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A sulfated nanofibrous mesh supporting the osteogenic differentiation of periosteum-derived cells

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Abstract

The periosteum is a thin fibrous membrane covering the surface of long bone and is known to play a critical role in bone development and adult bone fracture healing. Loss or damage of the periosteum tissue during traumatic long bone injuries can lead to retarded healing of bone graft-mediated repair. The regenerative potential of periosteum-derived progenitor cells (PDCs) has inspired their use as an alternative to bone marrow-derived mesenchymal stromal cells (MSCs) to augment scaffold-assisted bone repair. In this study, we first demonstrated that PDCs isolated from adult rat long bone exhibited innate advantages over bone marrow-derived MSCs in terms of faster proliferation and more potent osteogenic differentiation upon induction in plastic-adherent culture. Further, we examined the potential of two electrospun nanofibrous meshes, an uncharged regenerated cellulose mesh and a sulfated mesh, to support the attachment and osteogenic differentiation of PDCs. We showed that both nanofibrous meshes were able to support the attachment and proliferation of PDCs and MSCs alike, with the sulfated mesh enabling significantly higher seeding efficiency than the cellulose mesh. Both meshes were also able to support the osteogenic differentiation of adherent PDCs upon induction by osteogenic media, with the sulfated mesh facilitating more potent mineral deposition by adherent PDCs. Our study supports the sulfated nanofibrous mesh as a promising synthetic periosteal membrane for the delivery of exogenous PDCs to augment bone healing.

1. INTRODUCTION

Scaffold-assisted skeletal tissue repair aims to utilize bioactive constructs engineered with a proper biochemical and/or cellular microenvironment to promote healing.¹ The delivery of exogenous pluripotent cells via the biomaterial scaffold into a defect with impaired cellular functions could expedite the otherwise retarded healing.^{2,3} These exogenous progenitor cells, when supported by a proper microenvironment, can contribute to the healing process by differentiating into desired lineages, producing extracellular matrices, and/or exerting paracrine effects to impact the function of endogenous resident cells. The complex interplay between a biomaterial scaffold and its resident cells has been increasingly recognized, with many scaffolds engineered to impact cellular attachment and/or differentiation.^{4,5} For instance, scaffolds have been functionalized with chemical ligands mimicking those present

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in native extracellular matrices to enhance interactions with both exogenous growth factors/cells and endogenously recruited soluble factors/cells.^{4,6} Additionally, physical properties of biomaterials, such as stiffness, porosity, rate of degradation and surface topography, all shown to affect cellular behavior, have also been increasingly incorporated as design parameters of synthetic tissue scaffolds.^{6,7}

The choice of proper exogenous progenitor cells is just as important as the design of the biomaterial scaffold. Among the progenitor cells for bone repair, bone marrow-derived mesenchymal stromal cells (MSCs) have been the most commonly integrated with biomaterial constructs in scaffold-assisted bone repair and regeneration.^{6,8} However, variations in both isolation techniques (e.g. flushing of the marrow cavity,^{9–11} enzymatic digestion of whole bone marrow plug,¹² crushing whole cortical bone with intact bone marrow cavity)¹³ and the lack of a standardized method for enriching MSC populations prior to seeding/implantation (e.g. plastic-adherent cultures,^{9,14} colony-forming units^{15,16} or various combinations of cell surface markers in combination with fluorescence activated cells sorting, FACS, have all been used to enrich for or characterize MSC populations)^{13,17,18} have led to significant variations in their *in vivo* efficacies in promoting bone repair.^{19,20} Meanwhile, several alternative sources of bone progenitor cells have been under investigation including but not limited to the adipose tissue, skeletal muscle, periosteum, and umbilical cord.²¹ Overall, it is still difficult to generalize the superiority of one particular source of progenitor cells over the others due to incomplete understanding of their biology, variations of their intrinsic properties by species, as well as difficulty in obtaining highly purified populations.²²

