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The effect of heparin-functionalized PEG hydrogels on three-dimensional human mesenchymal stem cell osteogenic differentiation

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Abstract

Poly(ethylene glycol) (PEG) hydrogels functionalized with heparin were utilized as a three-dimensional culture system for human mesenchymal stem cells (hMSCs). Heparin-functionalized hydrogels supported hMSC viability, as quantified through live/dead imaging, and induced osteogenic differentiation, as measured by increased alkaline phosphatase (ALP) production and osteopontin (OPN) and collagen I (COL I) gene expression over the 5-week study. Further exploration of the potential mechanism of heparin-induced osteogenic differentiation was performed. Specifically, the availability of bone morphogenetic protein 2 (BMP2) and fibronectin (FN) in the culture system was controlled and hMSC osteogenic differentiation was evaluated as a function of the microenvironment. BMP2 availability increased both ALP production and OPN gene expression, while FN increased ALP production, but not OPN gene expression. Furthermore, immunostaining of integrin expression revealed that viability and differentiation were differentially affected by integrin production, where both $\alpha 5\beta 1$ and $\alpha v\beta 3$ integrin–ligand interactions supported viability, while only the $\alpha 5\beta 1$ integrin played a role in hMSC osteogenic differentiation.
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1. Introduction

The nature of cell adhesion to substrate materials has a tremendous effect on cell function and tissue development. Signaling via receptor–ligand interactions initiated by cell adhesion provides the cell with vital information about its extracellular environment, regulating a variety of events, including differentiation, tissue evolution, and apoptosis [1,2]. Understanding how cells interact with a substrate is crucial in the development of functional biomaterials.

Cell adhesion through cell surface receptors, such as integrins, selectins, and immunoglobulins, is mediated by interactions with proteins adsorbed on the material. However, a controlled adhesion environment is difficult to achieve when relying upon non-specific protein adsorption. Furthermore, modification of biomaterials with full proteins can be very complex; protein coupling to biomaterials requires mild reaction conditions, as the proteins are subject to both denaturation and degradation. As an alternative to inclusion of full proteins, significant interest has emerged in the design of cell scaffolds that incorporate moieties that can induce specific, controlled protein adsorption.

Relative to integrins, interactions of proteoglycans and glycosaminoglycans (GAGs) with their ligands have not been widely employed in the design of biomaterials that regulate cell adhesion or function. However, a few studies have demonstrated that proteoglycan or GAG interactions with ligands can be exploited to create unique biomaterials [3,4]. A GAG of particular significance is heparin. Many proteins contain heparin-binding domains. Thus,

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immobilized heparin can be utilized as a sequestering molecule for signaling or adhesive proteins produced by or delivered to cells. Controlled release of growth factors has been described from heparin–sepharose conjugates [5], heparin-carrying polystyrene-bound collagen substrata [6], acid gelatin hydrogels [7], alginate gels containing heparin [8], photocrosslinkable chitosan hydrogels [9], poly(ethylene oxide)/heparin-functionalized poly(ethylene oxide) electrospun fibers [10], heparin-functionalized poly(ethylene glycol) (PEG) hydrogels [11], heparin–PEG star copolymer hydrogels [12], and a fibrin-based system incorporating a heparin-binding peptide [13]. Heparin has been studied previously as a means to control the orientation of adsorbed fibronectin (FN) [14] and to impart anti-coagulant properties to stainless steel by deposition of polyethylenimine and heparin layer-by-layer films [15]. Furthermore, evidence exists that heparin can promote cell adhesion [16]. Interestingly, heparin is capable of interacting with numerous proteins associated with osteoblast and osteoblast progenitor cell adhesion (e.g., FN, vitronectin) and osteogenic differentiation (e.g., bone morphogenetic proteins (BMPs), pleiotrophin) [17].

In our previous work [18], heparin was modified with methacrylate groups, copolymerized with dimethacrylated PEG, and analyzed as a localized delivery vehicle for the model protein, bFGF. Results demonstrated that methacrylate-modified heparin retained its ability to bind heparin-binding proteins both in solution and when copolymerized with dimethacrylated PEG in a hydrogel. In addition, the heparin-functionalized gels sustained the delivery of biologically active bFGF for up to 5 weeks. Finally, the gels were examined as a potential osteogenic scaffold for human mesenchymal stem cell (hMSC) culture and found to promote adhesion, proliferation, and osteogenic differentiation when hMSCs were cultured on the gel surface.

Towards the goal of designing a three-dimensional osteogenic niche for hMSCs, the role of heparin as an advantageous gel component was explored. Specifically, macromolecular heparin monomers were synthesized and copolymerized with dimethacrylated PEG monomers in the presence of hMSCs to yield cell-laden hydrogels. For controls, hMSCs were encapsulated in both PEG and RGDS-functionalized PEG hydrogels. Encapsulated hMSCs were cultured in vitro in the various gel formulations and cell viability, alkaline phosphatase (ALP) production, and gene expression of osteopontin (OPN) and collagen type I were monitored as a function of time. Finally, the combined effects of RGDS and heparin were examined. Because heparin interacts with numerous proteins in the media, many of which are involved in hMSC adhesion and regulation of the osteogenic lineage, two-dimensional studies on heparin functionalized gels were conducted in the presence of media with defined, heparin-binding exogenous proteins and ALP production and OPN gene expression were monitored. Finally, after 5 weeks of culture, hMSC integrin expression was examined via immunohistochemistry to analyze expression in response to the availability of the specific ligands, RGDS and heparin.

2. Materials and methods

All chemicals were obtained from Sigma-Aldrich (St. Louis, MO) unless otherwise specified.

2.1. Synthesis of PEG dimethacrylate (PEGDM) and methacrylated heparin

PEGDM was synthesized as described previously [19]. ^{1}H NMR analysis (in chloroform-d, Cambridge Isotopes) on the PEGDM revealed an average of 95% methacrylation.

Heparin (sodium salt from porcine intestinal mucosa, MW$\sim$16 kDa) was methacrylated modified from a previous method [20]. Briefly, a 2% (w/v) solution of heparin in diH_2O was prepared and reacted with 5-fold molar excess of methacrylic anhydride. The pH of the reaction mixture was adjusted to 8.5 using 5 N NaOH, and the reaction was allowed to proceed overnight at 4 °C. The product, methacrylated heparin, was precipitated once in 95% ethanol, dried, and dialyzed (Spectrum, 1000 MW cutoff) for 48 h against diH_2O and lyophilized. ^{1}H NMR analysis (in D_2O, Cambridge Isotopes) revealed an average of 22% methacrylation. The percentage of methacrylation refers to the number of methacrylate groups per heparin disaccharide unit.

