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Potential of Centrifugal Seeding Method in Improving Cells Distribution and Proliferation on Demineralized Cancellous Bone Scaffolds for Tissue-Engineered Meniscus

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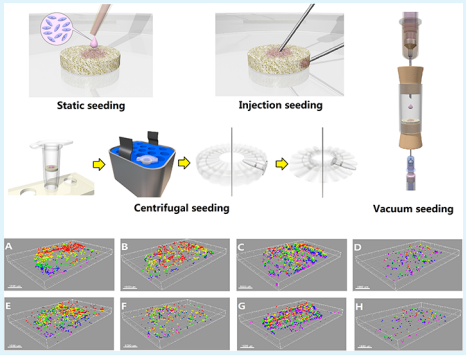
ABSTRACT: Tissue-engineered meniscus offers a possible solution to the regeneration and replacement problem of meniscectomy. However, the nonuniform distribution and declined proliferation of seeded cells on scaffolds hinder the application of tissue-engineered meniscus as a new generation of meniscus graft. This study systematically investigated the performances of different seeding techniques by using the demineralized cancellous bone (DCB) as the scaffold. Static seeding, injection seeding, centrifugal seeding, and vacuum seeding methods were used to seed the meniscal fibrochondrocytes (MFCs) and mesenchymal stem cells (MSCs) to scaffolds. Cell-binding efficiency, survival rate, distribution ability, and long-term proliferation effects on scaffolds were quantitatively evaluated. Cell adhesion was compared via cell-binding kinetics. Cell viability and morphology were assessed by using fluorescence staining. Combined with the reconstructed three-dimensional image, the distribution of seeded cells was investigated. The Cell Counting Kit-8 assay and DNA assay were employed to assess cell proliferation. Cell-binding kinetics and cell survival of the MFCs were improved via centrifugal seeding compared to injection or vacuum seeding methods. Seeded MFCs by centrifugation showed a more homogeneous distribution throughout the scaffold than cells seeded by other methods. Moreover, the penetration depth in the scaffold of seeded MFCs by centrifugation was 300−500 μm, much higher than the value of 100−300 μm by the surface static and injection seeding. The long-term proliferation of the MFCs in the centrifugal group was also significantly higher than that in the other groups. The results of the MSCs were similar to those of the MFCs. The centrifugal seeding method could significantly improve MFCs or MSCs distribution and proliferation on the DCB scaffolds, thus providing a simple, cost-effective, and effective cell-seeding protocol for tissue-engineered meniscus.



KEYWORDS: *tissue-engineered meniscus, seeding method, meniscal fibrochondrocytes, mesenchymal stem cells, demineralized cancellous bone*

INTRODUCTION

Meniscus plays a vital role in the biomechanical function and stabilization of the knee joint. Meniscal injuries are commonly associated with sports and age-related degeneration. Traditional surgery of the damaged meniscus, either a partial or total meniscectomy, or suture repair has been a challenge due to its poor healing potential.[1,2] Meniscus allograft transplantation came as an alternative but with the limitations of availability and possible disease transmission. Given the above situation, tissue-engineering (TE) strategies, which aim to regenerate menisci, offer a feasible solution.

Various strategies in tissue-engineered meniscus (TEM) have been adopted using different seed cells and natural or synthetic three-dimensional (3D) porous scaffolds, but problems in TEM are considerable mainly due to the lack of biomechanical or biocompatible performance.[3−5] For native meniscus, which is a thick and complex structured tissue, the initial hurdle in TEM is the inability to distribute the seeded cells throughout the scaffold. It has been found that there are not many cells in the inner parts of the scaffolds as anticipated, and cell distribution is not uniform throughout the scaffolds, where the center areas contain few cells.[6,7] Therefore, the premier allocation and following diffusion of cells on scaffolds are a prerequisite for successful TEM.

Seeding cells into 3D porous scaffolds is a passive process in which the cells disperse into the scaffolds by diffusion and attach through weak molecular level driven forces such as adhesion.[8] In contrast to simple monolayer culture, seeding cells into the 3D scaffolds is difficult due to their complicated structure. Moreover, highly efficient and uniform seeding could achieve reliable consistency in cell number, which is essential for functional tissue constructs.[9] In conclusion, how to make seeding cells efficient, uniform, and highly proliferative, especially in the inner parts of porous scaffolds, is an urgent need for TEM.

Seeding techniques have been classified into two categories: direct and indirect. Direct loading includes incubation of cells and scaffold in Petri dishes or injecting a small volume of cell suspension on the surface or into the center of the scaffold, which always results in unsuccessful synthesis.[10,11] Indirect

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seeding techniques were based on the attachment of cells suspended in a dynamic environment to improve their diffusion. The spinner flask technique, involving movement of the cells through the scaffold, has been demonstrated to provide better cell coverage and subsequent growth than static seeding.[12] However, previous studies have also shown contradictory results.[13,14] Above all, while the method for tissue engineering is extensive, the ideal technique has yet to be determined.

The present investigation focuses on the distribution and proliferation of meniscal fibrochondrocytes (MFCs) and mesenchymal stem cells (MSCs), which were classical seed cells for TEM, using four seeding techniques (static seeding, injection seeding, centrifugal seeding, and vacuum seeding). Demineralized cancellous bone (DCB) was chosen for the scaffold, which has been used for repair of articular cartilage in our previous studies but not seen in TEM.[15] Cell-binding efficiency, survival, distribution, and long-term proliferation on the scaffold were quantitatively evaluated using a combination of fluorescence cell staining and 3D image compilation. Our goal was to assess the parameters influencing the seeding and to offer insight into TEM.

EXPERIMENTAL SECTION

MFCs and MSCs Isolation and Culture. This study was approved by the animal ethics committee. MFCs and MSCs were isolated by using 3-month-old adult New Zealand White rabbits, average weight approximately 3 kg. The DCB scaffolds were prepared by using the extremities of large white pigs.

Both lateral and medial menisci were harvested from the knee joint in a sterile manner. After removal of adherent synovium from the meniscal rim, menisci were minced into 1 mm^3 (1 mm × 1 mm × 1 mm) pieces and plated onto tissue culture plastic. MFCs were released from these sections by incubation for 1 h at 37 °C in trypsin-EDTA (EDTA, ethylenediaminetetraacetic acid, 0.25%; Gibco BRL Co. Ltd., Gaithersburg, MD, USA) followed by 6 h at 37 °C in type I collagenase (0.2% w/v; Gibco BRL Co. Ltd.) in an expansion medium composed of low-glucose Dulbecco's modified Eagle's medium (LG-DMEM) containing 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 U/mL streptomycin.

