Tissue engineering of dermal substitutes based on porous PEGT/PBT copolymer scaffolds: comparison of culture conditions

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Previously, it was found that chondrocytes and fibroblasts could be efficiently seeded onto three-dimensional scaffolds in spinner flasks. In this study different culture conditions were compared to create a living dermal substitute as rapidly as possible. Human dermal fibroblasts were dynamically seeded onto biodegradable porous PEGT/PBT copolymer (PolyActive^{TM}) scaffolds for 24 h in spinner flasks. Subsequently, the cell-seeded scaffolds were cultured in two conditions: statically (without medium flow, S) and dynamically (with slow medium flow, D). Qualitative analyses (scanning electron microscopy and histology) and quantitative assays for DNA, total collagen (hydroxyproline) and glycosaminoglycans were done with samples cultured for 3, 7, 14 and 21 days. In dynamically cultured constructs, human dermal fibroblasts were uniformly distributed throughout the pores of the scaffolds and had deposited higher amounts of extracellular matrix (ECM). Significantly higher numbers of fibroblasts were found ($p < 0.001$), and significantly more collagen (hydroxyproline content) ($p < 0.001$) and glycosaminoglycan (GAG) ($p < 0.05$) were deposited at all the investigated time points when compared to static cultured constructs. In conclusion, medium flow stimulated the proliferation of human dermal fibroblasts and accelerated the ECM deposition in PolyActive^{TM} dermal substitutes when compared to static culture. Dynamic culture reduced the time to create a dermal substitute containing autologous fibroblasts.

1. Introduction

Cell seeding onto 3-D scaffolds and subsequent culture are fundamental steps in tissue engineering [1, 2]. Efficient seeding to reduce cell loss and optimal culture conditions to shorten the *in vitro* production time are critical for skin tissue engineering when using autologous cells because the patients wounds should be closed as quickly as possible.

Optimization of seeding and culture conditions using different bioreactors, e.g. spinner flasks [3], perfusion cartridges [4, 5], rotating vessels [6], were studied for a variety of cell types such as chondrocytes [6, 7], myofibroblasts [8], and aortic endothelial cells [9], but little data is available for dermal fibroblasts. In these studies, porous block, tube or fibrous scaffolds were usually investigated [8–11]. However, thin porous scaffolds (PEGT/PBT copolymer based) with a large surface area are required for dermal skin substitution. Previously we showed that high seeding efficiencies were obtained for fibroblasts that were seeded onto thin porous scaffolds using spinner flasks [12]. In cartilage tissue engineering, it was found that the existence of medium flow in spinner flasks favored the cartilage formation [13]. However, it is known that fibroblasts secrete most newly formed ECM molecules into the culture medium instead of depositing as a tissue [14]. A continuous medium flow through scaffolds will accelerate this dispersion process and could finally result in less deposition of ECM when compared to static medium culture conditions.

In this study, human dermal fibroblasts were dynamically seeded onto biodegradable porous PEGT/PBT copolymer (PolyActive^{TM}) scaffolds for 24 h in spinner flasks. Subsequently, the cell-seeded scaffolds were cultured in two conditions, statically (without medium flow, S) and dynamically (with slow medium flow, D). ECM formation and cell proliferation were compared between these two culture systems as a first step to develop the optimal system to engineer dermal substitutes using autologous fibroblasts.

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2. Materials and methods

2.1. Human dermal fibroblast isolation and culture

HDFs were isolated from dermis of adult human *breast tissue* ($n = 3$) by enzymatic digestion. Briefly, surgically removed dermis was minced into small pieces and digested with a freshly prepared enzymatic solution containing 0.25% (w/v) collagenase (Gibco BRL, Paisley, Scotland) and 0.25% (w/v) dispase (Gibco) at 37 °C for 2.5 h. The digestion mixture was filtrated through a 70 μm cell strainer (Falcon, Becton Dickingson, Mountainview, AL), and rinsed with fibroblast culture medium (FCM) consisting of Dulbecco's modified minimum essential medium (D-MEM, Gibco), 5% (v/v) foetal bovine serum (FBS, Sigma, St. Louis, MO) and 100 U ml^{-1} penicillin/100 μg ml^{-1} streptomycin. The filtrated solution was centrifuged, and the cell pellet resuspended prior to plating into culture flasks. The cultures were placed in an incubator at 37 °C in a humidified atmosphere with 5% CO_2. Culture medium was refreshed every 2–3 days. This method typically rendered confluent cultures of fibroblasts within one week.