The amino acid sequence Arg–Gly–Asp–Ser (RGDS) was synthesized using solid-phase methods on an ABI 433A Peptide Synthesizer (Applied Biosystems, Foster City, CA) and following procedures for HBTU (2-(1H-benzotriazol-1,1,3,3-tetramethyluroniumhexafluorophosphate) activation coupling. The peptides, after UV-monitored synthesis, were cleaved from the solid support with a cocktail consisting of 5% phenol, 5% water, and 2.5% triisopropylsilane in trifluoroacetic acid (TFA). The peptide was then washed with copious amounts of ice-cold diethyl ether, redissolved in distilled water, and dialyzed (Spectrum, 500 MW cutoff) over 24 h with two exchanges of distilled water.

RGDS was coupled to acrylated-PEG following a previously reported method [21]. Structures of PEGDM, methacrylated heparin, and acrylated RGDS are shown in Fig. 1.

2.2. hMSC culture

hMSCs were purchased from Cambrex and cultured in growth medium: low-glucose Dulbecco's modified eagle medium (Gibco) supplemented with 10% FBS (Invitrogen), 1% penicillin/streptomycin (Gibco), 0.25% gentamicin (Gibco), and 0.25% fungizone (Gibco). hMSCs at passage 3 were used in this study.

2.3. Encapsulation of hMSCs

All hydrogels were formulated by dissolving PEGDM in phosphate-buffered saline (PBS) to achieve a final monomer concentration of 10wt%. The photoinitiator 2-hydroxy-1-[4-(hydroxyethoxy) phenyl]-2-methyl-1-propanone (Ciba-Geigy) was added to a final concentration of 0.05 wt%. hMSCs were combined with sterile macromer/initiator solutions (with 1 mM of methacrylated heparin for the heparin-functionalized gels, 5 mM of acrylated-PEG–RGDS for the RGDS-funtionalized gels, and a combination of heparin and RGDS at the same concentrations for heparin + RGDS-functionalized gels) at a concentration of 25×10^6 cells/ml and photoencapsulated using a long-wave ultraviolet lamp (UVP, model XX-20) at an intensity of $\sim$4 mW/cm^2 for 10 min [22]. Without any adhesive ligands, hMSCs are known to undergo apoptosis after <2 weeks of culture [23-25]. Therefore, RGDS was incorporated as a positive control, as it has formerly been shown to rescue hMSC viability under the same culture conditions [24]. The resulting cell–hydrogel constructs were

Fig. 1. Chemical structures of (a) dimethacrylated macromer PEGDM, (b) methacrylated heparin, and (c) acrylated-PEG–RGDS.

cultured in 12-well plates in growth medium supplemented with 100 nM dexamethasone, 10 mM β-glycerophosphate, and 0.05 mM ascorbic acid phosphate. Constructs were removed from culture at 4, 10, 21, and 35 days for live/dead, metabolic activity, ALP production, and gene expression analysis.

2.4. Viability of hMSCs encapsulated in heparin- and RGDS-functionalized PEG hydrogels

hMSC/polymer constructs were cultured for up to 5 weeks. At each time point, a sample of constructs were removed from culture and stained using the live/dead assay. Constructs were placed in 1.5 ml tubes with 1 ml of growth media and treated with 0.5 μl Calcein substrate (Calbiochem) and 2 μl of ethidium homodimer substrate (Calbiochem) and incubated at 37 °C for 30 min. Cell viability and distribution were visualized using confocal microscopy at 4, 10, 21, and 35 days. The images were analyzed using ImageJ to determine the percent of live cells present at each time point.

2.5. ALP production of hMSCs encapsulated in heparin and RGDS-functionalized hydrogels

ALP production was measured using an assay based on the change in absorbance of *o*-nitrophenol as it is enzymatically cleaved by ALP. Cell/hydrogel constructs were removed from culture at 4, 10, 21, and 35 days, lysed with Cell-glo lysis buffer (Promega), and manually homogenized. The assay was performed by combining 100 μl of sample with 100 μl of the ALP substrate. By performing the assay using known concentrations of ALP in parallel with the samples, the concentrations of ALP for the samples were calculated.

Metabolic activity, which is an indirect measure of the number of living cells, of the encapsulated hMSCs was analyzed at 4, 10, 21, and 35 days with the AlamarBlue assay (Serotec). A 10 vol% solution of AlamarBlue in media was added to the constructs. Active mitochondria convert resazurin, the active ingredient in AlamarBlue, to resorufin, a fluorescent molecule. After 4 h of incubation, the media/AlamarBlue solution was analyzed for fluorescence (excitation at 560 nm, emission at 590 nm) with a plate reader (Victor^2, Perkin Elmer). AlamarBlue readings were utilized to normalize ALP production in order to eliminate error due to cell number fluctuations.

2.6. hMSC gene expression encapsulated in heparin and RGDS-functionalized PEG hydrogels

Gene expression of encapsulated hMSC gene expression was analyzed using reverse-transcription polymerase chain reaction. After 4, 10, 21, and 35 days, constructs were removed from culture and rinsed three times with PBS. Total RNA was isolated using a guanidinium thiocyanate/phenol reagent (TRI reagent, Sigma) and standard manufacturer's protocols. After allowing the RNA pellet to dry, it was resuspended in nuclease-free water, and any residual genomic DNA in the samples was digested (DNase I, Invitrogen). RNA was then quantified using the RiboGreen assay (Molecular Probes) based on the manufacturer's instructions.

