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# Delivering Heparin-Binding Insulin-Like Growth Factor 1 with Self-Assembling Peptide Hydrogels

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Heparin-binding insulin-like growth factor 1 (HB-IGF-1) is a fusion protein of IGF-1 with the HB domain of heparin-binding epidermal growth factor-like growth factor. A single dose of HB-IGF-1 has been shown to bind specifically to cartilage and to promote sustained upregulation of proteoglycan synthesis in cartilage explants. Achieving strong integration between native cartilage and tissue-engineered cartilage remains challenging. We hypothesize that if a growth factor delivered by the tissue engineering scaffold could stimulate enhanced matrix synthesis by both the cells within the scaffold and the adjacent native cartilage, integration could be enhanced. In this work, we investigated methods for adsorbing HB-IGF-1 to self-assembling peptide hydrogels to deliver the growth factor to encapsulated chondrocytes and cartilage explants cultured with growth factor-loaded hydrogels. We tested multiple methods for adsorbing HB-IGF-1 in self-assembling peptide hydrogels, including adsorption prior to peptide assembly, following peptide assembly, and with/without heparan sulfate (HS, a potential linker between peptide molecules and HB-IGF-1). We found that HB-IGF-1 and HS were retained in the peptide for all tested conditions. A subset of these conditions was then studied for their ability to stimulate increased matrix production by gel-encapsulated chondrocytes and by chondrocytes within adjacent native cartilage. Adsorbing HB-IGF-1 or IGF-1 prior to peptide assembly was found to stimulate increased sulfated glycosaminoglycan per DNA and hydroxyproline content of chondrocyte-seeded hydrogels compared with basal controls at day 10. Cartilage explants cultured adjacent to functionalized hydrogels had increased proteoglycan synthesis at day 10 when HB-IGF-1 was adsorbed, but not IGF-1. We conclude that delivery of HB-IGF-1 to focal defects in cartilage using self-assembling peptide hydrogels is a promising technique that could aid cartilage repair via enhanced matrix production and integration with native tissue.

## Introduction

**I**NJURIES TO CARTILAGE are common and have a limited ability to self-repair.<sup>1,2</sup> One approach to enhancing the native healing response is to deliver proanabolic growth factors to the damaged area using a biomaterial scaffold such that the growth factor is locally delivered at the site of injury and in a sustained manner over time. Insulin-like growth factor 1 (IGF-1) is known to enhance aggrecan production and minimize aggrecan catabolism, making it an exciting candi-

date for delivery to cartilage tissue.<sup>3–5</sup> Unfortunately, unmodified IGF-1 has a short half-life and easily diffuses out of the joint, creating the potential for unwanted side effects.<sup>6–8</sup>

Recently, we have shown that a fusion protein combining the heparin-binding (HB) domain of heparin-binding epidermal growth factor-like growth factor (HB-EGF) and IGF-1, called heparin-binding insulin-like growth factor 1 (HB-IGF-1), is a promising candidate for sustained, local delivery to cartilage. HB-IGF-1 binds to heparan sulfate (HS) with a  $K_D$  of 21 nM and to chondroitin sulfate with a  $K_D$  of 160 nM, whereas

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Parts of this work have been published in the form of abstracts at the Orthopedic Research Society Meeting:

Florine, E.M., Miller, R.E., Lee, R.T., Patwari, P., Grodzinsky, A.J. Heparan sulfate is retained in peptide hydrogel scaffold via ionic interactions: potential for delivery of HB-IGF-1. Abstract presented at the 58th Orthopedic Research Society Meeting, San Francisco, CA, 2012. Abstract no. 1648.

Florine, E.M., Lee, R.T., Patwari, P., Grodzinsky, A.J. HB-IGF-1 Adsorbed to Self-Assembling Peptide Enhances Matrix Production by Encapsulated Chondrocytes and Cocultured Cartilage Explants. Abstract presented at the 59th Orthopedic Research Society Meeting, San Antonio, TX, 2013. Abstract no. 0159.

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This work was performed at the Massachusetts Institute of Technology, Cambridge, MA and the Brigham and Women's Hospital, Cambridge, MA.

IGF-1 does not bind to either.<sup>9</sup> The mechanism of binding is thought to be via interactions between the positively charged HB domain of HB-IGF-1 and the negatively charged sulfated glycosaminoglycan (sGAG) chains in cartilage. An intra-articular injection of HB-IGF-1 into the joints of rats showed that the growth factor bound to sGAG-containing articular cartilage and the meniscus, but was not detected in tendon, patella, or muscle.<sup>9</sup> In addition, sustained bioactivity of a single dose of HB-IGF-1 was demonstrated by stimulation of increased proteoglycan synthesis in cartilage explants 6 days after the washout of unbound growth factor.<sup>10</sup>

Given the promising interactions between HB-IGF-1 and cartilage tissue, we investigated several methods of delivering HB-IGF-1 using the self-assembling peptide (RADA)<sub>4</sub>, or RAD. We have found self-assembling peptide hydrogels to be advantageous in a rabbit model of cartilage repair,<sup>11</sup> supportive of chondrogenesis for bone marrow stromal cells,<sup>12,13</sup> and capable of delivering growth factors *in vitro*.<sup>14</sup> The RAD peptide sequence consists of alternating hydrophobic and hydrophilic amino acids, where the hydrophilic amino acids have alternating acidic and basic residues. Since the reversible binding interaction between HB-IGF-1 and chondroitin sulfate chains in cartilage is likely based on charge, we hypothesized that we could take advantage of the charged amino acids in RAD to bind negatively charged HS in complex with HB-IGF-1, or positively charged HB-IGF-1 alone, to deliver HB-IGF-1. We also tested adsorbing HB-IGF-1 to RAD either before self-assembly or after the formation of the hydrogel.

After testing these different methods of adsorbing HB-IGF-1 to the peptide, we selected the most promising approach and tested it with chondrocyte-seeded hydrogels and explants cocultured with functionalized hydrogels to see whether we could stimulate proteoglycan synthesis. The ability to deliver a proanabolic growth factor to both the cells encapsulated in the tissue engineering scaffold and the native cartilage tissue surrounding a cartilage defect could be a key step in achieving integrative cartilage repair. Here, we present work showing that adsorption of HB-IGF-1 to RAD self-assembling peptide is a promising method for reaching this goal.

