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DYNAMIC CULTURE ENHANCES STEM CELL INFILTRATION AND MODULATES EXTRACELLULAR MATRIX PRODUCTION ON ALIGNED ELECTROSPUN NANOFIBROUS SCAFFOLDS

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Abstract

Electrospun nanofibrous scaffolds have become widely investigated for tissue engineering applications, owing to their ability to replicate the scale and organization of many fiber-reinforced soft tissues such as the knee meniscus, the annulus fibrosus of the intervertebral disc, tendon, and cartilage. However, due to their small pore size and dense packing of fibers, cellular ingress into electrospun scaffolds is limited. Progress in the application of electrospun scaffolds has therefore been hampered, as limited cell infiltration results in heterogeneous deposition of extracellular matrix and mechanical properties that remain below native benchmarks. In the present study, dynamic culture conditions dramatically improved the infiltration of mesenchymal stem cells into aligned nanofibrous scaffolds. While dynamic culture resulted in a reduction of glycosaminoglycan content, removal from dynamic culture to free swelling conditions after 6 weeks resulted recovery of glycosaminoglycan content. Dynamic culture significantly increased collagen content, and collagen was more uniformly distributed throughout the scaffold thickness. While mechanical function was assessed and tensile modulus increased with culture duration, dynamic culture did not result in any additional improvement beyond free swelling culture. Transient dynamic (6 weeks dynamic followed by 6 weeks free swelling) culture significantly enhanced cell infiltration while permitting GAG accumulation. In this study, we demonstrated that a simple modification to standard *in vitro* culture conditions effectively improves cellular ingress into electrospun scaffolds, resolving a challenge which has until now limited the utility of these materials for various tissue engineering applications.

Keywords

electrospinning; functional tissue engineering; dynamic culture

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1. INTRODUCTION

The mechanical function of load-bearing tissues is primarily determined by their composition and organization. Therefore if an engineered tissue is to successfully replace ailing and compromised tissues, anatomic form must be replicated. As such, there is a growing interest in the application of biomaterials that mimic the micro-architecture of native tissues. Electrospinning generates nanofibrous polymeric scaffolds with controllable fiber diameter, alignment, and composition [1]. Due to their ability to mimic the scale and organization found within the extracellular matrix (ECM) of many fiber-reinforced soft tissues, electrospun scaffolds have become widely investigated for the engineering of such tissues as cartilage [2,3], the annulus fibrosus of the intervertebral disc [4,5], tendons [6], and the knee meniscus [7,8].

Perhaps the most important feature of these materials is their ability to direct the alignment of cells and subcellular structures [4–7]. Cell alignment precedes the deposition of collagen rich, aligned extracellular matrix on electrospun scaffolds [7,8]. Ultimately, this ordered collagen deposition results in demonstrable increases in tensile properties, on the order of tens of MPa [7–10].

Despite this, for most applications even mature constructs fail to reach functional equivalence with their native counterparts. Due to the dense packing of nanofibers and a relatively small pore size, cell ingress into nanofibrous meshes has been limited to the periphery of even 0.5 mm thick scaffolds [7,12]. As a result, ECM deposition is heterogeneous. Moreover, because the eventual application of these materials clinically will likely require much thicker constructs, this limited cellular infiltration poses a significant challenge to the electrospinning approach for functional tissue engineering. To address this limitation, cells have been incorporated into forming scaffolds during electrospinning [2]. While this approach shows promise, technical difficulties associated with solvent cytotoxicity and construct sterility may limit its efficacy, particularly when relatively thick scaffolds must be formed over the time-course of hours. Alternatively, porogens have been incorporated into nanofiber mats during electrospinning. Recently, composite nanofiber scaffolds have been developed that intermingle a sacrificial, water soluble fiber population with a slow-degrading one, resulting in scaffolds whose pore size increases upon hydration [3]. This approach enhanced cellular infiltration as a function of sacrificial fiber density, and is preferable to salt-leaching [4] and similar porogenic approaches that may fail to preserve the original nanofibrous architecture and organization of the scaffold. Although sacrificial fibers enhanced infiltration, cellular ingress was hastened at the expense of tensile properties. In fact, the most dramatic improvements in infiltration were observed under conditions where cell-generated forces deformed and contorted the scaffold [3].

Enhanced cell proliferation and ECM production have been achieved on a range of materials by culturing tissue constructs under dynamic conditions, whereby media flow is induced by agitation in order to enhance nutrient and waste exchange by convective transport [5–9]. In one study, direct perfusion has been used to enhance cellular infiltration into electrospun scaffolds [10], and recently dynamic culture has been suggested to enhance proliferation of mesenchymal stem cells on electrospun nanofibrous scaffolds [21]. While perfusion dramatically improved infiltration into scaffolds consisting of larger micro-fibers, the addition of even very thin layers of nano-fibers mitigated this effect, further illustrating the challenge associated with cell migration in nanofibrous scaffolds. In the present study, dynamic culture conditions were applied by orbital shaker to enhance infiltration and tissue deposition by mesenchymal stem cells (MSCs) on aligned, electrospun nanofibrous scaffolds. We show here that dynamic culture serves as a simple and effective modification

to free-swelling culture conditions, reducing the cell and extracellular matrix heterogeneity that is common to electrospinning-based tissue constructs.

2. METHODS

2.1 Electrospun nanofibrous scaffold fabrication

Aligned nanofibrous scaffolds were generated by electrospinning as described previously [3,11]. Briefly, 8 g poly(ε-caprolactone) (PCL, Sigma Aldrich, batch # 00702CE, MW 80kDa) was dissolved at 37°C overnight in 56 mL of equal parts tetrahydrofuran and N, N-dimethylformamide. The PCL solution was ejected via syringe pump at 2.5 mL/h through a spinneret charged to +13 kV, generating a nanofibrous jet that was collected on a grounded mandrel rotating at 10 m/s at a distance of 20 cm from the spinneret tip. Aluminum shields on either side of the spinneret were charged to +9 kV in order to focus the jet toward the mandrel, while the spinneret was fanned back and forth axially to ensure uniform deposition along the mandrel [3]. As demonstrated in previous work, this results in the formation of aligned mesh of fibers ranging in diameter from 300 nm up to 1 μm, with a fiber volume fraction of approximately 0.15 [1,5,7,9,20]. Fibers were collected onto the mandrel for 6 to 8 hours, resulting in an aligned nanofibrous mesh of approximately 0.75 mm thickness. Rectangular samples (5 mm × 30 mm) were excised from the mesh with the long axis parallel to the prevailing fiber direction.