Reverse transcription was performed using the iScript cDNA Synthesis Kit (Bio-Rad). A 15 ng total RNA sample was used for the single-strand cDNA synthesis. The reverse transcription reaction was incubated at 25 °C for 5 min, 42 °C for 30 min, and terminated at 85 °C for 5 min. PCR was conducted using the iCycler Real-Time PCR machine (Bio-Rad), and primers and probes were designed using the Beacon Designer primer design program (Table 1). Primers (Invitrogen) and probes (Integrated DNA Technologies) for OPN, collagen type I (COL I), and glyceralde-

Table 1
Primer and probe sequences designed by Beacon designer software and utilized for real-time PCR

Gene	Sense primer	Anti-sense primer	Probe
Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	5′-GCAAGAGCACAAGAGGAAGAG-3′	5′-AAGGGGTCTACATGGCAACT-3′	5′-ACCCTCACTGCTGGGGAGTCC-3′
Collagen type I (Col I)	5′-GGGCAAGACAGTGATTGAATACA-3′	5′-GGATGGAGGGAGTTTACAGGAA-3′	5′-CCAAGTCCTCCCGCCTGCCCATC-3′
Osteopontin (OPN)	5′-ATTCTGGGAGGGCTTGGTTG-3′	5′-TCTGGTCCCGACGATGCT-3′	5′-CTCTGCCTCCTCCTGCTGCTGCTG-3′

hyde-3-phosphate dehydrogenase (GAPDH) were used in a multiplex format. The following PCR parameters were utilized: 95 °C for 90 s followed by 45 cycles of 95 °C for 30 s and 55 °C for 60 s. Threshold cycle (C_T) analysis was used to quantify PCR products, normalized to GAPDH and relative to expression of hMSCs encapsulated in the homopolymer PEGDM gels.

2.7. Mechanism of osteogenic differentiation: effect of BMP2 and FN on hMSCs cultured on heparin-functionalized PEG hydrogels

To examine heparin-binding protein-dependent osteogenic differentiation, hMSCs were trypsinized from culture plates, counted, centrifuged, resuspended, and seeded onto sterile hydrogel disks formulated from 10% PEGDM (control gels) or 10% PEGDM and 0.025 mM methacrylated heparin at a density of 5×10^3 cells/cm^2. hMSCs were cultured with five different media compositions: growth media supplemented with 100 nM dexamethasone, 10 mM β-glycerophosphate, and 0.05 mM ascorbic acid phosphate (osteogenic media), osteogenic media with all heparin-binding proteins removed by filtration through a heparin column, heparin-binding protein-depleted media with FN added at 30 ng/ml (the concentration of FN in osteogenic media, which contains 10% FBS [27]), heparin-binding protein-depleted media with BMP2 added at 84 pg/ml (the concentration of BMP2 in osteogenic media as analyzed with an ELISA, R&D Systems), and heparin-binding protein-depleted media with FN and BMP2 added at 30 ng/ml and 84 pg/ml, respectively.

After 2 and 14 days, cells were removed from culture, rinsed three times with PBS, and analyzed for ALP production and OPN gene expression, as described above, to evaluate the osteogenic differentiation of the cells under the different culture conditions. In addition, the number of cells was evaluated by quantification of DNA using the PicoGreen assay (Molecular Probes) for normalization of ALP production. After 2 and 14 days of in vitro culture, gels with attached cells were removed from culture, the gels were rinsed three times with PBS, and 0.5 ml of Glo Lysis Buffer was added (Promega). The samples were assayed for ALP production and DNA content. In addition, reverse transcriptase PCR was utilized, as described above, to assess OPN gene expression.

2.8. Integrin expression by hMSCs encapsulated in heparin and RGDS-functionalized PEG hydrogels

The expression of integrins $\alpha 5\beta 1$ and $\alpha v\beta 3$, associated with osteoblast-ECM interactions, osteogenic differentiation, and/or cell survival [28,29], was monitored using immunohistology. FN-mediated cell adhesion is controlled mainly by $\alpha 5\beta 1$ (recognizing the RGD sequence). In addition, $\alpha v\beta 3$ binds RGD motifs in extracellular matrix proteins such as FN, OPN, and laminin, among others [30,31]. At day 35, cell–hydrogel constructs were fixed overnight in 10% formalin (Fisher), transferred to 22 wt% sucrose (Aldrich) for 72 h, frozen in Cryo-gel (Instrumedics Inc.), and cryosectioned (10 μm sections). Antigen retrieval was performed by incubating sections in 0.1% trypsin for 5 min. The slides were blocked in 10% normal goat serum and 0.5% BSA for 30 min, incubated separately in primary antibodies (mouse anti-human $\alpha 5\beta 1$ (Covance Research) or $\alpha v\beta 3$ (Abcam)) at 1:1000 for 4 h, then incubated in secondary antibody [goat anti-mouse HRP (Chemicon)] at 1:750 for 2 h. The sections were developed using Vector NovaRED Substrate Kit (Vector Labs), and nuclei were stained with DAPI. Finally, the sections were mounted with Vectashield (Vector Labs), imaged using conventional fluorescence microscopy (Nikon Eclipse TE300 and associated SPOT software), and evaluated for number of integrin-positive cells.

2.9. Statistical analysis

Statistical analysis was performed using a one-way ANOVA. Data are presented as mean ± standard deviation.

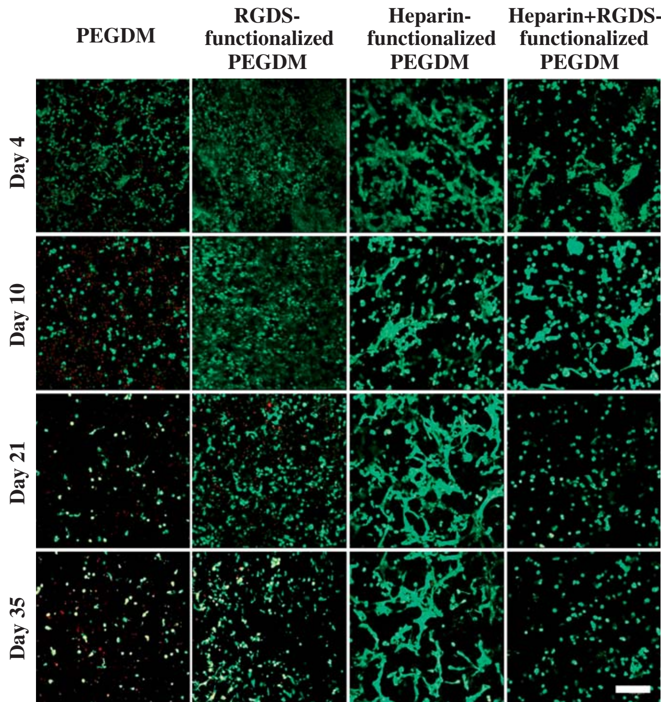

Fig. 2. Effect of RGDS, heparin, or heparin + RGDS incorporation on viability of hMSCs encapsulated within PEGDM hydrogels. hMSCs were encapsulated in 10% PEGDM, 10% PEGDM containing 5 mM acrylated-PEG–RGDS, 10% PEGDM containing 1 mM methacrylated heparin, or 10% PEGDM containing 1 mM methacrylated heparin + 5 mM acrylated-PEG–RGDS. Encapsulated cells were cultured for 5 weeks and viability was assessed at days 4, 10, 21, and 35 using the live/dead assay (bar = 200 μm). Representative live/dead images are shown for each time point.

