Flow perfusion culture of mesenchymal stem cells for bone tissue engineering

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Abstract
Due to the limited supply of and numerous potential complications associated with current bone grafting materials, a tremendous clinical need exists for alternative biologically active implant materials capable of promoting bone regeneration in orthopaedic applications. Recent advances in tissue engineering technology have enabled the coating of biologically inactive materials, such as titanium fiber meshes, with a biologically active bone-like extracellular matrix produced by mesenchymal stem cells during in vitro culture. The resulting constructs can then be implanted as acellular scaffolds or as transplantation vehicles for mesenchymal stem cell populations to guide bone tissue regeneration in vivo. Such a novel tissue engineering strategy marks a paradigmatic shift in drug delivery approaches from delivering bioactive factors from a scaffold to generating constructs that contain biological signaling moieties produced by cells under engineered conditions in vitro. This chapter provides a brief introduction to general bone tissue engineering strategies and an overview of the seminal work from our laboratory in the application of mesenchymal stem cells in the in vitro generation of biologically active bone-like extracellular matrix constructs for bone tissue engineering.

Prevalence and significance of bone defects
A commonly encountered challenge for reconstructive surgeons is the treatment of large bony defects resulting from traumatic injury, tumor resection, degenerative diseases, and congenital deformities. In 2005, Nationwide Inpatient Statistics show that over 1,000,000 surgical procedures addressing the partial excision of bone, bone grafting, spinal fusion, and inpatient fracture repair were performed with total charges from these procedures exceeding $40 billion (2005; PMID Unavailable), and beyond financial considerations, multiple studies have examined the psychosocial impact of orthopedic trauma and surgery (Crichlow et al., 2006; PMID 16951107; Starr et al., 2004; PMID 15173282). The cost of surgical treatment and subsequent impact on patient quality of life illustrate well the need to better address the functional and social impact of such defects.

Current clinical strategies for bone regeneration
Surgeons and other researchers have long sought a synthetic material capable of accelerating the bone healing process, integrating with the surrounding tissue, and later allowing or

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encouraging tissue remodeling such that the material resembles or is replaced by native bone. Historically, a variety of alloplastic materials have been investigated toward this end, including celluloid, aluminum, gold, vitallium, tantalum, stainless steel, titanium, methylmethacrylate resins, polyethylene, silicone elastomers, and hydroxyapatite ceramics (Artico et al., 2003; PMID 12865021). When compared with autologous bone grafts, the current material gold standard for these applications, alloplastic materials are deficient in a number of areas. For example, a non-degradable alloplastic material may not respond to mechanical stress in the same manner as the surrounding host bone, resulting in structural failure of the implant under load or pathologic changes in the surrounding bone, as seen in stress shielding (Konttinen et al., 2005; PMID 15662301). Biologically inactive materials may facilitate inflammatory scarring, neoproliferative reactions in the neighboring tissues, and may serve as a nidus for bacteria, resulting in infectious complications (Mercuri and Giobbie-Hurder, 2004; PMID 15346359). Bioactive implants such as demineralized bone matrix obtained from allogeneic (cadaver) or xenogeneic sources have shown promise as reconstructive materials because of their high osteoinductivity and propensity for remodeling (Pou, 2003; PMID 14515070), although drawbacks include the theoretical risk of disease transmission as well as cost and availability. The benchmark for comparison of new bone grafting materials continues to be autogenous bone as a result of its potential for growth and remodeling, as well as the ability to osseointegrate and resist infection.

**Tissue engineering-based clinical approaches and considerations for bone regeneration**

The tissue engineering paradigm typically incorporates three components for tissue regeneration – a degradable support or scaffold material, bioactive factors such as growth factors or other pharmaceuticals, and cells. The clinical strategies outlined in the previous section generally do not include components of this paradigm, with the exception of autogenous bone and demineralized bone from other sources, which may contain a number of bioactive factors (Reddi, 1998; PMID 9528003). Recently, a number of new products for bone regeneration have entered into widespread clinical use that incorporate key elements of the tissue engineering paradigm. One such product, Infuse® (Medtronic, USA) incorporates a bioactive factor, bone morphogenetic protein-2 (BMP-2) into a degradable, acellular collagen sponge and is indicated for clinical use in a number of applications including spinal fusion, traumatic tibial fractures, and certain oral-facial applications. The clinical success of this material (Govender et al., 2002; PMID 12473698) illustrates the potential for tissue engineering-based therapies in the clinic.

In the laboratory setting, continued work expanding on and developing new technologies has led to important advances within all three components of the tissue engineering paradigm. New materials, specifically tailored for applications such as cell encapsulation, injectable delivery, and composite tissue regeneration have been developed, new bioactive factors and efficient delivery methods are being studied, and readily available, easily obtainable cell sources have been identified. Advances in all of these areas as related to bone tissue engineering will be expanded upon in the subsequent sections.