About 3 mL of bone marrow aspirate was collected in a sterile manner from the femoral shafts of the rabbits under anesthesia. Each aspirate sample was diluted 1:1 with phosphate buffered saline (PBS), then loaded into an equivalent volume of Ficoll solution (1.084 g/mL; GE Healthcare, Uppsala, Sweden), and finally isolated using density gradient centrifugation at 2000 rpm for 20 min. The collected cells were resuspended in an α-minimum essential medium (αMEM) complete medium with 10% FBS, 100 U/ml penicillin, and 100 U/ml streptomycin and incubated at 37 °C with a 5% humidified CO_2 incubator. A rabbit MSCs chondrogenic differentiation medium (RBXMX-90041; Cyagen Biosciences Inc., Santa Clara, CA, USA) was used for chondrogenic culturing. The trilineage differentiation potential assay and immunophenotypic identification of MSCs were described in our previous study.[15]

The protocols were the same in the following: medium change was performed twice a week, and when cells reached confluence, first passage (P1) cells were detached with trypsin-EDTA and split 1:2 to produce second passage cells (P2) as previously described. MFCs and MSCs were used between the second passage (P2) and third passage (P3).

Scaffolds and Scanning Electron Microscopy. As previously described by Urist et al.,[16] cancellous bone at the metaphysis of extremities in large white pigs from other unrelated studies was frozen for 72 h with a temperature of −80 °C. The muscles and periosteums were discarded, and the bone was decalcified in 5% HCl for 72 h, with stirring every 12 h. Then the bone was defatted for 24 h in ether and 95% alcohol (1:1 v/v). After deproteinization for 4 h in 3% hydrogen peroxide, the bone was rinsed and soaked in sterile distilled water until the soak solution became neutral. Until now, the cancellated bone looked like a white sponge, which can be deformed and self-recovered to its original shape. The DCBs were sterilized by cobalt-60 for 24 h and reserved under the condition of −80 °C. The scaffolds measured 10 mm in diameter and 1 mm thick (Figure 1). The minimum

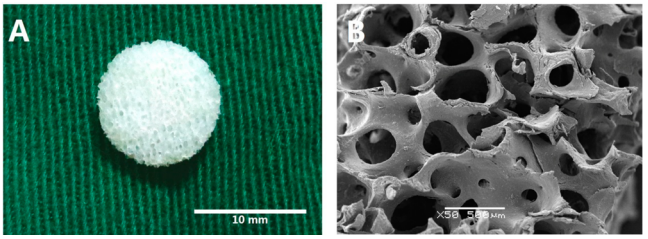

Figure 1. Macroscopic (A) and scanning electron microscopy (B) images of demineralized cancellous bone (DCB). (A) Scale bar represents 10 mm; (B) scanning electron microscopy of scaffold at ×50, and scale bar represents 500 μm.

porosity of these scaffolds was maintained above 80%, with the measured mean pore size of 268 μm on a scanning electron microscope (Figure 1). We chose the scaffolds with similar parameters to allow comparison of all seeding techniques.

Seeding Techniques. For seeding efficiency, we developed a novel protocol to avoid cells attaching to the Petri dishes using the caps of 15 mL centrifugal tubes. Briefly, scaffolds were placed into the sterile caps before seeding and culturing, and then the caps were implanted into 12-well plates to prevent cells attaching to Petri dishes. All the following seeding or culturing protocols were operated in this way (Figure 2).

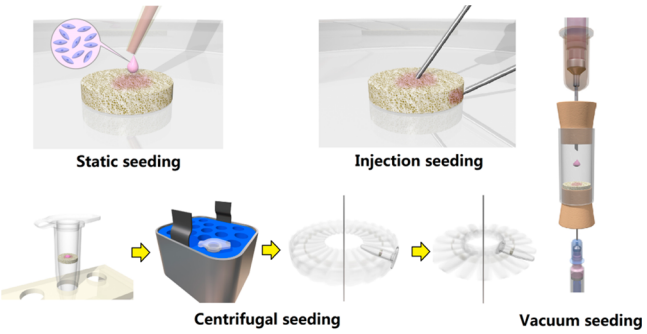

Figure 2. Schematic illustration of seeding techniques.

Static Surface Seeding. A 50 μL cell suspension containing 2.5 × 10^5 MFCs or MSCs was loaded onto the top of the scaffold and then incubated for 15 min in a 5% CO_2, 37 °C incubator for attachment. The uninfiltrated cell suspension (about half original volume) was collected and loaded on the bottom of the scaffold when the scaffold was subsequently overturned. After cell attachment, the seeded scaffolds were cultured in 1 mL of LG-DMEM or αMEM containing 10% FBS.

Injection Seeding. Cell suspension injection was performed by injecting a 50 μL concentrated cell solution (2.5 × 10^5 cells) into the scaffold using a 25-gauge needle, as described in other reports.[10] Briefly, the cells were injected into the top/bottom/side face of the scaffold with a 25-gauge needle syringe.

Centrifugal Seeding. The cells were cytocentrifuged onto scaffolds using an Eppendorf 5810 R benchtop centrifuge (radius = 17.6 cm). The scaffold was placed at the bottom of the 1.5 mL centrifugal tube and 50 μL concentrated cell solution (2.5 × 10^5 cells) was added. The tube was centrifuged at 500 rpm (relative centrifugal force = 49.2g) for 2 min and then turned over for centrifuging as one cycle. The process

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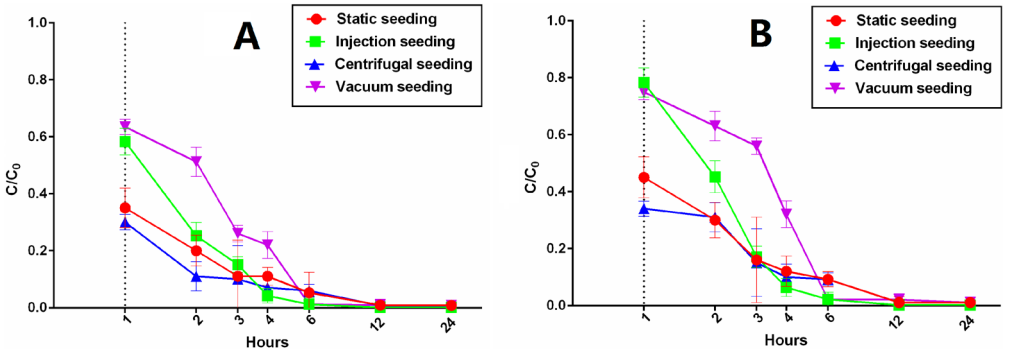

Figure 3. Cell-binding kinetics: (A) Seeded with mesenchymal stem cells (MSCs); (B) seeded with meniscal fibrochondrocytes (MFCs). The formula used in computing cell-binding kinetics is C/C_0, in which C is the concentration of the remaining live cells in the medium at each time point and C_0 is the concentration of the live cells at time 0. Data represent mean ± SEM.

was repeated three consecutive times. The scaffold was then transferred to a 12-well plate with a complete culture medium.

