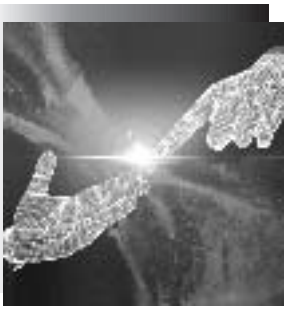

HANDS: ©IMAGESTATE.
BACKGROUND: ©ELSEVIER (SEE P. 1).

Osteoblast and Chondrocyte Interactions During Coculture on Scaffolds

Examining Matrix and Substrate-Dependent Effects on the Formation of Functional Bone-Cartilage Interfaces

JEFFREY P. SPALAZZI, KATHIE L. DIONISIO, JIE JIANG, AND HELEN H. LU

In the United States, over 21 million Americans currently suffer from osteoarthritis. Due to limitations associated with existing treatment modalities, there is a growing interest in tissue-engineered cartilage grafts. One aspect of our approach to treating osteoarthritis is to reconstruct a functional interface between the tissue-engineered cartilage and bone. The interface is designed to mimic the native osteochondral interface, and it is reconstructed by coculturing osteoblasts and chondrocytes on degradable scaffolds. The objectives of the current study are, first, to examine osteoblast-chondrocyte interactions in a novel, indirect coculture model and, second, to evaluate both matrix- and substrate-dependent effects on chondrocyte response during coculture on two-dimensional (2-D) and three-dimensional (3-D) substrates.

Overview

Arthritis is the leading cause of physical disability in the United States, with osteoarthritis being the most common form of arthritis [1]. The prevalence of this joint-degenerative disease is reported in adults 45 years or older. The medical and social cost of this condition is exacerbated by the fact that there is an increase in the number of elderly Americans. By 2020, over 53 million Americans will be 65 years or older [2], and the majority of them will be at risk for osteoarthritis. With this aging yet still physically active population, an increase in the incidence of age-related diseases such as osteoarthritis will present a significant challenge to the medical and research communities.

In osteoarthritis, it is believed that long-term joint loading leads to the degradation of articular cartilage. Unfortunately, articular cartilage loses the ability to repair itself over time [3]-[6]. Current treatment modalities for the operative repair of degraded articular cartilage include the use of allografts and autografts. While these methods can provide short-term pain relief and partially restore joint function, none of these approaches has resulted in a long-term functional solution [5]-[9]. Moreover, there are a variety of complications associated with each of these types of grafts. Some of the challenges facing the use of allografts include the scarcity of donor tissue and the risks of disease transmission and host immune response [3]. With autogenous grafts, in addition to insufficient supply, donor site pain and morbidity are major limiting factors [4]. Another problem in utilizing biological or synthetic grafts is the inability of these grafts to integrate with cartilage or bone at the implant site. Due to limitations associated with existing grafting systems, tissue-engineered constructs for cartilage repair and regeneration have been investigated during the past decade [10]-[15]. However, the integration of tissue-engineered cartilage to the subchondral bone and to the native cartilage remains a significant challenge in the clinical implementation of this type of grafting system [16]-[17].

The difficulty in the adhesion of two tissue types leads to one of the critical research interests in cartilage tissue engineering, which is the development of scaffolds or constructs capable of interfacing different tissue types. Several groups have reported on osteochondral grafts [18]-[21], and these grafts have demonstrated significant potential. The long-term goal of our research is to facilitate the integration of bone and cartilage by developing tissue graft systems that will lead to the reconstruction of the osteochondral interface. We believe that a functional interface can be formed between bone and cartilage by coculturing osteoblasts and chondrocytes on three-dimensional scaffolds that have been optimized for the growth and differentiation of both cell types.

The native osteochondral interface is composed of multiple tissue types, specifically cartilage, mineralized cartilage, and bone [22]. Chondrocytes and osteoblasts are the predominant cell types found at the osteochondral interface. Lacombe-Gleize et al. cocultured osteoblasts and chondrocytes using an insert system where, although these cells were not in contact with each other, they were grown in the same well [23]. Conditioning media from the single-cultured as well as cocultured wells were added to osteoblast and chondrocyte-only cultures. An increase in chondrocyte proliferation was measured during coculture and after conditioning with osteoblast media. These results suggest that osteoblasts may direct chondrocyte growth or differentiation through secreted factors. Weng et al. reported that when osteoblasts and chondrocytes are both preseeded on an osteochondral scaffold, in vivo healing may be enhanced [55]. Schaefer et al. reported on an osteochondral graft system consisting of two independently seeded scaffolds. One of the scaffolds was seeded with primary chondrocytes and the other with periosteal cells, and these constructs were later sutured together after an initial culture period. In this system, the

 0739-5175/03/$17.00©2003IEEE

The long-term goal is to facilitate the integration of bone and cartilage by developing tissue graft systems that will lead to the reconstruction of the osteochondral interface.

chondrocytes and osteoblasts only came into contact when the constructs were sutured together. It was observed that when the scaffolds were sutured together at earlier time points, there was improved integration between the two tissue types [18]. These results suggest that the sooner the chondrocytes and osteoblasts can interact with each other, the more likely that the tissue types will integrate effectively.

Osteoblast-chondrocyte interactions may play a significant role in the eventual integration of cartilage with bone. Takai et al. reported on the development of an osteochondral graft consisting of a chondrocyte-embedded agarose hydrogel combined with osteoblast-preseeded devitalized trabecular bone [21]. It was observed that chondrocytes migrated into the bony substrate containing osteoblasts, suggesting that their interaction may be important in modulating the interfacial zone. At this time, it is not yet well understood how osteoblasts and chondrocytes interact to form the osteochondral interface due to the lack of in vitro and in vivo models of osteoblast-chondrocyte interactions. In order to tissue engineer the osteochondral interface, cell-cell interactions during coculturing must first be examined. In a previous study, we developed a novel and direct coculture system of the two cell types by using a chondrocyte micromass and osteoblast monolayer model [24]. It was observed that the chondrocyte phenotype was maintained while osteoblast mineralization was diminished. In this model, osteoblasts are directly exposed to the chondrocytes, while they are more likely to interact through the extracellular matrix in vivo. Building upon this direct coculture model, the goal of the current study is to examine the response of chondrocytes to an extracellular matrix pre-elaborated by osteoblasts grown on a degradable, polymer-ceramic composite scaffold. This scaffold is a composite of poly-lactide-co-glycolide (PLAGA) and bioactive glass (BG). This microsphere-based, degradable scaffold exhibits an inter-connected porous network and it has been shown by Lu et al. [29] to support osteoblast proliferation and elaboration of a mineralized, type I collagen matrix by these cells.