**Bone regeneration by progenitor cell transplantation**

Bone regeneration by autogenous osteoblast or osteoblast progenitor transplantation is one of the most promising new techniques being developed because it would eliminate problems of donor scarcity, immune rejection, and pathogen transfer (Bancroft and Mikos, 2001; Bruder and Fox, 1999; PMID 10546637; Mistry and Mikos, 2005; PMID 15915866). Osteoblasts and osteoblast progenitors obtained from patient bone marrow can be expanded in culture (Haynesworth et al., 1992; PMID 1581112) and seeded onto an appropriate

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degradable scaffold, which will slowly degrade as cells grow and secrete new bone in vivo (Yoshikawa et al., 1996; PMID 8897155). In addition to bone marrow progenitors, other cell sources have recently been identified as promising precursors capable of differentiation into osteoblast-like cells. Kern et al. (Kern et al., 2006; PMID 16410387) compared mesenchymal stem cells isolated for bone marrow aspirates, adipose tissue, and umbilical cord blood and found that while umbilical cord blood-derived progenitor cells were the most difficult to isolate and were incapable of adipogenic differentiation, all three cell sources were promising for bone regeneration. The transcription profiles of differentiating bone marrow and adipose derived progenitor cells have been shown to be very similar, although bone marrow mesenchymal stem cells more efficiently differentiate into fully mature osteoblasts (Liu et al., 2007; PMID 17095706). Additional cells with multipotent or “stem-like” potential have been isolated from various tissues including dental tissues (Zhang et al., 2006; PMID 17518650) and dermal fibroblasts (Chen et al., 2007; PMID 17652163), and the selection of the most appropriate cell source likely depends on the intended clinical application and any present comorbidity.

Three-dimensional architecture is thought to be critical to new bone formation both in embryogenesis and for regenerative purposes; spheroids of osteoblasts and marrow derived osteoblast precursors express bone related protein and form microparticles of bone at significantly greater levels than identical cells cultured on two-dimensional plates (Kale et al., 2000; PMID 10973215). While bone formation in the absence of a scaffold is clearly possible when three dimensional architecture is established, the use of a scaffold provides controlled and necessary support for cells to maintain their differentiated function and defines the ultimate shape of the new bone (Temenoff et al., 2000; PMID Unavailable). Several substrates are being investigated for this strategy including ceramics, poly(α-hydroxy esters), polyanhydrides, polyanhydrides, polyanhydrides, and collagen. Collagen sponges have been successful in supporting osteoblast growth and function in vitro, but the low mechanical strength of these materials excludes them from use in load bearing sites (Schoeters et al., 1992; PMID 1457607). Hydroxyapatite and β-tricalcium phosphate, which have superior mechanical strength characteristics, also support osteogenesis in vivo (Kadiyala et al., 1997; PMID Unavailable); however, their remodeling is limited by a very slow degradation rate. The poly(α-hydroxy esters) such as poly([ɛ]-lactic-co-glycolic acid) (PLGA) copolymers have been extensively investigated because they are FDA approved for certain clinical uses, easily fabricated with controlled pore size and porosity, and degrade in a controllable manner over a period of weeks to years to products that can be easily metabolized (Thomson et al., 1995; PMID Unavailable; Vacanti et al., 1995; PMID Unavailable). Indeed, the ability of osteoblasts and osteoblast progenitors to attach, proliferate, migrate, and perform differentiated function on two-dimensional PLGA films in vitro has been demonstrated (Ishaug et al., 1996; PMID Unavailable; Ishaug et al., 1994; PMID 7876284). Further, results of three-dimensional flow perfusion culture studies of rat mesenchymal stem cells seeded on non-woven poly([ɛ]-lactic acid) (PLLA) fiber mesh scaffolds demonstrated the feasibility of osteoblasts and osteoblast progenitors to attach, proliferate and perform osteogenic differentiated function on the three-dimensional scaffolds in vitro (Sikavitsas et al., 2005; PMID 15709706). Poly([ε]-caprolactone) (PCL) is another poly(α-hydroxy ester) that has been widely investigated for use in tissue engineering scaffolds due, in part, to FDA approval for its use in certain clinical applications and its established biodegradability (Pachence and Kohn, 2000; PMID Unavailable). A key advantage presented by PCL is that it can be used to fabricate tissue engineering scaffolds of a wide range of controllable architectures, including scaffolds composed of nanofibers, microfibers or blends of nanofibers and microfibers through the process of electrospinning (Figure 1) (Pham et al., 2006; PMID 16771634; Pham et al., 2006; PMID 17025355). The generation of scaffolds with nanometer scale fibers or other nanoscale features may facilitate osteogenic differentiation of cells by providing an environment with features that mimic the
scale of native bone extracellular matrix molecules. Many natural materials including bone utilize nanoscale building block within a composite matrix that have been shown to provide optimum strength and minimize stress concentration at material flaws (Gao et al., 2003; PMID 12732735). Bone marrow derived mesenchymal stem cells can undergo osteoblastic differentiation in response to nanoscale material surface features such as pits, grooves, and nanoscale symmetry or disorder (Dalby et al., 2007; PMID 17891143; Dalby et al., 2006; PMID 16143393), and genomic expression by these cells is similar but via a different mechanism in these cells when compared to cells cultured in media supplemented with dexamethasone (Dalby et al., 2008; PMID 18270147). In addition to the nanofiber composite scaffolds mentioned above, a number of other scaffold materials with nanoscale elements such as self assembling peptide nanofibers (Hosseinkhani et al., 2006; PMID 16600365; Storrie et al., 2007; PMID 17662383) and composites incorporating hydroxyapatite nanocrystals (Leeuwenburgh et al., 2007; PMID 17988519) have been fabricated to potentially utilize this nanoscale cell-material interaction.

