Towards Cellular Sieving: Exploring the Limits of Scaffold Accessibility for Cell Type Specific Invasion

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Coordinating the behavior of multiple cell types provides a challenge for the tissue engineer, since response to structure is highly cell type dependent. Here, human primary fibroblast invasion is compared with that of the MC3T3 and HT1080 cell lines to demonstrate proof-of-concept that controlled changes in 3D collagen scaffold architecture can modify the invasion response of one cell type, while keeping that of other cells constant. While complete invasion is seen for all three cell types where pore size and percolation diameter are sufficiently high, both primary fibroblasts and MC3T3 require a percolation diameter above roughly 40 µm to permit efficient invasion by day 3. Conversely, substantial HT1080 invasion is observed in all scaffold conditions. By day 7, MC3T3 and fibroblast invasion responses to structure are also distinct, with only MC3T3 inhibited by low percolation diameters. Collagen scaffolds can therefore act as cellular sieves, using structural control to promote the invasion of some cell types, while restricting the movement of others. As well as holding promise for the design of tissue engineering scaffolds for complex applications, this result is highly relevant for the development of in vitro systems for studying in vivo phenomena such as cancer cell invasion and metastasis.

As the methods available for tissue engineering scaffold fabrication become ever more sophisticated, it is increasingly important to understand how scaffold design may be used to promote the desired biological outcome. In particular, the elusive goal of regenerating complex hybrid tissues requires control of the behavior of multiple cell types.[1,2] An effective therapy for hybrid tissue regeneration should enable invasion of the appropriate progenitor cells to each region, while simultaneously preventing the ingrowth of undesirable cells.[1,4] To create a scaffold capable of directing the regeneration of such complex tissues, a detailed understanding of the cell type dependent link between scaffold structure and cell motility is therefore required. In this work, we describe how this cell type dependence may be exploited toward cellular sieving: scaffold structures that permit differing degrees of invasion according to cell type. By comparing the invasion behavior of cells from three different sources, in scaffolds of systematically increasing accessibility, we probe the structural requirements for cell movement. In this way, we demonstrate that highly occlusive structures may be used to allow differential invasion according to cell type, therefore physically separating cells according to their invasive capacity.

Freeze-dried collagen scaffolds are promising materials in the tissue engineering field, since their highly tunable structure and properties provide potential for the regeneration of a wide range of tissues.[5-9] In recent work, we have shown that measurement of pore size in these scaffolds is insufficient for predicting accessibility to cell invasion, and that characterizing the transport pathways through the pore space is also necessary.[10,11] These transport pathways can be characterized by measurement of percolation diameter, which describes the maximum object diameter that can move through a structure unimpeded. Percolation diameters below 40 µm have been shown to inhibit the invasion of primary fibroblasts, even in structures of constant pore size and collagen wall orientation.[11] In this study, we present simultaneous analysis of pore size and percolation diameter, meaning that, for the first time, we are able to compare their relative influences on the invasion potential of each cell type. The cells chosen for investigation reflect three distinct tissue engineering applications: human primary fibroblasts, for soft tissue regeneration;[12] the MC3T3 mouse osteoprogenitor cell line, for bone regeneration;[13] and the HT1080 cell line from human fibrosarcoma, reflecting the use of 3D models for studying tumor cell invasion.[14] As well as providing a tool for assaying the invasive capability of different cells in response to defined structures, this approach demonstrates how the informed design of tissue engineering scaffolds could be used to control the directed invasion of multiple cell types. The differing species, phenotype and origin of these cells is also relevant for demonstrating how the application of interest may determine the structural design criteria required for tissue engineering scaffolds, as well as the choice of cell line needed to provide an accurate biological assessment of such materials.

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To produce defined scaffolds with systematic changes in structure, collagen suspensions were freeze-dried using controlled temperature regimes. Controlling the freezing process allows the pore structure to be altered, by changing the morphology of the solidifying ice crystals, which later form the pore space. In this case, controlled freeze-drying methods were used to produce collagen scaffolds with successively increasing pore sizes and percolation diameters, measured by X-ray micro-computed tomography (micro-CT) analysis as shown in Figure 1. Four scaffolds were produced, covering a range of pore sizes between 66 and 99 μm, and percolation diameter ($d_c$) values between 44 and 118 μm. For simplicity, these scaffolds have been ranked from (1) to (4) in order of increasing accessibility. Independent changes in both pore size and $d_c$ were achieved, with an increase in $d_c$ from 44 to 60 μm between scaffolds (1) and (2) at constant pore size, and an increase in pore size from 67 to 82 μm between scaffolds (2) and (3) at constant $d_c$. Scaffold (4) contained the highest values of both pore size and $d_c$, and also highly anisotropic pore channels as apparent from the micro-CT images in Figure 1a, which also shows the corresponding pore wall structure in each scaffold imaged using bright field microscopy. These images reveal that the pore wall structure was relatively fibrous in the lower accessibility scaffolds, becoming smoother in the scaffolds with higher pore sizes and percolation diameters as displayed in Figure 1b. Quantitative analysis of each scaffold cross-section imaged in bright field also revealed that, in all cases, the collagen pore walls were oriented into the bulk of the scaffold, i.e., in the direction of cell invasion (see the Experimental Section).