2.2 MSC isolation and seeding onto aligned nanofibrous scaffolds

Marrow was isolated and combined from femurs and tibiae of two 3–6 month old calves as described previously [12] and plated on tissue culture plastic in basal medium (high glucose DMEM containing 1% Penicillin, Streptomycin, Fungizone and 10% Fetal Bovine Serum). Colony formation was observed by two weeks; subsequently, cells were expanded to passage 2 in basal medium (changed twice weekly). Scaffolds (above) were hydrated by 1 hour sequential washes in 100%, 70%, 50% and 30% ethanol, followed by 1 hour in Phosphate Buffered Saline (PBS). Prior to seeding, scaffolds were incubated overnight in 20 μg/mL fibronectin in PBS. Scaffolds were rinsed with PBS and transferred to untreated six-well plates before seeding. 50 μL of cell solution (1×10^7 cells/mL) were applied to one side of the scaffold, followed by incubation at 37°C for one hour. Scaffolds were turned and an additional 50 μL of cell solution applied to the opposite side. After an additional two hour incubation, wells were flooded with 3 mL of chemically defined growth media (DMEM, 0.1μM dexamethasone, 40 μg/mL L-Proline, 100 μg/mL Sodium Pyruvate, 1% Insulin, Transferrin, Selenium/Premix, and 1% penicillin, streptomycin and fungizone supplemented with 10 ng/mL Transforming Growth Factor β-3) [13]. Constructs were fed twice weekly with chemically defined growth medium for the duration of the study.

2.3 Dynamic culture of MSC-seeded nanofibrous scaffolds

After seeding, all groups were pre-cultured for 3 days in free-swelling conditions to allow for cell attachment and colonization of the scaffold surface. Following this pre-culture, dynamic culture was initiated (0 weeks) by incubating constructs on an orbital shaker (Bellco, 7744-01000). The orbital shaker was operated continuously at a rate of 1.2 Hz. A second group of constructs was subject to free-swelling culture in the absence of media agitation. Finally, to determine the benefit of temporary or transient dynamic culture, additional constructs were cultured under dynamic conditions for 6 weeks, followed by 6 weeks of free-swelling culture.

2.4 Mechanical testing and biochemical analyses

At bi-weekly intervals up to 12 weeks, free-swelling, dynamic culture, and transient dynamic culture (after 6 weeks) constructs were taken for mechanical testing followed by biochemical analyses (n = 5 per group per time point). Cross-sectional area was determined using a custom non-contact laser device as described previously [14,15]. Samples were clamped with custom-built serrated grips and loaded into an Instron 5542 testing device for uniaxial tensile testing [16]. In brief, samples were subjected to a pre-load of 0.1 N applied at 0.1% per second, and held for five minutes, followed by preconditioning for 15 cycles of 0.1% strain at a rate of 0.05% strain per second, and finally a ramp to failure at a strain rate of 0.1% per second. Failure properties were omitted from the analysis due to the high occurrence of failure near the grips. All testing was performed in a PBS bath. Stress and strain were computed as force divided by undeformed cross-sectional area and displacement divided by original gage length, respectively, and the modulus was calculated as the slope of the linear region of the stress-strain curve. Stiffness was computed from modulus by multiplying the modulus by area and dividing by gage length.

Biochemical assays were performed to determine sulfated glycosaminoglycan (GAG) and collagen content as described previously [13]. After tensile testing, samples were digested for 16 hours in papain at 60°C. Digested samples were analyzed for GAG content using the 1, 9-dimethylmethylene blue dye-binding assay, and for orthohydroxyproline (OHP) content (after acid hydrolysis) to determine collagen content by reaction with chloramine T and dimethylaminobenzaldehyde. OHP content was converted to collagen with a ratio of 1 : 7.14 (OHP:collagen) [17].

2.5 Histology and quantification of infiltration

Samples from each group (n = 3 per time point) were collected for histologic analyses. Samples were embedded in OCT freezing medium and flash frozen in liquid nitrogen. Embedded samples were cryo-sectioned in the plane perpendicular to the fiber direction (i.e., in cross-section). Sections were fixed in 4% paraformaldehyde and stained for cell nuclei (DAPI), GAG (Alcian Blue) and collagen (Picrosirius Red). DAPI-stained sections were visualized on a Nikon T30 inverted fluorescent microscope. Alcian Blue and Picrosirius Red stains were visualized on an upright Leica DMLP microscope. Infiltration was measured by analysis of DAPI stained sections using a custom MATLAB script described previously [3]. The distance of each DAPI-stained nucleus from the scaffold surface was measured, and data were binned by % of thickness infiltrated: 0 – 25%, 25 – 50%, 50 –75%, and 75 – 100%, where 100% infiltration indicates arrival of a cell at the center of the scaffold.

2.6 Statistics

Statistical significance was determined by two-way ANOVA, with factors of culture duration (0 – 12 weeks) and culture condition (free swelling, dynamic culture, and transient dynamic culture) using the Systat software. Tukey's post hoc testing was used to establish significance between groups ($p < 0.05$).

3. RESULTS

Consistent with previous findings, *in vitro* culture of MSC-seeded nanofibrous scaffolds under free-swelling conditions resulted in only partial infiltration of cells through the scaffold thickness, even after 12 weeks (Fig. 1D). Although cellular ingress did increase with culture duration (Fig. 1), by 12 weeks the central third of the scaffold was nearly devoid of cells. In dynamically cultured constructs, however, cells were visible near the center of scaffolds as early as 6 weeks after seeding (Fig. 1C) and continued to infiltrate

until full colonization was achieved by 12 weeks when the study was terminated (Fig. 1E). Even at this point, however, cell density was non-uniform, with the periphery containing more cells than the central portion. A third group was exposed to transient dynamic culture, consisting of 6 weeks under dynamic culture followed by free-swelling culture for the remaining 6 weeks of the study. In transient dynamic constructs, cells were observed throughout the scaffold thickness (Fig. 1E, F).