**Bone tissue induction into polymer scaffolds**

Alternative approaches to regenerate bone rely on the induction of bone into biomaterials (Bostrom and Mikos, 1997; PMID Unavailable). When the scaffold material is implanted adjacent to bone tissue, cells from the tissue begin to invade and populate the material, lay down new matrix, and eventually form new bone (Thomson et al., 1999; PMID 10535812; Yaszemski et al., 1995; PMID Unavailable). Synthetic polymers on their own usually lack the factors required to make the material osteoinductive but may be used as templates to enhance bone growth for fracture healing (Hedberg and Mikos, 2001; PMID Unavailable). However, synthetic and natural scaffold materials can be made osteoinductive by combining them with bioactive molecules such as bone morphogenetic proteins (Babensee et al., 2000; PMID 10888299; Holland and Mikos, 2006; PMID 17089790). It has been demonstrated that recombinant human bone morphogenetic protein-2 (rhBMP-2) in a demineralized bone matrix carrier will induce the formation of endochondral bone when placed in rat femoral defects (Yasko et al., 1992; PMID 1378056; Yasko et al., 1992; PMID Unavailable). Bone formation occurred in a dose dependent manner, and the experiment demonstrated the feasibility of using recombinant growth factors in orthopedic sites to obtain successful union. Successful results have also been obtained when rhBMP-2 was implanted in a PLGA carrier into large segmental defects created in rat femurs (Lee et al., 1994; PMID 7829545) or when genetically modified cells expressing BMP-2 were used with a demineralized bone matrix carrier in the same model (Lieberman et al., 1998; PMID 9671928). Additionally, enhanced bone formation was observed in a critical sized cranial defect model in rats when genetically modified cells expressing BMP-2 seeded in titanium fiber meshes were implanted (Blum et al., 2003; PMID 14670121). Recent work has demonstrated that mesenchymal stem cells can be seeded within scaffolds and cultured under flow perfusion conditions to synthesize a bone-like extracellular matrix coating containing an array of osteogenic and angiogenic growth factors (Gomes et al., 2006; PMID 16499454). Similar bone-like extracellular matrix constructs generated by mesenchymal stem cells under flow perfusion culture have been shown to be osteoinductive in both ectopic (Holtorf et al., 2005; PMID 15921737) and orthotopic (Sikavitsas et al., 2003; PMID 14613243) sites *in vivo*. Indeed, the osteogenic differentiation of the seeded mesenchymal stem cells has been demonstrated in a number of studies by tracking various markers of differentiation, such as alkaline phosphatase activity, calcium deposition, and gene expression with time. The conditions of the flow perfusion culture, including the fluid flow perfusion rate, the duration of culture, the presence of a pre-generated extracellular matrix among many other factors have been shown to influence the osteogenic potential of mesenchymal stem cells seeded within the scaffolds and the consequent osteogenicity of the resulting constructs. The following sections of this chapter provide an overview of the highlights of studies from our
In initial studies from our laboratory, it was hypothesized that mesenchymal stem cells seeded onto a three-dimensional osteoconductive scaffold and cultured in a flow perfusion bioreactor in vitro could be differentiated down an osteogenic lineage and begin secreting a bone-like extracellular matrix throughout the scaffold. It was further hypothesized that flow perfusion culture, which forces the flow of culture medium through the porosity of a scaffold (see Figure 3), would enhance mesenchymal stem cell proliferation and differentiation, as well as mineralized matrix production, by mitigating external and internal mass transport limitations and applying fluid shear forces to cultured cells. Mechanical stimulation of mesenchymal stem cells has been established as a powerful tool for inducing osteogenic differentiation of stimulated cells (Mauney et al., 2004; PMID 14961210), an effect that may be partially controlled by the inhibition of adipogenesis in cells experiencing mechanical stimulation (Rubin et al., 2007; PMID 17959771). Matrix elasticity in the absence of active mechanical stimulation has also been found to direct mesenchymal stem cell differentiation (Engler et al., 2006; PMID 16923388); however, the flow perfusion system is one of the only ways of producing mechanical stimulation within a system that simultaneously ameliorates mitigates diffusional transport limitations, making it an ideal system for the ex vivo production of large bone constructs.

A flow perfusion bioreactor culture system was designed and built to investigate the aforementioned hypotheses (Bancroft et al., 2003; PMID 12857422). As an initial test, titanium fiber mesh scaffolds were seeded with rat bone mesenchymal stem cells and cultured under flow perfusion or static conditions for up to 16 days (van den Dolder et al., 2003; PMID 12522809). Results showed a greater cell number for the flow perfusion system at 4 and 8 days, but similar cell numbers for each culture system at 16 days. Flow perfusion culture conditions resulted in the covering of the scaffolds with layers of proliferating cells and mineralized bone-like extracellular matrix penetrating deep within the scaffolds at day 16. Static culture, however, resulted in a thin coating of extracellular matrix limited to the upper surface of the scaffolds at day 16. Comparable results were obtained in studies exploring flow perfusion culture of mesenchymal stem cells seeded upon three-dimensional scaffolds of other osteoconductive materials, including starch-based fiber meshes (Gomes et al., 2003; PMID 14517865), non-woven poly(-lactic acid) fiber meshes (Sikavitsas et al., 2005; PMID 15709706), and porous biphasic calcium phosphate ceramics (Holtorf et al., 2005; PMID 16133930). Together these studies demonstrate that a flow perfusion system can enhance the early proliferation, differentiation, and mineralized matrix production of mesenchymal stem cells seeded in three-dimensional osteoconductive scaffolds.

To further investigate the effect of flow perfusion culture on osteogenic differentiation of mesenchymal stem cells and extracellular matrix development, mesenchymal stem cells were cultured on titanium fiber mesh scaffolds under flow perfusion with different rates of fluid flow (Bancroft et al., 2002; PMID 12242339). The calcium content of the constructs increased with increasing fluid flow rate, and in all cases, mineral deposition was higher for scaffolds cultured in flow perfusion conditions when compared to those cultured in static...
conditions. Pore-like structures were observed in the extracellular matrix generated on scaffolds in flow perfusion culture, indicating de novo tissue modeling (Figure 4). Further, flow perfusion culture resulted in greater distribution of cells and extracellular matrix throughout the scaffolds. This study demonstrates the effects of increasing fluid flow on mesenchymal stem cells and indicates that the flow perfusion of culture medium through a scaffold has a broad influence on the osteogenic differentiation of mesenchymal stem cells in vitro.