Vacuum Seeding. Seeding chambers were made from untreated 15 mL centrifugal tubes, rubber stoppers, and syringes. The tubes were cut to create cylinders measuring 5 cm in length with rubber stoppers that occluded each end. A 24-gauge needle was inserted into the bottom stopper, with the needle tip barely emerging from the inner surface of the stopper. A 21-gauge needle was implanted in the upper stopper. The scaffold was then placed on the surface of the bottom stopper in a sterile manner. A 1 mL negative pressure syringe was placed on the upper needle, and 5 mL negative pressure syringe was connected with the bottom needle to create a closed chamber. For seeding protocol, a 50 μL cell suspension (2.5×10^5 cells) was slowly injected into the upper surface of the scaffold and retrieved at the bottom of the syringe. The medium was reinjected into the upper surface of the scaffold through the upper needle. The cell suspension was passed three times through the chamber, and then the seeded scaffolds were returned and cultured in a 12-well plate.

Cell-Binding Kinetics Assessment in Scaffolds. Cell-binding kinetics was evaluated according to the previous protocol.[17] Briefly, after the scaffolds were transferred to their respective culture conditions with fresh complete medium (time 0), the cell concentration in the medium was evaluated (C_0). The concentration of cells and their viability were detected every 1 h for the first 6 h, and at 12 and 24 h, via Trypan blue exclusion. The formula used in computing cell-binding kinetics is C/C_0 in which C is the concentration of the remaining live cells in the medium at each time point and C_0 is the concentration of the live cells at time 0.

Cell Viability and Morphology Assessment in Scaffolds. To observe the effects of varying seeding methods, cell viability in the scaffolds was evaluated with a LIVE/DEAD Viability/Cytotoxicity Kit assay (Invitrogen, Carlsbad, CA, USA) after 24 h of culture. Leica TCS-SP8 confocal microscopy (Leica, Nussloch, Germany), was used for image analysis. After being washed three times in sterile PBS for 2 min, each scaffold was sectioned perpendicularly into quarter slices. Each slice containing the periphery and center part was immersed in 250 μL of PBS solution with 2 mM calcein AM and 4 mM ethidium homodimer-1 reagents before incubation for 1 h at room temperature. Excitation wavelength of 568 or 488 nm was used to detect the visualization of ethidium homodimer-1 (labeling dead cells = red fluorescence) or calcein AM (labeling live cells = green fluorescence). Nonseeding scaffolds were also stained as blank control to avoid scaffolds background effect.

The morphology of the cells in the scaffolds was also observed by using confocal microscopy. Briefly, after 1 week of culture, the scaffolds were washed with PBS, sectioned into slices, and fixed with 4% paraformaldehyde for 10 min. After utilization of 1% Triton X-100, the nuclei were stained using Hoechst33258 (1:800; Fanbo, Beijing, China). The cytoskeleton of the cells was stained by rhodamine phalloidin (100 nM; Cytoskeleton Inc., Denver, CO, USA) for 30 min at 37 °C.

Image Compilation and Data Analysis. The stained scaffolds were imaged using Leica TCS-SP8 confocal microscopy. When the focal plane was moved into the depth of the scaffolds surface, consecutive series of sections were acquired at intervals of 10 μm to a depth of approximately 500 μm. When the focus was horizontally moved to the next adjoining regions, the whole distribution of seed cells could be obtained. Finally, the 3D structure was obtained from different planes and the total volume of each region of interest was $6550 \times 5000 \times 500$ μm^3 (x, y, z axes, respectively). The microscope settings and optimal camera exposure settings were fixed and for all scaffold groups and time points. Image z-stacks were stitched together and converted to an 8-bit RGB stack, and then noise outliers were removed. With use of Image-pro Plus software (6.0; Media Cybernetics), the numbers of live cells and dead cells were quantified, and then the percentage of live cells was calculated.

To quantitatively assess the distribution of live cells, the volume data were used to create 3D renderings of the seeded scaffolds using Imaris software (7.4.2; Bitplane). This software provides many means to inspect the 3D arrangements of various cell types by measuring spots which were manually created and placed in a volume image. Briefly, the 3D structure was reconstructed from multispectral fluorescence data using Imaris. Then the spots-creation algorithm was utilized to locate live cells (green fluorescence) at intervals of 100 μm. The voxel intensities data were exchanged to spot coordinates data to quantitatively assess the 3D cell distributions by using the Imaris XT module which integrates MATLAB applications (R2014a; MathWorks).

Seeded Cell Proliferation Analysis. The metabolic activity of cells was quantified using a Cell Counting Kit-8 assay (CCK-8; Dojindo Laboratories, Kamimashiki Gun, Kumamoto, Japan). At each time point, the cell-seeded scaffolds were thoroughly washed in PBS and then submersed in 20 μL of CCK-8 working solution with 200 μL of fresh medium at 37 °C for 2 h. For the subtraction of medium background, four wells were filled with 20 μL of CCK-8 working solution and 200 μL of fresh medium only. The optical density was then observed at 450 nm using a plate reader. The cell content was normalized with each standard curve of seed cells.

The DNA content was measured using a fluorometric assay. For each group, these specimens were weighed and then digested in a preprepared papain solution (containing 0.5 M EDTA, 0.05 M cysteine-HCl, and 1 mg/mL papain enzyme) (Sigma) at 60 °C overnight. Aliquots of the sample digestion were stained at 37 °C for 20 min with 200 μL of Hoechst33258 working solution (2 $\mu g\ mL^{-1}$). The fluorescence intensities were then detected at 360 nm for excitation and 460 nm for emission. The DNA content was normalized with a standard curve of calf thymus DNA (Sigma).

Statistical Analysis. All data were expressed as mean ± standard deviation (SD)/standard error of mean (SEM). All experiments were repeated at least three times. The significance of the results was determined by using ANOVA test and repeated measure tests with Bonferroni correction. Data analysis was performed with SPSS

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statistical software (15.0; SPSS Inc.). $P < 0.05$ was considered statistically significant.

RESULTS

Cell-Binding Kinetics. The formula used in computing cell binding kinetics is C/C_0, in which C is the concentration of the remaining live cells in the medium at each time point and C_0 is the concentration of the live cells at time 0. For example, after seeding for 1 h, the ratio of the concentration of the remaining live cells in the medium (C) to that of the live cells at time 0 (C_0) was calculated as C/C_0 at this time point. The number of live cells in suspension in the four seeding techniques decreased exponentially from 1 to 6 h and remained constant within a C/C_0 range of 1−5%. This indicates that a majority of seeded cells attached scaffolds within 6 h with these methods (Figure 3). However, the initial ratio (1 h) in injection or vacuum seeding was significantly different compared with static surface or centrifugal seeding, indicating a higher cell-binding efficiency in centrifugal or static surface methods at the beginning. In addition, after 12 h, though the C/C_0 were similar in all groups, the dead cells in the medium around the scaffolds were increasing in the injection and vacuum groups (data not shown), which could also indicate that the cells attachment affected their survival.

Cell Viability Analysis. To determine the effects of the seeding method on cell viability, the seeded scaffolds were stained for live and dead cells. DCB has not marked with the fluorescent dye, which indicates there were no residual cells (data not show). The LIVE/DEAD assay showed that all four seeding methods could support cell activity (Figure 4). Scaffolds seeded with the centrifugal method sustained the greatest amount of live cells in all four groups. Moreover, the distribution of MSCs or MFCs was more even and extensive using this method (Figure 4C,G).

