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Nanofiber/Microsphere Hybrid Matrices *In Vivo* for Bone Regenerative Engineering: A Preliminary Report

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Abstract

The demand for bone grafts has led to advances in regenerative engineering, a field at the intersection of advanced biomaterials, stem cell science, physics, developmental biology, and clinical translation. In this work, the authors evaluated a hybrid nanofiber/microsphere matrices both *in vitro* and *in vivo* for its ability to promote bone regeneration. Quantitative measures of cellular characteristics *in vitro* showed a higher fraction of marrow stromal cells with collagen promoter activity on hybrid matrices compared to control matrices (41% vs. 24%, $p = 0.02$). Control and hybrid matrices were then implanted for 6 weeks in calvarial defects of mice, and the animals received a single injection of calcein 1 day prior to sacrifice to visualize bone formation. Cryohistology of the undecalcified implants were evaluated for markers of bone mineralization, which revealed evidence of higher levels of bone tissue formation in hybrid matrices compared to controls. These data provide support that nanofiber-permeated, sintered, composite microsphere matrices may be a particularly useful matrix for the regenerative engineering of bone.

Keywords

Nanofiber; microsphere; hybrid; *in vivo*

Introduction

Autograft bone is widely used in orthopaedic clinics to augment bone loss, but its access and isolation a painful procedure that can add to the morbidity of a surgery that already carries a risk of high complications.¹² Because of this, researchers from diverse backgrounds

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including materials science and chemical engineering have sought to create synthetic bone graft substitutes that perform as well as or better than autografts. In designing these advanced materials, groups have sought to mimic the physical, chemical, biological, and material characteristics of natural human bone.

One such example from our group has been to use sintered, composite ceramic/polymer microspheres.³ The sintered microspheres provide mechanical integrity on par with human trabecular bone, an interconnected pore network to allow cellular infiltration and nutrient exchange, a reservoir of calcium and phosphate ions, and predictable degradation that allows the body time to regenerate lost tissue at the site of injury.^{4–6} Recently our group has created matrices with nanofibers placed in the pore spaces of these sintered microsphere matrices.³ Since natural human bone has large quantities of extracellular matrix (ECM), incorporation of such matrix elements in an advanced biomaterial matrix is well reasoned.

Early studies of this nanofiber-microsphere matrix demonstrated that the process for fabricating nanofibers within the matrices did not impact their mechanical integrity. These hybrid matrices were shown to support the adherence and proliferation of the immortalized mouse pre-osteoblast cell line MC3T3-E1.³ Furthermore, these constructs were shown to influence the expression of Runx2 and osteocalcin, two important markers of osteoblast differentiation and function.⁸ To have a successful clinical application, however, the efficacy of these matrices needs to be tested both *in vitro* and *in vivo* to determine their ability to function as bone graft substitutes. Furthermore, when selecting appropriate models with which to test these advanced materials, the clinical application must be kept in mind. While the mouse calvaria-derived MC3T3-E1 cells are widely available and well-studied, they are immortalized and therefore have limited clinical application.⁹ *In situ*, the matrices are likely to encounter a diverse, non-immortalized, and heterogeneous population of cells, more than one of which may potentially mitigate a favorable regenerative response *in vivo*. It is for this reason that the authors decided to use bone marrow derived stem cells (BMSCs) obtained from fresh bone marrow. Matrices implanted in a critical sized defect are likely to potentially encounter populations of cells that more closely resemble BMSCs, and thus any conclusions that we may draw from the material interaction with these cells may potentially be more likely to translate *in vivo*.

Recent genetic advances in the field of developmental biology have led to tools that can evaluate growth and differentiation of particular groups of cells (fluorescent reporters). Col3.6 mice have the ability to express fluorescent proteins in a bone-restricted lineage, and thus can offer a number of advantages over wild type mice with respect to monitoring phenotypic progression, and α SMA-mCherry mice have the ability to express fluorescent proteins maintaining a stem cell phenotype.^{10,11} The work presented in this paper seeks to investigate the biological effects of nanofiber-permeated, sintered, composite microspheres through *in vitro* analysis with mouse-derived fluorescent reporter BMSCs and *in vivo* analysis in calvarial defects in wild-type mice.