2.2. Polymer scaffolds

Porous 30055PEGT/45PBT copolymer scaffolds (the weight ratio of PEGT/PBT is 55/45 and molecular weight of PEG is 300D, produced at IsoTis NV, Bilthoven, The Netherlands), whose suitable mechanical properties and biocompatibility have been shown both *in vitro* and *in vivo* [15, 16], were used as a 3-D template to engineer dermal substitutes (Fig. 1 left corner, cross-section of PolyActive™ scaffolds). This copolymer consisting of hard segments (polybutylene therephthalate, PBT) and soft segments (polyethylene oxide therephthalate, PEGT) was fabricated into porous films (300 μm thick) by combining methods of solution casting and salt leaching [17, 18]. The porosity of the PolyActive™ scaffolds was 70–80%, and the pore size varied from 50 to 200 μm. Small round discs (1.55 cm in diameter) cored from the film were used.

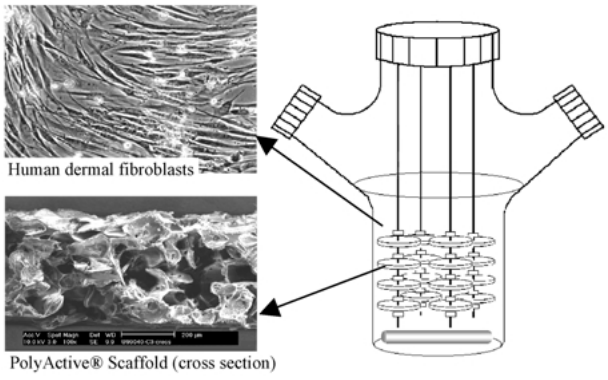

Figure 1 Schematic representation of seeding and culture system for tissue engineering of dermal substitutes in spinner flask. The scanning electron micrograph is the cross section image of the PolyActive™ scaffolds used in this study.

2.3. Seeding of human dermal fibroblast onto scaffolds

PolyActive™ discs were fixed onto 22-gauge needles in spinner flasks (Bellco Biotechnology Inc., Vineland, NJ), and positioned 3–6 mm apart with polyethylene spacers as shown in Fig. 1. The spinner flask and scaffolds were steam-sterilized for 20 min at 121 °C. Before seeding the PolyActive™ scaffolds were hydrated overnight at 37 °C in 100 ml of FCM. The medium was replaced with 145 ml FCM and 5 ml HDF suspension (1.6×10^6 cells/ml, passage 2–4). HDFs were dynamically seeded into PolyActive™ scaffolds while stirring with a magnetic stirring bar (0.9 cm in diameter and 5 cm long) at 40 ± 1 rpm for 24 h. The calculated seeding density was 5×10^5 fibroblasts per disc for all experiments.

2.4. Culture of cell-polymer constructs

After dynamic seeding, PolyActive™ scaffolds seeded with HDFs were continuously cultured in spinner flasks under two different conditions: statically (S, no stirring, i.e. no medium flow) and dynamically (D, stirring at 13 ± 1 rpm, resulting in continuous movement of medium). The FCM was supplemented with 0.2 mM ascorbic acid-2-phosphate (Sigma), and refreshed every 3 to 4 days. Constructs ($n = 4$ at every time point) were harvested on days 3, 7, 14 and 21, respectively. One construct was used for histological evaluation, and the other three were used for quantitative assays.