In order to confirm these findings quantitatively, a custom analysis procedure was used to determine the % of cells residing within the depth of the constructs. At the time dynamic culture was initiated (3 days after seeding), 95% of cells were restricted to the outer quarter of scaffold thickness (Fig. 2A). By 12 weeks, 70% were in the outer quarter of the scaffold in free-swelling constructs, indicating that the majority of cells were still restricted to the periphery of the scaffold (Fig. 2G). However, under dynamic culture conditions, a significantly higher percentage of the population was observed within the 50–75% and central 75%–100% quartiles, while only 34% of the cells remained in the outer region (Fig. 2G). In fact, full infiltration was observed under dynamic culture conditions by 10 weeks, with a nearly uniform distribution of cells across each of the four quartiles (Fig. 2F). Finally, no significant differences in % of cells were observed between constructs subjected to continuous and transient dynamic culture (Fig. 2E–G). This indicates that 6 weeks exposure to dynamic culture conditions is sufficient to enhance and sustain cellular ingress into aligned nanofibrous scaffolds.

Biochemical analyses revealed significant increases in GAG content with culture duration for all groups (Fig. 3A). However, by as early as 2 weeks, a significant reduction in GAG content was observed for dynamically cultured constructs compared to free-swelling constructs. Interestingly, GAG content was recovered within 2 weeks after removal from dynamic culture, as demonstrated by equivalent GAG contents between free-swelling and transient dynamic culture groups at 8 weeks (Fig. 3A). From this point, however, GAG content continued to increase in free-swelling constructs, but plateaued for transient dynamic culture constructs. Alcian Blue staining at 12 week revealed a concentration of GAG in the hypercellular layer surrounding the scaffold in each culture condition (Fig. 3B–D). The above biochemical findings were corroborated, within the scaffold as well, with staining throughout free-swelling constructs (Fig. 3B, E) and reduced staining near the center of scaffolds cultured under transient dynamic culture (Fig. 3D, G). In dynamically cultured constructs, GAG was only observed in the hypercellular layer surrounding the scaffold, with little or no staining within the scaffold (Fig. 3C, F). These findings suggest that dynamic culture severely depletes GAG content in mature constructs.

Collagen content increased significantly with culture duration for all groups (Fig. 4A). Dynamic culture resulted in a 2.5-fold increase in collagen content compared to free-swelling controls. At 10 weeks transient dynamic culture also resulted in improved collagen content compared to free-swelling; however, no measurable difference between the two was observed at 12 weeks (Fig. 4A). As indicated by Picrosirius Red staining of 12 week samples, collagen was also found in the hypercellular layer surrounding the scaffold in each culture condition (Fig. 4B–D). However, intense staining was observed uniformly through the thickness of dynamically cultured constructs Fig. 4F), while less staining was observed under transient dynamic culture (Fig. 4G), and even less under free-swelling conditions (Fig. 4E).

Uniaxial tensile testing revealed significant increases in the tensile modulus (Fig. 5A) and stiffness (Fig. 5B) with culture duration for all conditions. Despite variations in cellular ingress, GAG, and collagen contents, no significant differences were observed between tensile moduli of free-swelling, dynamic, and transient dynamic culture conditions. At 12

weeks, the stiffness of transient dynamic constructs was significantly higher than free swelling constructs.

4. DISCUSSION

In this study, dynamic culture conditions provided via orbital shaking was employed to enhance cellular infiltration into aligned electrospun nanofibrous scaffolds. Improved cellular ingress was achieved without compromising mechanical properties or functional growth. In fact, dynamic culture enhanced collagen deposition by nearly two-fold compared to free-swelling conditions. While this occurred at the expense of GAG content, removal from dynamic culture conditions resulted in a rapid recovery of GAG content. This indicates that transient dynamic culture can effectively improve cell infiltration while retaining the matrix formation and retention capacity of free-swelling cultures.

The reasons underlying poor ingress of cells into nanofibrous scaffolds under free-swelling conditions have not yet been fully elucidated. However, it may be possible that nutrient delivery and waste removal, processes driven primarily by diffusion *in vitro*, occur less efficiently in the central regions of the scaffold than at the periphery. The resulting gradients may discourage cell migration away from the nutrient-rich periphery. Alternatively, these gradients may positionally favor cell proliferation at the periphery and cell death at the center. Agitation of the culture medium via dynamic culture may enhance nutrient-waste exchange, where convective transport would attenuate these gradients. Indeed, we observed a pronounced improvement in cellular ingress/survival throughout the scaffold under dynamic conditions. In addition to altered transport, media agitation results in an altered mechanical environment, with cells exposed to increased fluid shear stress. Although small in magnitude, these forces may illicit a response that drives cells into the scaffold. Because many variables including scaffold geometry can influence the transmission of fluid shear stress through engineered tissues [18], additional work is necessary to fully interpret the mechanical implications of this simple dynamic culture system. Further investigation is necessary to determine the precise mechanism by which improved infiltration is achieved under dynamic culture conditions. Understanding the underlying reasons for improved cell migration into the scaffold under dynamic culture conditions may help to optimize the culture system for further enhancement of functional growth.

In addition to altered infiltration, dynamic culture had pronounced effects on the composition of ECM that accumulated within constructs. While dynamic culture depleted GAG, collagen content was significantly increased. Based on previous findings, collagen typically co-localizes with cells in nanofibrous scaffolds, while GAG is observed throughout the construct, even in regions where cells are absent. This suggests that GAG may be free to diffuse through the construct, while collagen is incorporated into immobile networks near the site of synthesis. As such, it is probable that dynamic culture results in leaching of GAG from the construct. Although media fractions were not tested for GAG content in this study, doing so in future work will indicate whether GAG is being lost to the media under dynamic conditions, or whether cells are simply failing to produce GAG at the same rate. These results are in contrast to a previous study in which dynamic culture of chondrocyte-seeded nonaligned scaffolds resulted in improved GAG content [17]. However, this discrepancy could arise from one of many variables that were disparate between this work and the present study, including cell-type, fiber alignment and composition, media formulation, and method of dynamic culture. In the present study, removal from dynamic culture resulted in a rapid recovery of GAG content to free-swelling levels within two weeks. However, following this point, GAG content continued to increase in free-swelling constructs, but plateaued in transient dynamic constructs. The reason for this is unclear; however it is possible that differences in collagen content, cell localization, and stimulation during

dynamic culture may alter the phenotypic stability of differentiated fibrochondrocytes in this system. It is well established from previous work that in the presence of TGF-β3, MSCs on electrospun scaffolds express genes associated with fibrocartilagenous ECM, including collagen types I and II and aggrecan, and that expression of these genes is dramatically reduced in the absence of TGF-β3 [19,20]. However, how MSC phenotype is altered under dynamic culture conditions is not known. Further study is necessary to determine the effect of dynamic culture on gene expression and protein production. Moreover, it is interesting that GAG rich regions were observed at the periphery, and not the center, of constructs subject to continuous and transient dynamic culture. This further indicates that there may be phenotypic shifts that occur with dynamic stimulation, and these shifts may occur heterogeneously throughout the construct. Therefore, temporal exposure to dynamic culture conditions can be used to enhance infiltration without sacrificing final GAG content.

