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ABSTRACT

Craniosynostosis is a congenital disorder of premature ossification of cranial sutures, occurring in one of approximately every 2500 live human births. This work addressed a hypothesis that a cranial suture can be tissue-engineered from autologous cells. Dermal fibroblasts were isolated subcutaneously from growing rabbits, culture-expanded, and seeded in a gelatin scaffold. We fabricated a composite tissue construct by sandwiching the fibroblast-seeded gelatin scaffold between two collagen sponges loaded with recombinant human BMP2. Surgically created, full-thickness parietal defects were filled with the composite tissue construct in the same rabbits from which dermal fibroblasts had been obtained. After four-week *in vivo* implantation, there was *de novo* formation of tissue-engineered cranial suture, microscopically reminiscent of the adjacent natural cranial suture. The tissue-engineered cranial suture showed radiolucency on radiographic images, in contrast to radio-opacity of microscopically ossified calvarial defects filled with fibroblast-free, BMP2-loaded constructs. This approach may be refined for tissue engineering of cranial sutures for craniosynostosis patients.

KEY WORDS: suture, bone, osteoblast, craniofacial, tissue engineering.

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INTRODUCTION

Cranial sutures are a soft connective-tissue interface between mineralized calvarial bones, with the primary function of allowing expansive bone and brain growth (Mao, 2002; Mao and Nah, 2004). Sutures consist of an array of cell lineages, such as mesenchymal cells and fibroblast-like cells, residing in a vascularization-rich matrix that is sandwiched between two osteoblast-lined bone formation fronts (Opperman, 2000; Mao, 2002). With the progression of sutural osteogenesis, a physiological mechanism exists to maintain the vitality and presence of suture mesenchyme, and to prevent the suture from undergoing complete mineralization (Cohen and MacLean, 2000; Warren and Longaker, 2001). Mechanical stresses play important roles in suture development at different levels of organization (Mao, 2002). Although tensile stresses hypothetically resulting from brain enlargement have long been attributed to allowing the separation of cranial sutures and subsequent sutural growth, analysis of recent data showing anabolic sutural responses upon compressive stresses generated by either mastication or exogenous forces suggests the diverse nature of mechanical stimuli capable of stimulating sutural growth (Rafferty and Herring, 1999; Kopher and Mao, 2003; Collins *et al.*, 2004).

Much of our knowledge of suture development has derived from studies of heterogeneous congenital disorders collectively known as craniosynostosis or premature suture ossification prior to the completion of craniofacial growth (Cohen and MacLean, 2000; Opperman, 2000). Although many craniosynostosis phenotypes are linked to an increasing number of gene mutations, such as FGFRs and MSX2, over 50% of current craniosynostosis cases appear to be sporadic (Liu *et al.*, 1995; Cohen and MacLean, 2000; Wilkie and Morriss-Kay, 2001; Ignelzi *et al.*, 2003). Craniosynostosis occurs in one of approximately every 2500 live human births, and clinical phenotypes may manifest as visible craniofacial disfigurations, high intracranial pressure, and severe neurological disorders such as mental retardation, blindness, and seizure (Cohen and MacLean, 2000). Craniofacial surgery is the primary choice for correcting visible craniofacial disfigurations and relieving abnormally high intracranial pressure (Marsh, 2000; Tessier, 2000). Surgeons typically perform craniotomy in early childhood by dissecting fused sutures and leaving gaps of empirical size between involved calvarial bones, anticipating that the surgically created gaps will accommodate both brain growth and calvarial bone growth (Marsh, 2000; Tessier, 2000). Due to the unpredictability of craniofacial growth from the time of the first surgical correction of craniosynostosis, frequently performed in infants, to growth completion in adolescents, surgically created gaps may re-synostose. Secondary surgeries are then needed for re-synostosis, probably attributable to the fact that a synostosed suture with missing mesenchymal and fibrous interface is replaced by a surgically created gap still lacking a sustainable mesenchymal and fibrous interface (Marsh, 2000; Tessier, 2000).