Materials and Methods

Matrix Fabrication

17% hydroxyapatite (HA)/poly(L-lactide) (PLLA) microspheres were fabricated according to the modified procedure of Borden *et al.*⁵ Briefly, high molecular weight PLLA (Purac, Netherlands) was purchased and dissolved in dichloromethane (DCM) (ThermoFisher Scientific, Waltham, MA). 17% by weight nanocrystalline HA (Berkeley Biomaterials, Berkeley, CA) was then suspended by agitation. The resulting suspension of HA and PLLA in DCM was then added dropwise into a solution of polyvinyl alcohol (PVA) and stirred at a speed of 250 RPM. The DCM was then allowed to evaporate overnight as the composite microspheres formed in the oil/water immersion. The composite microspheres were then vacuum filtered from the PVA solution, washed with DI water, and lyophilized for at least 24 hours to remove any excess solvent. Once dried, the microspheres were placed in a stainless steel sintering mold and heated for 90 minutes to sinter adjacent microspheres. The resulting sintered, composite microspheres were used for control groups in this study. To create hybrid matrices, nanofibers were created in the pore spaces of the sintered microspheres matrices. To precipitate nanofibers, the thermally induced phase separation (TIPS) procedure of Ma *et al.*, was followed.¹² Briefly, the sintered composite microspheres were submerged in a separate 1% PLLA solution in N,N-dimethylformamide (DMF). The solutions were then rapidly cooled and submerged in deionized water for three days with a water change every day. The hybrid matrices were then placed in the freezer and subsequently lyophilized to remove all water.

Animal Studies

All animal studies were accomplished in compliance with the Institutional Animal Care and Use Committee at the University of Connecticut Health Center. Three types of mice were used in this study: (1) wild type mice (WT) for the *in vivo* implantation study, (2) mice with a cyan fluorescent protein under the control of a collagen promoter fragment (Col3.6-cyan) (residence study), (3) and mice with both Col3.6-cyan and a mCherry fluorescent protein under the control of an alpha smooth muscle actin promoter (α SMA-mCherry) (cell residence images). For the WT mice, 4 female animals were used of age 6 to 8 weeks. For the Col3.6-cyan and Col3.6-cyan/mCherry studies, one single female mouse of age 6 weeks was used to extract and culture bone marrow stromal cells (BMSCs).

Cell Culture

BMSCs were isolated from adult (>6 weeks of age) tibias and femurs of mice. After isolation, BMSCs were cultured in growth medium (α MEM, 10% FBS, 1% penicillin/streptomycin) on tissue culture polystyrene (TCP). After 3 days of cell culture, half of the medium was exchanged for fresh cell culture medium, and all of the media was changed at 6 days post-harvest. After 7 days in culture, the cells were released from the TCP surface using trypsin (ThermoFisher), counted, and 5×10^4 cells were implanted on 5 mm $\times$ 10 mm cylindrical matrices. The cells were then allowed to adhere to the matrix for one hour at 37 °C in a 24 well plate (CytoOne, USA Scientific) in wells coated with poly(2-hydroxyethyl methacrylate) (polyHEMA) (Sigma-Aldrich, St. Louis, MO) to prevent cells that did not adhere to the matrix from occupying the surface of the well. The wells were then

filled with 2 mL of a differentiation medium of α MEM, 10% FBS, 1% penicillin/streptomycin (ThermoFisher), 50 μ g/mL ascorbic acid (Sigma), and 10 nM dexamethasone (Sigma), with media changed every three days.

BMSC phenotypic fluorescence assay

Cell migration and phenotype was determined by seeding 10^6 Col3.6-cyan BMSCs on the 3.5 mm x 1 mm cylindrical matrix either with nanofibers (1% PLLA/DMF) or no nanofibers (control) and allowing the cells to adhere for 1 hour in a 24 well plate coated with poly(2-hydroxyethyl methacrylate) (polyHEMA) to prevent cells from adhering to the underlying TCP. After 21 days in culture, the cell-seeded matrices were fixed with 10% formalin for one hour, soaked in 30% sucrose in PBS for overnight, then embedded in cryomatrix (ThermoFisher) for cryosectioning. 10 μ m sections were taken every 100 μ m through the matrix, and the three sections closest to the middle (500 μ m, 600 μ m, and 700 μ m) were plated on charged microscope glass (Denville Scientific) with a 1% chitosan (Sigma) mixture. The slides were allowed to dry, and then cover slipped in a 50% glycerol (Sigma)/PBS with a 1:1000 dilution of Hoechst 33342 dye (Sigma). The resulting slides were then imaged for either early osteogenic activity (Col3.6-cyan) or nuclear signal (Hoechst).