2.5. Histological evaluation

Samples (1/4 of each construct) for histological analysis were fixed in 4% formalin – phosphate buffer solution (PBS, Gibco). After rinsing in PBS, the samples were dehydrated using a graded series of ethanol solutions. The dehydrated samples were first equilibrated in 50% glycol methacrylate (GMA) in ethanol and secondly in 100% GMA solution overnight at 4 °C. The GMA embedding was initiated with benzoyl peroxide. The embedded blocks were sectioned (3–4 μm thick) with a microtome (Microm GmbH, Walldorf, Germany). The sections were subsequently stained with hematoxylin and eosin (H&E) (Sigma) to analyze cell distribution, morphology and ECM deposition. Samples (half of each construct) for general microscopic evaluation were fixed in 1.5% glutaraldehyde/0.14 M cacodylate buffer, pH 7.2–7.4. After rinsing in deionized water, samples were directly stained with 1% methylene blue and rinsed in deionized water. The cell distribution was analyzed with stereo light microscopy (Nikon SMZ-10A). Images were captured using CCD camera (Sony Corp., Tokyo, Japan) and Matrix Vision software (MATRIX vision GmbH, Oppenweiler, Germany). For scanning electron microscopy (SEM), 1/4 of each construct was fixed in 1.5% glutaraldehyde/0.14 M cacodylate buffer, pH 7.2–7.4, and dehydrated with graded ethanol series. After critical-point drying and gold-sputtering, samples were analyzed by scanning electron microscopy (FEI & Philips, Eindhoven, NL).

2.6. Quantitative analytical assay

Samples (triplicate per time point) were rinsed with PBS, and then digested in proteinase K (1 mg/mL proteinase K, 1 mM iodoacetamide and 10 μg/mL pepstatin-A in a buffer solution of 50 mM Tris with 1 mM EDTA, pH 7.4) for 16 h at 56 °C under gentle shaking to release cells and ECM molecules from scaffolds [19]. The number of fibroblasts per construct was assessed by measuring the amount of DNA in the enzyme-digested solution using a commercially available assay kit (CyQuant™ cell proliferation assay kit, Molecular Probes, Eugene, OR). For measurement, 100 μL of digested solution was added into a 3 ml tube with 400 μL CyQuant lysis buffer containing 1.35 Kunitz/ml RNAse A (Sigma), and incubated at R.T. for 1 h. A 100 μL aliquot of the sample (duplicate) was pipetted into a 96 well microplate and mixed with 100 μL of GR dye solution. After 15 min, the fluorescence was measured using fluorometer (Perkin Elmer Ltd., Connecticut, USA) (excitation at 480 nm, emission at 520 nm). DNA content was calibrated using a DNA standard (calf thymus DNA, Sigma). A fibroblast cell standard curve was also obtained by measuring DNA of a series of number known fibroblast stocks. Cell number per construct was calculated based on fibroblast cell standard curve and checked with the DNA standard.

Collagen content in the construct was determined by hydroxyproline assay. In brief, proteinase K digested sample was hydrolyzed in 6 M HCl (100 μL each of sample and 12 M HCl) at 110 °C for 16 h. HCl solution was removed by evaporation under a stream of N_2 at 37 °C. When evaporation was complete, the hydrolysate was dissolved in 0.5 mL of demineralized water, and 100 μL of hydrolysis samples dispensed into a 96-well plate. To each well, 50 μL of chloramines-T solution (14.1 g/L chloramines-T in 33.3% isopropanol, 0.16 M citric acid, 0.59 M anhydrous sodium acetate, 0.13 M acetic acid and 0.4 M sodium hydroxide with a pH of 6.0) was added. Following a 20 min incubation at room temperature, 50 μL of p-dimethyl-amino-benzaldehyde solution (p-dimethyl-amino-benzaldehyde 176.5 g/L in isopropanol/perchloric acid (60%) (12:5 v/v)) was carefully added. The microplate was incubated for 30 min at 60 °C in a water bath and subsequently cooled down. Absorbance of each sample was measured at 570 nm with a BioRad microplate reader and the hydroxyproline content of the samples was calculated using a standard curve constructed with a range of hydroxyproline concentrations.