The periosteum is a thin, bilayer membrane (outer fibrous layer and inner cambium layer) surrounding bone that is essential for bone development (e.g. appositional bone growth) and adult bone fracture repair.^{23–25} The cambium layer is a rich source of vasculature, progenitor cells, and osteoblasts. Thus, periosteal-derived cells (PDCs) have garnered recent attention as an excellent source of progenitor cells for bone repair.²⁶ PDCs contain multipotent cells that may have been “primed” for osteo/chondral differentiations.¹⁹ For instance, during the initial inflammation stage post-fracture, PDCs quickly participate in the external callus formation and subsequent endochondral ossification.^{25,27} PDCs have also been shown to facilitate bone graft-mediated repair of critical-size long bone defects in mice and have been increasingly recognized as a critical source of progenitor cells that one should reconstitute for the successful repair of traumatic long bone injuries where the natural periosteum is often severely damaged or lost.^{28–32} Indeed, PDCs have been combined with biomaterials in lieu of the bone marrow-derived MSCs for various bone tissue engineering applications.³³ Like bone marrow-derived MSCs, the differentiation of PDCs can also be profoundly impacted by the scaffold environment that they adhere to, underscoring the importance of choosing a suitable biomaterial scaffold for the delivery of exogenous PDCs to promote bone healing.³⁴ In this study, we demonstrate the advantages of rat PDCs over bone marrow-derived MSCs in terms of their *in vitro* proliferation and osteogenic differentiation potential. Further, we compare the efficacy of an uncharged electrospun cellulose fibrous mesh and a negatively charged sulfated electrospun fibrous mesh recently

developed in our laboratory³⁵ in supporting the cellular attachment, proliferation and osteogenic differentiation of PDCs.

2. MATERIALS AND METHODS

2.1 MSC and PDC isolations

All animal procedures were approved by the University of Massachusetts Medical School Animal Care and Use Committee. Bone marrow derived MSCs and PDCs were isolated from long bones of 4-week old male Charles River SASCO SD rats.

MSCs were isolated as previously described.³⁶ Briefly, marrow was gently flushed from the marrow cavity of femurs and tibiae with a syringe containing alpha-minimum essential medium (α -MEM) without ascorbic acid (Invitrogen, Grand Island, NY). The whole bone marrow was pelleted and red blood cells were lysed with sterile water. The cells were pelleted again by centrifugation and resuspended in expansion media (α -MEM without ascorbic acid supplemented with 20% FBS, 1% penicillin-streptomycin and 1% l-glutamine), and passed through a sterile swinney filter. Cells were plated on tissue culture plates at an initial seeding density of 10 million cells per 100-mm plate. Media were changed on day 4 and 3 times a week thereafter prior to replating cells for subsequent experiments.

PDCs were isolated from the rat femurs and tibiae by enzymatic digestion. Skeletal muscles were first trimmed away from the bones to expose the periosteal tissue. Periosteum was then carefully scraped off from the bone surface and digested in an α -MEM (without ascorbic acid) solution of 2.5-mg/mL type 2 collagenase (Worthington, Lakewood, NJ) under agitation for 2 h at 37°C. Cells were pelleted from the digestion solution, resuspended in expansion media and plated on 100-mm tissue culture plates (PDCs isolated from each leg were plated on 2 plates). Media were changed on day 4 and 3 times a week thereafter before the cells were replated for subsequent experiments.

2.2 Growth curve and beta gal staining

Passage 2 PDCs and MSCs were plated in 6-well tissue culture plates (n=3) with an initial seeding density of 20,000 cells/well, and cultured in expansion media for up to 7 days with media changes 3 times a week. Cell numbers were quantified daily using a Beckman Coulter Z1 particle counter. In a parallel set of experiments (n=2), cells were stained on days 1, 4, and 6 for β -galactosidase activity as an indicator for senescence as previously described.³⁷

2.3 Osteogenic differentiation induction of cells adhered on tissue culture plates

Passage 2 MSCs and PDCs were plated in 6-well plates at a seeding density of 25,000-cells/cm² in expansion media. When 90% confluence was reached, the expansion media were replaced with osteogenic media (expansion media supplemented with 10 nM dexamethasone, 20 μ M β -glycerol phosphate, and 50 μ M 1-ascorbic acid 2-phosphate) and cultured for 21 days with media changes 3 times a week before they were fixed for alizarin red staining.

2.4 Electrospun nanofibrous mesh preparations

Cellulose and sulfated nanofibrous meshes were prepared by electrospinning and subsequent chemical modifications as previously described.³⁵ Briefly, electrospun cellulose acetate (CA) fibrous mesh was prepared by ejecting a trifluoroethanol solution of CA (150 g/L) at a rate of 2.4 mL/h under 15 kV with a distance of 10 cm between the ejection tip and the collection plate. After 4 h of electrospinning, the mesh was annealed on a Carver hydraulic hot press under 25.85-MPa compressive loading for 10 min at 90 °C. The CA meshes were hydrolyzed with NaOH solution (0.1 N, 1:4/EtOH:H₂O) at room temperature for 12 h to obtain the regenerated cellulose (RC) mesh. To produce sulfated cellulose (SC) fibrous meshes, the RC meshes were oxidized in a PBS solution of NaIO₄ (5 mg/mL) at room temperature for 10 h, followed by reductive amination with 2-aminoethyl sulfate (0.05 g/mL, PBS, pH 7.4) and NaBH₃CN (2.5 mg/mL, PBS, pH 7.4) at room temperature for 12 h. All meshes were extensively washed in MilliQ water following each chemical modification step. All meshes were sterilized with 70% ethanol and ultraviolet light, then equilibrated in PBS prior to cell culture use.