Cell Residence Image analysis

To examine the effect of nanofibers leading to enhanced cell residence, histological sections were taken at the midpoint of the matrix in cross sections to measure (1) the total number of cells present on the section as measured by the nuclear staining Hoechst dye, (2) the number of cells undergoing early osteogenic differentiation as measured through cyan-GFP expression, and (3) the distances of all cells from the periphery of the matrix to the interior. To determine the distribution of evidence for collagen promoter activity as a measure for osteogenic differentiation, the image analysis program ImageJ and its associated software bundle (Fiji) were used. A region of interest was drawn around the periphery of the cross section of the matrix. The fluorescent images of the cross section were then converted to a binary image, and the distance from each particle to the boundary for the region of interest was calculated in pixels and then normalized to the size of the scaffold. The resulting histogram for the distances of all of the particles was then plotted, allowing comparison between all cells (positive for Hoechst) and cells undergoing early osteogenic differentiation (positive for cyan-GFP) as shown in figure 2A-I. Gating on the signal for Hoechst allowed removal of false-positive signal from matrix autofluorescence.

Matrix Implantation

For *in vivo* biocompatibility studies, 4 CD-1 background (WT) mice were selected. The mice were provided ad libitum access to food and water. The mice were harvested by CO₂ asphyxiation, and the calvaria were resected and placed in 10% formalin solution for fixation. After fixation, the crania were submerged in 30% sucrose/PBS solution prior to cryofixation for frozen sectioning in Cryomatrix™ (ThermoFisher). Sections were then taken in the coronal plane from each mouse that contained both matrix types (hybrid and control) and stained for tartrate resistant acid phosphatase (TRAP) activity, alkaline phosphatase activity (AP), calcein (new bone formation), Hoechst (to stain nuclei), and hematoxylin.

Fluorescence Staining and Analysis

Fluorescent images of histological sections were taken using a Z1 microscope (Carl Zeiss AG, Germany) using the Zen Pro (Zeiss) software suite. Fluorescent signals were captured using a grayscale image pseudocolored to provide visual contrast between separate filter types. Sections were initially imaged for endogenous fluorescence, which included Col3.6-cyan, and calcein, a marker for active bone mineral formation in the 24 hours prior to sacrifice. Separate images were also taken simultaneously for differential interference contrast (DIC). After endogenous fluorescence, the slides were removed and stained with ELF97 (Sigma) and imaged a second time for tartrate resistant acid phosphatase (TRAP) which indicates the activity and population of osteoclasts. After TRAP, the slides were stained with Fast Red TR salt (#F8764, Sigma) and Naphthol AS-MX phosphate (#N-4875, Sigma) and Hoechst dye (Hoechst #33342, Thermo Scientific) at the same time to visualize alkaline phosphatase (AP), a marker to highlight a key protein in mineralization of collagen networks, and nuclear localization respectively. The slides were then rinsed again and stained for hematoxylin (Ricca Chemical Company) for basophilic structures and glycosaminoglycans. AP and mCherry were detected with a TRITC filter (#31002, Chroma Technology, Bellows Falls, VT), Col3.6-cyan with a CFP filter (#49001, Chroma Technology), Col3.6-topaz with a topaz filter (#49003, Chroma Technology), Hoescht with a DAPI filter (#49000, Chroma Technology), and TRAP with a custom tetracycline filter (Chroma Technology Custom HQ409sp, 425dcxr, HQ555/30, set lot C-104285). Hematoxylin was imaged using light microscopy through DIC.

Statistical Analysis

For statistical analysis between the different matrix designs, a Student's *t*-test was used, with $p < 0.05$ set as the point of criticality. No statistical analysis was made between the matrix types *in vivo*.

Results

The images of BMSCs seeded onto matrices, and imaged 14 days later through fluorescence microscopy are shown in figures 1A-C. The matrices that contained no nanofibers showed increased α SMA-mCherry fluorescent signal indicating the maintenance of stem cell phenotype after culture for 14 days in osteogenic media. In contrast the 1% nanofiber-infused matrix showed relatively small amounts of α SMA-mCherry expression, but high amounts of cyan-GFP, indicating the incorporation of ECM-mimetic fibers progresses the population of stem cells from an undifferentiated state towards the osteoblast lineage.