3. Results

3.1. Viability of hMSCs encapsulated in heparin- and RGDS-functionalized PEG hydrogels

hMSCs were encapsulated in heparin-, RGDS-, or a combination of heparin- and RGDS-functionalized hydrogels and cultured for up to 5 weeks. Previously, PEG-based hydrogels have lead to apoptosis of gel-encapsulated hMSCs, thought to be due to lack of cell–material interactions. The addition of RGDS increased viability substantially [32]. In this work, we found that all of the ligands studied, heparin, RGDS-, and their combination, led to high levels of hMSC viability over a prolonged culture time, up to 5 weeks. Qualitative images are shown in Fig. 2, and quantification analysis is presented in Fig. 3. For quantification, series of live/dead images for each time point was analyzed using ImageJ to determine the relative fraction of live versus dead cells by the fluorescence intensities. After 35 days, viability of hMSCs in heparin-functionalized hydrogels, assessed via the live/dead assay, was 94%, which is increased over unmodified PEGDM gels and RGDS-modified gels, which exhibited 63% and 87% viability over 5 weeks, respectively. As expected, the combination of heparin and RGDS had the greatest survivability (95%) over the 5 weeks (not statistically different from heparin-functionalized gels). Cells between days 4 and 10 in the unmodified PEGDM gels exhibited the greatest cell death. Interestingly, by examining the pictures in column 3 of Fig. 2, it is apparent that the hMSCs encapsulated in heparin-functionalized hydrogels are able to attach and spread to some extent and appear to be proliferating, although that was not

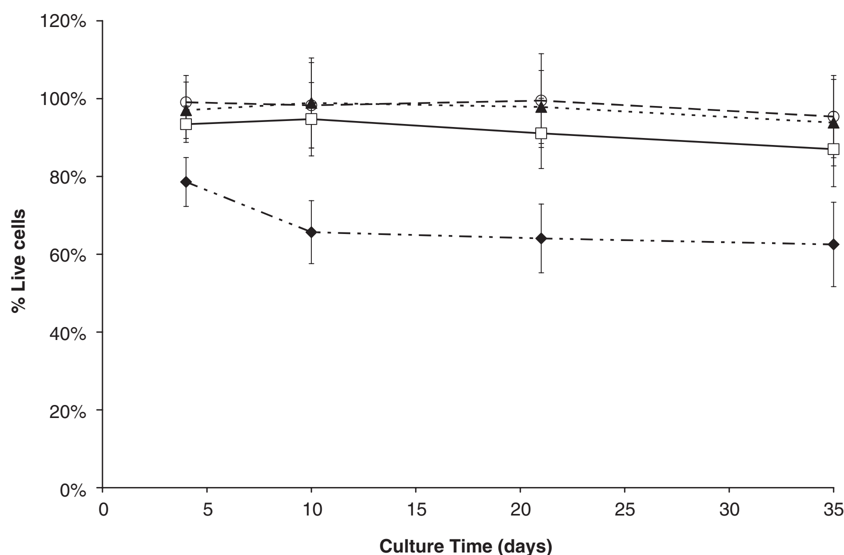

Fig. 3. Effect of RGDS, heparin, or heparin + RGDS incorporation on viability of hMSCs encapsulated within PEGDM hydrogels. Series of live/dead images for each time point and treatment were quantified using ImageJ and the results are shown over time. hMSCs were encapsulated in 10% PEGDM (- –◆– -), 10% PEGDM containing 5 mM acrylated-PEG–RGDS (–□–), 10% PEGDM containing 1 mM methacrylated heparin (- -▲- -), or 10% PEGDM containing 1 mM methacrylated heparin + 5 mM acrylated-PEG–RGDS (… ○ …). Encapsulated cells were cultured for 5 weeks and viability was assessed at days 4, 10, 21, and 35 using the live/dead assay.

quantified specifically. However, this same phenomena is reversed in the heparin + RGDS-functionalized hydrogels.

3.2. ALP production by hMSCs encapsulated in heparin and RGDS-functionalized hydrogels

To evaluate the function of the hMSCs in the various gel formulations, ALP production was monitored at 4, 10, 21, and 35 days, and the results shown in Fig. 4 were normalized to a relative measure of cell number (Alamar-Blue fluorescence measurement of metabolic activity). ALP production, which is shown relative to ALP production by cells encapsulated in PEG hydrogels, increased with culture time in the heparin-functionalized gels in the presence or absence of RGDS. ALP production increases 4- and 3-fold, respectively, for hMSCs encapsulated in heparin-functionalized and heparin + RGDS-functionalized hydrogels, indicating that heparin promoted osteogenic differentiation of hMSCs in three-dimensional culture. Cells encapsulated in RGDS-functionalized gels maintained a constant ALP production over the culture period, indicating that RGDS is not significantly affecting osteogenic differentiation. Interestingly, in the presence of both heparin and RGDS, the ALP production is lower at all time points than that of cells in the presence of only heparin, but greater than that of cells in RGDS-functionalized gels. It is possible that the inclusion of RGDS in the heparin-functionalized gels suppresses osteogenic differentiation, reversing the effects of heparin alone. hMSCs encapsulated in heparin-functionalized gels exhibited a greater than 6-fold increase in ALP production, and hMSCs encapsulated in heparin + RGDS-functionalized gels showed a greater than 4-fold increase in ALP production compared with cells in RGDS-functionalized gels at day 35.