It is likely that in vivo, instead of being exposed to each other directly, osteoblasts and chondrocytes interact through the preformed extracellular matrix. In this study, both matrix- and substrate-dependent effects will be investigated during osteoblast and chondrocyte coculture on 2-D and 3-D scaffolds. Specifically, we will examine cellular attachment and temporal morphological changes of chondrocytes when cocultured with osteoblasts. Our hypothesis is that the presence of the preformed extracellular matrix produced by the osteoblasts on the composite scaffold will modulate the attachment and growth of chondrocytes during coculture.

Materials and Methods

Polymer-Ceramic Composite

The composite was fabricated as both 2-D discs and 3-D porous scaffolds following the methods of Lu et al. [25]. Polylactide-co-glycolide 85:15 (PLAGA 85:15, American Cyanamide, Sunnyvale, California) and 45S5 bioactive glass (BG, 10 μm, MO-SCI Corporation, Rolla, Maryland) were used to fabricate the 2-D composite (PLAGA-BG) discs, and PLAGA 50:50 combined with BG were used to fabricate the 3-D scaffolds. Briefly, PLAGA 85:15 granules were dissolved in methylene chloride (EM Science, Gibbstown, New Jersey), and BG particles were added to achieve a 25% mixture. The solution was then slowly poured into a Teflon® mold and the solvent was allowed to evaporate at −20 °C. The resulting polymer film was bored into 10-mm diameter discs.

To form the PLAGA-BG composite microspheres, PLAGA 50:50 granules were first dissolved in methylene chloride and BG granules were added to achieve a 25% mixture. The mixture was then poured into a 1% polyvinyl alcohol (Sigma Chemicals, St. Louis) solution, the suspension was stirred, and the spheres were allowed to harden. The resulting microspheres were washed, vacuum filtered, and dried. The microspheres were then sifted into different sizes (Retsch AS200, Haan, Germany). The 3-D scaffold constructs (7.5 mm in diameter and 3 mm in height) were formed by sintering the microspheres (300–350 μm) at 70 °C for 20 hours.

Cells and Cell Culture

Bovine articular chondrocytes were isolated using an enzymatic digestion method previously described by Mauck et al. [14]. Briefly, articular cartilage specimens were harvested from the carpo-metacarpal joints of freshly slaughtered calves (2-6 month) obtained from a local abattoir. The cartilage specimens were then rinsed with Dulbecco's Modified Essential Medium (DMEM) supplemented with 2% antibiotics (both from Mediatech, Herdon, Virginia). The chondrocytes were isolated from the cartilage specimen by serial digestions with protease (PRONASE®, EMD Biosciences, San Diego, California) and collagenase (Sigma Chemicals, St. Louis, Missouri). The primary bovine osteoblast cultures were established from explant cultures of trabecular bone fragments. Both the cartilage and bone fragments were harvested from the same animal. All cells were cultured in fully supplemented DMEM containing 10% fetal bovine serum, 1% non-essential amino acid, and 1% penicillin streptomycin (all from Mediatech, Herdon, Virginia). The cultures were maintained at 37 °C in a 5% CO_2 incubator.

Osteoblast-Chondrocyte Coculture

Osteoblast-chondrocyte coculture was examined on three different substrates: PLAGA-BG 3-D constructs, PLAGA-BG 2-D thin films, and tissue-culture-treated plastic coverslips (Thermanox™, Fisher Scientific, Fair Lawn, New Jersey). The coverslips had a surface area of 176.7 mm^2, and the composite discs had a surface area of 39.2 mm^2. All substrates were UV sterilized and washed in ethanol prior to seeding. All three substrates had preformed osteoblast matrix, where bovine osteoblasts were first seeded onto the substrate at a density of $2.0x10^5$ cells per scaffold and cultured in fully supplemented media for two days. Chondrocytes at a density of $4.0x10^5$ cells per scaffold were then seeded onto the 3-D scaffold with the pre-elaborated matrix. Scaffolds seeded with only osteoblasts or chondrocytes at the same densities served as controls. Similar to the 3-D scaffold, the 2-D disc was seeded with $1.5x10^5$ osteoblasts and twice as many chondrocytes ($3.0x10^5$). For the 2-D controls, the seeding density for both cell types was approximately 100,000 cells per coverslip.

Both short-term and long-term coculture experiments were conducted. The 3-D scaffolds were analyzed at 0.5, 3, and 8 hours, as well as at 1, 3, and 7 days, and the 2-D scaffolds were analyzed at 0.5, 3, 6, and 24 hours. In addition, to differentiate the two cell types, chondrocytes on the 2-D and 3-D substrates were prelabeled with Chloromethylbenzamido (CellTracker™ CM-DiI, Molecular Probes, Eugene, Oregon) before seeding. The labeling was performed following the manufacturer's suggested protocol.

Characterization of Cocultured Composite Substrates

Cell attachment and growth morphology was monitored using scanning electron microscopy (SEM, JEOL-5600LV and LEO 1455) at designated time points. The incident electron energy varied from 2 kV to 7 kV depending on the instrument. The samples were first fixed in Karnovsky fixative followed by serial dehydration in ethanol prior to imaging. In addition, cell viability and growth morphology were also assessed using the Calcein-AM, Ethidium homodimer two-color fluorescence cell viability assay (LIVE/DEAD® Viability/ Cytotoxicity Assay Kit, Molecular Probes). The presence and location of the prelabeled chondrocytes were tracked using both a fluorescence microscope (Zeiss Axiovert 25C, Carl Zeiss, Germany) and a confocal microscope (Olympus IX70, Melville, New York).

Results

The objective of this study is to examine the interaction of osteoblasts and chondrocytes on 2-D and 3-D composite scaffolds. Positive live stain (green) demonstrated that the composite PLAGA-BG scaffold supported chondrocyte viability and growth when cultured alone or with osteoblasts. Extensive SEM analyses revealed that significant interactions occurred between osteoblasts and chondrocytes during coculture.