In the cell-residence/phenotype assay, the resulting values of both total cell number and GFP-positive cell number were plotted as a function of distance from the periphery of the matrix (figure 2C-2G) in order to determine which matrix was more suitable to assist in the differentiation of cells farther into the interior of a matrix. The total population of nuclei was higher in control matrices compared to hybrid matrices (figure 2F, $p < 0.01$). Using both the average distance of all nuclei (positive with Hoechst) and those that had positive collagen promoter activity as determined through GFP expression (positive for 3.6cyan), the number and fraction of these total cells that had the effect of nanofiber-permeated matrices on cell

phenotype in the interior of the matrices is shown in figure 2H. These data indicate that the average distance of a cell with positive collagen promoter activity was found further from the periphery in hybrid matrices compared to control matrices ($p = 0.02$) and provides evidence that this matrix is providing differentiation cues in the declining nutrient and waste exchange milieu of the interior matrix.

The osteocompatibility of these matrices were tested *in vivo* to clarify their ability to interface with host bone. Light micrographs and X-rays were taken and shown below in figures 3A-H. The light micrographs of the calvaria was that the sintered composites (control matrices) were not as well integrated into the host tissue defect compared to hybrid matrices. In two of the cases, shown as animals one and three (figures A/E and C/G respectively), the control matrices did not preferentially adhere to the calvarial defect but rather to the overlying skin on the defect, and as a result the matrices were not able to remain seated in the defect when the skin was dissected away from the calvaria. In one case (3C/G) the matrix was completely adhered to the skin, and thus is not visible in either the X-ray or light micrographs. Also noticeable in figure 3D was a slightly erythematous region around the control scaffold, shown in detail in figure 3I below. For the X-rays taken of the matrices, no extensive or excessive change in radiolucency could be appreciated for any of the matrix types in any of the animals. In figure 3H, there may be preliminary evidence for slight radiographic changes in the hybrid matrices. A detailed radiograph of the animal is presented below in figure 3J. These results provide evidence the nanofiber-permeated matrices interface better with host bone.

To establish the cytological changes these hybrid matrices impart on their host cells, in depth histological examination of only mouse number 4 was undertaken and shown in figure 4A. The first stain investigated in these images was the calcein stain, shown as the top layer in figure 4A. A detail of the calcein stain for the interior of the hybrid matrix overlaid with ALP signal is shown below in figure 4B indicating active bone mineralization during the previous day before sacrifice. Additionally, the alkaline phosphatase activity in the control matrix was limited to the surface of the matrix in contact with the mouse dura, and no activity was seen on the interior of the matrix. The alkaline phosphatase activity on the hybrid matrix, however, was strong throughout the interior of the matrix. The overlay of these two signals, calcein and ALP, provide evidence that both bone has been formed and is likely in the process of forming more.

The third stain from the top in figure 4A is TRAP, which indicates osteoclasts. No significant amount of activity was found in any of the matrices, but a region of interest in mouse number four is highlighted with a blue rectangle and shown in detail in figure 4D. This shows what appear to be large multinucleated cells on the medial border of the matrix with the host calvaria. A continuing concern of implanted matrices is that they may evoke an unfavorable immune response from the host that may cause a large amount of degradation of the surrounding host bone. The results from 4D indicate that these matrices may be inducing an immune response in the form of increased osteoclast activity. The relatively low signal, however, may simply mean that the host bone is undergoing natural, balanced turnover of bone. The activation of TRAP will be investigated more deeply in future studies.

A detail of the hematoxylin blue stain for mouse number 4 is shown in figure 4C to indicate cellularity, glycosaminoglycans, as well as bone matrix. This figure shows that the red signal of alkaline phosphatase displaying osteogenic activity correlates well with the areas of bone matrix in the interior of the hybrid matrix. Hematoxylin also shows well the cellularity of the mineralized tissue in mouse number 4 and what appear to be osteocytes embedded in bone matrix (figure 4C).

Discussion

The purpose of this study is to evaluate the utility of incorporating ECM-mimetic fibers into the pore spaces of a sintered composite microsphere matrix can support the phenotypic progression and cell residence *in vitro*, and biocompatibility of these matrices *in vivo*. The potential advantages of using these ECM-mimetic fibers is that that may be able to more accurately represent the ECM milieu of physiological bone as well as provide enhanced physical and chemical cues to local stem cells to progress down the osteoblastic lineage instead of forming scar tissue.