2.5 Cell viability on fibrous meshes

The viability of passage 2 PDCs and MSCs adhered on the RC and SC meshes over time was determined using the Cell Counting Kit-8 (CCK-8) assay according to vendor instructions (Dojindo, Japan). The absorbance was read at 450 nm with a background correction at 620 nm on a Multiskan FC Microplate Photometer (Thermo Scientific, Billerica, MA).

2.6 Osteogenic differentiation of PDCs on fibrous meshes

Passage 2 PDCs were seeded at a density of 100,000 cells/cm² on sterile fibrous RC and SC meshes in expansion media and allowed to adhere overnight before being further cultured in osteogenic media or in expansion media (negative control) for 7 and 14 days. Medium was changed 3 times a week until the cells were fixed with 4% formaldehyde for staining or subjected to RNA isolation at these time points.

2.7 Alizarin red and alkaline phosphatase staining

Following osteogenic differentiation induction, cells were fixed on their respective substrates (tissue culture plates, RC or SC meshes) with 4% formaldehyde for 1 h at room temperature. Fixed cells were then stained with alizarin red (1 g/100mL MilliQ H₂O, Sigma-Aldrich, St. Louis, MO), a negatively charged dye for the detection of mineralized matrix, or alkaline phosphatase (Sigma-Aldrich, St. Louis, MO) for qualitative assessment of the extent of the induced osteogenic differentiation.

Alizarin red staining from RC and SC meshes after 2 weeks of osteogenic differentiation was solubilized by acetic acid³⁸ followed by hydrochloric acid and quantified by absorbance at 405 nm. Briefly, the dyes absorbed on the meshes were first extracted by 10% acetic acid. As 10% acetic acid was not able to completely solubilize the dye tightly bound to the negatively charged SC mesh, all stained meshes were further treated with 0.5N HCl (aq.) to completely solubilize the mesh-bound dye. The combined acidic extracts were neutralized

with 10% ammonium hydroxide before the absorbance was read at 405 nm on a Multiskan FC Microplate Photometer.

2.8 RNA isolation and qPCR

Following 7 and 14 days in osteogenic culture, total RNA from the PDCs adhered on the RC and SC meshes as well as PDCs prior to seeding on meshes (time 0 control) was isolated using TRIzol (Invitrogen, Carlsbad, CA) and purified by Direct-Zol miniprep (Zymo Research, Irvine, CA) following vendor instructions. RNA was reverse transcribed into cDNA with SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA) according to vendor instructions on a GeneAmp 2700 PCR system (Applied Biosystems, Foster City, CA). qPCR was carried out on an Applied Biosystems 7500 Fast Real-Time PCR system with TaqMan Gene Expression Master Mix (Applied Biosystems, Foster City, CA) and inventoried TaqMan probes for bone gamma-carboxyglutamate protein (BGLAP), also known as osteocalcin, and housekeeping gene GAPDH. Gene expression was quantified using the delta-delta Ct method. Expression of each gene of interest at each time point (n=3) was normalized using GAPDH and plotted as expression relative to that of PDCs at time 0.

3. RESULTS AND DISCUSSION

3.1 Proliferation and osteogenic differentiation of PDCs and MSCs on tissue culture plastics

To compare the proliferation rates of PDCs and MSCs, passage 2 cells were plated on tissue culture plastics and their proliferations were monitored over 7 days. PDCs proliferated faster than MSCs, with approximate 2.8-fold more PDCs than MSCs obtained by day 4 (Fig. 1A). Confluence of PDC culture was reached by day 5 while that of the MSC culture was not reached until day 6. The confluence of the PDC culture was also accompanied by some positive (blue) staining for β -galactosidase activity on day 6 due to contact inhibition (Fig. 1B). Both PDCs and MSCs adhered on tissue culture plastics were able to undergo robust osteogenic differentiation, with PDCs exhibiting more intense staining for mineralized matrix by alizarin red after a 21-day osteogenic induction (Fig. 1C). Overall, the more rapid *in vitro* proliferation and more potent osteogenic differentiation of PDCs upon culture induction could be advantageous for both the culture expansion of this progenitor cell and its subsequent use for bone tissue engineering applications.