3.3. hMSC gene expression encapsulated in heparin and RGDS-functionalized PEG hydrogels

To more closely examine osteogenic differentiation of the encapsulated hMSCs as a function of gel chemistry, gene expression profiles for OPN and collagen type I (COL I) were monitored and normalized to GAPDH. Results were quantified over time and with gel composition, and the results are shown in Fig. 5. OPN (Fig. 5a) gene expression was at the same level for all treatments at day 4. A substantial increase in gene expression was found at day 10 for cells encapsulated in heparin-functionalized gels to nearly 3-fold over the RGDS- and heparin + RGDS-functionalized gels. OPN gene expression remained at this elevated level throughout the duration of the study for hMSCs encapsulated in heparin-functionalized gels and was greater than that of cells in all other treatments.

COL I (Fig. 5b) gene expression of hMSCs in heparin- and heparin + RGDS-functionalized gels was greater than all treatments and steady over the culture days 4, 10, and 21 (1.6-fold, 2-fold, and 2-fold greater, respectively) than expression in RGDS-functionalized hydrogels. COL I expression decreased at day 35 for cells in both heparin-functionalized gels to about the same level of expression by cells in RGDS-functionalized gels. One potential reason for

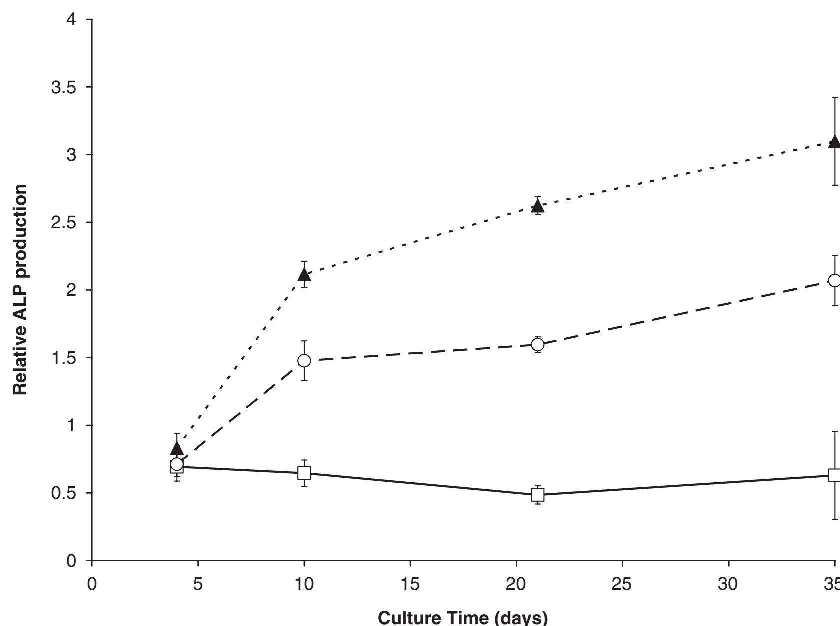

Fig. 4. Alkaline phosphatase (ALP) production of hMSCs encapsulated in heparin functionalized (…▲…), RGDS-functionalized (–□–), and heparin + RGDS-functionalized (- -○- -) PEGDM hydrogels as a function of time. Relative ALP production is presented as relative to ALP production by cells encapsulated in unmodified PEG hydrogels. Values are reported as the average of three samples per composition (error bars designate standard deviation).

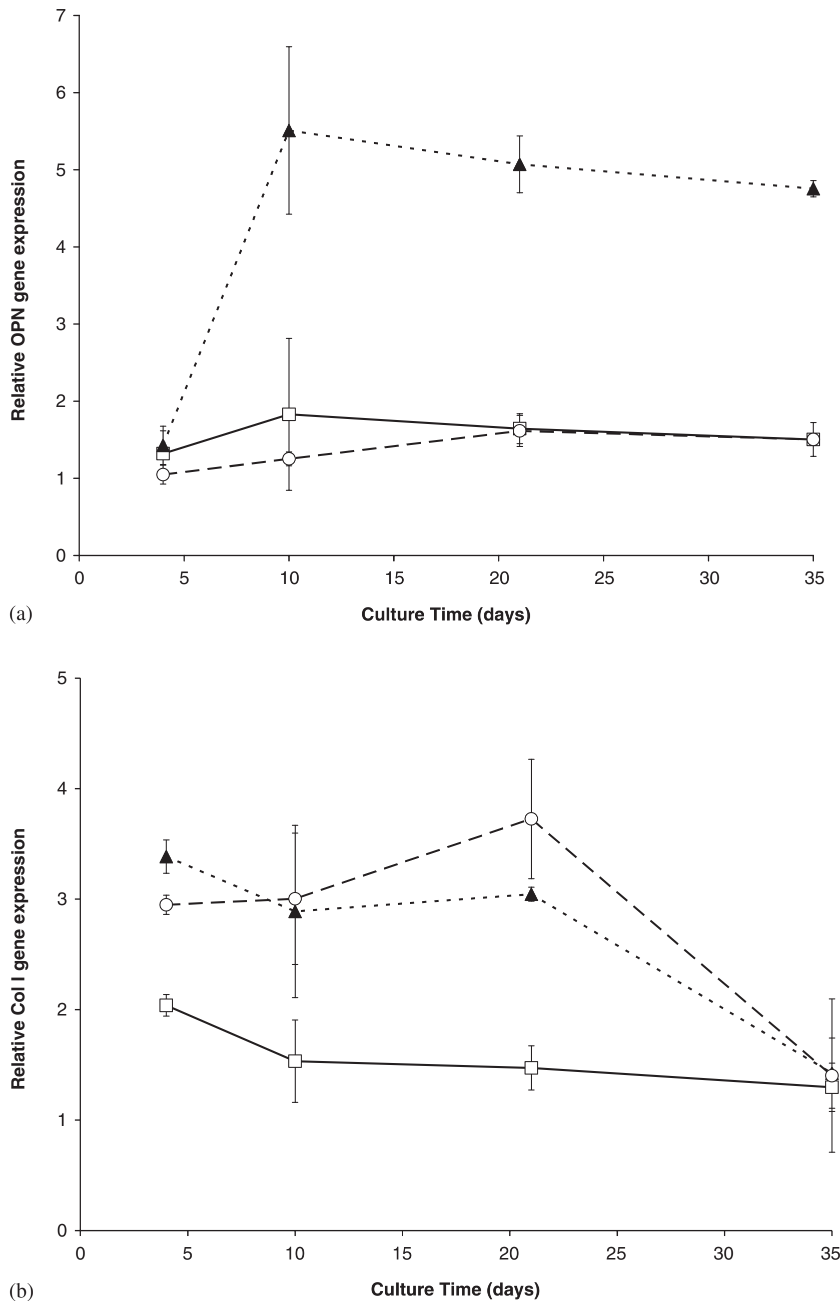

Fig. 5. Gene expression, as measured by real-time RT-PCR, of osteopontin (OPN) (a) and collagen type I (Col I) (b) normalized to levels of GAPDH and relative to expression in PEGDM of hMSCs encapsulated in RGDS-functionalized PEGDM (–□–), heparin-functionalized PEGDM (…▲…), and heparin + RGDS-functionalized PEGDM (---○---). Relative gene expression is presented as normalized to gene expression by cells encapsulated in unmodified PEGDM hydrogels. Values are reported as the average of three samples per composition (error bars designate standard deviation).