Dynamic culture resulted in a two-fold increase in collagen content. The reason for this increase is unknown; however it is possible that enhanced nutrient/waste transport may have resulted in improved metabolic activity and ECM formation by cells throughout the scaffold. While mechanical stimulation via fluid-shear stress may also be involved, it is unlikely that such forces would exist within the central portion of the scaffold (Fig. 4F), where improved collagen deposition was observed. Nonetheless, fluid-shear stress has been previously shown to result in increased collagen deposition by chondrocytes when cultured as scaffold-free constructs [21]. Increased collagen content may also be a consequence of improved infiltration: if collagen deposition is regulated by a negative feedback mechanism, cell-dense regions may become saturated with collagen early, resulting in a reduced rate of collagen synthesis. On the other hand, under dynamic conditions cells are better distributed throughout the scaffold, such that saturation may only occur after a larger amount of collagen has been produced. Whether such a mechanism regulates collagen synthesis by cells on nanofibrous scaffolds is not known. It is also possible that the reduced GAG that accompanies dynamic culture may alter cell behavior, favoring a phenotype that permits greater collagen deposition. Regardless of mechanism, dynamic conditions resulted in collagen contents as high as 25% of the total dry weight (which includes the weight of polymeric scaffold), exceeding, to our knowledge, all previous reports of collagen content for MSC and fibrochondrocytes in nanofibrous scaffolds [4,7,8,11]. It is unclear why a reduction in collagen was observed after removal from dynamic culture in the transient group. In future work, it will be important to assess the types of collagen produced by cells in these culture conditions in order to clarify the biologic effects of dynamic culture.

Consistent with previous studies of bovine MSCs on aligned nanofibrous scaffolds, tensile modulus and stiffness increased with culture duration for all groups. Unfortunately, despite improved infiltration and collagen content, dynamic culture failed to produce any gain in mechanical function beyond free-swelling culture. GAGs have been implicated in collagen fibrillogenesis during development [22], and as such, GAG depletion under dynamic conditions may adversely effect collagen assembly, and therefore limit functional gains. Although GAG may also contribute directly to the tensile response of engineered and native fibrocartilages, recent work suggests that the mechanical interplay between GAG and collagen within mature tissues is quite complex [28–32]. Although no differences were observed for modulus, tensile stiffness was significantly higher in constructs that were cultured dynamically for 6 weeks and then removed to free-swelling conditions, when compared to continuously free-swelling or dynamically cultured constructs. Because these constructs possessed both the infiltration of dynamic culture and the GAG content of free-swelling culture, it is possible that both are important for functional growth. These results highlight the importance of functional outcome measures in assessing the success or failure of engineered replacements for load-bearing tissues [33, 34]. Failure to achieve enhanced mechanical function despite significant improvements in cell infiltration and collagen

content suggests that GAGs, which were reduced with dynamic culture, may play an important role in the function and formation of extracellular matrix. Alternatively, although collagen content improved with dynamic culture, it may necessary to closely examine collagen organization (e.g. fiber bundling and alignment) in order to determine why collagen increases did not yield improved tensile function.

Dynamic culture conditions have been widely employed for engineering a broad range of tissues. While in the present work, agitation of the culture medium was achieved using orbital shakers, spinner flasks and rotating wall bioreactors have also been used to enhance matrix formation in engineered tissues, as well as matrix retention/maintenance in native tissues [17, 35, 36]. While each method appears to confer enhanced nutrient/waste exchange to the culture system, an advantage of the orbital shaker is the ease of its implementation. Conversely, it is possible that the state of micro-gravity that is achieved in a rotating-wall bioreactor may further enhance tissue formation while potentially reducing GAG loss [23]. While in the current study, our attention is focused on infiltration of electrospun scaffolds, it is likely for many applications that some form of enhanced nutrient/waste transport will be necessary as constructs approach geometries and length scales of clinical relevance. This is especially true for load-bearing and fiber-reinforced tissues, which are typically quite dense and poorly vascularized. For example, we have recently employed MSC-seeded nanofibrous scaffolds in concert with MSC-containing hydrogels to engineer composite intervertebral discs that contain both engineered nucleus pulposus and annulus fibrosus sub-regions [24]. In these constructs, pronounced heterogeneity was observed, with the annulus fibrosus region restricting nutrient waste exchange within the nucleus pulposus region. In tissues like the intervertebral disc, that despite their size are largely avascular, engineered replacements may require dynamic culture conditions in order to provide uniform cell infiltration and matrix deposition.

Although the dynamic culture approach employed here was successful in enhancing cell infiltration, tensile moduli obtained in the present study (~35 MPa) remain below that of native fibrocartilages like the annulus fibrosus (80 – 120 MPa) and the knee meniscus (100 – 300 MPa) [25–27]. However, there are several avenues by which to modify this approach in order to further enhance functional growth. For instance, the magnitude of media agitation applied via dynamic culture has yet to be optimized; it is possible that slower frequencies of gyration could achieve enhanced infiltration without sacrificing GAG content. Additionally, dynamic culture could be applied intermittently instead of continuously. Dynamic culture could also be coupled with other methods of enhancing infiltration and growth on nanofibrous scaffolds, such as dynamic loading bioreactors [28] or nanofibrous composite scaffolds with porogenic sacrificial fibers [3]. Sacrificial fibers have recently been used to successfully enhance infiltration into nanofibrous scaffolds by increasing porosity, but did so at the expense of mechanical properties [3]. Through a union of such approaches with dynamic culture, it may be possible to further enhance infiltration and tissue deposition and homogeneity in large tissue constructs without compromising mechanical performance. Finally, as the electrospun scaffold-based strategies move toward clinical implementation, it will be important to understand how the present findings for bovine MSCs translate to aged, human donor cells.