In terms of cell response to these structures, first to be established was which, if any, of the structures permitted complete cell invasion and colonization of the entire scaffold. This was evaluated by cell seeding onto the surface of samples cut from each scaffold, and examining the cell distribution in cross-section after 3 days of culture, as shown in Figure 2. Near complete cell invasion from the seeded to the opposite surface occurred in scaffold (4), for all three cell types. This result indicates that when pore size and $d_c$ are sufficiently high, there is no substantial structural barrier to invasion of any of the cell types. However, as illustrated by the schematic in Figure 2a and shown qualitatively by the scaffold cross-sections in Figure 2b, the decreased pore size and $d_c$ values in scaffolds (1), (2), and (3) corresponded to varying degrees of invasion, in all cases considerably lower than that observed in scaffold (4). For the primary fibroblasts, it is clear that the extent of invasion was by far the lowest in scaffold (1), and increased in scaffolds (2) and (3), which show similar extents of invasion. This pattern was also seen in the MC3T3 cells, indicating that the invasion of both these cell types was primarily inhibited by the low $d_c$ structure of scaffold (1). However, for HT1080, all of scaffolds (1), (2), and (3) produced a comparable level of invasion. These results are also displayed quantitatively in Figure 2c, which shows the median position from the seeding surface measured for the invading fraction of the cell population, i.e., not including those trapped at the surface. The independent change in pore size between scaffolds (2) and (3) did not affect median cell position for any of the cell types. However, the independent change in $d_c$ from scaffold (1) to (2) produced a significant increase in median cell position for both the fibroblasts and MC3T3 cells ($p < 0.05$). This was not observed for the HT1080 cells, which showed an intermediate value of median cell position for all of scaffolds (1) to (3). Therefore, by day 3, the response of the HT1080 cell line is distinctive in that it is the only cell type that was insensitive to low values of $d_c$, with substantial (but not complete) invasion occurring even in highly occlusive structures.

With this result in mind, it remained to be seen whether the invasion response to structure differed between the fibroblasts and MC3T3 cells at longer time points. To examine this, the surfaces of each of scaffolds (1), (2), and (3) were seeded with both cell types as before, and kept in culture until day 7.
The cross-sectional images in Figure 3a display qualitative views of the cell distribution achieved in each scaffold. Although scaffolds (1) and (2) both showed evidence of fibroblast aggregation at the seeding surface, a limited number of fibroblasts were able to reach the opposite surface in each scaffold. Conversely, the maximum invasion distance achieved by MC3T3 was much lower, particularly in scaffold (1), in which the entire MC3T3 population seemed to be restricted to the upper half of the scaffold, close to the seeded surface. This cell type dependent behavior is also apparent from the numerical measurements of median cell position at day 7 in Figure 3b. In particular, whereas the median cell position at day 7 was significantly greater in scaffold (2) compared with scaffold (1) for MC3T3 ($p < 0.05$), there was no significant difference observed between the different scaffold conditions for the fibroblasts. Therefore, the inhibitory effect of low $d_c$ on cell invasion only persisted to day 7 for the MC3T3 cells.