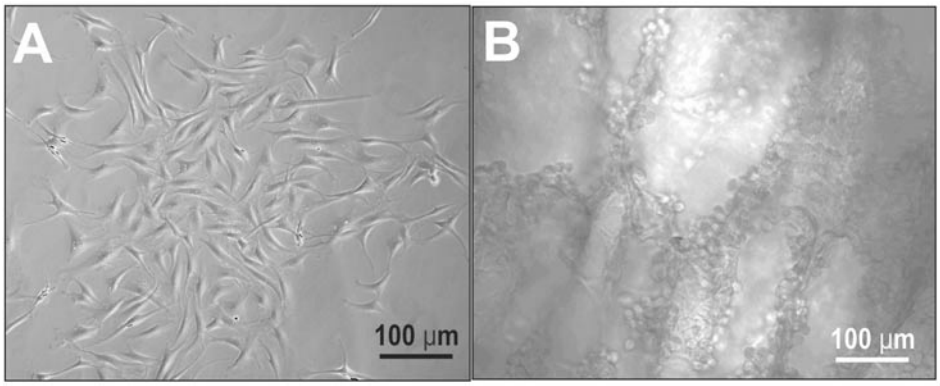

Figure 1. Isolation, culture-expansion, and seeding of autologous dermal fibroblasts. A 5-mm incision was made in the anterior tibia. A small piece of subcutaneous fibrous tissue (approx. 3 x 3 mm^2) was removed. The isolated dermal fibroblasts were plate-cultured and expanded **(A)**. **(B)** An absorbable gelatin scaffold was trimmed to 2 x 2 x 6 mm^3 and immersed in the suspension of culture-expanded dermal fibroblasts at a density of 10^7 cells/mL.

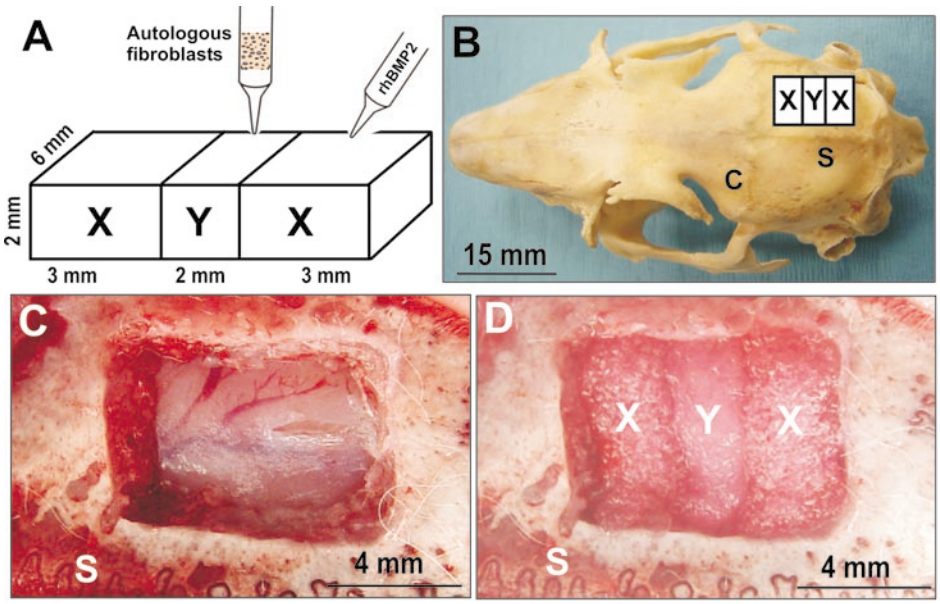

Figure 2. Experimental procedures. **(A)** Dermal fibroblast-seeded gelatin scaffold (Y component) is sandwiched between two microporous collagen sponges loaded with rhBMP2 (two X components). **(B)** Location of surgically created calvarial defect in the parietal bone in relation to the natural sagittal suture (s) and coronal suture (c). **(C)** Surgical creation of full-thickness calvarial defect with a dimension of 6 x 2 x 8 mm^3 in the center of the rabbit parietal bone devoid of natural cranial sutures. The adjacent sagittal suture (s) is shown. The dura mater was kept intact. Following creation of surgical calvarial defects in the corresponding rabbits from which dermal fibroblasts had been obtained, composite tissue grafts were implanted into the center of the parietal bone devoid of natural cranial sutures (N = 3). In one control group, tissue grafts consisting of two rhBMP2-loaded collagen sponges without autologous fibroblasts were implanted in age- and sex-matched rabbits (N = 3). In another control group, tissue grafts consisting of two rhBMP2-loaded collagen sponges with an intervening fibroblast-free gelatin scaffold were implanted in age- and sex-matched rabbits (N = 3). **(D)** The surgically created calvarial defect was filled with a composite tissue construct consisting of autologous autologous fibroblast-seeded gelatin scaffold (Y component) that was sandwiched between two rhBMP2-loaded microporous collagen sponges (two X components). The adjacent sagittal suture (s) was not part of the surgically created calvarial defect.