In the present study a simple modification to our culture system successfully increased cellular infiltration, resolving a challenge that has thus far hampered progress in the application of aligned electrospun scaffolds for tissue engineering. Future work will be directed towards probing collagen assembly through evaluation of fiber diameters and connectivity, as well as tuning dynamic culture conditions to optimize mechanical functionality.

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REFERENCES

1. Mauck RL, Baker BM, Nerurkar NL, Burdick JA, Li WJ, Tuan RS, et al. Engineering on the Straight and Narrow: The Mechanics of Nanofibrous Assemblies for Fiber-Reinforced Tissue Regeneration. Tissue Eng Part B Rev 2009;15(2):171–193. [PubMed: 19207040]
2. Stankus JJ, Guan J, Fujimoto K, Wagner WR. Microintegrating smooth muscle cells into a biodegradable, elastomeric fiber matrix. Biomaterials 2006 Feb;27(5):735–744. [PubMed: 16095685]
3. Baker BM, Gee AO, Metter RB, Nathan AS, Marklein RA, Burdick JA, et al. The potential to improve cell infiltration in composite fiber-aligned electrospun scaffolds by the selective removal of sacrificial fibers. Biomaterials 2008 May;29(15):2348–2358. [PubMed: 18313138]
4. Nam J, Huang Y, Agarwal S, Lannutti J. Improved cellular infiltration in electrospun fiber via engineered porosity. Tissue Eng 2007 Sep;13(9):2249–2257. [PubMed: 17536926]
5. Mahmoudifar N, Doran PM. Chondrogenic differentiation of human adipose-derived stem cells in polyglycolic acid mesh scaffolds under dynamic culture conditions. Biomaterials May;31(14):3858–3867. [PubMed: 20153043]
6. Mahmoudifar N, Doran PM. Effect of seeding and bioreactor culture conditions on the development of human tissue-engineered cartilage. Tissue Eng 2006 Jun;12(6):1675–1685. [PubMed: 16846362]
7. Li WJ, Jiang YJ, Tuan RS. Cell-nanofiber-based cartilage tissue engineering using improved cell seeding, growth factor, and bioreactor technologies. Tissue Eng Part A 2008 May;14(5):639–648. [PubMed: 18419231]
8. Obradovic B, Martin I, Freed LE, Vunjak-Novakovic G. Bioreactor studies of natural and tissue engineered cartilage. Ortop Traumatol Rehabil 2001 Apr 30;3(2):181–189. [PubMed: 17986981]
9. Araujo JV, Cunha-Reis C, Rada T, da Silva MA, Gomes ME, Yang Y, et al. Dynamic culture of osteogenic cells in biomimetically coated poly(caprolactone) nanofibre mesh constructs. Tissue Eng Part A Feb;16(2):557–563. [PubMed: 19728792]
10. Pham QP, Sharma U, Mikos AG. Electrospun poly(epsilon-caprolactone) microfiber and multilayer nanofiber/microfiber scaffolds: characterization of scaffolds and measurement of cellular infiltration. Biomacromolecules 2006 Oct;7(10):2796–2805. [PubMed: 17025355]
11. Nerurkar NL, Mauck RL, Elliott DM. ISSLS prize winner: Integrating theoretical and experimental methods for functional tissue engineering of the annulus fibrosus. Spine (Phila Pa 1976) 2008 Dec 1;33(25):2691–2701. [PubMed: 19018251]
12. Baker BM, Mauck RL. The effect of nanofiber alignment on the maturation of engineered meniscus constructs. Biomaterials 2007 Apr;28(11):1967–1977. [PubMed: 17250888]
13. Mauck RL, Yuan X, Tuan RS. Chondrogenic differentiation and functional maturation of bovine mesenchymal stem cells in long-term agarose culture. Osteoarthritis Cartilage 2006 Feb;14(2):179–189. [PubMed: 16257243]
14. Peltz CD, Perry SM, Getz CL, Soslowsky LJ. Mechanical properties of the long-head of the biceps tendon are altered in the presence of rotator cuff tears in a rat model. J Orthop Res 2009 Mar;27(3):416–420. [PubMed: 18924143]
15. Favata, M. Bioengineering PhD Thesis. University of Pennsylvania; 2006. Scarless healing in the fetus: implications and strategies for postnatal tendon repair.
16. Nerurkar NL, Elliott DM, Mauck RL. Mechanics of oriented electrospun nanofibrous scaffolds for annulus fibrosus tissue engineering. J Orthop Res 2007 Aug;25(8):1018–1028. [PubMed: 17457824]
17. Neuman RE, Logan MA. The determination of hydroxyproline. J Biol Chem 1950 May;184(1):299–306. [PubMed: 15421999]

18. Jungreuthmayer C, Donahue SW, Jaasma MJ, Al-Munajjed AA, Zanghellini J, Kelly DJ, et al. A comparative study of shear stresses in collagen-glycosaminoglycan and calcium phosphate scaffolds in bone tissue-engineering bioreactors. Tissue Eng Part A 2009 May;15(5):1141–1149. [PubMed: 18831686]

19. Nerurkar, NL.; Elliott, DM.; Mauck, RL. Architecture of nanofibrous scaffolds influences fibrocartilagenous gene expression of annulus fibrosus and mesenchymal stem cells. 6th Combined meeting of the Orthopaedic Research Society; October 20–24; 2007. 0388

20. Baker BM, Nathan AS, Gee AO, Mauck RL. The influence of an aligned nanofibrous topography on human mesenchymal stem cell fibrochondrogenesis. Biomaterials Aug;31(24):6190–6200. [PubMed: 20494438]

21. Gemmiti CV, Guldberg RE. Shear stress magnitude and duration modulates matrix composition and tensile mechanical properties in engineered cartilaginous tissue. Biotechnol Bioeng 2009 Nov 1;104(4):809–820. [PubMed: 19591192]

22. Zhang G, Young BB, Ezura Y, Favata M, Soslowsky LJ, Chakravarti S, et al. Development of tendon structure and function: regulation of collagen fibrillogenesis. J Musculoskelet Neuronal Interact 2005 Mar;5(1):5–21. [PubMed: 15788867]

23. Unsworth BR, Lelkes PI. Growing tissues in microgravity. Nat Med 1998 Aug;4(8):901–907. [PubMed: 9701241]

24. Nerurkar NL, Sen S, Huang AH, Elliott DM, Mauck RL. Engineering disc-like angle-ply structures for intervertebral disc replacement. Spine. 2009 (in press).