It is also of note that both cell types were insensitive to the change in pore size between scaffolds (2) and (3), when measured in terms of median cell position. This indicates that the distance achieved by the invading fraction was constant. However, as revealed by the cross-sections in Figure 3a, the fraction of fibroblasts remaining at the surface appeared to have pore size dependence, with a high cell density at the surfaces of scaffolds (1) and (2), i.e., where pore size was below 80 µm. It is interesting to note that a pore size of 80 µm has been previously suggested as a criterion for efficient fibroblast movement through collagen sponges.[16] Although we have shown that a subset of the cell population may still be invasive in such scaffolds, it is important to realize that high cell density at the surface of a tissue engineering scaffold may limit the transport of nutrients and oxygen to cells within the scaffold interior, potentially limiting its ability to support tissue synthesis.
Taken together, these results indicate that the invasion response of the MC3T3 cell line was distinct from the invasion responses of both the primary fibroblasts and the HT1080 cell line, in that the low $d_c$ structure of scaffold (1) provided a structural barrier to invasion that persisted up to day 7. The schematic in Figure 3c summarizes these findings, demonstrating that whereas scaffold (4) permitted complete invasion of all cell types by day 3, a scaffold with a structure similar to scaffold (1) may be used to physically separate regions of cells according to their biological origin. Such a structure could act as a cellular sieve, allowing successive invasion of HT1080, followed by primary fibroblasts, while trapping the majority of the MC3T3 cells close to the scaffold surface.

Low $d_c$ values below a threshold value of $\approx 40 \mu m$ have previously been observed to inhibit the invasion of primary fibroblasts at day 3.[11] Here, we have shown that scaffolds with low $d_c$ values have a cell type dependent influence on cell invasion, with MC3T3 the most and HT1080 the least affected by such structures among the cell types tested here. It is possible that the dimensions of the migrating cells may have an influence on these results, with high cell elongations (over $100 \mu m$) observed only in the primary fibroblasts and MC3T3 cells, and lower typical sizes for the HT1080 cells, as previously reported.[17] However, cell size cannot account for the fact that the MC3T3 cells showed the greatest dependence on scaffold structure. Furthermore, the cell–matrix interaction involved in the migration of all three cell types, observed in preliminary experiments (data not shown) and as previously reported, indicate a more active mechanism of migration than passive flow through the scaffold.[18] The adhesion and migration behavior of HT1080 cells has, however, previously been shown to be unrepresentative of primary fibroblasts. Instead, these cells show properties unique to the transformed phenotype, such as contractility-dependent motility rather than a strong dependence on matrix adhesion.[18] The destruction of collagen extracellular matrix components by HT1080 has also been previously recorded to be high in comparison with primary fibroblasts.[14] One or both of these factors may explain the lack of sensitivity to low values of $d_c$, since barriers to invasion may be more easily overcome. Conversely, previous literature has shown MC3T3 movement to be highly influenced by occlusive structures, with poly(lactic acid)-based scaffolds requiring the presence of interconnected porosity to allow complete infiltration through the scaffold cross-section.[19] Here, we have been able to quantify the interconnecting networks required for complete invasion, by assessing the invasion response to $d_c$.

This cell type dependent response to occlusive structures of low pore size and $d_c$ provides proof-of-concept that careful architectural design may encourage the invasion of some cell types, while restricting the movement of others. In addition to the threshold $d_c$ value required for efficient invasion, it also...

Figure 3. Invasion analysis after 7 days’ culture, showing cell position as revealed by Phalloidin staining in cross-section (scale bar: 1 mm). a) Although substantial fibroblast invasion was seen in all scaffolds, both scaffolds (1) and (2) contained visible cell aggregation at the scaffold surface; an effect much less apparent for MC3T3. b) Quantitative measurement of median cell position at day 7 shows a significant increase in MC3T3 invasion as $d_c$ increased between scaffolds (1) and (2), but unlike at day 3, this was not observed for the fibroblasts. Data are shown as mean ± SEM ($n = 6$). * indicates $p < 0.05$ as evaluated using one-way ANOVA, along with the Games-Howell post hoc test. The schematic in (c) illustrates how the results of this study could be used to inform the design of structures to separate different cell types according to their invasive capacity.
appears that structures of much higher $d_c$ and pore size, as in scaffold (4), may promote a further increase in invasion, due to their high availability of continuous, mechanically stable pore channels. This is likely to contain a contribution from enhanced cell spreading and directionality in response to the 3D contact guidance cues, as has been observed in previous studies with porous collagen structures.[20,21] Therefore, where a scaffold structure allowing uninhibited invasion of multiple cell types is required, high pore size and $d_c$ are logical design criteria.

