either stained with hematoxylin eosin or immunostained with specific markers (*SI Materials and Methods*).

Immunofluorescence and Confocal Microscopy. hESC colonies grown on MEFs, hESC colonies grown on MEFs and exposed to UV for 10 min and 5 h, and HA-hESC-differentiated constructs were fixed *in situ* with Accustain (Sigma) for 20–25 min at room temperature. After blocking with 5% FBS, cells were permeabilized (when intracellular markers were examined) and stained with one primary antibody (see list of antibodies in *SI Materials and Methods*). Cells were then rinsed three times with PBS (Invitrogen) and incubated for 30 min with suitable FITC- (R & D Systems) or Cy3-conjugated (Sigma) secondary antibodies. DAPI (2 μg/ml; Sigma) or To-pro 3 (1:500; Molecular Probes/Invitrogen) were added during the last rinse. IgG isotype-matching using mouse or goat (both from R & D Systems) or secondary antibody alone served as controls. The immunolabeled cells were examined by using either fluorescence microscopy (TE300 inverted microscope; Nikon) or confocal laser scanning microscopy (LSM 510; Zeiss).

HA Binding, Uptake, and Blocking. The binding assay of FL-HA was performed as described (26). Briefly, hESCs were cultured on coverslips and gently washed. Human FL-HA at 100 μg/ml (Sigma) was added to the growth medium for 16 h at 4°C. In some cases, to study the role HA receptor, anti-human CD44 was added to the cultures or incubation with FL-HA. After three washes with ice-cold PBS, the cells were fixed in 100% ice-cold acetone for 10 min, air-dried, and rehydrated for 15 min in PBS. Processed cells were further stained with anti-CD44 or anti-CD168 and examined. To block HA receptors, hESCs were passaged and seeded with or without the addition of a mixture of anti-CD44 (clones A3D8 and P3H9) and anti-CD168 ($n = 4$). Colony formation and morphologically differentiating colonies were quantified and documented after 24–48 h.

RT-PCR. RNA was extracted and analyzed as described (40). Please see *SI Materials and Methods* for details.

Karyotyping Analysis. Cells were prepared and analyzed as described and recommended (41). Please see *SI Materials and Methods* for details.

We thank Drs. Hyoungshin Park, Chris Cannizzaro, and Nicola Elvassore for help with cell analyses; Kathy Cormier for help with histology; and Zhitong Zhang for technical assistance. This work was supported by the Juvenile Diabetes Research Foundation fellowship (to S.G.) and grant (to G.V.-N.), National Institutes of Health Grants K22 DE-015761 (to J.A.B.), P41 EB002520-01A1, and 1R01HL076485-01A2 (both to G.V.-N.), and Fundação para a Ciência e Tecnologia Grant SFRH/BPD/14502/2003 (to L.S.F.).

1. Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, Jones JM (1998) *Science* 282:1145–1147.
2. Xu C, Inokuma MS, Denham J, Golds K, Kundu P, Gold JD, Carpenter MK (2001) *Nat Biotechnol* 19:971–974.
3. Richards M, Fong CY, Chan WK, Wong PC, Bongso A (2002) *Nat Biotechnol* 20:933–936.
4. Stoikovic P, Lako M, Przyborski S, Stewart R, Armstong L, Evans J, Zhang X, Stojkpvic M (2005) *Stem Cells* 23:895–902.
5. Itskovitz-Eldor J, Schuldiner M, Karsenti D, Eden A, Yanuka O, Amit M, Soreq H, Benvenisty N (2000) *Mol Med* 6:88–95.
6. Gerecht-Nir S, Itskovitz-Eldor J (2006) in *Embryonic Stem Cells: A Practical Approach*, eds Notarianni E, Evans M (Oxford Univ Press, Oxford).
7. Turksen K, Troy TC (2006) *Methods Mol Biol* 331:1–12.
8. Czyz J, Wobus A (2001) *Differentiation (Berlin)* 68:167–174.
9. Abbott A (2003) *Nature* 424:870–872.
10. Yang S, Leong KF, Du Z, Chua CK (2001) *Tissue Eng* 7:679–689.
11. Yao S, Chen S, Clark J, Hao E, Beattie GM, Hayek A, Ding S (2006) *Proc Natl Acad Sci USA* 103:6907–6912.
12. Li YJ, Chung EH, Rodriguez RT, Firpo MT, Healy KE (2006) *J Biomed Mater Res A* 79:1–5.
13. Gerecht-Nir S, Cohen S, Ziskind A, Itskovitz-Eldor J (2004) *Biotechnol Bioeng* 88:313–320.
14. Levenberg S, Huang NF, Lavik E, Rogers AB, Itskovitz-Eldor J, Langer R (2003) *Proc Natl Acad Sci USA* 100:12741–12746.
15. Levenberg S, Burdick JA, Kraehenbuehl T, Langer R (2005) *Tissue Eng* 11:506–512.
16. Baharvand H, Hashemi SM, Kazemi Ashtiani, Farrokhi A (2006) *Int J Dev Biol* 50:645–652.
17. Gerami-Naini B, Dovzhenko OV, Durning M, Wegner FH, Thomson JA, Golos TG (2004) *Endocrinology* 145:1517–1524.
18. Ferreira L, Gerecht S, Fuller J, Shieh HF, Vunjak-Novakovic G, Langer R (2007) *Biomaterials* 28:2706–2717.
19. Toole BP (2004) *Nat Rev Cancer* 4:528–539.
20. Toole BP (2001) *Semin Cell Dev Biol* 12:79–87.
21. Rooney P, Kumar S, Ponting J, Wang M (1995) *Int J Cancer* 60:632–636.
22. Slevin M, Krupinski J, Kumar S, Gaffney J (1998) *Lab Invest* 78:987–1003.
23. Slevin M, Kumar S, Gaffney J (2002) *J Biol Chem* 277:41046–41059.
24. Tammi R, Rilla K, Pienimaki JP, MacCallum DK, Hogg M, Luukkonen M, Hascall VC, Tammi M (2001) *J Biol Chem* 276: 35111–35122.
25. Londono I, Bendayan M (1998) *J Histochem Cytochem* 36:1005–1014.
26. Evanko SP, Parks WT, Wight TN (2004) *J Histochem Cytochem* 52:1525–1535.
27. Campbell S, Swann HR, Aplin JD, Seif MW, Kimber SJ, Elstein M (1995) *Hum Reprod* 10:425–430.
28. Yang B, Hall CL, Yang BL, Savani RC, Turley EA (1994) *J Cell Biochem* 56:455–468.
29. Stojkovic M, Krebs O, Kölle S, Prelle K, Assmann V, Zakhartchenko V, Sinowatz F, Wolf K (2003) *Biol Reprod* 68:60–66.
30. McGuire PG, Castellot JJ, Orkin RW (1987) *J Cell Physiol* 133:267–276.
31. Stern R (2003) *Glycobiology* 13:105R–115R.
32. Burdick JA, Chung C, Jia X, Randolph MA, Langer R (2005) *Biomacromolecules* 6:386–391.
33. Chung C, Mesa J, Randolph MA, Yaremchuk M, Burdick JA (2006) *J Biomed Mater Res A* 77A:518–525.
34. Amit M, Carpenter MK, Inokuma MS, Chiu CP, Harris CP, Waknitz MA, Itskovitz-Eldor J, Thomson JA (2000) *Dev Biol* 227:271–278.
35. Qin H, Yu T, Qing T, Liu Y, Zhao Y, Cai J, Li J, Song Z, Qu X, Zhou P, *et al*. (2007) *J Biol Chem* 282:5842–5852.
36. Heng BC, Cao T, Liu H, Rufaihah AJ (2005) *Cell Biochem Funct* 23:141–146.
37. Carpenter MK, Chunhui Xu, Daight CA, Antosiewiczm JE, Thomson JA (2003) in *Human Embryonic Stem Cells*, eds Chiu A, Rao MS (Humana, Totowa, NJ).
38. Schatten G, Smith J, Navara C, Park JH, Pedersen R (2005) *Nat Methods* 2:455–463.
39. Dang S, Gerecht-Nir S, Chen J, Itskovitz-Eldor J, Zandstra PW (2004) *Stem Cells* 22:275–282.
40. Gerecht-Nir S, Osenberg S, Nevo O, Ziskind A, Coleman R, Itskovitz-Eldor J (2004) *Biol Reprod* 71:2029–2036.
41. Cowan CA (2004) *N Engl J Med* 350:1353–1356.