25. Skaggs DL, Weidenbaum M, Iatridis JC, Ratcliffe A, Mow VC. Regional variation in tensile properties and biochemical composition of the human lumbar anulus fibrosus. Spine 1994 Jun 15;19(12):1310–1319. [PubMed: 8066509]

26. Holzapfel GA, Schulze-Bauer CA, Feigl G, Regitnig P. Single lamellar mechanics of the human lumbar anulus fibrosus. Biomech Model Mechanobiol 2005 Mar;3(3):125–140. [PubMed: 15778871]

27. Fithian DC, Kelly MA, Mow VC. Material properties and structure-function relationships in the menisci. Clin Orthop 1990 Mar;(252):19–31. [PubMed: 2406069]

28. Baker, BM.; Nathan, A.; Mauck, RL. Tensile Loading Modulates Collagen Gene Expression in MSC-Laden Nanofibrous Scaffolds. 54th Annual Meeting of the Orthopaedic Research Society; March 2–5, 2008; San Francisco, CA. 2008.

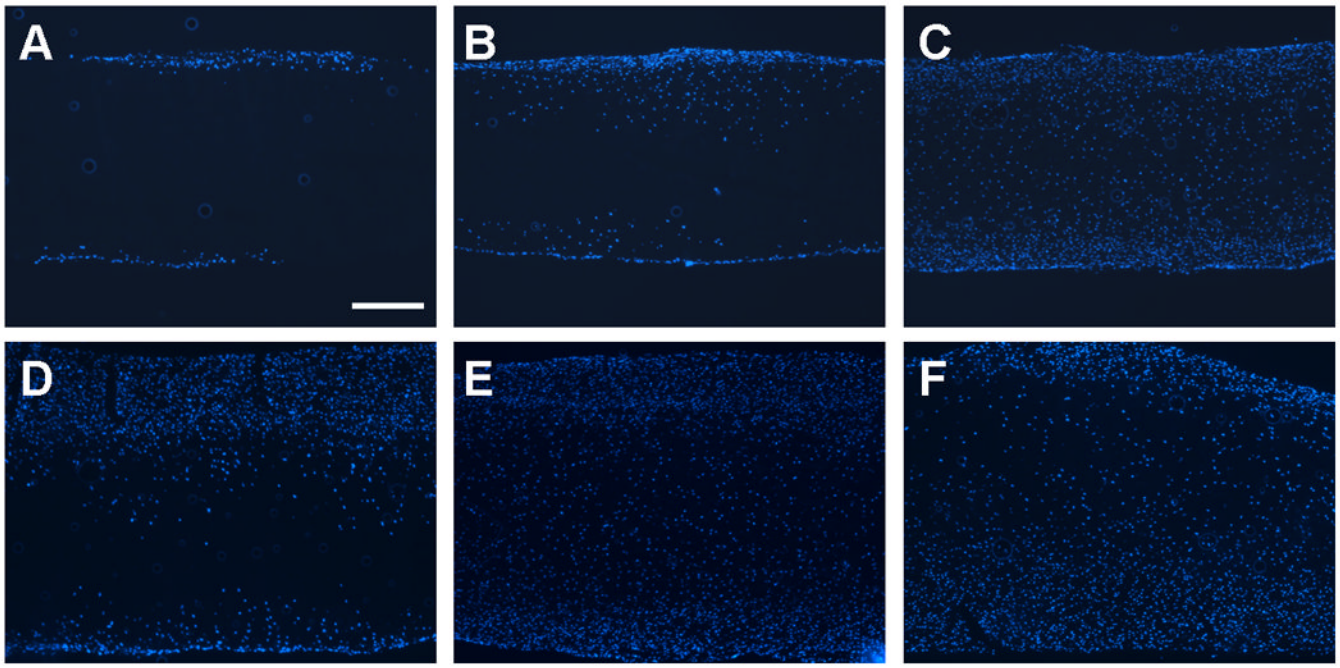

Fig. 1. MSC infiltration into aligned nanofibrous scaffolds

DAPI staining of cell nuclei reveals cell localization at the scaffold periphery at 0 weeks (A), partial infiltration at 6 weeks under free-swelling conditions (B), and enhanced infiltration with 6 weeks of dynamic culture (C). When the study was terminated at 12 weeks, free-swelling constructs still lacked cells in the central portion of the scaffold (D) while continuous (E) and transient (F) dynamic culture conditions resulted in full infiltration. Scale bar = 250 μm.