The main implications of this research are threefold. First, our work demonstrates the importance of careful biological assay design according to application, with choice of a suitable cell type that is representative of the expected behavior in the implant site. Both the MC3T3 and HT1080 cell lines are well-characterized lines, and are therefore frequently used in assessing biological response to naturally derived materials.[22]

However, our results show that in this case, neither the MC3T3 nor the HT1080 response to structure was completely representative of the primary fibroblast response, indicating the limitations of this cell line in evaluating the biological properties of collagen scaffolds. Second, our model has implications for in vitro models of cancer metastasis. Local collagen organization is known to influence the invasion of cancer cells, but the relative effects of collagen arrangement and cell phenotype are unclear.[23]

Our results provide a potential platform for independent assessment of the influence of collagen arrangement according to the invasive capacity of different cancer cells. Finally, our results are also highly relevant for the design of biological materials for the regeneration of complex tissues in vivo. For example, many applications require the simultaneous regeneration of hard and soft tissue. Existing approaches to this problem involve delivery of multiple cell types,[24] which is likely to be a complex and cost-inefficient process, or the fabrication of multilayer constructs.[25]

The results of this study, however, suggest the future possibility of directed cell invasion according to cell type by the use of a single scaffold structure. Clearly, more complex coculture experiments are required to explore this intriguing possibility, but this study has made an important first step by demonstrating, for the first time, the independent role of percolation diameter in determining cell-type dependent invasion.

In summary, using collagen scaffold structures with incremental changes in pore size and $d_c$, we have shown that the structural criteria for cell invasion have a strong dependence on cell type. Whereas open, anisotropic structures with high pore size and $d_c$ produced rapid invasion of all three cell types, occlusive structures with low pore size and $d_c$ produced differential rates of cell invasion according to the cell type under investigation. In particular, the initial invasion of both primary fibroblasts and MC3T3 cells were limited by $d_c$ values of roughly 40 $\mu$m and below, whereas HT1080 invasion was insensitive to such structural variations. Furthermore, whereas MC3T3 invasion became increasingly impeded by low $d_c$ values at later time points, primary fibroblasts showed a greater sensitivity to pore size, with surface aggregation observed where the scaffold pore size was below 80 $\mu$m. This approach has crucial implications for the use of architectural control to promote the invasion of some cell types while restricting the movement of others. Equally, these results demonstrate the importance of cell type in determining biological response to structure, emphasizing the need for biomaterial screening methods that are representative of the in vivo cellular environment.

**Experimental Section**

**Scaffold Fabrication**: Scaffolds were fabricated by freeze-drying suspensions of type I collagen from bovine Achilles tendon (Sigma-Aldrich, UK). Collagen was suspended at 1% w/v in either hydrochloric acid (HCl, Sigma-Aldrich), diluted to 0.001 M, or acetic acid (Alfa-Aesar, UK), diluted to 0.001 or 0.05 M. All suspensions were left to hydrate overnight, before homogenization and centrifugation to remove air bubbles. Freeze-drying took place in a VirTis AdVantage benchtop freeze-drier (Biopharma Process Systems, UK), with a final freezing temperature controlled between −20 and −35 °C. Suspensions were contained in moulds made of stainless steel (filling height 10 mm) or silicone (filling height 25 mm). The cooling rate of the freeze-dryer shelf was controlled using the system software, and was set at 0.5 °C min$^{-1}$, 1.2 °C min$^{-1}$, or a quench using a precooled shelf. Drying took place at 0 °C and 80 mTorr. The resulting scaffolds were cross-linked in a solution of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC, Sigma-Aldrich) and N-hydroxysuccinimide (NHS, Sigma-Aldrich) in 95% ethanol, at a molar ratio of 5:2:1 (EDC:NHS:COOH), before drying using a repeat of the initial freeze-drying protocol.

**Scaffold Selection and Morphological Characterization**: Scaffolds with systematic changes in pore structure were identified by rigorous characterization using X-ray micro-CT. A Skyscan 1172 system (Bruker, BE) was used to image scaffold samples at 25 kV and 138 $\mu$A, with 0.2° rotation steps and 1.5 s image acquisition time, averaged over 2 frames. Image pixel size was set at 3.1 $\mu$m. Shadow projections were processed into 3D datasets using the Skyscan reconstruction software NRecon, which were binarized using the Trainable Segmentation plugin in the ImageJ software distribution Fiji. Image noise was removed using the FIJ despeckle function in 2D, followed by a 2 $\times$ 2 $\times$ 2 median filter in 3D. For pore size analysis, 2D slices were sampled at 100 $\mu$m spacing in the scaffold seeding plane. The average pore size over all slices was calculated by ellipse fitting using Fiji, as previously described.[31] Briefly, outliers up to 2 pixels in size were removed, and the automated Watershed and Analyse Particles were used to identify the area of each ellipse, from which the diameter of the circle of equivalent area was calculated. The pore transport pathways for each scaffold were characterized for each scaffold by calculation of percolation diameter, $d_c$, as previously described.[32] Measurement of the accessible distance in the direction of cell invasion, L, for objects of varying diameter, $d_c$, allows calculation of $d_c$ according to the following relationship: $L = (d_c − d_c)$.[33] Measurements of L and d were obtained using the “ROI shrinkwrap” function in the Skyscan software CTAn. This procedure was carried out using cubic ROIs (regions of interest) of side length 2 mm. For qualitative examination of pore wall arrangement, dry scaffold sections were observed in bright field using a Zeiss Axio Observer Z1 inverted microscope. Quantitative assessment of pore wall arrangement took place after scaffold seeding and fixation, as described below.