The assay to determine if the hybrid matrices support phenotypic progression to the osteoblastic lineage is shown in figure 1 and shows that matrices that contained no nanofibers showed a strong α SMA-mCherry fluorescent signal indicating the maintenance of stem cell phenotype after culture for 14 days in osteogenic media. In contrast, the 1% nanofiber-infused matrix showed relatively small amounts of α SMA-mCherry expression, but high amounts of cyan-GFP, indicating the expression of collagen type I and commitment away from a more pluripotent state. The image detail shown in figure 1C shows evidence that a BMSC occupies the pore space between two adjacent microspheres, suggesting the ability of the permeated nanofiber networks to provide not only an additional surface for adhesion and migration than may be provided by the microspheres alone, but also that the fibers, as well as the entire matrix, are capable of supporting osteoblastic differentiation. Furthermore, these images provide evidence of cell adherence to the nanofibers because one would not expect the poly(L-lactic acid) nanofibers to degrade measurably at this early time point, as the degradation time, particularly for high molecular weight PLLA, is measured in months to years.¹³

The residence assay determined if the intraporous nanofibers were able to support phenotypic progression, and quantified the average distance of that phenotypic progression from the periphery of the matrices. Histological sections were taken at the midpoint of the matrix in cross sections to measure (1) the total number of cells present on the section as measured by the nuclear staining Hoechst dye, (2) the number of cells undergoing early osteogenic differentiation as measured through cyan-GFP expression, and (3) the distances of all cells from the periphery of the matrix to the interior to determine if nanofibers in the pore spaces of the matrix lead to enhanced cell residence and proportion of cells undergoing phenotypic progression. The resulting values of both total cell number and GFP positive cell number were plotted as a function of distance from the periphery of the matrix (figure 2C–2E) are consistent with both the previous *in vitro* data on matrix DNA content, the total population of nuclei was higher in control matrices compared to hybrid matrices (figure 2D, $p < 0.01$).³ Although the total number of cells was lower, the fraction of these total cells that

had positive collagen promoter activity as determined through GFP expression was significantly higher on hybrid matrices compared to control matrices (figure 2F, $p = 0.02$). Taken together, these data indicate that fewer cells are inhabiting the nanofiber-permeated matrices, but a higher fraction of those cells are producing type I collagen and are thus more likely to be in the process of committing to the osteogenic lineage and therefore may be more beneficial for regenerative engineering. A separate measurement was taken to determine the average distance of cells that were positive for Col3.6-GFP from the periphery of the matrix (figure 2G). These data indicated that the average distance of a cell with positive collagen promoter activity, evidence for osteogenic differentiation, was found further from the periphery in hybrid matrices compared to control matrices ($p = 0.02$) suggesting that the environment provided by the nanofiber-permeated matrices may be more permissive for osteogenic collagen promoter activity compared to control matrices.

The biocompatibility assay showed that the intraporous nanofibers did provide better osteointegration with surrounding calvarial tissue as well as evidence for bone mineralization. matrices did not promote bone formation, we would expect to see no changes with regard to X-ray signal, and poor adherence to the underlying calvarial defect.

Light micrographs and X-rays were taken and shown below in figures 3A-H. Evident on the light micrographs of the calvaria was that the sintered composites (control matrices) were not as well integrated into the host tissue defect compared to hybrid matrices. In two of the cases, shown as animals one and three (figures A/E and C/G respectively), the control matrices did not preferentially adhere to the calvarial defect but rather to the overlying skin on the defect, and as a result the matrices were not able to remain seated in the defect when the skin was dissected away from the calvaria. In one case (3C/G) the matrix was completely adhered to the skin, and thus is not visible in either the X-ray or light micrographs. Noticeable in figure 3D was a slightly erythematous region around the control matrix, shown in detail in figure 3I below. For the X-rays taken of the matrices, no extensive or excessive change in radiolucency could be appreciated for any of the matrix types in any of the animals. In figure 3H, there may be preliminary evidence for slight radiographic changes in the hybrid matrices that could potentially indicate matrix mineralization.