The four seeding methods had different effects on the survival of seeded cells (Figure 5). Scaffolds seeded using static surface and centrifugal seeding methods resulted in approximately 90% cell survival after 24 h. Rather unexpectedly, the injection or vacuum seeding method led to significant reduction in viability. Especially in seeding with MFCs, the cells death rate was more than 40% because the injection damaged the fine natural structure of the scaffold, compromised cell attachment, and then led to cell death.

Cell Morphology in Scaffolds. Cytoskeleton immunostaining images were consistent with cell viability assay. The number of seed cells in the centrifugal group was larger than the other groups (Figure 6C,G). Moreover, the distribution of cells in these groups was more even. Interestingly, seed cells in the injection group showed a tendency to aggregate as a high density of cells was present around the injection point (Figure 6B). In addition, after 1 week of in vitro chondrogenic culture, MSCs in all four groups were transformed into round chondrocyte-like cells, while MFCs typically showed fusiform fibroblast-like morphology.

3D Cell Organization and Distribution in Scaffolds. A dense layer of cells was found on the surface of the scaffold (100−300 μm) while a few were found in the center (300−500 μm) in the static surface or injection seeding group (Figure 7A,B,E,F). Consistent with the 3D images, we detected a higher percentage of cells in the top layer where the cells were seeded (Figure 8). The density diminished significantly in the next layers which indicated that these methods had poor cell penetration into the center of the scaffolds.

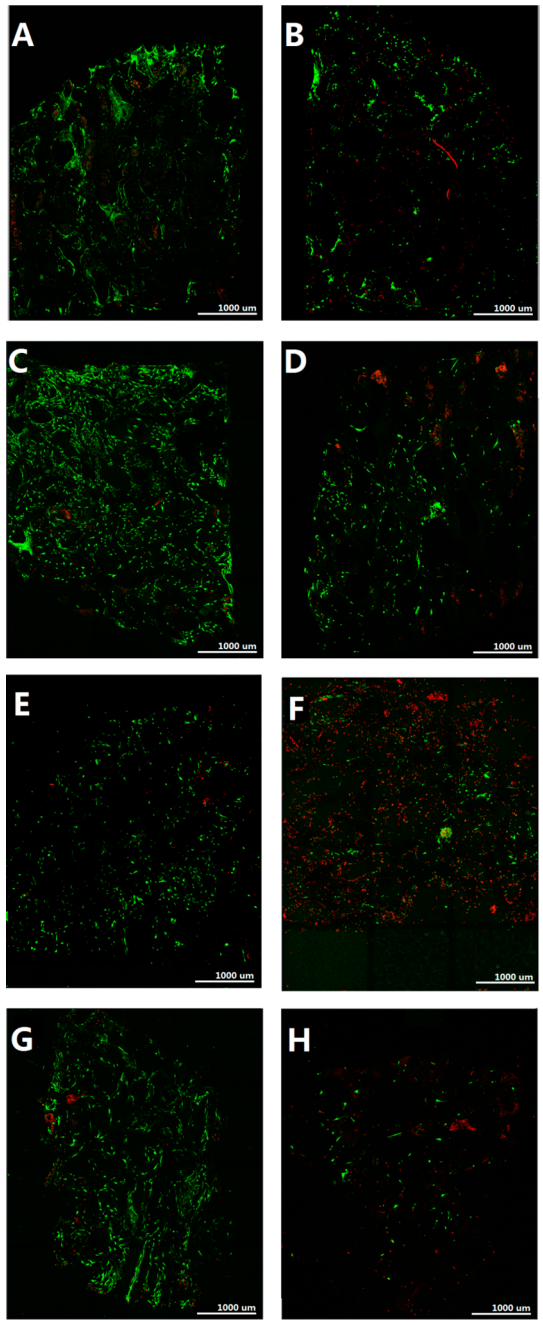

Figure 4. Representative images of live and dead cells on scaffolds using different cell seeding methods. (A)−(D) Seeded with MSCs; (E)−(H) seeded with MFCs. (A),(E) Surface static seeding; (B),(F) injection seeding; (C),(G) centrifugal seeding; (D),(H) vacuum seeding. According to LIVE/DEAD assay, all four seeding methods could support cell activity. Scaffolds seeded with the centrifugal method sustained the greatest amounts of live cells. Moreover, the distribution of MSCs (C) or MFCs (G) was more uniform.

In contrast to the static seeding methods investigated, the centrifuge group distributed cells evenly throughout the scaffold and all zones had an appreciable cell density (Figure 7C,G; $P > 0.05$). Although there was no significant difference among the layers, the deep layer (300−500 μm) showed more seed cells, which indicated that the centrifugal seeding method enhanced the cell infiltration (Figure 8). Compared to the centrifugal force, the vacuum method could also boost the cell penetration. However, this seeding method caused the cell distribution to be

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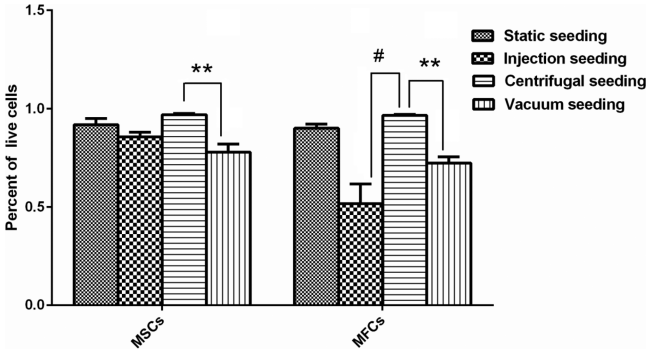

Figure 5. Effects of seeding methods on the survival of seeded cells. There was no significant difference in the percentage of live cells between surface static seeding and centrifugal seeding (n = 3, P > 0.05). The vacuum seeding method led to significant reduction in viability (n = 3, $^{**}P$ < 0.05 versus centrifugal seeding). Especially in seeding with MFCs, the cells death rate was more than 40% (n = 3, $^{\#}P$ < 0.05 versus centrifuge seeded scaffolds). Data represent mean ± SD.

uneven throughout the scaffold at a very low density (Figure 7D,H).

In Vitro Cell Proliferation and DNA Analysis. The proliferation and DNA assay results of seed cells cultured using different seeding methods are shown in Figure 9. In the CCK-8 test, either the MSCs or MFCs in all four groups presented an increasing tendency during the culture period of 1−5 days. Consistent with the above data, the total cell numbers in the centrifugal group were significantly higher than the other groups (Figure 9A,B; P < 0.05). Similar to the cell proliferation assay, assessment of DNA content showed that the centrifugal group had larger cell numbers compared with the other groups at 14 and 21 days (Figure 9C,D; P < 0.05). Either MSCs or MFCs exhibited proliferative potential in the centrifugal group or the static group, which indicated that seeding methods play an important role in enhancing the cell penetration and diffusion in the 3D microenvironment.