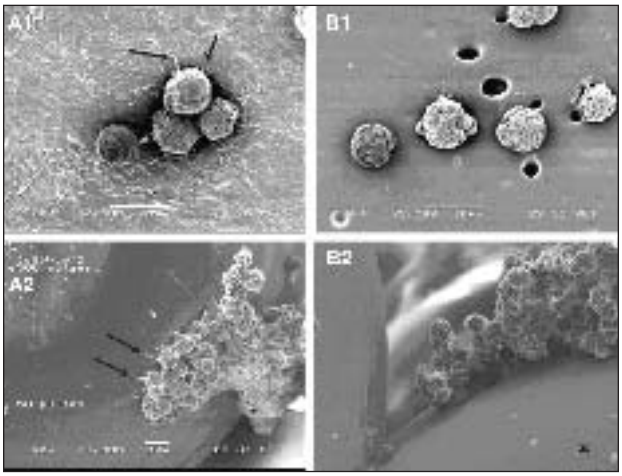

Fig. 1. Chondrocytes cocultured with an osteoblast matrix formed cell-matrix adhesions on both 2-D and 3-D substrate surfaces after only 30 minutes. Note the cell-matrix adhesions formed by the chondrocytes (arrows) on osteoblast preseeded scaffolds (A), as well as the absence of adhesions on control scaffolds (B). (A1) 2-D composite disc preseeded with osteoblasts for two days (x2,500, 30-min chondrocyte culture). (B1). 2-D composite disc with no osteoblasts preseeded (x2,500, three-hour chondrocyte culture). (A2). 3-D composite scaffold preseeded with osteoblasts for two days (x1,000, 30 min chondrocyte culture). (B2) 3-D composite scaffold with no osteoblasts preseeded (x1,000, 30-min chondrocyte culture).

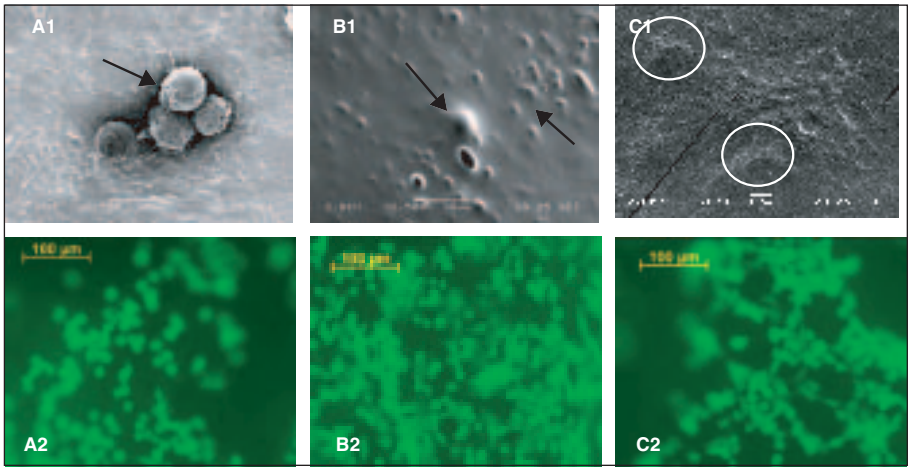

Fig. 2. Chondrocyte morphology changed gradually between eight hours and 24 hours in cultures with osteoblasts. Scanning electron micrographs (top) and fluorescent optical micrographs (bottom) of chondrocytes (indicated with arrows) seeded on osteoblast-preseeded composite 2-D discs are shown here. Note the change in chondrocyte morphology with respect to time from left to right: (A) 30 min, (B) 3 hours, and (C). 24 hours. Chondrocytes maintained a semi-spherical morphology during the first 24 hours of culture, and aggregates were also observed at this time point. Within the circles in C1 are aggregates of chondrocytes that can more clearly be seen in the optical micrograph of C2. (Magnifications: A1 at x2500; A2 at x20; B1 at x2500; B2 at x20; C1 at x1000; C2 at x20.)

Formation of Cell-Matrix Attachments

Differences in initial cellular attachment were observed between the cocultured scaffolds and the chondrocyte control scaffolds. As seen in Figure 1, the presence of the osteoblast-like cells or osteoblast-elaborated extracellular matrix induced the chondrocytes to form cell-matrix adhesion complexes on both 2-D (Figure 1 A1) and 3-D (Figure 1 A2) substrate surfaces after only 30 minutes of culture. In contrast, no comparable attachments were observed on the 2-D (Figure 1 B1) control discs after three hours of culture. Similarly, no cell- matrix adhesions were observed on 3-D (Figure 1 B2) chondrocyte controls without the preformed extracellular matrix after 30 minutes of culture.

Effects of the Preformed Extracellular Matrix on Chondrocyte Morphology

It was observed that chondrocyte morphology changed gradually between 8 hours and 24 hours in cultures with (Figures 2 and 4) and without osteoblasts (Figures 3 and 4). From the onset of seeding to eight hours, the chondrocytes on all substrates assumed a spherical or semi-spherical morphology. Differences in cell morphology were evident between the cocultured scaffolds and the chondrocyte control after one day of culture on both 2-D (Figures 2 and 3) and 3-D substrates (Figure 4). In the absence of the matrix, the chondrocytes spread and flattened on both the 2-D discs (Figure 3 C) and the 3-D microsphere-based scaffolds (Figure 4 B2).

In addition, the presence of the osteoblast-like cells and osteoblast-produced matrix was found to delay the spreading of chondrocytes in both 2-D and 3-D scaffolds. This delay was more pronounced in the 3-D scaffolds (Figure 4 B1) compared to the 2-D scaffolds (Figure 2 B1 and 2 C1). As shown in Figure 2 A1 and B1, semi-spherical chondrocytes can be seen on the surface of the cocultured discs. These observations were corroborated by the calcein-labeled images (Figure 2 A2, B2, and C2), which demonstrated the spherical morphology of the chondrocytes up to 24 hours of culture. However, on the chondrocyte controls shown in Figures 3 and 4, the chondrocytes have lost their phenotypic morphology and have conformed to the surface of the substrate in both 2-D and 3-D surfaces. In long-term culture, not surprisingly, it was found that more extensive cell growth and matrix elaboration were observed on the cocultured scaffolds (Figure 4 C1) as compared to the chondrocyte controls (Figure 4 C2) or the osteoblast controls.