**In vitro culture duration and stage of osteogenic differentiation of transplanted mesenchymal stem cells affect in vivo osteogenicity of in vitro generated bone-like extracellular matrix constructs**

Having observed that flow perfusion culture can promote the osteogenic differentiation of cells in vitro, it was hypothesized that the duration of the flow perfusion culture could influence the extent of osteogenic differentiation and that the osteogenic differentiation stage of mesenchymal stem cells could affect the ability of the cells transplanted with titanium fiber mesh scaffolds to regenerate bone in an orthotopic site. To investigate this hypothesis, rat mesenchymal stem cells were seeded on titanium fiber mesh scaffolds and cultured for 1, 4, and 8 days under either static or flow perfusion conditions (Sikavitsas et al., 2003; PMID 14613243). The cellular constructs were then implanted into critical size calvarial defects in a rat model for 7 or 30 days. Implants from the 7-day time point were filled with capillaries and fibrous tissue. Bone formation was not observed for any of the six treatment groups following 7 days of implantation, but was observed for all treatment groups following 30 days of implantation. Interestingly, the highest percentage of bone formation per implant and the highest ratio of defects resulting in bone union following 30 days of implantation was observed with the titanium scaffolds that had been seeded with mesenchymal stem cells and cultured in flow perfusion conditions for 1 day prior to implantation. This study demonstrates the potential of titanium fiber mesh scaffolds as a mesenchymal stem cell transplantation vehicle for bone regeneration. Further, the results illustrate that the stage of the osteogenic differentiation of the transplanted cells as well as the maturity of the in vitro generated extracellular matrix construct influence the osteogenic potential of the tissue engineering construct at an orthotopic site in vivo.

**Mineralized matrix deposition by mesenchymal stem cells in flow perfusion culture increases with increasing fluid shear forces**

Different mechanisms could be postulated to explain the observed effects of flow perfusion culture on mesenchymal stem cells, including both shear forces and enhancement of chemotransport. To elucidate the contributions of these two possible effectors, mesenchymal stem cells were cultured on three-dimensional titanium fiber mesh scaffolds with culture medium of different viscosities while maintaining a constant fluid flow rate (Sikavitsas et al., 2003; PMID 14657343). This methodology allowed exposure of the cultured cells to increasing levels of mechanical stimulation, in the form of fluid shear stress, whereas chemotransport conditions for nutrient delivery and waste removal remained essentially constant. It was found that the mineral content of the extracellular matrix produced during culture increased with increasing fluid shear forces, indicating that shear-induced mechanical stimulation of mesenchymal stem cells induces their osteogenic differentiation. It was also shown that fluid shear promotes osteogenic differentiation of mesenchymal stem cells cultured under flow perfusion on titanium fiber mesh scaffolds, even in the absence of the osteogenic culture medium supplement dexamethasone, and that fluid shear and the presence of dexamethasone have a synergistic effect on osteogenic differentiation (Holtorf et al., 2005; PMID 15965910). Additionally, the spatial distribution of the in vitro generated
extracellular matrix throughout the porosity of the titanium fiber mesh scaffolds was found to increase with increasing fluid shear forces (Sikavitsas et al., 2003; PMID 14657343). These studies demonstrate that mesenchymal stem cells are sensitive to fluid flow shear forces in three-dimensional culture and that increased shear forces result in enhanced mineralized extracellular matrix production with improved spatial distribution.

**Scaffold mesh size affects the osteogenic differentiation of mesenchymal stem cells cultured in a flow perfusion bioreactor**

To further investigate the effect of shear forces in flow perfusion culture, the effect of scaffold pore size on the osteogenic differentiation of mesenchymal stem cells seeded on titanium fiber mesh scaffolds and cultured in a flow perfusion bioreactor was evaluated (Holtorf et al., 2005; PMID 15965910). It was hypothesized that the difference in scaffold pore size would result in alterations in fluid flow and subsequently fluid shear stress experienced by the seeded cells that would affect their osteogenic differentiation. The results showed that differentiation was indeed dependent on scaffold pore size, however the dependence was not linear with respect to time. Larger scaffold pore sizes were associated with early osteogenic differentiation while smaller scaffold pore sizes were associated with delayed differentiation and matrix deposition. Comparable results were obtained in a similar study evaluating the influence of the scaffold porosity of starch-based fiber mesh scaffolds on the proliferation and osteogenic differentiation of bone mesenchymal stem cells cultured in a flow perfusion bioreactor (Gomes et al., 2006; PMID 16674293). Both of these studies indicate that physical properties of fiber mesh scaffolds have a large influence on the behavior of cells seeded onto their surfaces and that these properties can be tailored to induce the osteogenic differentiation pathway of progenitor cells harvested from bone marrow.