Glycosaminoglycan (GAG) content was determined with a dimethyl-methylene Blue (DMMB) dye based color assay [20, 21]. Briefly, 12.5 μL of the digested sample, 12.5 μL Na_2HPO_4-Na_2EDTA buffer (pH 6.5 ± 0.1), and 5 μL of 2.3 M NaCl solution were pipetted into a well of a 96-well plate. 150 μL of DMMB dye solution (16 mg/L) was added into the above solution and mixed well. Afterwards, the absorbance was measured spectrophotometrically at 520 nm, using chondroitin B sulfate (dermatan sulfate, Sigma) as a standard.

2.7. Statistical analysis

All quantitative results were obtained from triplicate samples, and the results were confirmed by three independent experiments. Data are expressed as the mean $\pm$SD. Statistical analysis was carried out using an unpaired t test. A value of $p < 0.05$ was considered to be statistically significant.

3. Results

3.1. Construct overview, histological analysis and SEM observation

Using stereo light microscopy, the distribution of the fibroblasts on the polymer constructs was visualized after staining with methylene blue (Fig. 2). After three days, a difference between dynamic and static culture was observed. In the dynamically cultured constructs, fibroblasts conglomerated on the surface and in the pores of scaffold, whereas the statically cultured constructs showed an even cell distribution. After 11 days, constructs were completely covered with a layer of fibroblasts (pictures not shown). SEM analysis and H&E staining showed that pores of dynamically cultured constructs were filled with more cells and ECM when compared to statically cultured ones. This difference was even more pronounced after 21 days (Fig. 3(A) *versus* (B) and (E) *versus* (F)). High magnification SEM indicated that the ECM fibers entangled together with cells in both dynamically and statically cultured constructs (Fig. 3(C), (D)). In addition, the cell layers

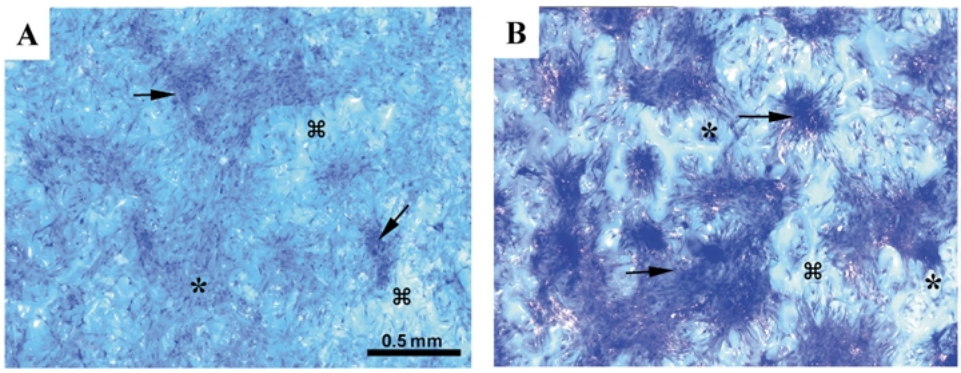

Figure 2 Block staining of dermal constructs with methylene blue after fixation. (A) Static cultured construct three days after dynamic seeding. Dermal fibroblasts (arrow) stained blue and spread on the scaffold homogeneously; (B) Dynamic cultured dermal construct three days after dynamic seeding. Cells (arrow) gather to form cell clusters and start to elongate. Scaffolds lightly stained blue ⌘ and (*) indicates the polymer and pores of the scaffold.