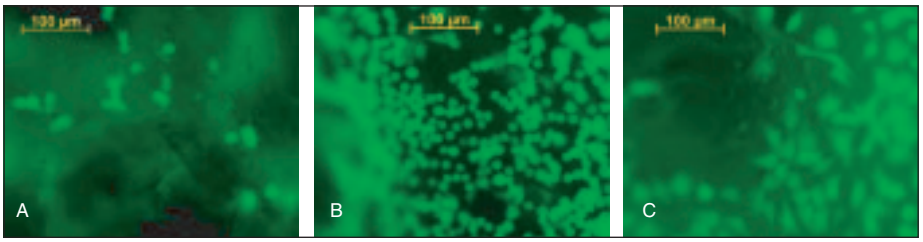

Fig. 3. Chondrocyte morphology changed gradually between eight hours and 24 hours when cultured on control 2-D scaffolds. Note the change in chondrocyte morphology with respect to time, from left: (A) 30 minutes, (B) 3 hours, and (C) 24 hours. These images show live chondrocytes (bright) that have completely spread by 24 hours in the absence of osteoblast preformed matrix. (All images are at 20x magnification.)

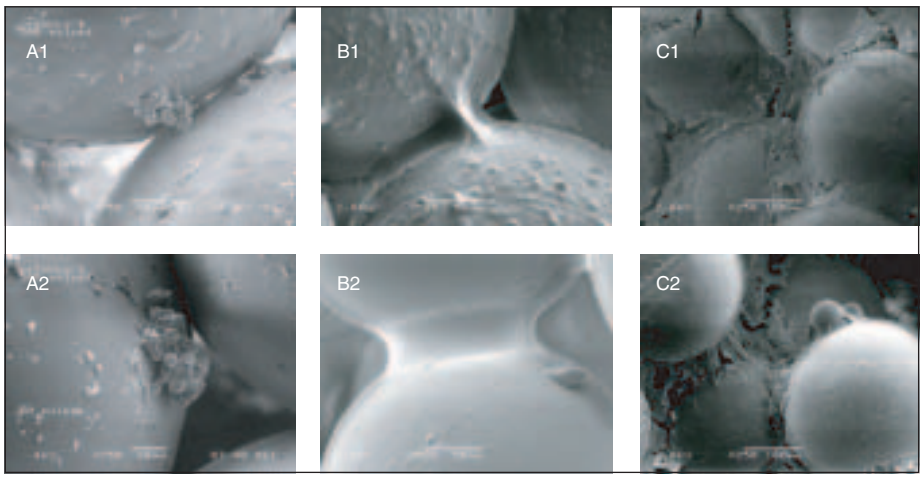

Fig. 4. In long-term culture, it was found that more extensive cell growth and matrix elaboration were observed on the cocultured scaffolds as compared to the chondrocyte control. Scanning electron micrographs of chondrocytes seeded on 3-D composite scaffolds in the presence (1) or absence (2) of osteoblast preformed matrix for (A) 8 hours, (B) 24 hours, (C) 7 days are shown here. Chondrocytes maintain a semi-spherical morphology on the preseeded scaffolds at 24 hours as seen in B1 but are almost completely spread at the same time on the control scaffold as seen in B2. Long-term cultures of osteoblasts-chondrocytes (C1) and chondrocyte alone (C2) revealed extensive matrix formation and coverage of the microspheres. (Magnifications: A1 at 750x; A2 at 750x; B1 at 500x; B2 at 500x; C1 at 250x; C2 at 250x).

Effect of Biomaterial Substrate on Chondrocyte Morphology

It was observed that the architecture or shape of the underlying substrate also had an effect on chondrocyte morphology, and this effect was found to be independent of the presence of a preformed extracellular matrix. Table 1 compares chondrocyte spreading on three different substrates (coverslips, 2-D composite disc, and 3-D composite scaffold) preseeded with osteoblasts, as well as on substrates without preformed extracellular matrix. The curvature of a surface is defined as $k = 1/r$, where k = curvature and r = radius, and the units of curvature are expressed in μm^{-1}. The coverslips have a flat surface and thus have a curvature of 0 μm^{-1}. Only

Both a matrix-dependent and substrate-architecture-related response in chondrocyte-morphology and spreading were observed when the two cell types were cocultured together.

microspheres from the 300-350 μm diameter size range were used to form the 3-D scaffold, thus the curvature of the microspheres ranged between 0.0057-0.0067 μm^{-1}. Chondrocytes range in size from 5-14 μm; therefore, only the curvature of an individual microsphere was considered to be relevant here. The curvature of the 2-D discs was estimated since the surface features of these discs were irregular. Their curvature is slightly greater than the flat coverslip control but significantly less than that of the microsphere. SEM micro- graphs confirmed the gross differences in curvature between the disc and the microsphere substrate.

In the presence of a preformed extracellular matrix, with increasing substrate curvature, it took longer for the chondrocytes to lose their phenotypic morphology and commence spreading. In the absence of the preformed extracellular matrix, the time to cell spreading was again found to be inversely dependent on substrate curvature. The chondrocytes seeded on flat cover slides commenced spreading by three hours, while these cells started to spread on the 2-D composite disc by six hours, and by eight hours on the 3-D composite scaffold (see Table 1).

Discussion

This is one of the first reported studies to examine the nature of osteoblast-chondrocyte interaction on a degradable scaffold in vitro. Based on our results, it was found that chondrocyte attachment and spreading are dependent on the presence of a preformed, osteoblast matrix. Indeed, chondrocytes are more likely to maintain their phenotypic morphology and take longer to spread in the presence of osteoblasts. In addition, construct geometry and chemistry may have an effect on chondrocyte response, as chondrocytes grown on 3-D scaffold took longer to spread compared to the two types of 2-D substrates tested.

It is evident that coculturing chondrocytes and osteoblasts on our PLAGA-BG composite scaffolds is a potential platform to elucidate the scaffold- or cell-dependent parameters that may ultimately dictate the formation of an osteochondral interface. Since the interaction of these two cell types has not been extensively examined, and since there are very few coculture models reported in the literature, the feasibility of this model system must first be validated. Although the PLAGA-BG composite scaffold has been shown to support the growth of osteoblasts [25], it was uncertain whether it could also support the growth of chondrocytes due to its mineral content. To promote integration with subchondral bone, Ca-P nodules were designed to form on the scaffold under physiological conditions. Thus, it was a potential concern that these nodules may affect the response of chondrocytes, since high concentrations of inorganic phosphate have been reported to induce chondrocyte apoptosis [26]. In this study, it was observed that chondrocytes maintained viability over the one-week culturing time on all substrates tested. An abundant production of extracellular matrix was observed during coculture on the 3-D scaffolds, with the matrix and cells filling the pores between the microspheres. More extensive matrix production and cell growth were seen in the coculture group compared to the chondrocyte and osteoblast controls. These results confirm the growth and development of chondrocytes on both the control and coculture scaffolds, and they establish the feasibility of coculturing chondrocytes and osteoblasts on the PLAGA-BG composite scaffolds.