## Materials and Methods

### Materials

(RADA)<sub>4</sub> self-assembling peptide, hereafter called RAD and also known as PuraMatrix, was a gift from 3DM (Cambridge, MA). Hydrogels were formed at a concentration of 0.5% (w/v) for all experiments, consistent with previous literature.<sup>12,13,15</sup> IGF-1 (Increlex) and HB-IGF-1 proteins were from Ipsen/Biomeasure, Inc. (Milford, MA). Bovine joints were from Research 87 (Marlborough, MA) and HS from bovine kidney (0.88 sulfates/disaccharide<sup>16</sup>) was from Sigma-Aldrich (St. Louis, MO, catalog number H7640). Figure captions describe the number of hydrogels, explants, or animals used in each experiment.

### Cartilage explant harvest

One millimeter thick, 3 mm diameter middle zone cartilage explants were harvested from the femoropatellar grooves of 1–2 week old bovine calves and allowed to rest for 24 h in basal

medium with low glucose Dulbecco's modified Eagle's medium (Mediatech, Inc., Manassas, VA) supplemented with penicillin, streptomycin, and amphotericin (PSA); 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Invitrogen, Carlsbad, CA); proline (Sigma-Aldrich); ascorbate-2-phosphate (Wake Chemicals, Richmond, VA); and non-essential amino acids (Sigma-Aldrich), as described previously.<sup>17</sup>

### Chondrocyte harvest

Cartilage from the femoral condyles of 1–2 week old bovine calves was collected and digested with pronase for 1 h and collagenase overnight.<sup>18</sup> Chondrocytes were filtered from remaining tissue with cell strainers having pore sizes of 70 and 40  $\mu$ m and seeded into hydrogel culture on the same day, as described below in the section "IGF-1 and HB-IGF-1 delivery to peptide-encapsulated bovine chondrocytes."

### <sup>14</sup>C-labeling of HB-IGF-1 and IGF-1

One percent (w/v) stock solutions of IGF-1 and HB-IGF-1 were each dialyzed overnight against 0.3 M sodium phosphate pH 7.2 at 4°C in a 2000 MW cutoff cassette. A volume of 13.4  $\mu$ L of 1.25  $\mu$ Ci/ $\mu$ L [1-<sup>14</sup>C]-acetic anhydride (American Radiolabeled Chemicals, Inc., St. Louis, MO) dissolved in 1,4-dioxane was added to 125  $\mu$ L of each stock protein and incubated for 30 min at room temperature. The samples were then redialyzed overnight against sodium phosphate buffer at 4°C to remove free radiolabel.

### HB-IGF-1 dose-response in cartilage explants

After a 24-h rest period in basal medium, medium on the cartilage explants was changed and replaced with new basal medium alone, or medium containing 50 nM IGF-1, or 0.5, 5, 50, or 100 nM HB-IGF-1 for a culture period of 48 h. Fifty nanomolar IGF-1 was chosen for the IGF-1 concentration because it is known to be effective at stimulating an anabolic response in chondrocytes and cartilage explants.<sup>3,17,19</sup> At the end of this period, soluble growth factor was removed by washing with phosphate-buffered saline (PBS) and replaced with basal medium. Medium changes with basal medium were performed every 2 days thereafter. For the final 24 h of culture on day 8, explants were radiolabeled with 5  $\mu$ Ci/mL <sup>35</sup>S-sulfate (Perkin Elmer, Inc., Waltham, MA) to measure proteoglycan synthesis, as described previously.<sup>20</sup> Unincorporated radiolabel was removed and explants were weighed wet and digested with Proteinase-K (Roche Applied Science, Indianapolis, IN). Digested samples were analyzed for radiolabel incorporation by liquid scintillation counting and DNA content by Hoechst dye binding.<sup>21</sup>

### HS release from acellular RAD peptide hydrogels

HS was mixed into unassembled RAD peptide solution at a concentration of 5  $\mu$ M. One hundred microliter of PBS with PSA was layered on top of 50  $\mu$ L of HS-functionalized RAD to initiate hydrogel self-assembly.<sup>22</sup> These volumes were chosen to allow predicted levels of HS release from the hydrogel to be detectable. After 15 min, the bath was removed and replaced with a bath of either PBS + PSA ("PBS alone"), PBS + PSA with 1 M NaCl added ("1 M NaCl"), or

PBS + PSA with the pH adjusted to 9 with NaOH ("pH 9"). All hydrogels were cultured at 37°C. Eighty microliter of the bath from each sample was collected and replaced every 24 h for days 1–7. The HS content of the bath was measured by 1,9-dimethylmethylene blue (DMMB) dye binding.<sup>23</sup>

#### *IGF-1 and HB-IGF-1 retained following adsorption to unassembled peptide*

Initial entrapment efficiency of HB-IGF-1 within the RAD hydrogel was assessed using methods as described previously.<sup>24</sup> Acellular RAD was mixed with HB-IGF-1 prior to gel assembly to yield a growth factor concentration of 50 ng/μL (4.92 μM), or a total of 2.5 μg HB-IGF-1 in 250 μg hydrogel. Fifty microliter of functionalized gel was then cast into low-binding microcentrifuge tubes. The gels were allowed to assemble in the tubes for 30 min, after which 500 μL of PBS was layered on top of the hydrogel samples to further induce self-assembly. The hydrogels were stored at 37°C for 3 h. As a control, 50 μL of non-functionalized RAD was prepared in the same manner. After 3 h, the PBS was sampled, and the amount of HB-IGF-1 released to PBS during the gel assembly was measured using an IGF-1 enzyme-linked immunosorbent assay (ELISA) known to bind HB-IGF-1 (R&D Systems, catalog number DY291). The standard curve for the ELISA was prepared using HB-IGF-1 and PBS to match gel/PBS samples. There was no intentional mechanical disruption of the gel. The amount of HB-IGF-1 released from the gel into PBS was subtracted from the amount that was initially pre-mixed into the gel. In this way, the initial entrapment efficiency of HB-IGF-1 was calculated.