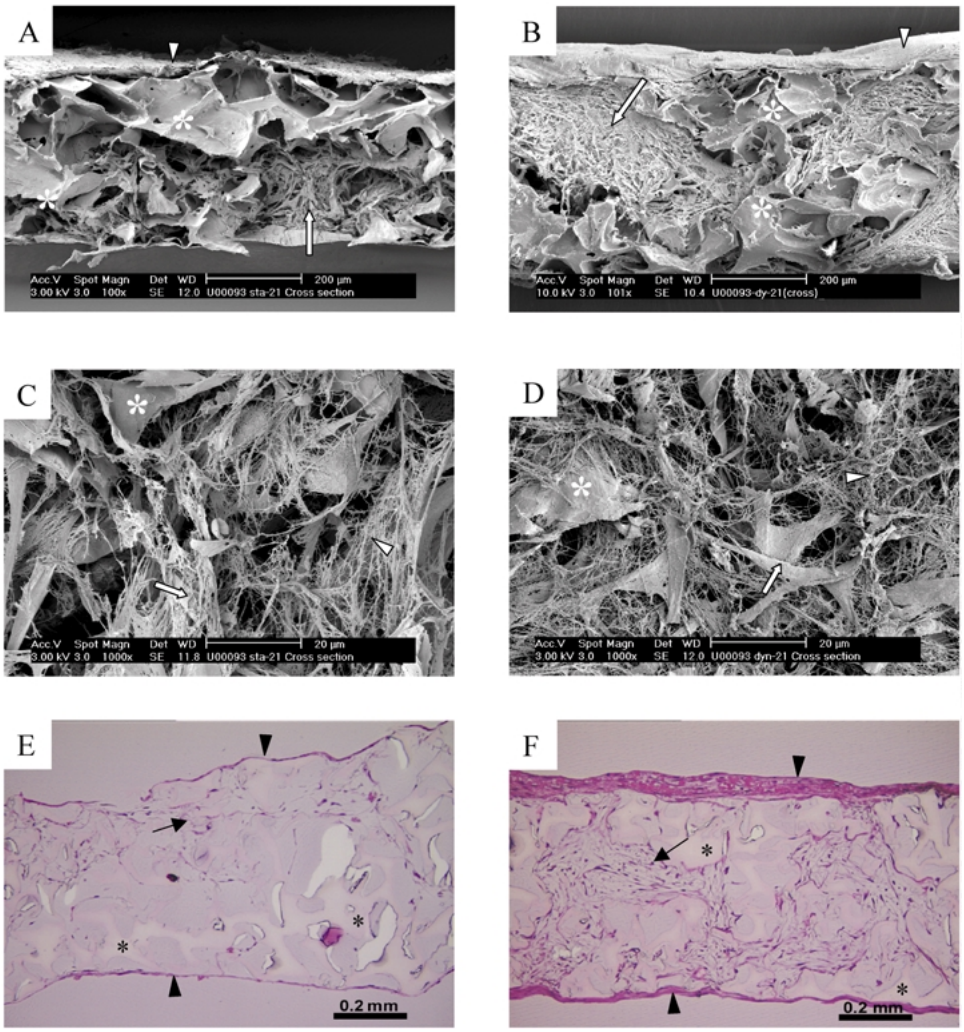

Figure 3 Cross section of dermal constructs cultured for 21 days. A–D are scanning electron micrographs, and E–F are hematoxylin-eosin staining. In static cultured constructs (A, C and E), fibroblasts grow on the surface (arrowhead) and in some of the pores of the scaffold (arrow), but not in all the pores. On the contrary, in dynamic cultured constructs (B, D and F), most of the pores are filled with dense ECM and cells (arrow) and the surface is covered with multi-layer of fibroblasts (arrowhead). In both cultures, higher magnification (C and D) shows that fibroblasts (arrow) are entangled by newly formed ECM fibers (arrowhead). The polymer scaffold is indicated with an asterisk (*) in the micrographs.

formed at the surface of the dynamically cultured constructs were thicker when compared to the statically cultured ones (Fig. 3(E) and (F), arrowheads).