DISCUSSION

In the present study, we demonstrated that (a) cell-binding kinetics and cell survival were improved via the centrifugal seeding method compared to the injection or vacuum seeding methods; (b) seeded cells were homogeneously distributed throughout the scaffold by using centrifugation; (c) seeding technique influences not only seed cells infiltration and attachment onto scaffolds but also the cells proliferation.

Although numerous biomaterials with different properties have been used to provide a substitute for TEM, the desirability of having cells attach to the scaffold material is a common goal.[18] This study was designed to seed MFCs and MSCs on DCB scaffolds by applying four different seeding techniques, which provides insight into the design of future experiments in TEM. Previous studies have demonstrated that the importance of cell distribution and proliferative behavior on scaffolds provides the key to ensuring the ultimate functionality in tissue-engineered constructs.[19,20] Therefore, a major aim in the initiation of meniscal construct is the achievement of a uniform distribution of cells in all three dimensions, as well as large quantity both on the surface and within the inner part of the scaffold. However, seeded cells tended to be concentrated on the surface of scaffolds and rarely penetrated the center of the scaffold.[10] As meniscal constructs become a substitute after partial or total meniscectomy, the issue of larger scaffolds and

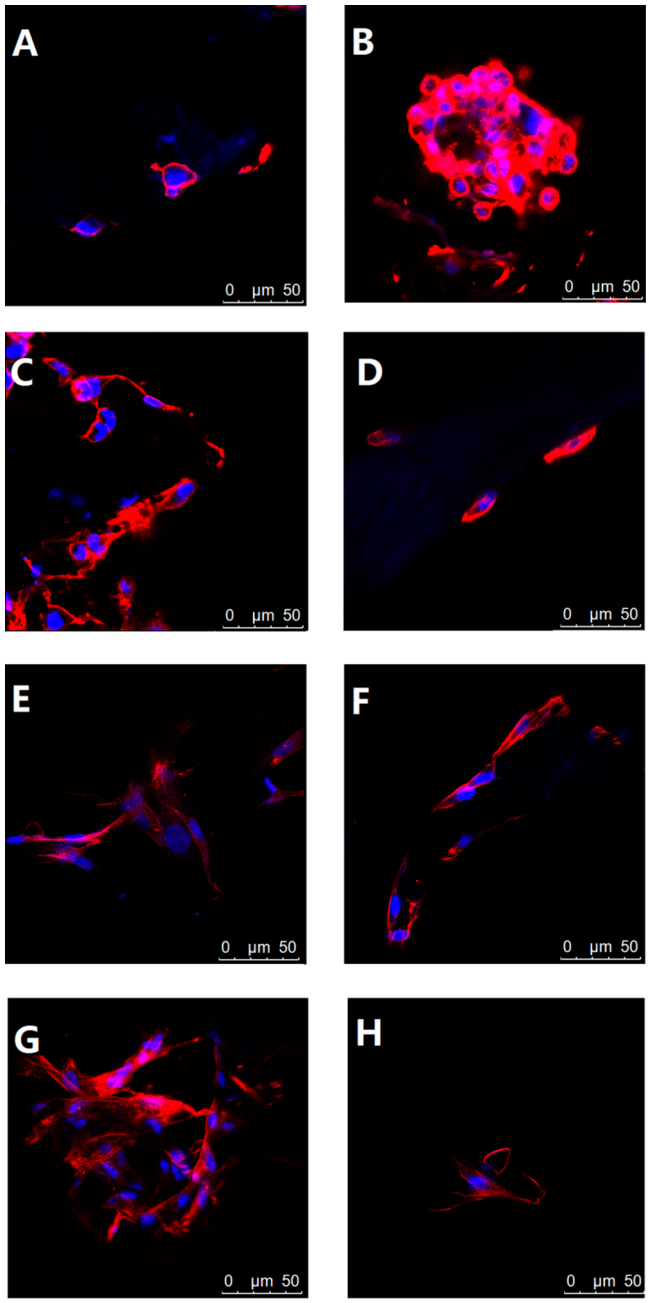

Figure 6. Representative images of the cells morphology on scaffolds using different cell seeding methods: (A)−(D) Seeded with MSCs; (E)−(H) seeded with MFCs. (A),(E) Surface static seeding; (B),(F) injection seeding; (C),(G) centrifugal seeding; (D),(H) vacuum seeding. (Red, Rhodamine phalloidin stained cytoskeleton; blue, Hoechst stained nuclei). Centrifugal group showed larger cell numbers and more uniform distribution of cells (Figure 6C,G).

corresponding seeding protocol will become more and more significant.

A large number of centrifugal seeding approaches have been applied to solve this problem with some degree of success.[6,7,21] Although all of these have shown an improved ability to deliver cells to the center of scaffolds, the choice of method would probably be dependent on other factors including sterility, cost, and simplicity. The centrifugal apparatus that we have used offered many advantages over the other centrifugal approaches described above: (a) The components are commercially

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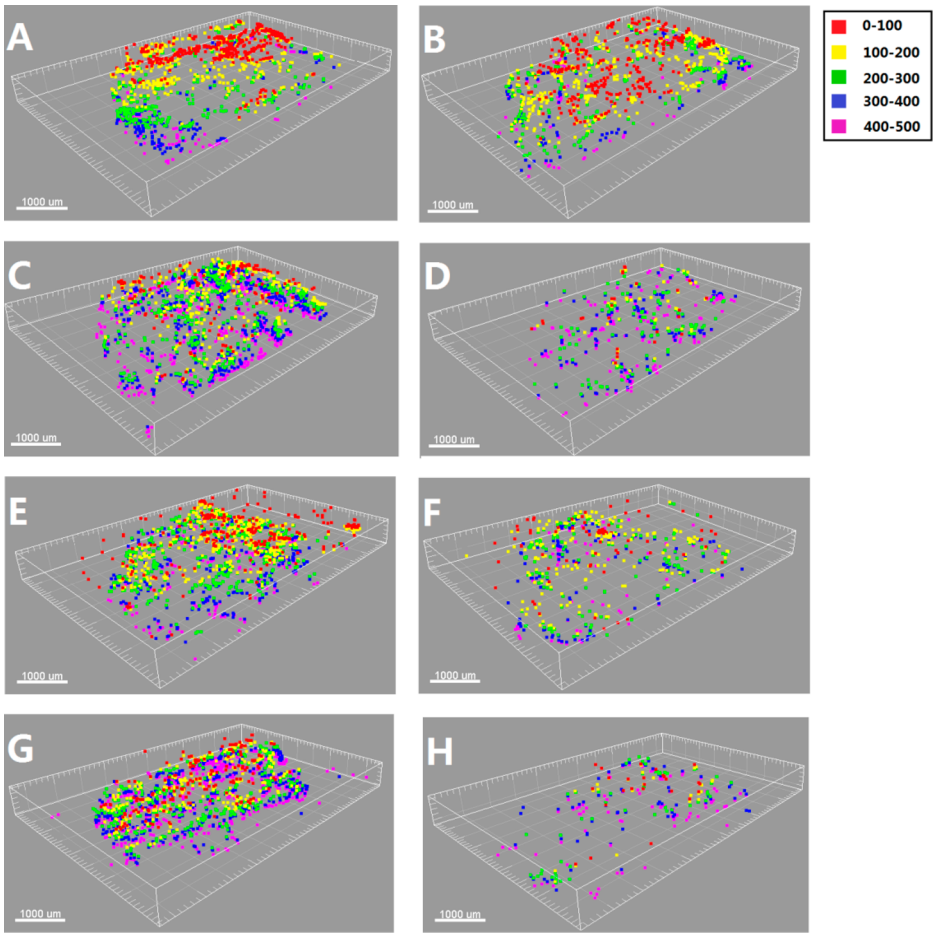