To test growth factor retention in unassembled peptide, 50 nM IGF-1 or 50 nM HB-IGF-1 was allowed to form a complex with 50 nM HS for 15 min before mixing with RAD peptides in solution. As additional separate conditions, 50 nM IGF-1 or 50 nM HB-IGF-1 alone were mixed with RAD peptides in solution. Seven hundred fifty microliter of PBS + PSA was layered on top of 50 μL of hydrogel samples to initiate self-assembly; hydrogels were then cultured at 37°C in low-binding microcentrifuge tubes. These volumes were chosen to match parallel experiments using chondrocyte-seeded hydrogels. The growth factor concentrations correspond to 19 ng IGF-1 or 25 ng HB-IGF-1 being mixed into the peptide. The bath was replaced every 24 h for 6 days. Hydrogel samples were frozen at each time point after removal of the bath. Determination of growth factor retention was made by western blotting. Hydrogel samples were mechanically disrupted and directly loaded into a 4–12% Bis-Tris gel (Invitrogen) and run at 200 V for 45 min. Proteins were transferred to a polyvinylidene fluoride membrane and probed with an anti-IGF-1 antibody (catalog number 9572; Abcam, Cambridge, MA), which recognizes both IGF-1 and HB-IGF-1.<sup>10</sup>

#### *HB-IGF-1 retained following adsorption to preassembled peptide hydrogel*

To determine the initial entrapment efficiency of HB-IGF-1 to preassembled peptide, 50 μL of RAD hydrogel was assembled overnight at 37°C in a bath of PBS + PSA. <sup>14</sup>C-HB-IGF-1 was added to the bath for a final concentration of 3.28 μM of radiolabeled protein in a bath volume of 300 μL.

Following overnight incubation at 37°C, the uptake baths were removed, and the hydrogels were gently rinsed twice with PBS + PSA. A fresh bath of 500 μL of PBS + PSA was added to the hydrogel, and protein was allowed to desorb from the hydrogel for 3 h. Following desorption, the bath was removed from the hydrogel, and the hydrogel was mechanically disrupted. Radiolabeled protein in the desorption baths and mechanically disrupted hydrogels were measured by liquid scintillation counting. Entrapment efficiency was determined as the amount of protein remaining in the hydrogel following the 3-h desorption, expressed as nanograms of protein retained/micrograms of hydrogel.

To test growth factor retention in preassembled hydrogels, 50 μL RAD hydrogels with or without 50 nM HS mixed in were allowed to assemble for 24 h in 750 μL PBS + PSA at 37°C in low-binding microcentrifuge tubes. After 24 h, the bath was replaced with PBS + PSA with 50 nM HB-IGF-1, which provides a total of 380 ng of HB-IGF-1 in the medium to potentially bind to the peptide. Twenty-four hours after addition of the HB-IGF-1 bath, soluble growth factor was washed out of the cultures using PBS and the bath was replaced with PBS + PSA. Hydrogel samples were frozen after bath removal every 24 h through day 8. Growth factor retention was assessed by western blotting, as described above.

#### *Release of <sup>14</sup>C-HB-IGF-1 and <sup>14</sup>C-IGF-1 following adsorption to unassembled peptide*

To determine a quantitative release profile, <sup>14</sup>C-IGF-1 or <sup>14</sup>C-HB-IGF-1 was mixed into unassembled RAD peptide solution at a concentration of 615 nM. Thirty microliters of PBS with PSA was layered on top of 50 μL of HB-IGF-1- or IGF-1-functionalized RAD to initiate hydrogel self-assembly. An additional 470 μL of PBS + PSA was added to the bath to reach a final volume of 500 μL, and the hydrogels were incubated at 37°C. Two hundred fifty microliters of the bath from each sample were collected and replaced every 24 h during days 1–8, and every 48 h between days 8 and 12. At day 12, the baths were completely replaced with 10× PBS. On day 14, the 10× PBS baths were removed and saved, and the hydrogels were mechanically disrupted and saved. Radiolabeled protein in the bath and mechanically disrupted hydrogels were measured by liquid scintillation counting.

#### *IGF-1 and HB-IGF-1 delivery to peptide-encapsulated bovine chondrocytes*

Bovine chondrocytes were encapsulated in RAD at a density of 30 million cells/mL. The RAD was either unaltered (for "Basal," "IGF soluble," and "HB soluble" conditions), or had one of the following adsorbed to RAD prior to cell encapsulation: 50 nM IGF-1 (for "IGF pre-mix" condition) or 50 nM HB-IGF-1 (for "HB pre-mix" condition). See Table 1 for a summary of the culture conditions. On day 0, the cell/hydrogel mixture was cast as 6 mm diameter, 1.5 mm thick disks into agarose rings pre-equilibrated in the basal medium described above, as described previously.<sup>12</sup> Some hydrogels were cast into 50 mM tris(hydroxymethyl)aminomethane (Tris) and 1 mM CaCl<sub>2</sub> at the time of casting to measure day-0 DNA levels. The hydrogel disks were cultured in basal medium for 24 h to

TABLE 1. FUNCTIONALIZED RAD HYDROGEL AND MEDIUM CONDITIONS FOR EXPERIMENTS WITH RAD PEPTIDE SEEDED WITH BOVINE CHONDROCYTES AND CARTILAGE EXPLANTS

Condition	HB/IGF source	HS	Time soluble GF present (days)
Basal	None	None	N/A
IGF soluble	Soluble in media	None	1–10
HB soluble	Soluble in media	None	1–10
IGF-pre-mix	Adsorbed to peptides prior to gel assembly	None	0
HB pre-mix	Adsorbed to peptides prior to gel assembly	None	0

Refer to Figure 6 for time course of experimental procedure.

HB, heparin-binding; IGF, insulin-like growth factor; HS, heparan sulfate; GF, growth factor; N/A, not applicable.

allow peptide assembly. After 24 h, on day 1, a medium change was performed on all hydrogels. Hydrogels for the “IGF soluble” and “HB soluble” conditions received 50 nM IGF-1 and HB-IGF-1, respectively, in the medium through the entire culture period. Hydrogels for the “Basal,” “IGF pre-mix,” and “HB pre-mix” conditions received basal medium for the entire culture duration. Hydrogel samples were cultured up to 10 days with medium changes every 2 days.