3.2. Biochemical composition of the constructs

Quantitative analyses were performed to compare cell proliferation and ECM formation between the two culture conditions. After only 3 days, dynamic culture had stimulated fibroblasts proliferation resulting in the presence of significantly more fibroblasts in dynamically cultured constructs at each evaluation time (Fig. 4(A)). In both culture conditions, the total collagen (hydroxyproline) and GAG content increased in time, but the total content was significantly higher in the dynamically cultured constructs when compared to the statically cultured ones (Fig. 4(B) and (C)). Interestingly, after 3 and 7 days, the GAG content per fibroblast under static culture conditions was significantly higher than that in dynamic culture conditions (10.9 ± 2.1 versus 6.9 ± 1.0 and 10.7 ± 1.8 versus 6.0 ± 1.5 pg/cell, respectively) indicating that medium flow did not favor GAG deposition in the scaffolds. The hydroxyproline content significantly increased only after 14 days and coincided with the time that fibroblasts reached ''confluence'' in the scaffold as judged from the H&E histological pictures.

4. Discussion and conclusion

The present study showed that dynamic culture with medium flow allows engineering of dermal tissue substitutes in a shorter time period when compared to culture under static medium conditions. The constructs dynamically cultured for three weeks showed an integral structure throughout the scaffold, and contained twice as many fibroblasts and interstitial collagen as the statically cultured ones. These results are clinically relevant because the production time of a dermal substitute based on autologous fibroblasts was reduced to a clinical acceptable timeframe (< 3 weeks) [22].

In our previous studies, dynamic seeding by stirring the medium at 40 rpm resulted in a high fibroblast-seeding efficiency compared to static seeding but did not further favor the growth of cells and ECM formation in 3-D scaffolds [12]. In simulation experiments using microspheres (unpublished data) it was found that a