In the present study, we attempted to tissue-engineer a composite construct utilizing autologous dermal fibroblastic cells isolated subcutaneously from the anterior tibial region, culture-expanding them, and seeding them in a gelatin scaffold sandwiched between two microporous collagen sponges loaded with recombinant human BMP2 (rhBMP2). The tissue-engineered composite construct was implanted into a surgically created calvarial defect in the center of the parietal bone devoid of natural sutures in the donor rabbit from which dermal fibroblasts had been isolated. The rationale for the present model was to create a tissue-engineered cranial suture from autologous cells and a potent osteogenic stimulant, BMP2, in an approach that is potentially aligned with eventual therapeutic application in patients with craniosynostosis. It was hypothesized that a cranial suture can be tissue-engineered from autologous fibroblasts and BMP2 loaded in biocompatible polymers.

MATERIALS & METHODS

Isolation of Dermal Fibroblasts and *ex vivo* Fabrication of Fibroblastic Construct

A total of 9 eight-week-old, male New Zealand White rabbits was used after institutional approval for animal use. Under general anesthesia and aseptic conditions, a 5-mm incision was made in the anterior tibial skin. A small piece of subcutaneous fibrous tissue (approx. 3 x 3 mm^2) was removed, minced, and digested with Accutase at 37°C for 1 hr, followed by neutralization with Dulbecco's Modified Eagle's Medium-Low Glucose (DMEM; Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS) (Biocell, Rancho Dominguez, CA, USA). The digested tissue solution was filtered through a cell restrainer (100-µm pore size). After centrifugation, the isolated dermal fibroblasts were plate-cultured (10^6 cells/100-mm dish) and expanded in DMEM with 10% FBS and 1% antibiotic-antimycotic (Gibco, Carlsbad, CA, USA) (Fig. 1A). Upon 90% confluence, the fibroblasts were trypsinized and subcultured at a density of 10^6 cells/100-mm dish. An absorbable gelatin scaffold (Pharmacia, Kalamazoo, MI, USA) was trimmed to 2 x 2 x 6 mm^3 and immersed in cell suspension at 10^7 cells/mL in DMEM supplemented with 10% FBS under a light vacuum created by a 20-mL syringe for 1 hr (Fig. 1B), followed by two-hour incubation at 37°C.

Ex vivo Preparation of Osteo-inductive Scaffold and Fabrication of Composite Tissue Construct

Individual absorbable collagen hemostatic sponges (Integra, Plainsboro, NJ, USA) were trimmed to 3 x 2 x 6 mm^3 size (Fig. 2A). We prepared recombinant human bone morphogenetic protein-2 (rhBMP2) by dissolving 2.5 μg rhBMP2 in 15 μL PBS at 4°C overnight (R&D Systems, Minneapolis, MN, USA) (Fig. 2A). The rationale for incorporating BMP2 was to simulate the high osteogenic potential of synostosed cranial sutures (De Pollack *et al.*, 1996). The fibroblast-gelatin construct as prepared above was sandwiched between two rhBMP2-loaded collagen sponges to create a composite construct with an overall dimension of 6 x 2 x 8 mm^3 (Fig. 2A).

Surgically Created Calvarial Defects and Implantation of Tissue-engineered Composite Construct

Under aseptic conditions and general anesthesia with ketamine (40 mg/kg) and xylazine (8 mg/kg), a 2-cm incision was made along the midsagittal plane in the calvarium. Upon deflection of the scalp, subcutaneous tissue, and periosteum, a full-thickness calvarial defect (6 x 2 x 8 mm^3) was created unilaterally in the center of the parietal bone by the use of a dental bur with constant PBS irrigation (Fig. 2B). Care was taken to ensure that the surgically created full-thickness calvarial defect was devoid of the adjacent coronal, sagittal, or lamboidal suture, and also to maintain the underlying dura mater intact (Fig. 2C). Three groups were devised: (1) two rhBMP2-loaded collagen constructs without either gelatin scaffold or autologous fibroblasts (no Y component in Fig. 2A) (N = 3); (2) two rhBMP2-loaded collagen constructs without autologous fibroblasts, but with an intervening gelatin scaffold (Y component without cells in Fig. 2A) (N = 3); and (3) two rhBMP2-loaded collagen constructs with autologous fibroblasts seeded in an intervening gelatin scaffold (Y component present in Fig. 2A) (N = 3). In the experimental group (Group 3), the tissue-engineered composite construct (Fig. 2D) was implanted into the surgically created calvarial defect of the corresponding donor rabbit from which the autologous fibroblasts had been harvested. In all 3 groups following placement of constructs, the previously deflected periosteum, subcutaneous tissue, and scalp were replaced. The incision was closed with 4-0 absorbable gut surgical suture. The rabbits were housed in a temperature- and light-controlled room (23-25°C, 14 hrs light/day), and given standard daily amounts of food and water.