For the final 24 h of culture, hydrogels were radiolabeled with 5  $\mu$ Ci/mL  $^{35}$ S-sulfate and 10  $\mu$ Ci/mL  $^3$ H-proline (Perkin Elmer, Inc.) to measure proteoglycan and total protein synthesis, respectively. Unincorporated radiolabel was removed and hydrogels were weighed wet, lyophilized, weighed dry, and digested with Proteinase-K (Roche Applied Science), as described previously.<sup>12</sup> Digested samples were analyzed for radiolabel incorporation by liquid scintillation counting, for sGAG content by DMMB dye binding,<sup>23</sup> for DNA content by Hoechst dye binding,<sup>21</sup> and hydroxyproline content as a measure of total collagen by reaction with *p*-dimethylaminobenzaldehyde.<sup>25</sup>

#### IGF-1 and HB-IGF-1 delivery to explants cultured with acellular functionalized peptide

Bovine cartilage explants were harvested and placed in basal medium to rest. One day after the harvest, 100  $\mu$ L RAD hydrogels were cast into 2 mL cryo-tubes and assembled with 300  $\mu$ L of basal medium. The hydrogel and medium conditions, and the schedule of medium changes, were identical to those described for the bovine chondrocyte experiment described above. The cartilage explants received new basal medium on day 1 and were added to the cryo-tubes with the acellular peptide on day 2.

Explants were radiolabeled with 5  $\mu$ Ci/mL  $^{35}$ S-sulfate (Perkin Elmer, Inc.) for the final 24 h of culture to measure proteoglycan synthesis. Explants were washed to remove unincorporated radiolabel, weighed wet, and digested with Proteinase-K (Roche Applied Science). Digested samples were analyzed for radiolabel incorporation by liquid scintillation counting and DNA content was measured by Hoechst dye binding.

#### Statistics

A general linear model with medium condition as an independent variable was used to analyze HB-IGF-1 dose-response data. HS release data were rank transformed and analyzed using a general linear model with medium condi-

tion and day as independent variables. For chondrocyte-seeded hydrogel experiments, a one-way analysis of variance with culture condition as an independent variable was used to analyze each time point from each of two replicate experiments separately for sGAG per DNA, proteoglycan synthesis, protein synthesis, and hydroxyproline content data. Statistical symbols are those that were consistent between the two experiments. A Kolmogorov–Smirnov test was used to test for normality and data were transformed as necessary for all the statistical tests above. A linear mixed model with animal as a random factor and culture condition as an independent variable was used to analyze data from the three functionalized hydrogel-cartilage explant coculture experiments together. Residuals were analyzed and data transformed as needed to ensure normality. Tukey *post hoc* tests with  $p < 0.05$  were used to evaluate statistical significance for all pairwise comparisons. Systat 12 software (Systat, Chicago, IL) was used for all statistical analyses.

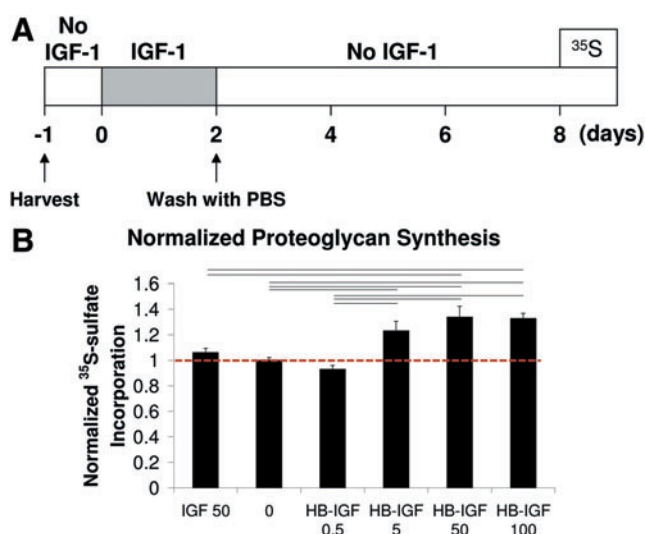
## Results

### HB-IGF-1 dose-response in cartilage explants

To determine a dose of HB-IGF-1 for use in later experiments, a dose-response study was first performed on cartilage explants. Explants received medium with either soluble IGF-1 or HB-IGF-1 at various doses for 48 h and then unbound growth factor was removed by washing with PBS. Six days later, explants were radiolabeled to measure proteoglycan synthesis (Fig. 1A). Six days after removing soluble growth factor, the 50 nM IGF-1 condition (“IGF 50”) was not statistically different from the no IGF (“0”) control (Fig. 1B), consistent with previous reports.<sup>10</sup> Doses of 5, 50, and 100 nM HB-IGF-1 showed significantly greater proteoglycan synthesis than the no IGF control and the dose of 0.5 nM HB-IGF-1. There was a trend on increasing proteoglycan synthesis as the HB-IGF-1 dose increased, although 5, 50, and 100 nM HB-IGF-1 were not statistically distinct. As the peak effect of HB-IGF-1 was seen with 50 nM HB-IGF-1, this dose was chosen for future experiments.

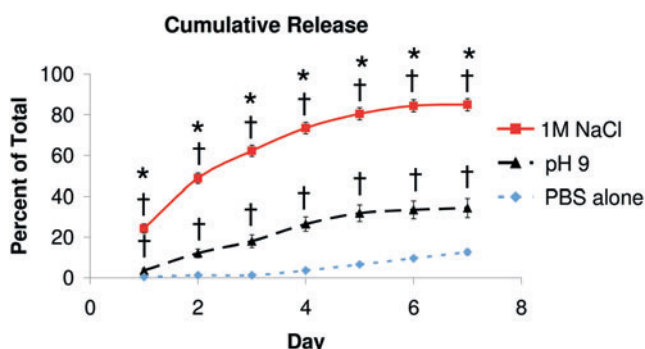
### HS release from acellular RAD peptide hydrogel

Because of the strong interaction between HS and HB-IGF-1, we hypothesized that including HS in the peptide gel could provide additional binding sites for the growth factor and enhance the longevity of its delivery. As an initial step toward this goal, we investigated whether HS alone could be retained in the peptide. HS was adsorbed to unassembled



**FIG. 1.** Heparin-binding insulin-like growth factor 1 (HB-IGF-1) dose-response in cartilage explants at day 8 following wash-out procedure. **(A)** Timeline of experimental procedure. Explants were treated with 50 nM IGF-1, or 0.5, 5, 50, or 100 nM HB-IGF-1 from day 0 to 2. **(B)** Proteoglycan synthesis normalized to DNA content ( $\mu\text{g}$ ) and the no-growth factor ("0") condition (dashed line) at day 8. Values are mean  $\pm$  standard error of the mean (SEM).  $N=6$  cartilage disks per condition, one animal. Solid lines indicate significant difference between two conditions,  $p < 0.05$ . Color images available online at [www.liebertpub.com/tea](http://www.liebertpub.com/tea)

RAD peptides in solution and cultured in a bath of either PBS alone, PBS + 1 M NaCl ("1 M NaCl"), or PBS with the pH adjusted to 9 by NaOH ("pH 9"). Release of HS from the peptide was measured over a 7-day period (Fig. 2). In PBS alone, <15% of the total HS was released from the RAD by day 7. The addition of 1 M NaCl or alteration of the pH to 9 resulted in increased release of HS compared with PBS alone, with the 1 M NaCl bath causing more release than the pH 9 bath.