this intriguing result is that the collagen levels in the pericellular environment become elevated in these non-degrading gels and subsequent collagen synthesis is reduced [33]. Cells in RGDS-functionalized gels exhibited the lowest COL I gene expression at all time points compared with the other treatments. OPN and COL I gene expression supports ALP production data supporting the hypothesis that heparin-functionalized gels are inducing the osteogenic differentiation of hMSCs in three-dimensional in vitro culture.

3.4. Mechanism of osteogenic differentiation: effect of BMP2 and FN on hMSCs cultured on heparin-functionalized PEG hydrogels

To evaluate the potential mechanism of the heparin-functionalized gels in inducing osteogenic differentiation of hMSCs, specific heparin-binding proteins were investigated in greater detail. Specifically, FN and BMP2 were selected for this study because of their prominent role in regulation of the osteogenic lineage [34,35]. In serum, which provides

Table 2
Relative alkaline phosphatase (ALP) production by hMSCs cultured on heparin-modified PEGDM hydrogels in osteogenic media and osteogenic media depleted of heparin-binding proteins

Media composition	Relative ALP production	
	Day 2	Day 14
Osteogenic media	0.85 ± 0.04	2.34 ± 0.43
Depleted osteogenic media	0.93 ± 0.06	1.38 ± 0.10
Depleted osteogenic media + 30 ng/ml fibronectin	1.68 ± 0.09	3.20 ± 0.22
Depleted osteogenic media + 84 pg/ml BMP2	1.91 ± 0.17	2.78 ± 0.43
Depleted osteogenic media + 30 ng/ml fibronectin + 84 pg/ml BMP2	1.11 ± 0.09	2.77 ± 0.36

In addition, fibronectin (30 ng/ml), bone morphogenetic protein 2 (BMP2, 84 pg/ml), and a combination of fibronectin and BMP2 (30 ng/ml and 84 pg/ml) were added to osteogenic media depleted of heparin-binding proteins and ALP production was assessed. Results are expressed as ALP production normalized to ALP production by hMSCs on PEGDM hydrogels cultured in osteogenic media. Values are reported as the average of three samples per composition.

Table 3
Osteopontin (OPN) gene expression normalized to levels of GAPDH and relative to expression on PEGDM of hMSCs cultured on heparin-functionalized PEGDM hydrogels in different culture conditions: osteogenic media, osteogenic media depleted of all heparin-binding proteins, osteogenic media depleted of all heparin-binding proteins but fibronectin at 30 ng/ml, osteogenic media depleted of all heparin-binding proteins but bone morphogenetic protein 2 (BMP2) at 84 pg/ml, osteogenic media depleted of all heparin-binding proteins but fibronectin at 30 ng/ml and BMP2 at 84 pg/ml

Media composition	Relative OPN expression	
	Day 2	Day 14
Osteogenic media	4.74 ± 1.28	6.29 ± 1.92
Depleted osteogenic media	1.83 ± 0.61	0.93 ± 0.20
Depleted osteogenic media + 30 ng/ml fibronectin	3.73 ± 1.18	3.78 ± 1.24
Depleted osteogenic media + 84 pg/ml BMP2	1.05 ± 0.21	3.53 ± 1.18
Depleted osteogenic media + 30 ng/ml fibronectin + 84 pg/ml BMP2	1.01 ± 0.29	0.58 ± 0.13

Values are reported as the average of three samples per composition.

the only available source of exogenous proteins in the previous cell culture studies, FN and BMP2 are present in 30 ng/ml and 84 pg/ml, respectively. To isolate the individual effects of FN and BMP2, media was first depleted of all heparin-binding proteins by filtration through a heparin column. Subsequent addition of either FN, BMP2, or both to the media allowed for examination of their specific effects on hMSC ALP production and OPN gene expression when cultured on the surface of heparin-functionalized gels. As a control, media that was not filtered and, therefore, containing all available heparin-binding proteins was utilized.

ALP production by hMSCs increased in all treatments from day 2 to day 14, as compared to levels on unmodified PEGDM (Table 2). The greatest increases, however, were found by hMSCs cultured in un-filtered osteogenic media (2.5-fold increase) and in depleted media supplemented with FN + BMP2 (2.5-fold increase). Both FN and BMP2 media, alone, had a strong impact on ALP production, where FN and BMP2 media, individually, caused a 2-fold increase and 1.6-fold increase, respectively, of ALP production by hMSCs. Even media without any heparin-binding proteins caused a significant, yet small (1.2-fold), increase in ALP production, indicating that heparin, by itself, could be initiating signal transduction pathways involved with cell differentiation.

In addition to ALP production, OPN gene expression was also investigated to elucidate which heparin-binding proteins might be most prominent in osteogenic differentiation. Results are shown in Table 3, relative to gene expression on PEGDM hydrogels. Overall, OPN gene expression was greatest at days 2 and 14 by cells cultured in media with all heparin-binding proteins. Without any heparin-binding proteins, however, OPN gene expression was low at day 2 and decreased 2-fold by day 14. Cells cultured in BMP2 media exhibited the greatest increase in OPN gene expression (4-fold) over the culture period. In the presence of FN, hMSC OPN gene expression was statistically the same at all time points as that of cells in the presence of all heparin-binding proteins but did not change over the time course of the study. Interestingly, although BMP2 media increased OPN gene expression, and FN media resulted in high and consistent OPN gene expression over the study, the combination of the two proteins resulted in the lowest gene expression at both time points and expression reduced 2-fold from days 2 to 14. FN and BMP2, therefore, do not act in combination to increase OPN gene expression but do have individual effects. Therefore, the temporal availability of these proteins may play a role in heparin-facilitated hMSC osteogenic differentiation.