A significant observation made in this study is that chondrocytes form cell-matrix adhesions when seeded on composite surfaces preseeded with osteoblasts. This was observed on both 2-D and 3-D surfaces, and these cell-matrix adhesions were notably absent on the substrates without the preformed osteoblast matrix. Cell-matrix adhesions are important complexes involved in cell migration, and they may affect downstream events such as cell signaling and gene expression [27]-[29]. The initial cell-matrix or cell-biomaterial interactions may directly influence cell spreading and matura-

Table 1. Summary of the effect of curvature on chondrocyte morphology over time. Note that with increase in curvature there is an increase in the time it takes for chondrocytes to spread.

Culture Conditions	Time for Cell Spreading on Substrate		
	Coverslip ($K = 0$)	2-D Composite Disc ($K \leq 0.0057\ \mu m^{-1}$)	3-D Composite Scaffold ($0.0057 \leq K \leq 0.0067\ \mu m^{-1}$)
Chondrocyte Control	$0.5 \leq x \leq 3$ hours	> 3 hours	$8 \leq x \leq 24$ hours
Co-Culture	≤ 0.5 hours	$0.5 \leq x \leq 3$ hours	≥ 24 hours

From the cellular perspective, the architecture of the underlying biomaterial substrate has a direct effect on chondrocyte adhesion and spreading.

tion on the surfaces [30], [31], [33], [34]. It has been reported that cell-matrix adhesions form as part of an integrin-mediated response to external environmental stimuli [35]. In our current study, the stimulus is the presence of the preformed osteoblast matrix. The presence of an extracellular matrix has been reported to affect cellular adhesion to biomaterials [36]-[41]. The lack of cell-matrix adhesion in the control group suggests that subsequent cellular response to the two substrates, in terms of cell differentiation, are likely to differ, and that cell adhesion to these substrates may be mediated by different types of integrins and adhesion complexes. El-Amin et al. compared integrin expression on 2-D polylactide (PLA) and PLAGA 50:50 discs, and reported significantly higher α_2, α_5, and β_1 integrin expressions on the PLAGA substrates compared to the PLA [34]. These results demonstrate that cell integrin expression is dependent on the chemistry of the biomaterial or substrate.

The extracellular matrix (ECM) formed by osteoblasts consists primarily of type I collagen and other ECM proteins such as fibronectin, laminin, vitronectin, osteopontin, and osteonectin [33], [42]-[44]. The two-day precultivation period allowed time for the preseeded osteoblasts to cover the composite surface with extracellular matrix. On PLAGA 2-D and 3-D substrates, human osteoblast-like cells have been shown to form a well-organized cytoskeleton [33] and produce a matrix that is rich in type I collagen [25] and ECM components listed above [33]. The observed differences in cell-matrix adhesion and spreading are likely due to the fact that the pre-elaboration of osteoblast extracellular matrix altered the surface chemistry and microstructure of the composite biomaterial surface. Therefore, the changes in surface chemistry and microstructure may lead to the development of the observed cell-matrix adhesions only in the cocultured group. It is to be expected that when compared to the preformed ECM, the native composite surface would elicit a different response from the chondrocytes. In addition, cell aggregates ranging from 10 μm during the initial culture times to around 100 μm after 24 hours were observed on the coculture groups. In contrast, well-spread, flat cellular sheets were seen on the control surfaces. The formation of these cell aggregates may be in part due to the early presence of cell-matrix adhesions, as these adhesions have been reported to mediate cell migration [45]-[48].

Both a matrix-dependent and substrate-architecture-related response in chondrocyte morphology and spreading were observed when the two cell types were cocultured together. First, the presence of the osteoblast preformed extracellular matrix on the surface of the composite scaffolds affected chondrocyte morphology over time. It is well established that when embedded in cartilage matrix or in suspension, chondrocytes maintain a phenotypic spherical morphology. These cells have also been shown to spread and assume a fibroblast-like morphology on 2-D substrates or during monolayer culture [49]-[53]. It is believed that chondrocyte morphology correlates with phenotypic expression. Specifically, spherical chondrocytes exhibit a differentiated phenotype expressing type II collagen and produce proteoglycans, while spread chondrocytes in a monolayer culture de-differentiate, expressing type I collagen and producing fewer proteoglycans [49]. In this study, it was observed that the presence of the preformed extracellular matrix delayed or prevented the spreading of the cultured chondrocytes. It is reasonable to suggest that the presence of the osteoblast extracellular matrix may provide an external environment beneficial to maintaining the phenotypic chondrocyte morphology. This may in part be due to the changes in surface chemistry, energy, and topography, which have been shown to direct cellular interaction with surfaces [43], [44], [54]. Recently, Chen et al. reported that when de-differentiated bovine articular chondrocytes were seeded on a porous PLAGA-type I collagen scaffold, these cells assumed a spherical morphology and reverted to the chondrocyte phenotype [50]. Specifically, these cultures reinitiated gene expression for type II collagen and aggrecan [50]. Since the osteoblast extracellular matrix consists primarily of type I collagen, we may be observing similar maintenance of chondrocyte phenotype due to the pre-elaboration of type I collagen matrix.