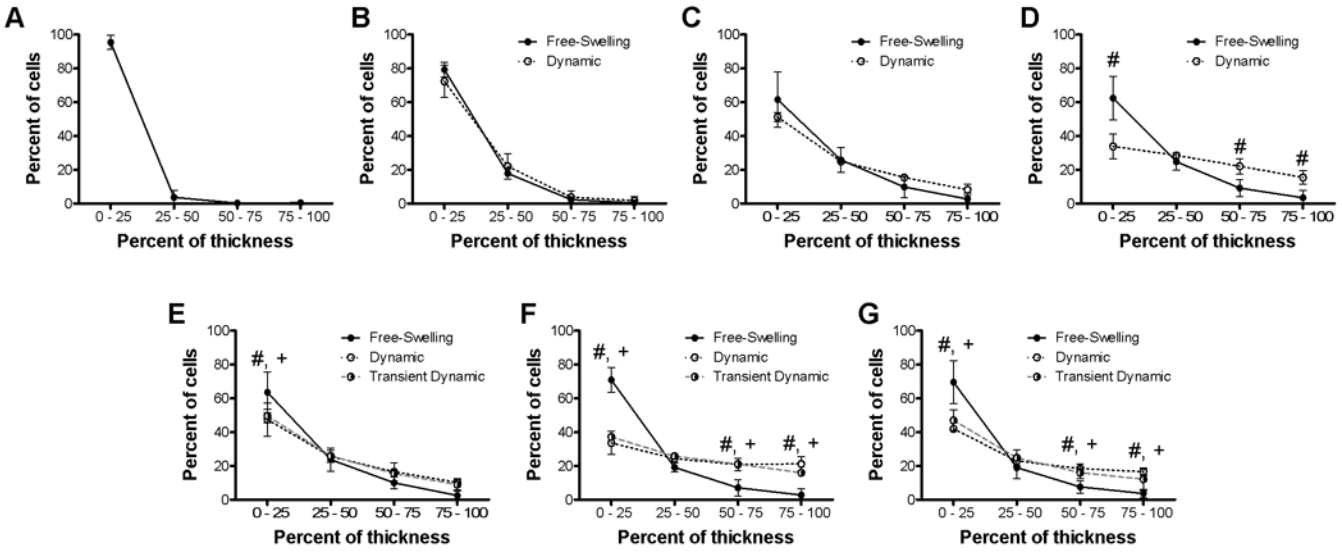

Fig. 2. Quantification of cellular infiltration

Shown at 0 (A), 2 (B), 4 (C), 6 (D), 8 (E), 10 (F), and 12 (G) weeks, infiltration is enhanced under dynamic and transient dynamic culture conditions. #, +, and † indicate $p < 0.05$ for free-swelling vs. dynamic, free-swelling vs. transient dynamic, and dynamic vs. transient dynamic, respectively, at a given time point.

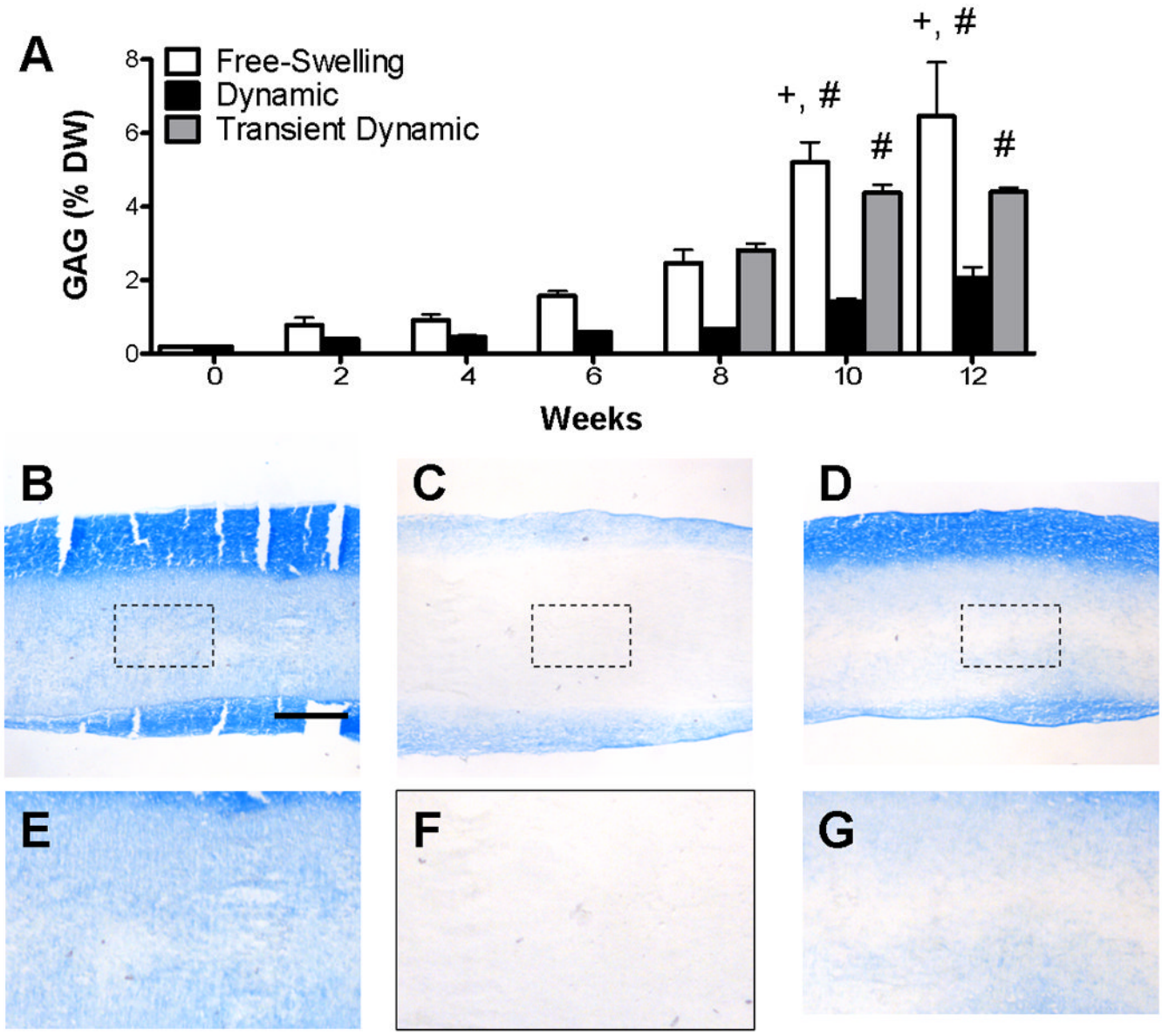

Fig. 3. Effect of dynamic culture on GAG content

Biochemical measurement (A) reveals depletion in GAG content due to dynamic culture conditions, and a partial recovery following removal from dynamic culture. In all three culture conditions, GAG increased significantly with time in culture ($p < 0.05$). Alcian Blue stains at 12 weeks confirm biochemical findings, with more intense staining of free-swelling (B) constructs compared to dynamic (C) and transient dynamic (D). Regions indicated in B, C, and D are enlarged in E, F, and G respectively. + and # indicate $p < 0.05$ vs. transient dynamic and dynamic culture, respectively. Scale bar = 250 μm (B–D) and 62.5 μm (E–G).