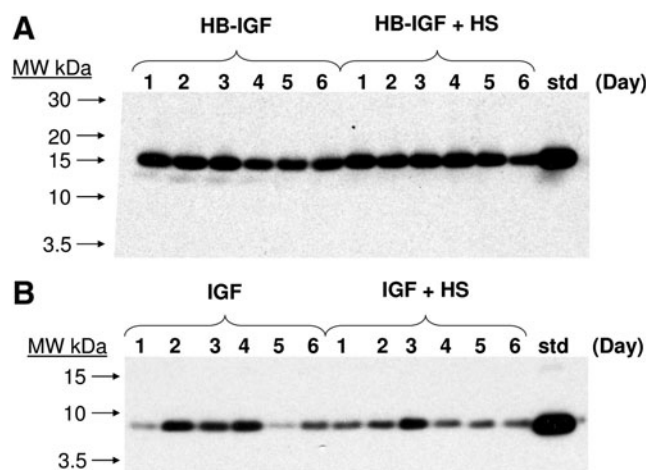
**FIG. 2.** Heparan sulfate (HS) release from acellular peptide hydrogels. Peptide with  $5 \mu\text{M}$  HS cultured in  $100 \mu\text{L}$  bath of phosphate-buffered saline (PBS), PBS with 1 M NaCl ("1 M NaCl"), or PBS with a pH of 9 (adjusted using NaOH, "pH 9"). Values are mean  $\pm$  SEM.  $N=6-8$ , except at day 6 where  $n=2$ . †Versus PBS alone, \*versus pH 9,  $p < 0.05$ . Color images available online at [www.liebertpub.com/tea](http://www.liebertpub.com/tea)

#### IGF-1 and HB-IGF-1 retained following adsorption to unassembled peptide

To test the initial entrapment efficiency of HB-IGF-1 in unassembled peptide,  $2.5 \mu\text{g}$  HB-IGF-1 was mixed with  $250 \mu\text{g}$  unassembled RAD and HB-IGF-1 release to the PBS bath was measured after 3 h, similar to methods described previously.<sup>24</sup> We found that the RAD peptide retains  $\sim 9 \text{ ng}$  HB-IGF-1 per microgram of peptide. We next tested whether HS improved retention of HB-IGF-1 in the peptide. To test this, 50 nM IGF-1 or HB-IGF-1 with or without 50 nM HS was mixed into unassembled RAD, PBS was added to initiate assembly, and the PBS bath was then changed daily. Each day, hydrogels were frozen after the bath was removed and their growth factor content assessed by western blotting. HB-IGF-1 was strongly retained in the hydrogel with or without HS for the entire culture duration (Fig. 3A). IGF-1 was also retained for the entire culture duration with or without HS (Fig. 3B).

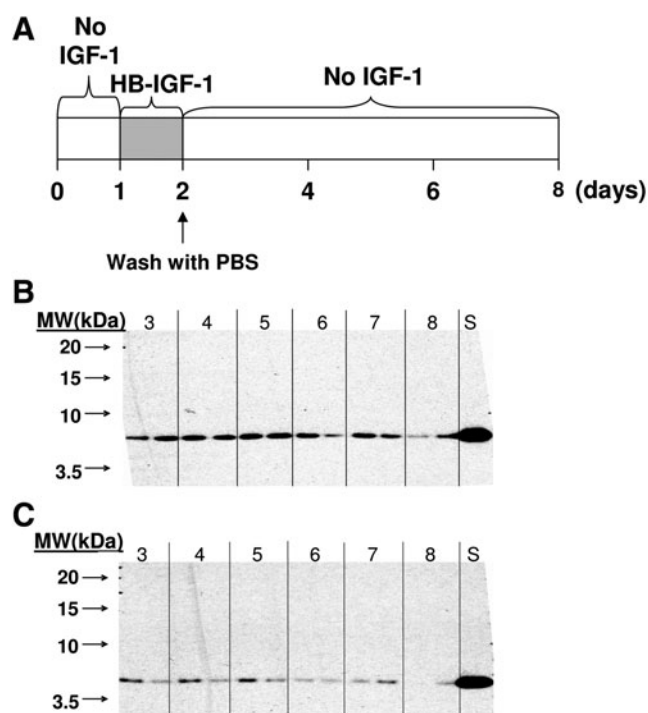
#### HB-IGF-1 retained following adsorption to preassembled peptide hydrogel

To test the initial entrapment efficiency of HB-IGF-1 to preassembled hydrogels,  $^{14}\text{C}$ -labeled HB-IGF-1 was added to a  $300 \mu\text{L}$  PBS bath at a concentration of  $3.28 \mu\text{M}$ . After 24 h at  $37^\circ\text{C}$ , the bath was removed, hydrogels were rinsed with PBS, and a  $500 \mu\text{L}$  bath of fresh PBS was added. After 3 h, the amount of radiolabeled protein in the bath and the mechanically disrupted gel were measured. About  $0.57 \text{ ng}$  of HB-IGF-1 per microgram of peptide was retained. To investigate retention over a longer time, 50 nM HB-IGF-1 was allowed to adsorb for 24 h to preassembled hydrogels of RAD alone or RAD with 50 nM HS mixed in prior to assembly. Soluble growth factor was then washed out of the culture using PBS (Fig. 4A). Time points were taken daily to assess retention of HB-IGF-1 by western blotting. HB-IGF-1 was retained in RAD (Fig. 4B) and RAD functionalized with HS throughout the culture period (Fig. 4C). Lower levels of



**FIG. 3.** Growth factor retention when mixed into initially unassembled acellular peptide. Western blots of **(A)** HB-IGF-1 and **(B)** IGF-1 retained in peptide with or without HS on days 1 through 6, using an anti-IGF-1 antibody. Std is a standard of 5 ng HB-IGF-1 or IGF-1 for **(A)** and **(B)**, respectively.