Figure 7. Representative 3D images of the cells distribution in scaffolds using different cell seeding methods. (A)−(D) Seeded with MSCs; (E)−(H) seeded with MFCs. (A),(E) Surface static seeding; (B),(F) injection seeding; (C),(G) centrifugal seeding; (D),(H) vacuum seeding. (Red, 0−100 μm; yellow, 100−200 μm; green, 200−300 μm; blue, 300−400 μm; purple, 400−500 μm).

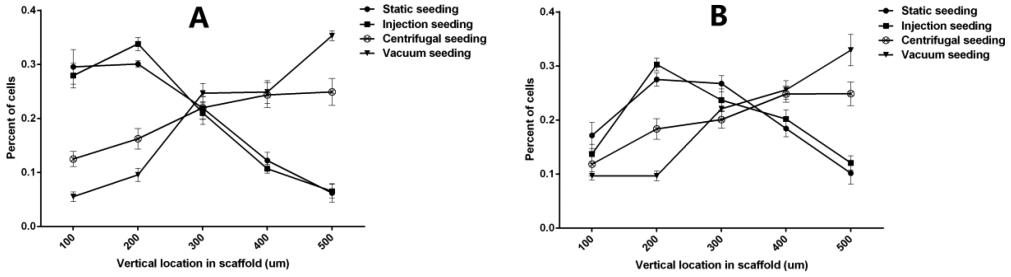

Figure 8. Analysis of the cells distribution in scaffolds using different cell seeding methods. (A) Seeded with mesenchymal stem cells (MSCs); (B) seeded with meniscal fibrochondrocytes (MFCs). In the static surface or injection seeding group, more cells were found on the surface of the scaffold (A, 0−300 μm; B, 100−300 μm) and a few in the center (300−500 μm); in the centrifuge group, there was no significant difference in each layer (n = 3, P > 0.05), and the deep layer (300−500 μm) showed more seed cells. Data represent mean ± SD.

available and constructed to a high standard. (b) There are plenty of centrifugal tubes in a variety of sizes fit for different scaffolds. This enables easy implantation and removal of scaffolds before and after cell seeding in a sterile manner. In addition, the ease of assembly and disassembly helps to maintain sterility of the construct. (c) The application of a centrifuge to seed MFCs and MSCs on DCB scaffolds provides another method in solving the seeding problem in TEM.

It has been indicated that centrifugation caused a temporary and reversible change in gene expression[22] and cell proliferation. Therefore, we analyzed cell development and DNA content for a long time and lasting effect has been demonstrated. In addition, the optimization of rotational speed and duration was another controversial issue. The rotational speed of 49.2g was optimal for seeding MFCs and MSCs into a DCB scaffold, as a higher rotation speed could result in cell lysis based on the observation of a large amount of cell debris in supernatants (data not shown), which were similar to a pioneering study.[6] In contrast, a lower rotation speed could lead to decreased seeding efficiency.[9] Other pioneering studies showed that the centrifugal forces of 52.5g was effective for seeding human bladder smooth muscle cells into polyglycolic acid scaffold.[6] Therefore, we believe that the parameters of the centrifugal method should be thoroughly optimized for

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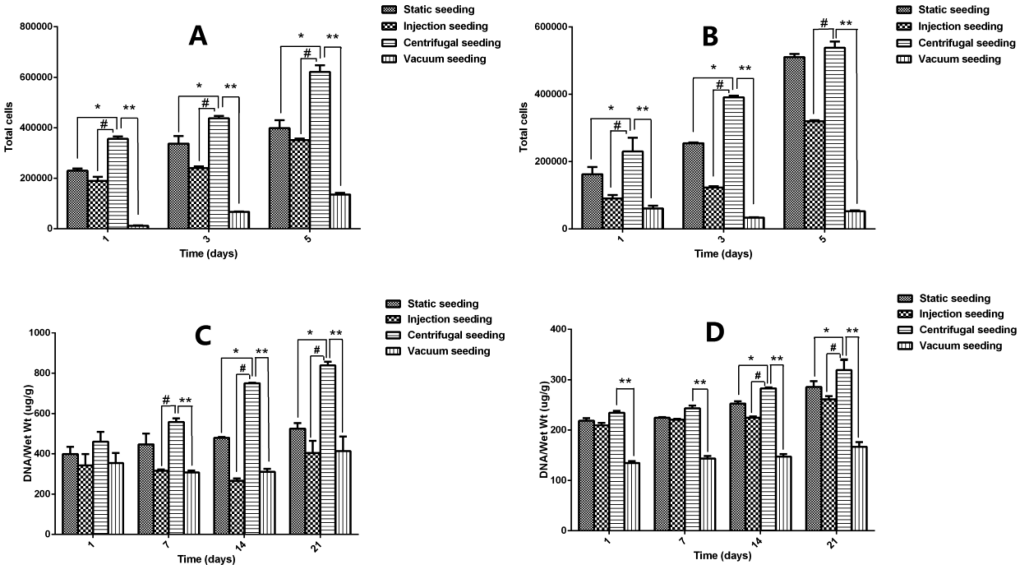

Figure 9. In vitro cell proliferation and DNA analysis: (A),(C) Seeded with MSCs; (B),(D) seeded with MFCs. (A),(B) Cell proliferation analysis: the total cell numbers in the centrifugal group were significantly higher than that in the other groups ($n = 3$, $^{*}P < 0.05$ versus static seeding; $n = 3$, $^{\#}P < 0.05$ versus injection seeding; $n = 3$, $^{**}P < 0.05$ versus vacuum seeding). (C),(D) DNA analysis: centrifugal group had larger cell numbers compared with the other groups at different time points ($n = 3$, $^{*}P < 0.05$ versus static seeding; $n = 3$, $^{\#}P < 0.05$ versus injection seeding; $n = 3$, $^{**}P < 0.05$ versus vacuum seeding). Data represent mean ± SD.

different kinds of cells and scaffolds, especially with distinct pore sizes, which would be systematically investigated in our future studies. Meanwhile, the scaffolds should also be taken into account in optimization of the centrifugal technique. In the case of previous work, the seeded scaffolds were centrifuged at 500 rpm for 2 min and then turned over for centrifugation as one cycle and then this process was performed twice. The seeded scaffolds were not damaged after cell distribution and proliferation analysis.