3.5. Integrin expression by hMSCs encapsulated in heparin- and RGDS-functionalized PEG hydrogels

To further explore how hMSCs might be interacting with heparin gels and influencing intracellular signaling, integrin expression was examined as a function of the gel functionality. Specifically, the distribution of integrins $\alpha5\beta1$ and $\alpha v\beta3$ were analyzed in cells encapsulated in hydrogels at day 35 by immunostaining and counterstaining with DAPI. Representative images of integrin immunostaining are shown in Fig. 6a. Quantification of integrin expression was performed by counting total number of cells and cells staining positive for the particular integrin. Therefore, as shown in Fig. 6b, the percentage of integrin-expressing cells for each hydrogel treatment was estimated. The greatest $\alpha5\beta1$ expression was found by cells

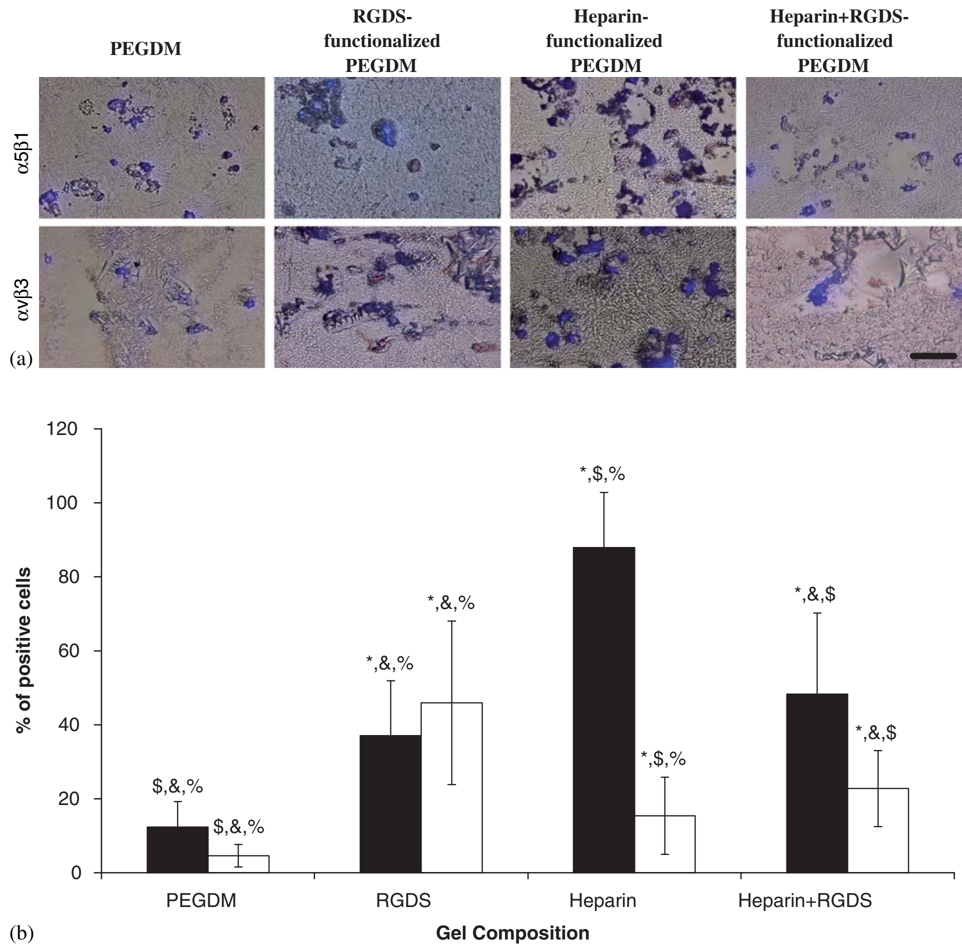

Fig. 6. Light micrographs (a) of NovaRED staining of integrins $\alpha5\beta1$ and $\alpha v\beta3$ (pink to red) merged with fluorescent micrograph of DAPI staining (nuclei – blue, bar = 50 μm) and (b) quantification of percent positively stained ($\alpha5\beta1$—black bars, $\alpha v\beta3$—white bars) cells of histological sections of hMSCs encapsulated in 10% PEGDM, 10% PEGDM containing 5 mM Ac-PEG–RGDS, 10% PEGDM containing 1 mM methacrylated heparin, or 10% PEGDM containing 1 mM methacrylated heparin + 5 mM Ac-PEG–RGDS after 35 days. *$p < 0.05$ of sample versus 10% PEGDM, $ $p < 0.05$ of sample versus 10% PEGDM + 5 mM Ac-PEG–RGDS, & $p < 0.05$ of sample versus 10% PEGDM + 1 mM methacrylated heparin, % $p < 0.05$ of sample versus 10% PEGDM + 1 mM methacrylated heparin + 5 mM Ac-PEG–RGDS at that time point. $n = 5$ samples per condition.

encapsulated in heparin-functionalized gels, with 88% positive cells. Heparin + RGDS-functionalized hydrogels elicited the second-greatest $\alpha5\beta1$ expression (48% positive cells), followed by RGDS-functionalized gels (37% positive cells), and PEGDM hydrogels (12% positive cells). When examining $\alpha v\beta3$ integrin expression, RGDS-functionalized hydrogels elicited the most expression, with 46% positive cells. Heparin + RGDS-functionalized gels induced 23% positive cells, followed by heparin-functionalized gels (15% positive cells), and PEGDM hydrogels (5% positive cells). Based on Figs. 2 and 3, and 6, integrin expression is linked to viability, as the hydrogels presenting RGDS and heparin show the greatest survivability of hMSCs and the greatest number of cells expressing integrins $\alpha5\beta1$ and $\alpha v\beta3$. In addition, based on data presented in Figs. 4 and 5, and 8, where heparin, specifically, is augmenting ALP production and OPN and Col I gene expression while also expressing the highest percentage of $\alpha5\beta1$-positive cells, it can be deduced that $\alpha5\beta1$ expression is positively correlated with the osteogenic differentiation of hMSCs.