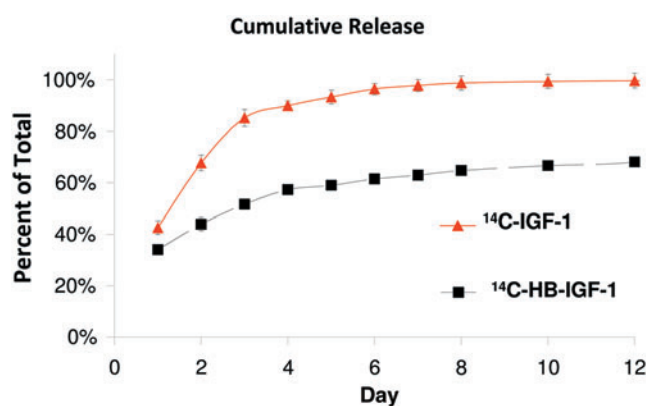
**FIG. 4.** HB-IGF-1 retention when adsorbed to preassembled acellular peptide. **(A)** Time course of experimental procedure where peptide  $\pm$  HS assembled from day 0 to 1 and HB-IGF-1 was adsorbed to peptide from day 1 to 2. Western blots of **(B)** HB-IGF-1 retained by days 3–8 after adsorption to peptide alone, and **(C)** HB-IGF-1 retained by days 3–8 after adsorption to peptide mixed with HS prior to assembly. S is a standard of 5 ng HB-IGF-1. Two lanes for each time point are repeats of the same condition.

protein were seen at day 8 in both conditions, perhaps suggesting release of the adsorbed HB-IGF-1.

Based on the results in Figures 3 and 4 we concluded that HS was not needed to retain HB-IGF-1 within the peptide hydrogel, so these conditions were not tested in the cell-based experiments. Furthermore, since assembling the peptide prior to adsorbing the HB-IGF-1 resulted in a lower initial entrapment efficiency and the only practical method of delivering the growth factor *in vivo* using the peptide is to mix them together prior to peptide assembly, we proceeded with the method of mixing the growth factor into the unassembled peptide solution.

#### Release of $^{14}\text{C}$ -HB-IGF-1 and $^{14}\text{C}$ -IGF-1 following adsorption to unassembled peptide

To assess the release profile of HB-IGF-1 as compared to IGF-1, we radiolabeled both growth factors with  $^{14}\text{C}$  and loaded them at a concentration of 615 nM into unassembled peptide. The release to a PBS bath was measured over the following 12 days (Fig. 5). Both growth factors were released from the peptide hydrogel, with the  $^{14}\text{C}$ -IGF-1 releasing from the peptide at a faster rate than the  $^{14}\text{C}$ -HB-IGF-1 over the first 4 days. Both growth factors had a slower rate of release in the second half of the time course, with the IGF curve approaching 100% release by day 7 and the HB-IGF curve at 65% of the material released by day 12.



**FIG. 5.**  $^{14}\text{C}$ -IGF-1 and  $^{14}\text{C}$ -HB-IGF-1 release from acellular peptide scaffold. Peptide with 615 nM labeled growth factor was cultured in a 500  $\mu\text{L}$  bath of PBS. Values are mean  $\pm$  standard deviation.  $N=4$ . Color images available online at [www.liebertpub.com/tea](http://www.liebertpub.com/tea)

These results are consistent with the western blots shown in Figure 3.

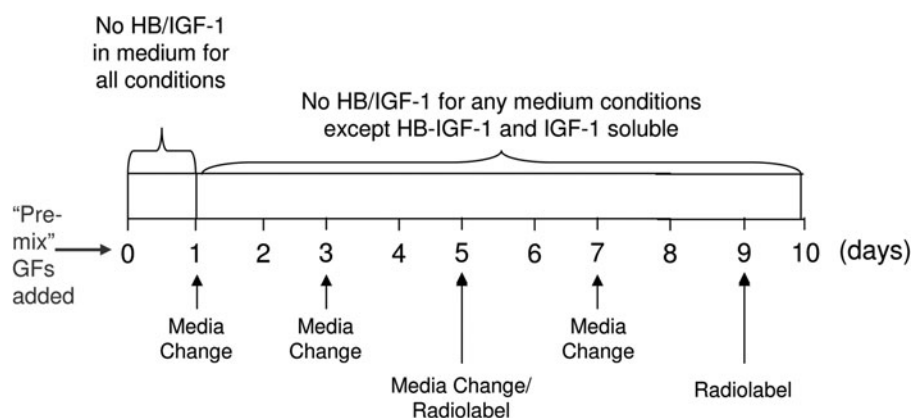
#### IGF-1 and HB-IGF-1 delivery to peptide-encapsulated bovine chondrocytes

We next tested the ability of HB-IGF-1 adsorbed to RAD to stimulate bovine chondrocytes encapsulated within the peptide hydrogel. For the “HB pre-mix” and “IGF pre-mix” conditions, 50 nM HB-IGF-1 or IGF-1, respectively, were adsorbed to unaltered RAD prior to peptide self-assembly and cultured in basal medium (Fig. 6). Unaltered RAD was cultured in basal medium as a negative control (“Basal”) or in medium containing 50 nM soluble IGF-1 or HB-IGF-1 throughout the 10-day culture period as positive controls (“IGF soluble” and “HB soluble,” respectively). Table 1 summarizes these peptide and medium conditions.

sGAG content normalized to DNA content was not significantly different between any conditions at day 6 (Fig. 7A). sGAG and DNA content are individually shown in Supplementary Figure 1 (Supplementary Data are available online at [www.liebertpub.com/tea](http://www.liebertpub.com/tea)). The soluble conditions, HB pre-mix, and IGF pre-mix were significantly greater than the Basal condition at day 10. HB pre-mix showed lower proteoglycan synthesis at day 6 than the HB soluble condition (Fig. 7B). By day 10, the soluble conditions were significantly higher than the Basal condition. Protein synthesis for HB pre-mix and IGF pre-mix were lower than IGF soluble and HB soluble at day 6 (Fig. 7C). At day 10, the soluble conditions exceeded the Basal condition, identical to proteoglycan synthesis. All conditions showed increased hydroxyproline content compared with the Basal condition at day 6 and 10 (Fig. 7D).