Another significant observation made in this study was that substrate architecture, i.e., 2-D versus 3-D, effected temporal morphological changes in chondrocytes. These effects were observed in both the coculture and control groups. Specifically, the time for spreading to occur was prolonged on the 3-D composite scaffolds compared to the 2-D composite discs. One potential explanation for the observed differences is the variation in curvature between the 2-D and 3-D substrates. The relatively higher curvature of the microspheres ($0.0057 \leq K \leq 0.0067\ \mu m^{-1}$) compared to the discs ($0 < K < 0.0057\ \mu m^{-1}$) may contribute to changes in chondrocyte spreading. Cukierman et al. evaluated human foreskin fibroblast adhesion to various 2-D and 3-D matrices [32]. Compared to the 2-D substrate or 3-D collagen gels, the cell-derived 3-D matrix was more effective by a factor greater than six in promoting cell adhesion. An enhanced cell migration rate was also reported in the 3-D matrix, accompanied by a complete triple co-localization of α_5 integrin, paxillin, and fibronectin similar to 3-D matrix adhesions observed in vivo. It is possible that in the current study, the observed differences in cell spreading on the 3-D compared to the 2-D matrix may be attributed to the expression of select surface integrins or

adhesion complexes that may lead to varied responses in long-term cultures.

In this study, an osteoblast matrix has been preformed on the scaffolds, thus minimizing the effects of the underlying surface by preventing direct contact between the chondrocytes and biomaterial. From the cellular perspective, the underlying biomaterial substrate may not have a direct effect on its adhesion. Another explanation for our observations may be that the preformed extracellular matrix on the 2-D substrate differs from the one formed on the 3-D scaffold and in turn elicits a distinct response from the chondrocytes. Indeed, the compositional variation between the two substrates may have resulted in differences in the preformed extracellular matrix elaborated by osteoblasts. These differences, coupled with the change from 2-D to 3-D structure, may have affected chondrocyte adhesion and spreading.

The current study focused on the initial interactions between chondrocytes and the preformed osteoblast matrix. Future studies will examine long term chondrocyte response, in particular chondrocyte growth and differentiation during coculture. It is critical to control the phenotypic expression of both chondrocytes and osteoblasts upon coculture in order to reproduce and maintain the multiple tissue zones found at the native osteochondral interface. In addition, the observed differences in cellular response to 2-D and 3-D substrates will be examined in depth in future studies.

Acknowledgment

This study was supported by the Whitaker Foundation Biomedical Engineer Research Grant (HHL). Scanning electron microscopy was performed at the Lamont-Doherty Earth Observatory (funded by the National Science Foundation, Lamont, and the American Museum of Natural History) and the Department of Applied Physics and Applied Mathematics (funded by the MRSEC program, NSF-DMR-9809687). Both facilities are located at Columbia University.

Jeffrey P. Spalazzi is a doctoral student in the Department of Biomedical Engineering at Columbia University. He received a B.S. degree in biomedical engineering with a concentration in materials science and engineering from Johns Hopkins University in May 2002. As a member of the Biomaterials and Interface Tissue Engineering Laboratory at Columbia, his current research interests include the design and application of novel scaffold systems for the tissue engineering of soft tissue-bone interfaces, as well as the mechanisms governing cell-cell interactions on a biomaterial substrate.

Kathie L. Dionisio received her B.S. degree in biomedical engineering and a minor in mechanical engineering from the Fu Foundation School of Engineering and Applied Science at Columbia University in 2003. While at Columbia, she worked as an undergraduate research assistant in the Biomaterials and Interface Tissue Engineering Laboratory. She is currently pursuing her doctoral degree with the Harvard-MIT Division of Health Sciences and Technology.

Jie Jiang is a Ph.D. candidate in the Department of Biomedical Engineering at Columbia University. He received his B.S. degree in biomedical engineering with minors in biology and chemistry from the University of California at San Diego in May 2001 and an M.S. degree in biomedical engineering at Columbia in May 2003. He joined the Biomaterials and Interface Tissue Engineering Laboratory at Columbia University in the spring of 2002, and his research focuses on examining cell to cell interactions and the development of novel coculturing models for cell types relevant in interface tissue engineering.

Helen H. Lu is an assistant professor of biomedical engineering in the Fu Foundation School of Engineering and Applied Science at Columbia University, and she is the director of the Biomaterials and Interface Tissue Engineering Laboratory. She received her B.S.E. and Ph.D. degrees in biomedical engineering from the University of Pennsylvania. Research in her laboratory focuses on developing functional solutions to the problems of soft tissue to bone integration. The goals of her research program are to tissue engineer novel biomaterial-based grafts that have the ability to direct cellular responses, and to elucidate the fundamental mechanisms that govern cell-biomaterials and cell-cell interactions.

Address for Correspondence: Helen H. Lu, Ph.D., Department of Biomedical Engineering, Columbia University, 351 Engineering Terrace Building, MC 8904, 1210 Amsterdam Avenue, New York, NY 10027 USA. E-mail: hl2052@columbia.edu.

References

[1] Center for Disease Control Statistics, 1999.

[2] Population Projections Program, Population Division U.S. Census Bureau (NP-T3-E), "Projections of the total resident population by 5-year age groups, and sex with special age categories: Middle series, 2016 to 2020," Jan. 13, 2000.

[3] T. Minas and S. Nehrer, "Current concepts in the treatment of articular cartilage defects," *Orthopedics,* vol. 20, pp. 525-538, 1997.

[4] J.E. Gilbert, "Current treatment options for the restoration of articular cartilage," *Amer. J. Knee Surg.,* vol. 11, pp. 42-46, 1998.

[5] F.S. Chen, S.R. Frenkel, and P.E. Di Cesare, "Repair of articular cartilage defects: Part II. Treatment options," *Amer. J. Orthop.,* vol. 28, pp. 88-96, 1999.

[6] F.S. Chen, S.R. Frenkel, and P.E. Di Cesare, "Chondrocyte transplantation and experimental treatment options for articular cartilage defects," *Amer. J. Orthop.,* vol. 26, pp. 396-406, 1997.

[7] S.D. Gillogly, M. Voight, and T. Blackburn, "Treatment of articular cartilage defects of the knee with autologous chondrocyte implantation," *J. Orthop. Sports Phys. Ther.,* vol. 28, pp. 241-251, 1998.

[8] J.A. Buckwalter, "Articular cartilage injuries," *Clin. Orthop.,* vol. 402, pp. 21-37, 2002.

[9] S.R. Frenkel and P.E. Di Cesare, "Degradation and repair of articular cartilage," *Front Biosci.,* vol. 4, pp. D671-D685, 1999.

[10] L.E. Freed, J.C. Marquis, A. Nohria, J. Emmanual, A.G. Mikos, and R. Langer, "Neocartilage formation in vitro and in vivo using cells cultured on synthetic biodegradable polymers," *J. Biomed. Mater. Res.,* vol. 27, pp. 11-23, 1993.