3.2 Seeding efficiency and proliferation of PDCs and MSCs on RC and SC meshes

To determine the feasibility of using electrospun RC or SC nanofibrous meshes recently developed in our laboratory (Fig. 2A)³⁵ to deliver skeletal progenitor cells, passage 2 MSCs and PDCs were seeded on these scaffolds and cultured in expansion media. We previously showed that the sulfated mesh, mimicking the naturally occurring sulfated polysaccharides abundantly present in skeletal tissue matrices,^{39–41} were able to better support the attachment and osteogenic differentiations of MSCs.³⁵ Here we show that MSCs and PDCs were able to attach to a given mesh type with comparable seeding efficiencies, but the SC mesh supported significantly higher seeding efficiencies for both MSCs and PDCs than the uncharged RC mesh (Fig. 2B). The negatively charged SC mesh may have enabled better sequestration of secreted soluble factors or serum proteins from the medium, mimicking the

protein-sulfated glycosaminoglycan interactions in natural ECM matrix,⁴¹ which in turn resulted in more efficient cell adhesion (higher seeding efficiency). All cells adhered on these meshes were able to proliferate at comparable rates, with no statistically significant differences detected between MSCs and PDCs adhered on a given mesh type or between RC and SC meshes for a given cell type (Fig. 2C). This observation, in contrast to the faster PDC proliferation observed on tissue culture plastics (Fig. 1A), may indicate that these progenitor cells are more poised for differentiation when adhered to the fibrous mesh microenvironment. Overall, these data suggest that the SC mesh is advantageous over the uncharged RC mesh in terms of supporting the attachment of PDCs and MSCs with significantly higher seeding efficiencies. This is an important consideration for the delivery of progenitor cells via a synthetic tissue scaffold.

3.3 Osteogenic differentiation of PDCs on RC and SC meshes

We have previously demonstrated that the SC mesh more readily support the osteogenic differentiation of MSCs than the uncharged RC mesh.³⁵ Given the advantages of PDCs over MSCs presented above, here we further explore how SC and RC may differentially support the osteogenic differentiation of PDCs. Alizarin red staining revealed far more bone mineral depositions by PDCs adhered on the SC mesh than on the RC mesh at 7 and 14 days post-osteogenic induction (Figs. 3A & B), suggesting that the sulfated mesh expedited the osteogenesis of PDCs and supported more potent bone matrix deposition. The ability of the sulfate residues of the SC mesh to sequester/retain endogenous factors secreted by the progenitor or differentiating cells and the soluble factors from the osteogenic media may have contributed to the more robust osteoblastic differentiation. Indeed, the ability of natural ECM component chondroitin sulfate to promote early bone healing^{42,43} has in part been attributed to its affinity for a wide range of endogenous proteins.^{41,43} Furthermore, the ability of the negatively charged SC mesh to sequester calcium ions may have also facilitated the more effective mineral deposition by the mature, terminally differentiated osteoblasts across the mesh (Fig. 3B). In comparison, the uncharged RC mesh supported the deposition of smaller clusters of minerals by the adherent cells (Fig. 3B). Staining for alkaline phosphatase (ALP), an earlier marker of osteogenic differentiation, revealed more ALP-positive (red) cells on the SC mesh than on the RC mesh at 1 week while the differences at 2 weeks were minimal (Fig. 3C). Both the number of ALP-positive cells and the intensity of the staining (stained over a more spread-out area) increased from 1 to 2 weeks on the RC and SC meshes (Fig. 3C), supporting more robust osteogenic differentiation of PDCs over time on both meshes.

CCK-8 proliferation assay revealed significantly more viable cells on SC mesh than on RC mesh after 1-week osteogenic induction (Fig. 4A), likely resulting from the significantly higher initial PDC seeding efficiencies supported by the SC mesh. Interestingly, only the RC mesh supported further increases of viable cells from 1 week to 2 week post-osteogenic induction, suggesting that the PDCs adhered to the SC mesh underwent more potent osteogenic differentiation at the expense of proliferation. This observation correlates well with the more intense alizarin red staining observed with the terminally differentiated mature osteoblasts on the SC mesh by 2 weeks.