#### IGF-1 and HB-IGF-1 delivery to explants cultured with acellular functionalized peptide

To ensure that the growth factor adsorbed to the RAD was bioactive and bioavailable, and also to test the hypothesis that HB-IGF-1 could be released from the peptide to stimulate nearby cartilage, we repeated the above experiment with acellular functionalized peptides cocultured with



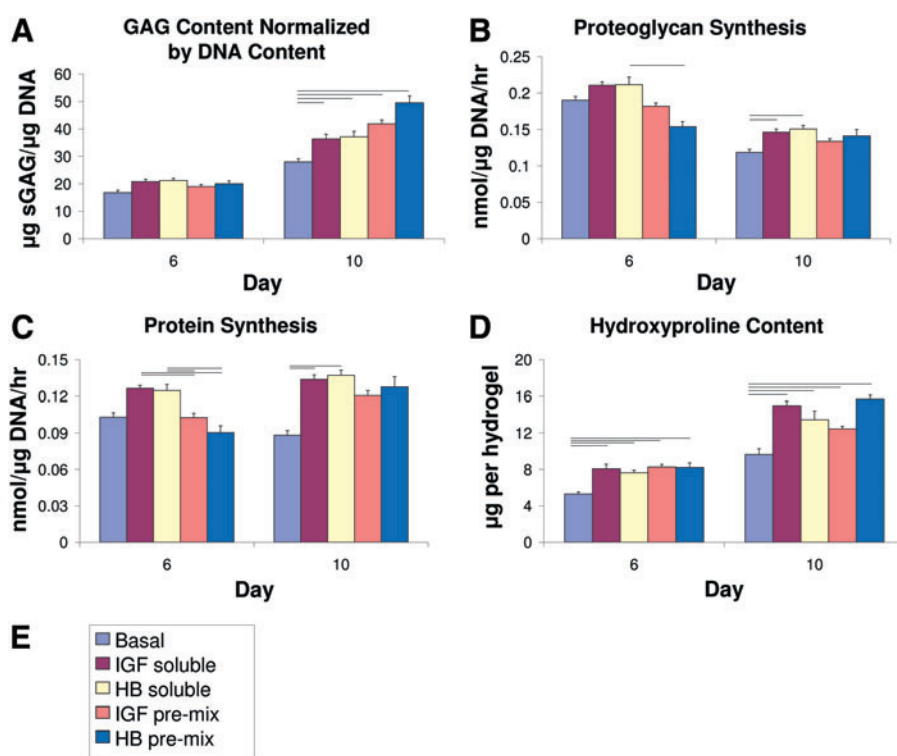
**FIG. 6.** Time course of experimental procedures for bovine chondrocytes in regular or functionalized peptide hydrogels. Identical time course was used for cartilage explants cultured with functionalized peptide gels, where explants were harvested at day-1, received a media change on day-1, and were added to gel cultures on day 2.

bovine cartilage explants. Explants from all conditions showed higher proteoglycan synthesis compared with the Basal condition at day 6 (Fig. 8). By day 10, the HB-IGF-1 condition maintained increased proteoglycan synthesis levels compared with the Basal condition. In contrast, the IGF pre-mix condition showed significantly lower synthesis levels compared with IGF soluble and was not statistically distinct from Basal at day 10.

## Discussion

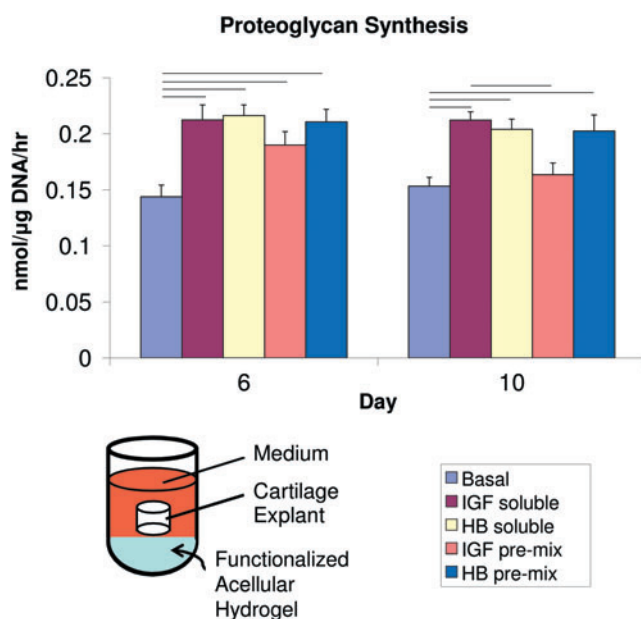
We tested the hypotheses that HB-IGF-1 adsorbed to RAD could stimulate increased sGAG accumulation in chondrocyte-seeded hydrogels and increased proteoglycan synthesis in cartilage explants cocultured with growth factor functionalized hydrogels. We established a dose-response of HB-IGF-1 and confirmed that IGF-1 does not stimulate

increased proteoglycan synthesis in cartilage explants after washing unbound growth factor out of the culture.<sup>10</sup> We found that HS could be retained in RAD when added to unassembled RAD. Since the HS was minimally released into a bath of PBS at pH 7.4, but could be released by increasing the pH or adding salt to the bath (Fig. 2), we conclude that HS is retained in RAD via ionic interactions, for example, the positively charged amino acids. Given the strong retention of HS in RAD and the nanomolar binding constant between HS and HB-IGF-1, we hypothesized that HS could be used to retain HB-IGF-1 in the peptide. Because the interaction between HS and RAD was based on charge, we also thought that positively charged HB-IGF-1 might be retained by the negatively charged amino acids in RAD by charge interactions without the presence of HS. We therefore pursued both of these delivery methods. HB-IGF-1 was retained in RAD with or without HS and regardless of



**FIG. 7.** Extracellular matrix content and biosynthesis by bovine chondrocytes encapsulated within peptide hydrogels. (A) Sulfated glycosaminoglycan (sGAG) content normalized by DNA content, (B) proteoglycan synthesis, (C) protein synthesis, (D) hydroxyproline content, and (E) key of culture conditions. Values are mean  $\pm$  SEM.  $N = 5-6 \times 2$  experiments with cells from different animal donors; data from one experiment shown above. Line indicates significant difference between two conditions consistent between both experiments,  $p < 0.05$ . See Figure 6 for time course of experimental procedure. Color images available online at [www.liebertpub.com/tea](http://www.liebertpub.com/tea)





**FIG. 8.** Proteoglycan synthesis within cartilage explants cultured with growth factor-functionalized acellular peptide hydrogel. Data normalized to DNA content. Values are mean  $\pm$  SEM.  $N = 11-12$  (3-4 explants  $\times$  3 animals). Line indicates significant difference between two conditions,  $p < 0.05$ . Color images available online at [www.liebertpub.com/tea](http://www.liebertpub.com/tea)

whether it was adsorbed to the peptide before or after it was assembled. HB-IGF-1 was found to release more slowly than untargeted IGF-1 when mixed into unassembled peptide (Fig. 5).