Only this one matrix, including both hybrid and control matrices, showed any calcein staining in the interior of the matrix, and it was the same hybrid matrix as shown in figures 3I-J. A detail of the calcein stain for the interior of the hybrid matrix overlaid with ALP signal is shown below in figure 4B. Notably, the green stain indicates active bone mineralization during the previous day before sacrifice. Due to the presence of the green activity on the interior of the matrix, it is unlikely that this stain is an artifact of host bone that was released during the formation of the cranial defect on the initial day of surgery and subsequently trapped in the scaffold. Additionally, the alkaline phosphatase activity in the control matrix was limited to the surface of the matrix in contact with the mouse dura, and no activity was seen on the interior of the matrix. The alkaline phosphatase activity on the hybrid scaffold, however, was strong throughout the interior of the matrix. The overlay of these two signals, calcein and ALP, provide evidence that both bone has been formed and is likely in the process of forming more.

The third stain from the top in figure 4 is TRAP, which indicates populations of active osteoclasts. No significant amount of activity was found in any of the matrices, but a region of interest in mouse number four is highlighted with a blue rectangle and shown in detail in figure 4D. This shows what appear to be large multinucleated cells on the medial border of the matrix with the host calvaria. A continuing concern of implanted matrices is that they may evoke an unfavorable immune response from the host that may cause a large amount of degradation of the surrounding host bone. The results from 4D indicate that these matrices may be inducing an immune response in the form of increased osteoclast activity. The relatively low signal, however, may simply mean that the host bone is undergoing natural, balanced turnover of bone. The activation of TRAP will be investigated more deeply in future studies.

The final stain visible in figure 4 is the hematoxylin stain, which is meant to show the cellularity, glycosaminoglycans, as well as bone matrix. These stains, however, allow investigation of the cellular nature of both the bone formed in the hybrid matrix and the TRAP activity in mouse number 4. An overlay of the hematoxylin stain with the alkaline phosphatase is shown in figure 4C. This figure shows that the red signal of alkaline phosphatase displaying osteogenic activity correlates well with the areas of bone matrix in the interior of the hybrid matrix. The hematoxylin also shows well the cellularity of the mineralized tissue in mouse number 4 and what appear to be osteocytes embedded in bone matrix.

No cells were added to the matrices before implantation because the purpose of this study was to prospect for any potentially adverse reactions against the host mouse. Furthermore, even without the addition of cells to the matrices, evidence for bone tissue formation on the interior of matrices was visible on one mouse (number 4). These may indicate that the fibers present on these matrices may be helping the host cells migrate and differentiate.

Conclusions

The purpose of this work was to evaluate nanofiber-permeated, hybrid polymer/ceramic matrices both *in vitro* and *in vivo* for their potential to be used in bone regenerative engineering. *In vitro*, the matrices were evaluated with a clinically relevant population of cells that the matrices are likely to encounter upon implantation to critical sized defects in human patients, BMSCs. The *in vitro* data provide evidence that although the nanofiber-permeated matrices did not prevent the adhesion or residence of BMSCs, the population is decreased. While the lower DNA content may be related to decrease cellular attachment, ingress, and/or migration, our theory was that the nanofibrous structures are driving differentiation of BMSCs. In support of the later idea, the proportion of cells that had evidence for osteogenesis as measured by collagen type I promoter activity was higher in hybrid matrices than control matrices. Additionally, the amount of calcein deposited on active bone forming surfaces as well as bone surface associated AP and GFP reporter activity was greater *in vivo* for the nanofiber-permeated matrix compared to control scaffold. The presence of areas of bone remodeling by osteoclasts further suggests this deposited tissue supports the idea that this newly formed tissue is biologically active. Taken together,

these *in vivo* studies support the ability of these matrices to direct cellular behavior in the absence of specifically cultured cells.

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Lay Summary

One idea to regenerate bone is to mimic the structure, composition, and feature size of natural human bone in a synthetic matrix to guide human stem cells. In this work, the authors combine an advanced material matrix with special genetically engineered stem cells to investigate if the special composition of the matrix can help improve existing matrices to support the regeneration of human bone. These results show that while the new matrices support a smaller population of cells, those cells are more likely to show evidence of growing into cells that may create bone.

Future Work

Future studies will include the implantation of transgenic, cell-seeded matrices of one fluorescent reporter mouse implanted in a host mouse that contains a separate fluorophore with a similar promoter. These studies will allow determination of the source of bone formation in these models: from host or from implant.

Fig. 1.