The experiments utilized DCB as scaffolds because they have been used in the field of TE to repair articular cartilage injury for their favorable biocompatibility, biodegradability, and nontoxicity.[15,23,24] However, there are still no reports about their application in TEM. The cancellous bone in large white pigs from other unrelated studies was used for xeno-donor because there was not enough cancellous bone in rabbit for preparation of large scaffolds. In fact, lymphocyte infiltration was rarely observed in meniscus transplantation of rabbits using treated xenogeneic (pig) meniscal tissue as previously published by us.[25] In addition, collagen, which is the main component of DCB, had low antigenicity and gamma irradiation and deep freezing can further reduce its immunogenicity.[26]

The experiments were conducted using two different cell types, MFCs and MSCs. MSCs are multipotent progenitor cells that had a high proliferation rate. As different cell proliferative activity directly affects migration rates under centrifugation, different cell types need testing to detect the optimal parameters for efficient seeding. This study demonstrated that the centrifugal seeding method could improve not only MFCs but also MSCs distribution and proliferation on scaffolds, which reveal its potential in TEM.

Though most studies quantified the cell behavior on tissue-engineered scaffolds using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide[27] or CCK-8 assay,[28] fewer approaches could provide information on the distribution, location, and infiltration of cells into scaffolds. By application of fluorescent dye to image cell−scaffold interactions, this method not only analyzed live or dead cells density by calculating each cell numbers but also detected cells distribution in each layer of the construct combined with the technique of reconstruction of scaffolds to 3D images. In addition, Figure 3 showed that scaffolds seeded with the centrifugal method sustained most amounts of live cells in all four groups which were discordant with our CCK-8 assay in 5 days (Figure 9B). This may be due to the fact that the seeded cells in the static surface group were mostly on the top and bottom layers of the scaffold, which were as far as CCK-8 solution could reach and react. CCK-8 solution would not be able to access the cells infiltrating deep in the scaffold in the centrifuge group.

The above seeding techniques have their unique advantages and drawbacks (Table 1). Static surface seeding has been the most commonly used in TE for its easy application.[10,28] However, the 3D images and the cells distribution data have demonstrated that seeded cells tend to locate on the surface and barely penetrate the scaffolds. Injection was proposed as a method of delivering cells into a specific area within the scaffold,[10] but the needle has to be inserted nearly half depth into the scaffold instead of staying on the surface of the scaffold. This may lead to damage of the fine natural structure of the

Table 1. Summary of Advantages and Drawbacks of Different Cell Seeding Methods

	advantages	drawbacks
static surface seeding	common and simple	seeding on scaffold surfaces; poor cells penetration
injection seeding	seeding in specific area of scaffolds	nonuniform; diminished cell attachment and increased cell death due to damage of the scaffolds
centrifugal seeding	homogeneous distribution; favors proliferation	parameters of centrifugal method affect the seeding efficiency
vacuum seeding	penetrated inner part of scaffolds	heterogeneous distribution and increased cell death

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scaffold, and the diminished attachment of MFCs onto the scaffold, resulting in increased incidents of cell death (Figure 4F). Although MSCs had a higher proliferation rate than MFCs due to the varying cell proliferative activities, the survival of seeded MSCs also decreased with the injection method (Figure 5). Our results suggest that the injection method may produce variable results, and therefore it could not be applied in a standardized way. The vacuum method meets the requirement of seeding cells penetrating the inner part of the scaffold as presented in previous research[29] and our data, but the vacuum seeding method showed a heterogeneous distribution of cells in large density because the initial distribution of cells by the vacuum method is nonuniform. Although the vacuum could deliver the cells into the inner part of the scaffold, the low pressure may also suck the cell solution out of the scaffold and lead to cell accumulation at the bottom. After 12 h, the unattached cells resulted in increased cell death because of their properties of anchorage-dependent growth.[30]

The major limitation of the present study is that we did not investigate the different material scaffolds with various mean pore sizes using the above seeding methods. Although we have used MFCs and MSCs as the seeding cells, we would investigate other kinds of cell lines in further research. In addition, for the limited visual depth of a confocal microscope at about 500 μm,[31] we ought to seek another imaging technique for thicker scaffolds. Moreover, further work is also required to evaluate the centrifuge method for TEM in vivo.

CONCLUSIONS

The centrifugal seeding method could improve cells distribution and proliferation on the DCB scaffolds. This information furthers our understanding of the MFCs and MSCs seeding protocol in our TEM research, which provides insight into the design of future TEM experiments.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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REFERENCES

(1) Paxton, E. S.; Stock, M. V.; Brophy, R. H. Meniscal Repair Versus Partial Meniscectomy: A Systematic Review Comparing Reoperation Rates and Clinical Outcomes. *Arthroscopy* **2011**, *27*, 1275−1288.

(2) Holzer, L. A.; Leithner, A.; Holzer, G. Surgery Versus Physical Therapy for Meniscal Tear and Osteoarthritis. *N. Engl. J. Med.* **2013**, *369*, 677.

(3) Buma, P.; Ramrattan, N. N.; van Tienen, T. G.; Veth, R. P. Tissue Engineering of the Meniscus. *Biomaterials* **2004**, *25*, 1523−1532.

(4) Cook, J. L.; Fox, D. B.; Malaviya, P.; Tomlinson, J. L.; Kuroki, K.; Cook, C. R.; Kladakis, S. Long-Term Outcome for Large Meniscal Defects Treated with Small Intestinal Submucosa in a Dog Model. *Am. J. Sports Med.* **2006**, *34*, 32−42.

(5) Kelly, B. T.; Robertson, W.; Potter, H. G.; Deng, X. H.; Turner, A. S.; Lyman, S.; Warren, R. F.; Rodeo, S. A. Hydrogel Meniscal Replacement in the Sheep Knee: Preliminary Evaluation of Chondroprotective Effects. *Am. J. Sports Med.* **2007**, *35*, 43−52.

(6) Godbey, W. T.; Hindy, S. B.; Sherman, M. E.; Atala, A. A Novel Use of Centrifugal Force for Cell Seeding into Porous Scaffolds. *Biomaterials* **2004**, *25*, 2799−2805.

(7) Roh, J. D.; Nelson, G. N.; Udelsman, B. V.; Brennan, M. P.; Lockhart, B.; Fong, P. M.; Lopez-Soler, R. I.; Saltzman, W. M.; Breuer, C. K. Centrifugal Seeding Increases Seeding Efficiency and Cellular Distribution of Bone Marrow Stromal Cells in Porous Biodegradable Scaffolds. *Tissue Eng.* **2007**, *13*, 2743−2749.

(8) Weinand, C.; Xu, J. W.; Peretti, G. M.; Bonassar, L. J.; Gill, T. J. Conditions Affecting Cell Seeding onto Three-Dimensional Scaffolds for Cellular-Based Biodegradable Implants. *J. Biomed. Mater. Res., Part B* **2009**, *91*, 80−87.

(9) Yang, T. H.; Miyoshi, H.; Ohshima, N. Novel Cell Immobilization Method Utilizing Centrifugal Force to Achieve High-Density Hepatocyte Culture in Porous Scaffold. *J. Biomed. Mater. Res.* **2001**, *55*, 379−386.