Having established that HB-IGF-1 could be retained in the peptide, we tested whether the growth factor could stimulate cells encapsulated within the hydrogel and chondrocytes within adjacent cartilage explants. We found that adsorbing HB-IGF-1 or IGF-1 to the peptide in solution, before it was assembled, increased sGAG content normalized by DNA content in chondrocyte-seeded hydrogels compared with the Basal condition at day 10. Hydroxyproline content was also enhanced compared with the Basal condition at day 6 and 10. Both proteoglycan and protein synthesis levels for the HB pre-mix condition were lower than the soluble conditions at day 6, but this did not correlate with the sGAG/DNA and hydroxyproline results. This could be explained by a release of growth factor from the functionalized peptide between the day 6 and 10 time points.

In the explant experiment (Fig. 8), there was increased proteoglycan synthesis for explants cocultured with all growth factor functionalized peptide conditions compared with the Basal peptide at day 6. While IGF-1 did not provide a sustained increase in proteoglycan synthesis in cartilage explants compared to the basal control at day 10, the presence of a binding arm (HB-IGF-1) resulted in sustained stimulation, analogous to results found previously where HB-IGF-1 was delivered in the medium to cartilage explants.<sup>10</sup> As the N-terminal tri-peptide sequence of IGF-1 is the region associated with the IGF-1 binding to IGF-binding proteins (IGFBPs) in native tissues,<sup>26</sup> the addition of the HB domain at this N-terminal region of IGF-1 may make the HB-IGF-1 fusion protein unable to bind to IGFBPs. As a

result, it is possible that the presence of IGFBPs in cartilage explants<sup>27</sup> would sequester more IGF-1 than HB-IGF-1. Interestingly, we observed that explants could be stimulated by certain growth factor-functionalized conditions (Fig. 8) that were not as clearly stimulatory for encapsulated chondrocytes *in vitro* (Fig. 7). We believe this may be due to the fact that there is less extracellular GAG-rich aggrecan surrounding the newly seeded chondrocytes to capture HB-IGF-1 released by the hydrogel.

While radiolabel assays have better sensitivity than other methods of detection, at day 12 only 65% of the radiolabeled HB-IGF-1 was released to the PBS bath. We attempted to recover growth factor remaining in the peptide by adding 10 $\times$  PBS and then mechanically disrupting the gel itself to measure the remaining HB-IGF-1 directly, but these procedures only detected an additional 10% of the material. We hypothesize that the HB-IGF-1 is generally more "sticky" due to the charged HB domain, resulting in small losses throughout the course of the experiment that account for the remaining material.

Ongoing studies focus on the use of radiolabeled HB-IGF-1 to characterize transport within hydrogels and native cartilage, including diffusion and binding in space and time. This approach may also be useful *in vivo* to demonstrate release of HB-IGF-1 from the hydrogel and binding to surrounding cartilage tissue. Additionally, in an experiment using a similar peptide, KLD<sup>12</sup>, where the hydrogel was cast into an annulus of cartilage tissue, 62% of the HB-IGF-1 loaded into the gel was recovered from the cartilage annulus after 9 days in culture, whereas only 4% of the initially loaded growth factor was recovered from the culture medium. Based on the medium volume and explant-medium geometry, these results suggest that the majority of the growth factor diffused laterally into the cartilage rather than being released to the medium and then adsorbed to the cartilage. Future work also remains for understanding how HS and HB-IGF-1 interact with unassembled and assembled RAD fibers. Though we hypothesize that the interactions are based on charge, the stoichiometry and how the interactions might change as self-assembly proceeds are as yet unknown.

We note that the work presented here utilized immature bovine cartilage for explants and chondrocytes due to availability of tissue. Confirmation of the translational value of these results using adult bovine tissue and importantly, mature human cartilage, would be very valuable, as important differences have been shown between cells from different species and ages of donors.<sup>13,28-31</sup> In the clinical application, the peptide would likely be used to fill in a cartilage defect following microfracture, so the cell population would be more similar to bone marrow stromal cells than chondrocytes.

We have shown that HB-IGF-1 adsorption to RAD self-assembling peptide hydrogels is a promising method for delivering the proanabolic, anticatabolic growth factor IGF-1 to cells encapsulated within hydrogels and to adjacent cartilage tissue. In terms of potential clinical usage, mixing HB-IGF with peptide solution prior to hydrogel self-assembly is a simple one step procedure for delivering a growth factor using a hydrogel. The finding that a single dose of HB-IGF-1 mixed into the unassembled peptide at day 0 was still stimulating statistically significant enhancement of proteoglycan synthesis in adjacent cartilage explants compared with Basal

control at day 10 demonstrates the utility of this approach (Fig. 8). We therefore suggest that HB-IGF-1 delivered via the peptide hydrogel to adjacent cartilage at the site of a focal defect *in vivo* would be capable of binding to sGAG chains within the cartilage and thereby enabling sustained bioactivity. The hypothesis that HB-IGF-1 would enhance the integration of peptide hydrogel-mediated neocartilage to adjacent native cartilage at a defect interface will be tested in future animal studies. Taken together, we believe that functionalized self-assembling peptide hydrogels provide a promising tissue engineering scaffold for enhancing cartilage repair.

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### Disclosure Statement

Brigham and Women's Hospital has filed for patents pertaining to the described HB-IGF-1 technology, listing P.P. and R.T.L. as inventors. Dr. Grodzinsky has equity in 3-D Matrix, Ltd., Japan. The other authors of this work have no competing financial interests.

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