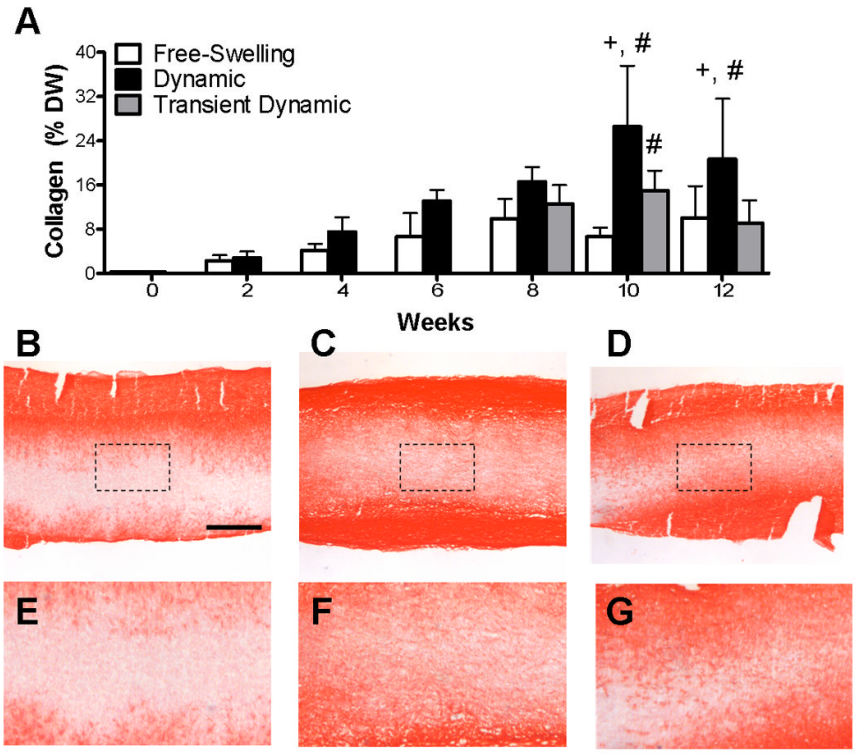

Fig. 4. Effect of dynamic culture on collagen content

Biochemical measurement (A) reveals an increase in collagen content due to dynamic culture conditions, and a partial loss following removal from dynamic culture. In all three culture conditions, collagen increased significantly with time in culture ($p < 0.05$). Picrosirius Red stains at 12 weeks confirm biochemical findings, with limited staining of free-swelling constructs (B) compared to dynamic (C) and transient dynamic constructs (D). Central scaffold regions indicated in B, C, and D are enlarged in E, F, and G respectively. + and # indicate $p < 0.05$ vs. transient dynamic and free-swelling culture, respectively. Scale bar = 250 μm (B–D) and 62.5 μm (E–G).

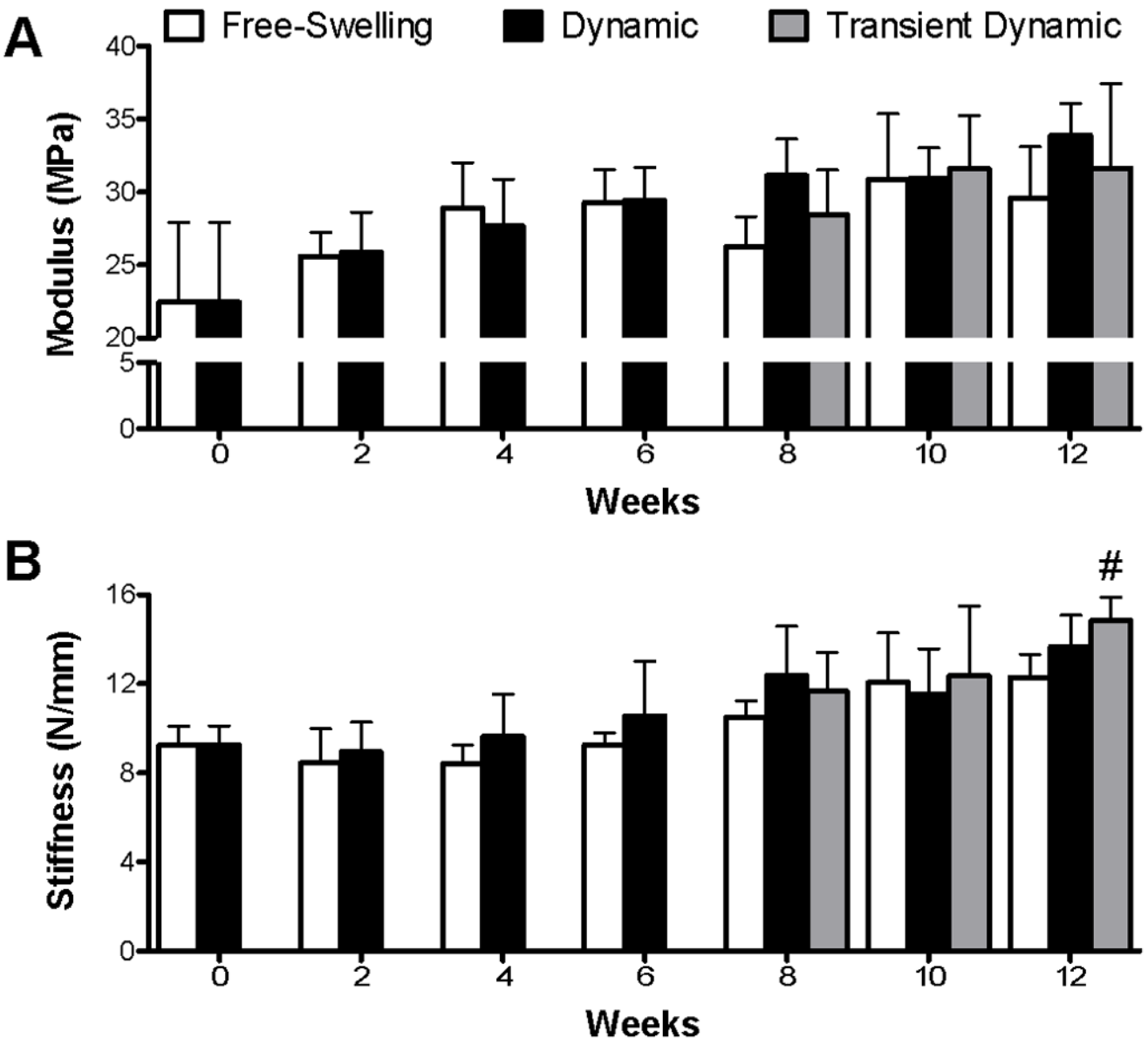

Fig. 5. Effect of dynamic culture on functional maturation

Tensile modulus (A) and stiffness (B) increased significantly with culture duration for all three conditions ($p < 0.05$). No differences in modulus was observed between free-swelling, dynamic, and transient dynamic culture at any time point, however at 12 weeks dynamic transient constructs were significantly stiffer than free swelling controls (indicated by #, $p < 0.05$).