**The initial cell phenotype and degree of cell adhesion to titanium fiber mesh scaffolds affects the ectopic bone formation response in vivo**

To determine if cell differentiation could be enhanced by promoting integrin specific cell binding to the scaffold, a titanium fiber mesh was coated with the adhesion peptide sequence RGD (Holtorf et al., 2005; PMID 15921737). Much stronger cell adhesion was observed with the RGD coated fiber mesh scaffolds, and this stronger adhesion tended to delay osteogenic differentiation, rather than promote it in static *in vitro* culture. The osteogenic potential of either plain titanium fiber mesh scaffolds or RGD peptide surface modified titanium fiber mesh scaffolds seeded with mesenchymal stem cells for 1 day prior to subcutaneous implantation in a rat model was also investigated. It was found that ectopic bone formation required the presence of mesenchymal stem cells that had already started down the osteoblast differentiation pathway. These results indicate that the osteogenic differentiation state of mesenchymal stem cells transplanted with tissue engineering scaffolds plays an important role in bone formation.

**In vitro generated bone-like extracellular matrix influences the osteogenic differentiation of mesenchymal stem cells in vitro**

It was then hypothesized that a combination of adhesion sequences and other extracellular matrix molecules would enhance the osteogenic differentiation of mesenchymal stem cells *in vitro*. To test this hypothesis, mesenchymal stem cells were cultured on titanium fiber mesh scaffolds under conditions known to result in the deposition of bone-like extracellular matrix (Datta et al., 2005; PMID 15369685). After 12 days of culture, this matrix was at the onset of mineralization. At this point, the cellular component of the scaffold/cell/ECM
constructs was removed to yield titanium fiber mesh scaffolds with a bone-like extracellular matrix deposited on its surface. This preformed mineralized extracellular matrix was shown to enhance the osteogenic differentiation of freshly seeded mesenchymal stem cells even in the absence of the osteogenic supplements normally required, specifically dexamethasone. This study demonstrates that preformed bone-like extracellular matrix enhances the osteogenic differentiation of freshly seeded mesenchymal stem cells in vitro.

In vitro generated bone-like extracellular matrix and fluid shear stress synergistically enhance osteogenic differentiation of mesenchymal stem cells in vitro

It was then hypothesized that the effects of mechanical stimulation in the form of fluid shear stress and the presence of bone-like extracellular matrix could be combined to have a synergistic effect on the osteogenic differentiation of mesenchymal stem cells. To evaluate this hypothesis, bone mesenchymal stem cells were cultured on titanium fiber mesh scaffolds for 12 days in a flow perfusion system to generate constructs containing bone-like extracellular matrix (Datta et al., 2006; PMID 16477044). These constructs were then decellularized, reseeded with fresh mesenchymal stem cells, and cultured in a flow perfusion bioreactor with culture medium either with or without supplementation with dexamethasone. Similarly, mesenchymal stem cells were seeded onto plain titanium fiber mesh scaffolds and cultured under flow perfusion conditions either with or without dexamethasone as experimental controls. The presence of the pregenerated mineralized extracellular matrix resulted in a 75-fold greater calcium content relative to plain titanium fiber mesh scaffolds following 16 days of culture. The culture medium supplement dexamethasone promoted mineral deposition in the constructs in a cooperative fashion with flow perfusion and the presence of the pregenerated extracellular matrix. Additionally, even in the absence of dexamethasone, flow perfusion culture together with the pregenerated extracellular matrix resulted in greater calcium deposition after 16 days of culture when compared to the plain titanium fiber mesh scaffolds. In order to confirm that bioactive molecules present in the pregenerated bone-like extracellular matrix contributed to the observed enhancement in the osteogenic differentiation (as measured by calcium deposition) of mesenchymal stem cells, pregenerated extracellular matrix constructs were decellularized, heat treated to denature the constituent proteins, and then reseeded with fresh mesenchymal stem cells for culture. It was found that the denatured titanium/extracellular matrix constructs resulted in significantly lower calcium content when compared to the untreated titanium/extracellular matrix constructs following 4 days of culture (Figure 5). These results demonstrate that fluid shear stresses act synergistically with the inherently osteogenic bone-like extracellular matrix generated during an initial (primary) flow perfusion culture period to enhance the osteogenic differentiation of mesenchymal stem cells seeded onto the extracellular matrix for a second (secondary) culture period.

Flow perfusion culture of mesenchymal stem cells results in the generation of an extracellular matrix containing bone-related growth factors

It was subsequently hypothesized that the osteogenicity of the extracellular matrix generated by mesenchymal stem cells cultured under flow perfusion conditions was due, in part, to the presence of osteogenic growth factors in the matrix. To examine this hypothesis, immunohistochemistry techniques were applied to investigate the presence and distribution of several bone-related growth factors within the bone-like extracellular matrix generated during flow perfusion culture of mesenchymal stem cells (Gomes et al., 2006; PMID 16499454). Specifically, two flow perfusion rates and two culture durations were employed to determine the effect of these culture parameters on the presence and spatial distribution of

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transforming growth factor-β1, platelet-derived growth factor-A, fibroblast growth factor-2, vascular endothelial growth factor, and bone morphogenetic protein-2. Each of the growth factors of interest, except platelet-derived growth factor-A, was found to be present in the constructs from each culture condition. Further, the presence and distribution of the various growth factors within the extracellular matrix was found to increase with increasing flow rate and culture duration. This study confirms the presence of osteogenic growth factors within extracellular matrix constructs generated under flow perfusion culture and indicates that the mechanical stimulation provided through flow perfusion enhances the biological signals present within scaffold/cell constructs to direct progenitor cell differentiation.