**Cell Culture and Seeding**: Human primary periodontal ligament fibroblasts (PDLf, Lonza, CH) were cultured in high glucose Dulbecco's modified Eagle medium (LifeTechnologies, CH) with 5% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S). The murine osteoblast precursor cell line MC3T3 and the human fibrosarcoma cell line HT1080 were chosen for comparison with these primary fibroblasts. Both cell lines were obtained from ATCC (LGC Standards). MC3T3 cells were cultured in minimum essential medium alpha, with 10% FBS and 1% P/S, while HT1080 were cultured in Eagle's minimum essential medium supplemented with 10% FBS and 1% P/S. Trypsin-EDTA (0.25%) was used to detach each cell-type at subconfluence before seeding. Scaffold samples were cut to $10 \times 10 \times 1$ mm$^3$ (dry dimensions), and sterilized in 70% ethanol. Sterilized samples were washed twice with phosphate buffered saline (PBS), prewetted in the medium specified above, before carefully removing excess medium to prevent cells spilling off the surfaces of the wet scaffolds. Scaffolds were seeded in triplicate, for each
time point, onto the 10 × 10 mm² face, at a concentration of 64 000 cells in 50 µL medium per scaffold. Extra medium was added after 1 h at room temperature. Culture conditions were then maintained at 37 °C and 5% CO₂ in a humidified atmosphere, with medium changed three times per week. Scaffolds were harvested at day 3 (all cells) and day 7 after seeding (PDLF and MC3T3 only), by fixing with 10% formalin (Sigma-Aldrich) after an initial wash in PBS.

Invasion Analysis: Fixed scaffold samples were stained with Alexa Fluor 488 Phalloidin (MolecularProbes, CH) to reveal the actin cytoskeleton, before gelatin embedding and slicing to reveal the scaffold cross-section. After permeabilization for 10 min in 0.1% Triton X-100/PBS (Sigma-Aldrich), Phalloidin was used at 2.5/200 µL in 1% bovine serum albumin/PBS (BSA, Sigma-Aldrich). Scaffolds were washed in PBS at each intermediate step. Gelatin (BioGel, CH) was dissolved in PBS at 15% to embed each scaffold section, and solidified at 4 °C overnight. The resulting blocks were fixed with 10% formalin, and sectioned with a Leica VT1000 S Vibratome at 200 µm thickness. A Yokogawa CV1000 Cell Voyager microscope system was used to record the maximum fluorescent intensity over 11 z-slices, spacing 20 µm, for each scaffold cross-section. Invasion distance was quantified by measurement of median cell position, relative to the seeded surface. The fluorescent intensity profile across the scaffold cross-section was used to measure the distance at which the cumulative intensity was half its total value, not including the closest 10 intensity values to the scaffold surface. In this way, the measurements of median cell position reflect the position of the invading cells only, rather than those trapped at the scaffold surface. Each section was also imaged in bright field to ensure that collagen wall orientation was kept constant across all analyzed sections. This was analyzed numerically using the OrientationJ plugin in FIJI, followed by calculation of orientation index, OI = 2cos² β - 1, where β is the angle between the invasion direction and the dominant direction of collagen wall alignment. All analyzed sections were chosen to ensure OI > 0.9, indicating high wall alignment in the direction of invasion.

Statistical Analysis: Fluorescent intensity profiles, with background intensity values (measured from an empty area of the image) subtracted, were normalized to the total summed intensity over the profile, before calculation of median cell position as described above. No other normalization or data transformation took place. All results are given as mean ± standard error of the mean (SEM), where n = 3 for pore size and d, and n = 6 for median cell position. Statistical significance was tested using one-way ANOVA, along with the Games-Howell test for pairwise comparisons (significance level p < 0.05). The software used for statistical analysis was SPSS Statistics 23.0.

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Conflict of Interest

M.M. and P.G.B. are employees of Geistlich Pharma AG, Switzerland. J.C.A. was the recipient of Ph.D. funding from Geistlich Pharma AG, with S.M.B. and R.E.C. as principal investigators.

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