[11] K. Masuda, R.L. Sah, M.J. Hejna, and E.J. Thonar, "A novel two-step method for the formation of tissue-engineered cartilage by mature bovine chondrocytes: The alginate-recovered-chondrocyte (ARC) method," *J. Orthop. Res.,* vol. 21, pp. 139-148, 2003.

[12] L.A. Solchaga, J.E. Dennis, V.M. Goldberg, and A.I. Caplan, "Hyaluronic acid-based polymers as cell carriers for tissue-engineered repair of bone and cartilage," *J. Orthop. Res.,* vol. 17, pp. 205-213, 1999.

[13] A.I. Caplan, M. Elyaderani, Y. Mochizuki, S. Wakitani, and V.M. Goldberg, "Principles of cartilage repair and regeneration," *Clin. Orthop.,* vol. 342, pp. 254-269, 1997.

[14] R.L. Mauck, M.A. Soltz, C.C. Wang, D.D. Wong, P.H. Chao, W.B. Valhmu, C.T. Hung, and G.A. Ateshian, "Functional tissue engineering of articular cartilage through dynamic loading of chondrocyte-seeded agarose gels," *J. Biomech. Eng.,* vol. 122, pp. 252-260, 2000.

[15] S. Nehrer, H.A. Breinan, A. Ramappa, S. Shortkroff, G. Young, T. Minas, C.B. Sledge, I.V. Yannas, and M. Spector, "Canine chondrocytes seeded in type I and type II collagen implants investigated in vitro," *J. Biomed. Mater. Res.,* vol. 38, pp. 95-104, 1997.

[16] T. Ahsan and R.L. Sah, "Biomechanics of integrative cartilage repair," *Osteoarthritis and Cartilage,* vol. 7, pp. 29-40, 1999.

[17] E.B. Hunziker, "Articular cartilage repair: Are the intrinsic biological constraints undermining this process insuperable?" *Osteoarthritis and Cartilage,* vol. 7, pp. 15-28, 1999.

[18] D. Schaefer, I. Martin, P. Shastri, R.F. Padera, R. Langer, L.E. Freed, and G. Vunjak-Novakovic, "In vitro generation of osteochondral composites," *Biomaterials,* vol. 21, pp. 2599-2606, 2000.

[19] J. Gao, J.E. Dennis, L.A. Solchaga, V.M. Goldberg, and A.I. Caplan, "Repair of osteochondral defect with tissue-engineered two-phase composite material of injectable calcium phosphate and hyaluronan sponge," *Tissue Eng.,* vol. 8, pp. 827-837, 2002.

[20] J.K. Sherwood, S.L. Riley, R. Palazzolo, S.C. Brown, D.C. Monkhouse, M. Coates, L.G. Griffith, L.K. Landeen, and A. Ratcliffe, "A three-dimensional osteochondral composite scaffold for articular cartilage repair," *Biomaterials,* vol. 23, pp. 4739-4751, 2002.

[21] E. Takai, E.G. Lima, H.H. Lu, G.A. Ateshian, X.E. Guo, and C.T. Hung, "Natural trabecular bone as a mineralized substrate for osteochondral tissue engineered hydrogel constructs," *Trans. Orth. Res. Soc.,* vol. 49, paper 307, 2003.

[22] V.C. Mow and W.M. Lai, "Some surface characteristics of articular cartilage. I. A scanning electron microscopy study and a theoretical model for the dynamic interaction of synovial fluid and articular cartilage," *J. Biomechanics,* vol. 7, pp. 449-456, 1974.

[23] S. Lacombe-Gleize, M. Gregoire, S. Demignot, C. Hecquet, and M. Adolphe, "Implication of TGF beta 1 in co-culture of chondrocytes-osteoblasts," *In Vitro Cell Dev. Biol. Anim.,* vol. 31, pp. 649-652, 1995.

[24] J. Jiang, S.B. Nicoll, and H.H. Lu, "Effects of osteoblast and chondrocyte co-culture on chondrogenic and osteoblastic phenotype in vitro," *Trans. Orhtop. Res. Soc.,* vol. 49, poster 579, 2003.

[25] H.H. Lu, S.F. El Amin, K.D. Scott, and C.T. Laurencin, "Three-dimensional, bioactive, biodegradable, polymer-bioactive glass composite scaffolds with improved mechanical properties support collagen synthesis and mineralization of human osteoblast-like cells in vitro," *J. Biomed. Mater. Res.,* vol. 64A, pp. 465-474, 2003.

[26] K. Mansfield, R. Rajpurohit, and I.M. Shapiro, "Extracellular phosphate ions cause apoptosis of terminally differentiated epiphyseal chondrocytes," *J. Cell Physiol.,* vol. 179, pp. 276-286, 1999.

[27] R.L. Vinall, S.H. Lo, and A.H. Reddi, "Regulation of articular chondrocyte phenotype by bone morphogenetic protein 7, interleukin 1, and cellular context is dependent on the cytoskeleton," *Exp. Cell. Res.,* vol. 272, pp. 32-44, 2002.

[28] K. Burridge, K. Fath, T. Kelly, G. Nuckolls, and C. Turner, "Focal adhesions: Transmembrane junctions between the extracellular matrix and the cytoskeleton," *Annu. Rev. Cell Biol.,* vol. 4, pp. 487-525, 1988.

[29] B.M. Jockusch, P. Bubeck, K. Giehl, M. Kroemker, J. Moschner, M. Rothkegel, M. Rudiger, K. Schluter, G. Stanke, and J. Winkler, "The molecular architecture of focal adhesions," *Annu. Rev. Cell Dev. Biol.,* vol. 11, pp. 379-416, 1995.

[30] R.K. Sinha and R.S. Tuan, "Regulation of human osteoblast integrin expression by orthopedic implant materials," *Bone,* vol. 18, pp. 451-457, 1996.

[31] R.S. Tuan, "Cellular and molecular events during bone-implant interaction," *Scripta Metallurgica et Materialia,* vol. 31, pp. 971-976, 1994.

[32] E. Cukierman, R. Pankov, D.R. Stevens, and K.M. Yamada, "Taking cell-matrix adhesions to the third dimension," *Science,* vol. 294, pp. 1708-1712, 2001.