The preculture period of mesenchymal stem cells in osteogenic media influences the in vivo bone forming potential of the cells

Having demonstrated that the initial cell phenotype affects the osteogenicity of titanium/cell tissue engineering constructs when implanted in an ectopic site (Holtorf et al., 2005; PMID 15921737), it was hypothesized that the in vitro preculture period in osteogenic media of rat mesenchymal stem cells influences their ability to regenerate bone when implanted at an orthotopic site in the absence of a pregenerated extracellular matrix. Mesenchymal stem cells were harvested from rats and expanded in vitro for different time periods (4, 10 and 16 days) in medium supplemented with dexamethasone, seeded on plain titanium fiber mesh scaffolds, and implanted into rat critical size cranial defects after twelve hours (Castano-Izquierdo et al., 2006; PMID 17269144). The alkaline phosphatase activity and calcium deposition of the mesenchymal stem cells from each pre-culture duration (4, 10, and 16 days) indicated that they were at different stages of osteogenic differentiation (pre-osteoblasts, committed osteoblasts, and mature osteoblasts, respectively). Mesenchymal stem cells cultured for six days in the absence of dexamethasone served as controls. Following four weeks of implantation, the highest degree of bone formation was observed with the implants seeded with cells from the shortest pre-culture duration (pre-osteoblasts), and the implants seeded with cells from the longest pre-culture duration (mature osteoblasts) resulted in the lowest degree of bone formation. The results of this study demonstrate that the in vitro preculture period of mesenchymal stem cells transplanted with a bone tissue engineering scaffold is a critical factor for their ability to regenerate bone when implanted in an orthotopic site.

An in vitro generated extracellular matrix supports tissue infiltration and promotes blood vessel formation at an ectopic site in vivo

Having demonstrated the osteogenic potential of an in vitro generated extracellular matrix on the osteogenic differentiation of mesenchymal stem cells in vitro, it was hypothesized that the extracellular matrix deposited by mesenchymal stem cells upon titanium fiber mesh scaffolds during flow perfusion culture in vitro would promote blood vessel and bone formation when implanted at an ectopic site in vivo, in the absence of transplanted mesenchymal stem cells. To evaluate this hypothesis, rat mesenchymal stem cells were cultured on titanium mesh scaffolds or 8, 12, and 16 days to generate constructs with varying degrees of extracellular matrix deposition and maturity (Ti/ECM/d8, Ti/ECM/d12, and Ti/ECM/d16, respectively) (Pham et al., 2008; PMID 18286641). The constructs were then decellularized and implanted for up to 56 days in an intramuscular site in a rat model. No ectopic bone formation was observed with any group after 56 days of implantation. However, tissue infiltration within the porosity of the constructs was observed for all groups, with the predominant regenerated tissue comprising blood vessel, fibroblasts, and/or fat cells. The maturity of the extracellular matrix constructs positively influenced the number of blood vessels formed within the constructs, as measured by histomorphometric analysis,
with Ti/ECM/d16 constructs resulting in a significantly higher number of blood vessels than plain titanium scaffolds. This study demonstrates that an acellular in vitro generated extracellular matrix construct alone may not be sufficient to promote bone formation at an ectopic site, however the constructs enhance blood vessel formation when implanted in vivo.

An in vitro generated bone-like extracellular matrix enhances the osteogenic gene expression of mesenchymal stem cells

Previous studies have shown that bone-like extracellular matrix constructs generated in vitro contain biologically active factors, including transforming growth factor-β1, fibroblast growth factor-2, vascular endothelial growth factor, and bone morphogenetic protein-2 (Gomes et al., 2006; PMID 16499454). Additional studies have demonstrated that the presence of a bone-like extracellular matrix enhances the osteogenic differentiation of mesenchymal stem cells seeded upon a scaffold, when compared to plain scaffolds (Datta et al., 2005; PMID 15369685; Datta et al., 2006; PMID 16477044). Considering these findings, it was hypothesized that the presence of a bone-like extracellular matrix enhances the osteogenic gene expression of mesenchymal stem cells cultured on the constructs in vitro. To test this hypothesis, rat mesenchymal stem cells were seeded onto titanium fiber mesh scaffolds and cultured for 12 days to generate a bone-like extracellular matrix, which was subsequently decellularized. Fresh mesenchymal stem cells were then seeded onto the decellularized bone-like extracellular matrix constructs and cultured for 1, 4, 8, 12 and 16 days, with mesenchymal stem cells seeded upon plain titanium scaffolds serving as controls. The expression of 45 bone-related genes was determined at each time point using real-time reverse transcriptase polymerase chain reaction (RT-PCR) (Pham et al., 2008; PMID 18367245). It was found that mesenchymal stem cells cultured upon bone-like extracellular matrix constructs upregulate expression of genes for a number of osteogenic markers, including collage type I, matrix extracellular phosphoglycoprotein with ASARM motif, parathyroid hormone receptor, and osteocalcin. The increase in bone-related gene expression by mesenchymal stem cells cultured upon bone-like extracellular matrix constructs, coupled with the concomitant increase in mineralized extracellular matrix deposition and downregulation of cartilage-related genes strongly indicates the osteogenic differentiation of the mesenchymal stem cells. The differentiation of the mesenchymal stem cells down an osteogenic lineage could be attributed to the interaction of the cells with various growth factors and bioactive matrix molecules present within the in vitro generated bone-like ECM. Indeed, genes for a number of growth factors and bioactive extracellular matrix molecules were found to be upregulated during the generation of the bone-like extracellular matrix constructs, including insulin-like growth factors 1 and 2, vascular endothelial growth factor, dentin matrix protein, collagen type IV, and matrix metalloproteinase 13. This study demonstrates that a biomaterial can be modified with a biologically active in vitro generated bone-like extracellular matrix capable of directing the gene expression and osteogenic differentiation of seeded progenitor cell populations.