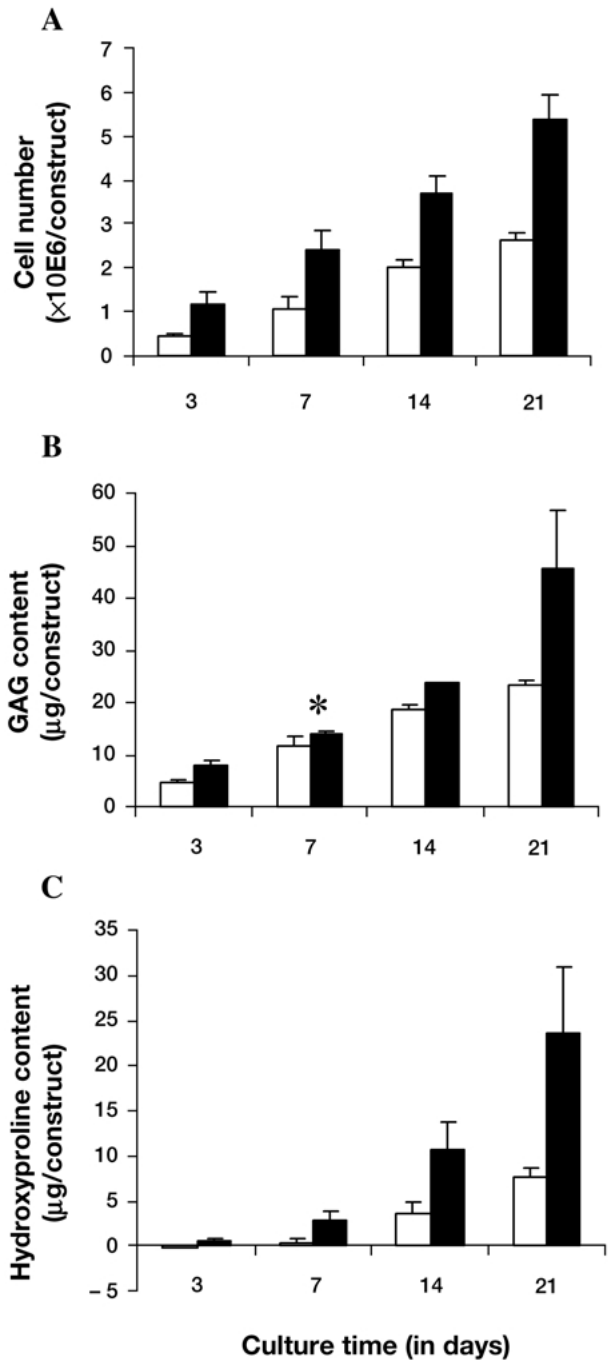

Figure 4 Fibroblast proliferation and ECM deposition in static (white bar) and dynamic (dark bar) cultured dermal constructs over time. (A) Fibroblast number within cultured substitutes, (B) the total glycosaminoglycan content and (C) the total hydroxyproline content deposited in the constructs. The difference between the static and dynamic cultured constructs was statistically significant ($p < 0.001$, and $^{*} < 0.05$, unpaired T test) at all evaluated time points and in all assays.

stirring speed of 13 rpm induced a laminar medium flow around the scaffolds, whereas 40 rpm induced a more turbulent medium movement with a high relative velocity between scaffold and medium. This is likely to create shear stresses that are too high for fibroblasts. These observations could explain why dermal fibroblasts grew better at a low speed (13 rpm) when compared to a higher speed (40 rpm).

During culture, a cell layer was formed on the scaffold surface. This cell layer could inhibit the exchange of nutrients and waste products between the interior and exterior of the construct. In static conditions, the transportation of nutrients and waste products mainly depends on diffusion, and this cell layer is likely to induce gradients of nutrients and accumulation of waste products within the scaffold [13]. This could explain why less cells and ECM formation were found inside the static cultured constructs. In dynamic culture, it is likely that the hydrodynamics of the medium enhances the nutrient and waste product exchange in the microenvironment of the scaffold. This may explain why a more homogeneous distribution of cells and ECM was found in these constructs.

Dynamic culture also induces shear stress on the fibroblasts, which plays a role in cell metabolism, mitosis, and in the production and degradation of extracellular matrix components which are important in control of tissue integrity. Dermal fibroblasts cultured dynamically were oriented and gathered in cluster three days after seeding, whereas statically cultured fibroblasts showed a more even distribution on the scaffold. Fibroblasts apparently clustered to avoid the shear stress present at the surface of dynamically cultured constructs, demonstrating to the apparent influence of shear stress on the behavior of fibroblasts. Pulmonary artery [23], lung [24] and cardiac [25, 26] fibroblasts were reported to replicate/proliferate in response to mechanical load such as extensile stress (also called axial stretch) [27, 28] or pressure overload [14, 26]. These are different forces from hydrodynamic shear stress produced in our study. In the present study, a stimulation of fibroblast proliferation was also observed. However, we are not able to discriminate if hydrodynamic shear stress has a direct influence on cell proliferation or if the stimulation of nutrient exchange was more important.

External forces also regulate the turnover of extracellular matrix proteins. Mechanical loading increased the level of procollagen α_1 (I) mRNA [14, 26] and of procollagen C-proteinase [14] in the presence of serum or TGF-β. This represents a net increase in production of insoluble collagen. On the other hand, it has also been reported that mechanical loading increased the level of metalloproteinases (MMPs), which are mainly involved in the degradation and remodeling of collagen [27, 29–32]. The collagen production in our dynamically cultured constructs experiencing hydrodynamic shear stress was found to be higher than statically cultured constructs. It is clear that different external forces have an effect on cellular transcriptional, translational, and posttranslational events in the pathways of collagen production. The net results could depend on the type of external force on the cell. Future studies will investigate if hydrodynamic forces directly stimulate collagen production and processing or if the improved nutrient exchange is more important for the observed effects.

In conclusion, medium flow stimulated fibroblast proliferation and ECM deposition in PolyActive^{TM} scaffolds when compared to static culture.

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