[33] S.F. El Amin, H.H. Lu, Y. Khan, J. Burems, J. Mitchell, R.S. Tuan, and C.T. Laurencin, "Extracellular matrix production by human osteoblasts cultured on biodegradable polymers applicable for tissue engineering," *Biomaterials,* vol. 24, pp. 1213-1221, 2003.

[34] S.F. El Amin, M. Attawia, H.H. Lu, A.K. Shah, R. Chang, N.J. Hickok, R.S. Tuan, and C.T. Laurencin, "Integrin expression by human osteoblasts cultured on degradable polymeric materials applicable for tissue engineered bone," *J. Orthopaedic Res.,* vol. 20, pp. 20-28, 2002.

[35] R.O. Hynes, "Integrins: Versatility, modulation, and signaling in cell adhesion," *Cell,* vol. 69, pp. 11-25, 1992.

[36] J.A. Schrier and P.P. DeLuca, "Recombinant human bone morphogenetic protein-2 binding and incorporation in PLGA microsphere delivery systems," *Pharm. Dev. Technol.,* vol. 4, pp. 611-621, 1999.

[37] W.G. Brodbeck, Y. Nakayama, T. Matsuda, E. Colton, N.P. Ziats, and J.M. Anderson, "Biomaterial surface chemistry dictates adherent monocyte/macrophage cytokine expression in vitro," *Cytokine,* vol. 18, pp. 311-319, 2002.

[38] E. Eisenbarth, P. Linez, V. Biehl, D. Velten, J. Breme, and H.F. Hildebrand, "Cell orientation and cytoskeleton organisation on ground titanium surfaces," *Biomolecular Eng.,* vol. 19, nos. 2-6, pp. 233-237, 2002.

[39] K. Webb, K. Caldwell, and P.A. Tresco, "Fibronectin immobilized by a novel surface treatment regulates fibroblast attachment and spreading," *Crit. Rev. Biomed. Eng.,* vol. 28, nos. 1-2, pp. 203-208, 2000.

[40] A.J. Garcia, M.D. Vega, and D. Boettiger, "Modulation of cell proliferation and differentiation through substrate-dependent changes in fibronectin conformation," *Molecular Biol. Cell,* vol. 10, no. 3, pp. 785-798, 1999.

[41] T.L. Seitz, K.D. Noonan, L.L. Hench, and N.E. Noonan, "Effect of fibronectin on the adhesion of an established cell line to a surface reactive biomaterial," *J. Biomed. Mater. Res.,* vol. 16, no. 3, pp. 195-207, 1982.

[42] J. Ivaska and J. Heino, "Adhesion receptors and cell invasion: Mechanisms of integrin-guided degradation of extracellular matrix," *Cell Mol. Life Sci.,* vol. 57, pp. 16-24, 2000.

[43] A.K. Shah, J. Lazatin, R.K. Sinha, T. Lennox, N.J. Hickok, and R.S. Tuan, "Mechanism of BMP-2 stimulated adhesion of osteoblastic cells to titanium alloy," *Biol. Cell,* vol. 91, pp. 131-142, 1999.

[44] K. Anselme, "Osteoblast adhesion on biomaterials," *Biomaterials,* vol. 21, pp. 667-681, 2000.

[45] E.S. Strobel, D. Mobest, S. von Kleist, M. Dangel, S. Ries, R. Mertelsmann, and R. Henschler, "Adhesion and migration are differentially regulated in hematopoietic progenitor cells by cytokines and extracellular matrix," *Blood,* vol. 90, no. 9, pp. 3524-3532, 1997.

[46] D.O. Freedman, S. Parker-Cook, M.C. Maia e Silva, C. Braga, and A. Maciel, "Very late antigen-4/vascular cell adhesion molecule-1 (VLA-4/VCAM-1) pathway is involved in the transendothelial migration of lymphocytes in bancroftian filariasis," *J. Immunology,* vol. 156, no. 8, pp. 2901-2908, 1996.

[47] S. Sankar, N. Mahooti-Brooks, G. Hu, and J.A. Madri, "Modulation of cell spreading and migration by pp125FAK phosphorylation," *Amer. J. Pathology,* vol. 147, no. 3, pp. 601-608, 1995.

[48] J.E. Hasson, D.H. Wiebe, J.B. Sharefkin, and W.M. Abbott, "Migration of adult human vascular endothelial cells: Effect of extracellular matrix proteins," *Surgery,* vol. 100, no. 2, pp. 384-391, 1986.

[49] P.D. Benya and J.D. Shaffer, "Dedifferentiated chondrocytes reexpress the differentiated collagen phenotype when cultured in agarose gels," *Cell,* vol. 30, pp. 215-224, 1982.

[50] G. Chen, T. Sato, T. Ushida, R. Hirochika, and T. Tateishi, "Redifferentiation of dedifferentiated bovine chondrocytes when cultured in vitro in a PLGA-collagen hybrid mesh," *FEBS Lett.,* vol. 542, pp. 95-99, 2003.

[51] L. Schuman, P. Buma, D. Versleyen, B. de Man, P.M. van der Kraan, W.B. van den Berg, and G.N. Homminga, "Chondrocyte behaviour within different types of collagen gel in vitro," *Biomaterials,* vol. 16, no. 10, pp. 809-814, 1995.

[52] S.S. Hughes, D.G. Hicks, R.J. O'Keefe, S.R. Hurwitz, I.D. Crabb, A.M. Krasinskas, L. Loveys, J.E. Puzas, and R.N. Rosier, "Shared phenotypic expression of osteoblasts and chondrocytes in fracture callus," *J. Bone and Mineral Res.,* vol. 10, no. 4, pp. 533-544, 1995.

[53] G.P. Bates, S.L. Schor, and M.E. Grant, "A comparison of the effects of different substrata on chondrocyte morphology and the synthesis of collagen types IX and X," *In Vitro Cellular and Developmental Biol.,* vol. 23, no. 5, pp. 374-380, 1987.

[54] M. Hott, B. Noel, D. Bernache-Assolant, C. Rey, and P.J. Marie, "Proliferation and differentiation of human trabecular osteoblastic cells on hydroxyapatite," *J. Biomed. Mater. Res.,* vol. 37, pp. 508-516, 1997.

[55] M. Weng, Y. Cao, C.A. Silva, M.P. Vacanti, and C.A. Vacanti, "Tissue-engineered composites of bone and cartilage for mandible condylar reconstruction," *J. Oral Maxillofac. Surg.,* vol. 59, pp. 185-190, 2001.