Flow perfusion culture promotes infiltration and spatial distribution of mesenchymal stem cells within electrospun poly(ε-caprolactone) microfiber and multilayer nanofiber/microfiber scaffolds

The majority of the initial work from our laboratory with flow perfusion culture of mesenchymal stem cells involved the application of non-degradable scaffolds, such as titanium fiber meshes, to isolate the effects of the culture parameters of interest while mitigating the potential effects of scaffold degradation during the studies. However, our laboratory envisions the use of flow perfusion culture of mesenchymal stem cells to coat degradable polymer scaffolds with a biologically active extracellular matrix, such that the
resulting constructs may be used as resorbable osteogenic implants for bone tissue engineering to result ultimately in complete filling of the defect with regenerated bone. Toward this goal, the generation of highly porous three-dimensional scaffolds through electrospinning of the degradable polymer poly(ε-caprolactone) for flow perfusion culture was explored (Pham et al., 2006; PMID 17025355). Scaffolds were fabricated reproducibly with fiber diameters ranging from the nanometer to the micrometer scale. Additionally, scaffolds were generated with layers of nanoscale fibers and microscale fibers through sequential electrospinning, with the thickness of each layer being controlled by the duration of the electrospinning of the respective layer. This technique was applied to generate bilayered scaffolds with a microscale fiber layer topped by a nanoscale fiber layer of varying thickness. These bilayered scaffolds were then evaluated for their potential to affect rat mesenchymal stem cell attachment, spreading, and infiltration in flow perfusion culture. It was found that the nanoscale fibers enhanced cell spreading, but increasing numbers of nanoscale fibers did not increase cell attachment. Further, cell infiltration within the scaffolds decreased with increasing thicknesses of nanoscale fiber layers under both static and flow perfusion conditions. However, for a given scaffold architecture, the presence of flow perfusion increased cell infiltration within the scaffolds relative to static culture. This work demonstrates the ability to reproducibly fabricate electrospun poly(ε-caprolactone) scaffolds of controlled architectures, and shows that these scaffolds can support rat mesenchymal stem cell attachment, spreading and infiltration under flow perfusion culture conditions.