4. Discussion

The development of functionalized cell delivery scaffolds that can elicit specific biological responses such as survivability, adhesion, proliferation, and differentiation is of interest in regenerative medicine. An important area of research includes strategies that control the density, clustering, and orientation of cell signaling epitopes, such as proteins and growth factors, to provide avenues for outside-in signaling. Heparin, a protein-binding GAG, possesses numerous attractive qualities with respect to functionalizing scaffolds for regenerative medicine applications. In this study, we built upon previous results that indicated that heparin-functionalized hydrogels promote the osteogenic differentiation of hMSCs. Utilizing heparin-functionalized PEG hydrogels, we encapsulated and cultured hMSCs in vitro. As a control, RGDS-functionalized hydrogels were utilized, as these conditions have previously promoted hMSC viability compared to unfunctionalized PEG.

Viability of the encapsulated hMSCs was verified over the time course of the experiment. Previous studies have examined hMSC viability in PEG hydrogels, and it was concluded that viability drops off dramatically with time [23]. Under the same culture conditions, except for ligand availability, less than 47% of hMSCs survive after 1 week of culture in PEG hydrogels [23]. Viability of hMSCs are dramatically improved (to $>95\%$ after a 4-week culture period) when either the well-known cell-adhesive ligand, RGDS peptide, or a phosphate-containing molecule is incorporated into the hydrogel network as pendant tethers [25,32]. With heparin functionalization, hMSC viability is improved as well (to $\sim 94\%$) which is statistically the same as with RGDS-functionalization in this particular study.

Beyond viability, the function of hMSCs was evaluated as a function of the biochemical environment. Specifically, the osteogenic differentiation of hMSCs was seen through increased ALP production and gene expression of OPN and collagen type I. The importance of sulfated GAGs for bone formation is inherent to their ability to bind most of the growth factors (e.g., FGFs, TGF-β1, BMP2 and 4, IGF-II) involved in the regulation of cells of the osteoblast lineage [26]. In vitro cultures of hMSCs in heparin-functionalized hydrogels follow the general osteogenic differentiation process with enhancement of various differentiation markers (e.g., ALP, COL I, and OPN). In general, differentiation begins with an increase in cell density, continues with augmented protein levels, a cascade that starts with an increase in ALP and OPN, followed by a heightened production of COL I, which continues at a high level until mineralization proceeds [36]. Shibata et al. [37] found that soluble heparin stimulates collagen synthesis in mineralized cultures of the osteoblast cell line, MC3T3-E1, and Saos-2 cells, an osteoblast-like cell line, exhibited increased ECM deposition in the presence of soluble heparin [38]. Gupta et al. [39] explored bone marrow stroma, the native hMSC niche, and found that it is the structural specificity of heparin that determines the selective colocalization of cytokines and ECM components that orchestrates their controlled growth and differentiation.

Based on the intriguing differences observed between the heparin and RGDS functionalities, further exploration of the potential mechanism of heparin-induced osteogenic differentiation was performed. Specifically, the availability of BMP2 and FN in the culture system was controlled and hMSC osteogenic differentiation was evaluated as a function of the microenvironment. BMP2 availability increased both ALP production and OPN gene expression, while FN increased ALP production, but not OPN gene expression. In vivo, BMPs are potent effectors of osteoblast function and hMSC osteogenic differentiation and have been used extensively to stimulate bone regeneration [34,40,41]. BMP2, a member of the TGF-β supergroup, is known to bind reversibly to heparin [26]. FN plays a critical role in the early stages of osteogenic differentiation. The localization of FN in the periosteum of rat calvaria [35,42] and in the osteoid surrounding implants [43] indicates that FN is synthesized and deposited in the areas of bone tissue where recruitment and commitment of osteoblast precursors appear. FN is also a well-known heparin-binding protein [26]. Therefore, increases in ALP production, an indicator of osteogenic differentiation, are plausible. However, OPN gene expression is not increased by the presence of FN. It is possible that, since OPN and FN are involved in cell adhesion and FN is exogenously present, OPN expression and synthesis is not required for hMSC adhesion.

To further understand, specifically, which integrins are produced and implicated in the viability of and osteogenic differentiation of hMSCs, $\alpha 5\beta 1$ and $\alpha v\beta 3$ integrin staining was analyzed and found to be dependent on ligand availability. Heparin-functionalized gels induced much higher $\alpha 5\beta 1$ expression compared to $\alpha v\beta 3$ while RGDS-functionalized gels induced $\alpha 5\beta 1$ and $\alpha v\beta 3$ expression to approximately the same extent. hMSCs cultured in the absence of adhesive ligands showed very little integrin expression. Integrin-mediated cell adhesion is known to be necessary for the survival of adhesion-dependent cell types, such as hMSCs, by activating a number of anti-apoptotic pathways [29]. Data presented here indicate that $\alpha 5\beta 1$ or $\alpha v\beta 3$ integrin-binding are sufficient to activate these pathways. Indeed, integrin-mediated signal transduction can also affect many other cell processes, including differentiation. Supportive of our findings that heparin induces hMSC osteogenic differentiation through $\alpha 5\beta 1$ integrin expression, Moursi et al. [43], found that the specific $\alpha 5\beta 1$ receptor mediates critical interactions between osteoblasts and the ECM required for both bone morphogenesis and osteoblast differentiation in an in vitro environment. Interestingly, both $\alpha 5\beta 1$ and $\alpha v\beta 3$ integrins are expressed equally by cells encapsulated in RGDS-functionalized hydrogels. Charo et al. [44] found that $\alpha v\beta 3$ is capable of recognizing the ECM in an RGDS-dependent manner, and that it and $\alpha 5\beta 1$ act in concert in mediating the attachment and spreading of human melanoma cells on a FN substrate. Thus, by careful design of hydrogel cell carriers with rationally targeted modification and incorporation of signaling molecules, niches can be synthesized that actively promote cell function such as viability and differentiation.

5. Conclusions

hMSCs, multipotent precursor cells, were encapsulated in heparin-functionalized PEG hydrogels and analyzed for viability and osteogenic differentiation. Heparin-functionalized hydrogels supported hMSC viability and induced osteogenic differentiation, likely through cell–material interactions established by heparin-binding proteins FN and BMP2. Furthermore, the viability and differentiation were differentially affected by integrin production, where both $\alpha 5\beta 1$ and $\alpha v\beta 3$ integrin–ligand interactions supported

viability, while only the $\alpha 5\beta 1$ integrin played a role in hMSC osteogenic differentiation.

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