Although the gene expression of osteocalcin, a late-stage marker of osteogenesis, increased over time for PDCs adhered on both meshes upon culture induction, its expression by cells adhered on the RC mesh were far more than by those adhered on the SC mesh at both 1 and 2 weeks (Fig. 4B). One possible explanation for this surprising observation that conflicts with the trends observed with the alizarin red staining is that the PDCs adhered on the RC mesh were attempting to compensate for the less effective mineral deposition (e.g. due to less calcium ions attracted to the uncharged mesh) by up-regulating the gene expression of osteocalcin, a protein known to regulate/template mineralization.⁴⁴ It was previously reported that the expression of osteogenic markers by PDCs upon osteogenic induction does not always correlate with the extent of mineral depositions,³⁴ and that similar osteocalcin expression by MSCs and PDCs in response to osteogenic induction does not necessarily translate into similar levels of mineral deposition.⁴⁵ Cautions should be exercised in assessing osteogenesis outcome. A more systemic examination of multiple early-, mid- and late-stage osteogenic markers at both gene expression and protein expression levels along with the profiling of secreted factors and mineralized matrix deposition in a temporally defined manner may help elucidate the mechanism by which the chemical and topological environment of these fibrous meshes impact the fate of the adherent progenitor cells.

4. CONCLUSIONS

Periosteal derived cells (PDCs) exhibited greater proliferation and osteogenic differentiation potential than bone marrow derived stromal cells (MSCs) on both tissue culture plastics and electrospun nanofibrous meshes. A sulfated nanofibrous mesh was shown to exhibit significant advantage over an uncharged nanofibrous cellulose mesh in giving rise to higher seeding efficiency of progenitor cells and supporting more abundant mineral deposition by PDCs upon osteogenic inductions. The sulfated nanofibrous mesh combined with PDCs holds great promise as a synthetic periosteum substitute for expediting the repair of skeletal defects. Current work in our laboratory explores the *in vivo* efficacy of this synthetic periosteum platform in augmenting allograft-mediated repair of critical-size long bone defects.

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Figure 1. Proliferation and osteogenic differentiation of PDCs and MSCs on tissue culture plastics
 (A) Growth curves over 7 days in expansion media; Error bars: standard deviation of the mean (n=3). (B) β -Galactosidase staining of cultures in expansion media (5x objective), with arrows indicating β -Galactosidase positive (blue) cells. (C) Alizarin red staining 21 days after osteogenic induction; Scale bars: 100 μ m.

Figure 2. Attachment and proliferation of PDCs and MSCs on RC and SC meshes
 (A) Chemical structures of RC and SC and corresponding SEM micrographs of the meshes. Scale bars: 10 μ m for higher resolution insets; 40 μ m for lower resolution micrographs. (B) Seeding efficiencies of PDCs and MSCs at day 1 on RC or SC mesh as determined by CCK-8 assay (n=3). (C) Proliferation rate of MSCs and PDCs on RC or SC mesh as determined by fold of change in cellular activity over 72 h (from day 1 to day 4 after initial cell seeding) by CCK-8 assay (n=3). Error bars: standard deviation of the mean. *p<0.05, NS: not significant (student's *t* test).

Figure 3. Alizarin red and alkaline phosphatase (ALP) staining of PDCs on RC and SC meshes upon osteogenic induction
 (A) Macroscopic images of alizarin red stained RC and SC meshes with and without seeded PDCs 1 and 2 weeks after osteogenic induction; Scale bar: 6.8 mm. No cell mesh controls revealed minimal non-specific absorption of alizarin red dye on the meshes. (B) Top: Micrographs of the alizarin red stained meshes 2 weeks after osteogenic induction (zoomed-in over the squared regions shown in (A)), scale bars: 100 μm; Bottom: quantification of alizarin red staining 2 weeks after osteogenic induction; Error bars: standard deviation of the

mean (n=3); *p<0.05 (student's *t* test). (C) Micrographs of ALP-stained RC and SC meshes with seeded PDCs 1 and 2 weeks after osteogenic induction, with arrows indicating ALP positive (red) cells at 1 week; scale bars: 100 μ m.

Figure 4. Cellular proliferation and osteocalcin gene expression of PDCs on RC and SC meshes upon osteogenic induction

(A) PDC proliferation on RC and SC meshes from 1 to 2 weeks upon osteogenic induction as determined by CCK-8 assay (n=3). (B) Osteocalcin gene expression of PDCs on RC and SC meshes at 1 and 2 weeks upon osteogenic induction relative to time 0. Data are normalized with housekeeping gene GAPDH and plotted as expression relative to that prior to seeding onto the meshes (time 0). Error bars: standard deviation of the mean. *p<0.05; **p<0.001 (student's *t* test).