**Summary**

Flow perfusion bioreactor systems, which force the flow of media through the porosity of a scaffold, provide several primary advantages for tissue engineering applications; including, (1) a mitigation of the nutrient transport limitations inherent in static culture and (2) the introduction of mechanical stimulation in the form of fluid shear to cells within the scaffold. Our laboratory has cultured mesenchymal stem cells on a variety of scaffolds in flow perfusion bioreactors as well as in static culture to generate mineralized extracellular matrix constructs for bone tissue engineering. Modulation of fluid shear forces through modification of specific flow perfusion culture parameters, such as media flow rate and viscosity, and elements of scaffold architecture, such as mesh size, has demonstrated that osteogenic differentiation of mesenchymal stem cells and subsequent mineralized extracellular matrix production increase with increasing fluid shear stress. Further studies have shown that, although fluid flow enhances mineralized matrix deposition even in the absence of the osteogenic culture medium supplement dexamethasone, the combination of the two elements has a synergistic effect on mesenchymal stem cell differentiation and mineralized matrix production. Immunohistochemistry studies have demonstrated that the mineralized matrix produced during flow perfusion culture contains bioactive factors, including bone morphogenetic protein-2 and transforming growth factor-β1. Additional studies have shown that the extracellular matrix constructs promote osteogenic differentiation of mesenchymal stem cells when decellularized following the initial culture and subsequently seeded with fresh mesenchymal stem cells for a second culture period. This effect was not observed, however, when the extracellular matrix constructs had been heat treated prior to the secondary culture, indicating that the bioactive factors present within the extracellular matrix constructs induce osteogenic differentiation of mesenchymal stem cells. As these examples demonstrate, flow perfusion bioreactors provide an effective environment for the controlled culture of bone progenitors cells under conditions optimized to promote the osteogenic differentiation of the cells and the production of a mineralized scaffold containing osteogenic bioactive factors for bone tissue engineering.
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**Figure 1.**
Micro- and nanofiber composite PCL scaffolds fabricated by electrospinning. Panels B, C, and D are falsely colored to show nanofibers (yellow) dispersed between layers of microfibers (green). The scale bars in panel A and B represent 100 μm and 25 μm, respectively. Reprinted with permission from (Pham et al., 2006; PMID 17025355). Copyright (2006) American Chemical Society.
Figure 2.
MSC osteogenic differentiation on ordered and disordered nanotopographies. After 21 days of culture, MSCs on control (a, f) and symmetrically arranged 100 nm deep pits (b, g) showed appeared fibroblastic (k) and did not stain positively for osteopontin (OPN) or osteocalcin (OCN). Cells cultured on surfaces with pits displaced ± 20 nm (c, h) and ± 50 nm (d, i) from the true center expressed OPN and OCN, formed nodules (arrow), and had an osteoblast-like morphology with mineral nodules after 28 days (l). Cells cultured on surfaces with random pit placement (e, j) had morphology similar to those on displaced surfaces but did not express OPN or OCN. These findings illustrate the sensitivity of cells and MSC differentiation to nanoscale surface features. Reprinted with permission from (Dalby et al., 2007; PMID 17891143). Copyright (2007) Nature Publishing Group.
Figure 3.
Schematic representation of a flow perfusion bioreactor system, which forces the flow of culture medium through a porous scaffold.
Figure 4.
SEM images of scaffold surfaces after culture for 16 days in static culture (A), flow perfusion of 0.3 ml/min (B), flow perfusion of 1 ml/min (C), and flow perfusion of 3 ml/min (D). Note the increasing matrix generation and pore formation at higher flow rates. At the highest flow rate (D), pores appeared to be clogged with matrix (arrows). An expanded view of the newly formed pores from (C) is presented (E, F). Adapted from (Bancroft et al., 2002; PMID 12242339). Copyright (2002) The National Academy of Sciences of the USA.
Figure 5. Calcium content of titanium (Ti), titanium/ECM composites (Ti/ECM), and titanium/denatured ECM composites (Ti/ECM*) after 4 days of culture (A) and after 16 days of culture (B) in the presence or absence (-) of dexamethasone. Error bars represent standard deviation, * represents statistical differences ($p < 0.05$) between constructs cultured under similar conditions, and # denotes statistical differences ($p < 0.05$) between the designated group and all other groups. The presence of ECM but not denatured ECM resulted in significantly increased calcium deposition in all cases, even when cultured in the absence of dexamethasone, showing the osteoinductive capability of the generated ECM. Reprinted from (Datta et al., 2006; PMID 16477044). Copyright (2006) The National Academy of Sciences of the USA.
| Study                                      | Scaffold material         | Culture conditions                        | Results                                                                 |
|-------------------------------------------|---------------------------|-------------------------------------------|------------------------------------------------------------------------|
| Bancroft et al. Proc Natl Acad Sci U S A 99 (2002) 12600-12605 | Titanium fiber meshes     | Static and flow for 4, 8, 16 days         | Increasing flow rate enhances osteogenic differentiation and improves the distribution of bone-like matrix |
| Castano-Izquierdo et al. J Biomed Mater Res A 82 (2007) 129-138 | Titanium fiber meshes     | Pre-culture for 4, 10, 16 days Cranial defect for 1 month | Shorter pre-culture duration in vitro results in more bone formation in vivo |
| Datta et al. Biomaterials. 26 (2005) 971-977 | Titanium fiber meshes     | Static for 1, 4, 8, 12, 16 days           | Bone-like matrix enhances osteogenic differentiation even without dexamethasone |
| Datta et al. Proc Natl Acad Sci U S A 103 (2006) 2488-2493     | Titanium fiber meshes     | Flow for 4, 8, 12, 16 days                | Bone-like matrix and flow have a synergistic effect on osteogenic differentiation even without dexamethasone |
| Gomes et al. J Biomed Mater Res A 67 (2003) 87-95              | SEVA-C and SPCL           | Static and flow for 3, 7, 15 days         | Flow enhances osteogenic differentiation even without dexamethasone distribution on SEVA-C and SPCL |
| Gomes et al. Tissue Eng 12 (2006) 177-188                       | SPCL                      | Flow for 10 and 16 days                   | Higher flow rate and longer culture duration increases the presence and distribution of growth factors |
| Gomes et al. Tissue Eng 12 (2006) 801-809                       | SPCL                      | Static and flow for 7 and 15 days         | Larger scaffold pores promote earlier osteogenic differentiation on SPCL |
| Holtorf et al. Ann Biomed Eng 33 (2005) 1238-1248              | Porous biphasic calcium phosphate ceramics | Static and flow for 4, 8, 16 days         | Flow enhances osteogenic differentiation even without dexamethasone distribution on porous biphasic calcium phosphate ceramics |
| Holtorf et al. Biomaterials 26 (2005) 6208-6216                | Titanium fiber meshes     | Static and flow for 2, 4, 6, 8, 16 days Subcutaneous for 28 days | RGD delays osteogenic differentiation in vitro but has no affect on bone formation in vivo |
| Holtorf et al. J Biomed Mater Res A 72 (2005) 326-334           | Titanium fiber meshes     | Static and flow for 8 and 16 days          | Flow and dexamethasone have a synergistic effect on osteogenic differentiation |
| Holtorf et al. J Biomed Mater Res A 74 (2005) 171-180           | Titanium fiber meshes     | Static and flow for 4, 8, 16 days          | Larger scaffold pores promote earlier osteogenic differentiation on titanium fiber meshes |
| Pham et al. Biomacromolecules 7 (2006) 2796-2805                | Electrospun PCL           | Static and flow for 12 days                | Less nanoscale fibers relative to microscale fibers results in better cell infiltration |
| Pham et al. Biomaterials 29 (2008) 2729-2739                   | Titanium fiber meshes     | Static for 1, 4, 8, 12, 16 days           | Bone-like matrix enhances osteogenic gene expression |
| Pham et al. J Biomed Mater Res A (2008) in press                 | Titanium fiber meshes     | Flow for 8, 12, 16 days                   | Longer culture duration in vitro results in more blood vessel formation in vivo |
| Sikavitsas et al. J Biomed Mater Res A 67 (2003) 944-951        | Titanium fiber meshes     | Static and flow for 1, 4, 8 days          | Shorter culture duration in vitro results in more bone formation in vivo |
| Sikavitsas et al. Proc Natl Acad Sci U S A 100 (2003) 14683-14688 | Titanium fiber meshes     | Static and flow for 4, 8, 16 days         | Increasing fluid viscosity and thus fluid shear stress enhances osteogenic differentiation |
| Sikavitsas et al. Ann Biomed Eng 33 (2005) 63-70                | Non-woven PLLA            | Static and flow for 4, 8, 16 days         | Flow enhances osteogenic differentiation and improves distribution on non-woven PLLA |
| Study | Scaffold material | Culture conditions | Results |
|-------|-------------------|--------------------|---------|
| van den Dolder et al. J Biomed Mater Res A 64 (2003) 235-241 | Titanium fiber meshes | Static and flow for 4, 8, 16 days | Flow enhances osteogenic differentiation and improves distribution on titanium fiber meshes |