A-C, seeding of fluorescent reporter cells on scaffold constructs; For these fluorescent reporter cells, red indicates the mCherry fluorescent protein under the promoter activity of α -SMA (a marker for mesenchymal stem cells) and blue represents the cyan fluorescent protein under the promoter activity of collagen type I (a marker for early osteogenesis). A(left) shows control scaffolds which contain no intraporous nanofibers displaying cellular contents with a strong stem cell character; B(middle) shows hybrid scaffolds with strong stem cell character and weak expression of stem cell markers; C(right) shows a detail indicated in 1B showing a BMSC indicated with an arrow adhering to a nanofiber in the pore space of the scaffold reporting activity of the collagen type I promoter.

Fig. 2.

A-I (A) Fluorescence image of an axial slice of a microsphere matrix. Image shows the relative position of the sintered, composite microspheres, as well as a nuclear stain (Hoescht) that stains all cells, and a green signal that indicates progression toward the osteoblastic lineage (Col3.6-cyan). White rectangle indicates detail for figure 2B (B) Detailed image of fluorescent image of axial slice of cell-seeded, sintered, composite microsphere matrices. The circles indicate microspheres that are filled with hydroxyapatite crystals, the blue signal is a nuclear stain (Hoescht) that stains all cells, and the green signal indicates progression toward the osteoblastic lineage (Col3.6-cyan). This image is representative in that most Col3.6 signal is shown near the periphery and only a portion of the Hoescht- positive cells are positive for Col3.6 (C) Representative axial section of a control scaffold showing how the distances are measured from the periphery radially inward for the following data graphs (D) Distribution of all cells (red curve) and the distribution of cells undergoing early osteogenic differentiation (blue curve) as a function of distance from the periphery of a 3.5 mm diameter control scaffold (E) Distribution of all nuclei (red curve) and cells undergoing early osteogenic differentiation (blue curve) as a function of distance from the periphery for hybrid scaffolds. Comparing the control and hybrid scaffolds, there are fewer cells at any given section taken through the scaffold (F), and no difference in the average distance between any given cell and the perimeter of the scaffold (G). Importantly, a higher fraction of those cells has active collagen type I promoter activity on the hybrid

scaffold (**H**) which is evidence for early osteoblastic differentiation, and the average distance of the cell that expresses the osteoblastic differentiation is greater in hybrids compared to controls (**I**). Taken together, these data provide evidence for increased phenotypic progression at increasing distances from the periphery of the scaffold in hybrid scaffolds compared to control scaffolds. Although not conclusively shown here, these data may foreshadow an increased ability of intraporous nanofibers to permit increased phenotypic progression in situations of low nutrient and waste exchange.

Fig. 3.

A-J Fluorescent reporter mice calvaria at four-week time point with skin dissected. On each of these four separate mice (**A-D**), the scaffolds on the reader's left are control scaffolds (no nanofibers) and the scaffolds on the reader's right are hybrid scaffolds (nanofibers in the pore spaces). In two of the cases (**A and C**), the control scaffolds adhered to the skin more than the defect site, causing the scaffolds to become dislodged during dissection. Notably this did not happen to any hybrid scaffold. (**E-F**) shows each of the corresponding X-ray images for the calvaria of mice shown above in light microscopy (**I**) A detail in light microscopy of mouse number four (as shown in **D**) has noted erythema around periphery of scaffold near the calvarial interface and where the scaffold interfaced with the scalp (**J**) A detail in X-ray of the same scaffold in mouse number four showing evidence of increased density, a potential marker of increased bone deposition.

Fig. 4.

A-D (A) Pilot *in vivo* study. Clockwise from top left (A) Composite image showing how histological slices were taken in the coronal plane of the calvaria of mouse number 4 and imaged for (top to bottom) calcein, alkaline phosphatase (ALP) activity, tartrate-resistant acid phosphatase (TRAP) and Hoechst stain, and hematoxylin along with the representative X-ray photograph of the denuded mouse calvaria. Areas with yellow rectangles indicate regions of interest for colocalization images shown in figure 4B and 4C. Area with blue rectangle indicates region of interest for panel D; (B) Overlay of ALP and calcein fluorescent images showing colocalization of calcein signal and alkaline phosphatase, evidence for active bone deposition and growth; (C) Overlay of hematoxylin and ALP images showing relative morphology of areas with strong ALP activity; (D) Image showing colocalization of Hoechst dye and TRAP indicating that the area in question likely has osteoclast activity which when significant may be detrimental to host bone tissue, but at low levels of activity may be a sign of health bone turnover and integration.