(10) Thevenot, P.; Nair, A.; Dey, J.; Yang, J.; Tang, L. Method to Analyze Three-Dimensional Cell Distribution and Infiltration in Degradable Scaffolds. *Tissue Eng., Part C* **2008**, *14*, 319−331.

(11) Manbachi, A.; Shrivastava, S.; Cioffi, M.; Chung, B. G.; Moretti, M.; Demirci, U.; Yliperttula, M.; Khademhosseini, A. Microcirculation within Grooved Substrates Regulates Cell Positioning and Cell Docking inside Microfluidic Channels. *Lab Chip* **2008**, *8*, 747−754.

(12) Chastain, S. R.; Kundu, A. K.; Dhar, S.; Calvert, J. W.; Putnam, A. J. Adhesion of Mesenchymal Stem Cells to Polymer Scaffolds Occurs Via Distinct ECM Ligands and Controls Their Osteogenic Differentiation. *J. Biomed. Mater. Res., Part A* **2006**, *78*, 73−85.

(13) Xiao, Y. L.; Riesle, J.; Van Blitterswijk, C. A. Static and Dynamic Fibroblast Seeding and Cultivation in Porous PEO/PBT Scaffolds. *J. Mater. Sci.: Mater. Med.* **1999**, *10*, 773−777.

(14) Kim, B. S.; Putnam, A. J.; Kulik, T. J.; Mooney, D. J. Optimizing Seeding and Culture Methods to Engineer Smooth Muscle Tissue on Biodegradable Polymer Matrices. *Biotechnol. Bioeng.* **1998**, *57*, 46−54.

(15) Fu, W. L.; Zhou, C. Y.; Yu, J. K. A New Source of Mesenchymal Stem Cells for Articular Cartilage Repair: Mscs Derived from Mobilized Peripheral Blood Share Similar Biological Characteristics in Vitro and Chondrogenesis in Vivo as Mscs from Bone Marrow in a Rabbit Model. *Am. J. Sports Med.* **2014**, *42*, 592−601.

(16) Urist, M. R.; Mikulski, A.; Boyd, S. D. A Chemosterilized Antigen-Extracted Autodigested Alloimplant for Bone Banks. *Arch. Surg. (Chicago, IL, U. S.)* **1975**, *110*, 416−428.

(17) Vunjak-Novakovic, G.; Obradovic, B.; Martin, I.; Bursac, P. M.; Langer, R.; Freed, L. E. Dynamic Cell Seeding of Polymer Scaffolds for Cartilage Tissue Engineering. *Biotechnol. Prog.* **1998**, *14*, 193−202.

(18) Rongen, J. J.; van Tienen, T. G.; van Bochove, B.; Grijpma, D. W.; Buma, P. Biomaterials in Search of a Meniscus Substitute. *Biomaterials* **2014**, *35*, 3527−3540.

(19) Lutolf, M. P.; Hubbell, J. A. Synthetic Biomaterials as Instructive Extracellular Microenvironments for Morphogenesis in Tissue Engineering. *Nat. Biotechnol.* **2005**, *23*, 47−55.

(20) Baker, B. M.; Mauck, R. L. The Effect of Nanofiber Alignment on the Maturation of Engineered Meniscus Constructs. *Biomaterials* **2007**, *28*, 1967−1977.

(21) Ng, R.; Gurm, J. S.; Yang, S. T. Centrifugal Seeding of Mammalian Cells in Nonwoven Fibrous Matrices. *Biotechnol. Prog.* **2010**, *26*, 239−245.

DOI: 10.1021/acsami.5b03129
ACS Appl. Mater. Interfaces 2015, 7, 15294−15302

(22) Li, J.; Jiang, L.; Liao, G.; Chen, G.; Liu, Y.; Wang, J.; Zheng, Y.; Luo, S.; Zhao, Z. Centrifugal Forces within Usually-Used Magnitude Elicited a Transitory and Reversible Change in Proliferation and Gene Expression of Osteoblastic Cells Umr-106. *Mol. Biol. Rep.* **2009**, *36*, 299−305.
(23) Dai, L.; He, Z.; Zhang, X.; Hu, X.; Yuan, L.; Qiang, M.; Zhu, J.; Shao, Z.; Zhou, C.; Ao, Y. One-Step Repair for Cartilage Defects in a Rabbit Model: A Technique Combining the Perforated Decalcified Cortical-Cancellous Bone Matrix Scaffold with Microfracture. *Am. J. Sports Med.* **2014**, *42*, 583−591.
(24) Huang, H.; Zhang, X.; Hu, X.; Shao, Z.; Zhu, J.; Dai, L.; Man, Z.; Yuan, L.; Chen, H.; Zhou, C.; Ao, Y. A Functional Biphasic Biomaterial Homing Mesenchymal Stem Cells for in Vivo Cartilage Regeneration. *Biomaterials* **2014**, *35*, 9608−9619.
(25) Jiang, D.; Zhao, L. H.; Tian, M.; Zhang, J. Y.; Yu, J. K. Meniscus Transplantation Using Treated Xenogeneic Meniscal Tissue: Viability and Chondroprotection Study in Rabbits. *Arthroscopy* **2012**, *28*, 1147−1159.
(26) Goble, E. M.; Kohn, D.; Verdonk, R.; Kane, S. M. Meniscal Substitutes–Human Experience. *Scand. J. Med. Sci. Sports* **1999**, *9*, 146−157.
(27) Gong, Y.; He, L.; Li, J.; Zhou, Q.; Ma, Z.; Gao, C.; Shen, J. Hydrogel-Filled Polylactide Porous Scaffolds for Cartilage Tissue Engineering. *J. Biomed. Mater. Res., Part B* **2007**, *82*, 192−204.
(28) Huang, H.; Zhang, X.; Hu, X.; Dai, L.; Zhu, J.; Man, Z.; Chen, H.; Zhou, C.; Ao, Y. Directing Chondrogenic Differentiation of Mesenchymal Stem Cells with a Solid-Supported Chitosan Thermogel for Cartilage Tissue Engineering. *Biomed. Mater.* **2014**, *9*, 035008.
(29) Dai, W.; Dong, J.; Chen, G.; Uemura, T. Application of Low-Pressure Cell Seeding System in Tissue Engineering. *BioSci. Trends* **2009**, *3*, 216−219.
(30) Jung, S.; Panchalingam, K. M.; Rosenberg, L.; Behie, L. A. Ex Vivo Expansion of Human Mesenchymal Stem Cells in Defined Serum-Free Media. *Stem Cells Int.* **2012**, *2012*, 123030.
(31) Doroski, D.; Tittmann, B.; Miyasaka, C. Study of Biomedical Specimens Using Scanning Acoustic Microscopy. In *Acoustical Imaging*; Andre, M. P., Ed.; Springer: Amsterdam, The Netherlands, 2007; Vol. *28*, pp 13−20.

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ACS Appl. Mater. Interfaces 2015, 7, 15294−15302