Assessment of *de novo* Formation of Tissue-engineered Constructs

Four weeks after *in vivo* implantation, all rabbits were killed by pentobarbital overdose (300 mg/kg, i.v.). The entire calvarium was harvested with an orthopedic saw, fixed in 10% paraformaldehyde, demineralized in equal volumes of 20% sodium citrate and 50% formic acid, and embedded in paraffin. Serial 8-μm-thick sections were cut in the parasagittal plane by means of a microtome.

Mineral deposition in surgically implanted grafts was grossly examined by radiophotographic imaging at 90 kV and 15 mA with 10-second exposure. Three 8-μm-thick microscopic sections in the parasagittal plane were stained with hematoxylin and eosin: one from the center of the implanted graft and two from both sides of the graft, each 2 mm from the parasagittal midline. Quantitative histomorphometric analysis was performed on H&E-stained microscopic sections under a research microscope equipped with a digital camera. The widths of the fibrous interface between two active bone formation fronts were measured from no fewer than 6-8 lines *per* specimen, by computerized image analysis (*cf.* Fig. 3A′). By ANOVA with Bonferroni tests at an alpha level of $P < 0.05$, the average widths of the fibrous interface (f in Fig. 3C′) in the tissue grafts were compared among those with seeded autologous fibroblasts in an intervening gelatin scaffold and those either without autologous fibroblasts or fibroblast-free gelatin scaffold.

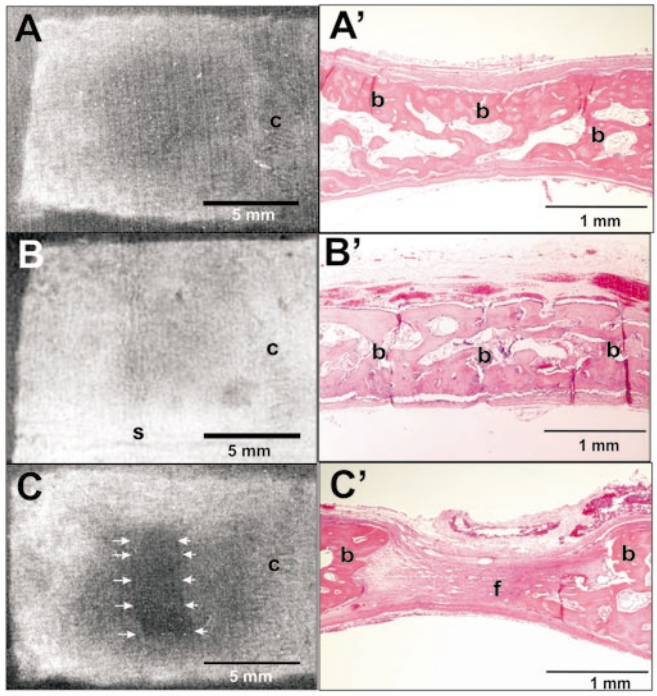

Figure 3. Radiographic and photomicrographic images of tissue-engineered cranial suture and controls. **(A)** Representative radiographic image showing a lack of radiolucency in the tissue graft with two rhBMP2-loaded microporous collagen sponges without intervening autologous fibroblasts loaded in a gelatin scaffold. The adjacent coronal suture is labeled as 'c'. **(A′)** Corresponding microscopic image showing ossification of the surgically created calvarial defect without delivery of autologous fibroblasts or an intervening gelatin scaffold. **(B)** Representative radiographic image showing a lack of radiolucency in the tissue grafts with an intervening fibroblast-free, gelatin scaffold between two rhBMP2-loaded microporous collagen sponges. The adjacent coronal and sagittal sutures are labeled as 'c' and 's', respectively. **(B′)** Corresponding microscopic image showing ossification of the surgically created calvarial defect with fibroblast-free gelatin scaffold. **(C)** Representative radiographic image showing a band of radiolucency (between opposing white arrows) corresponding to the area of autologous fibroblasts loaded in a gelatin scaffold intervening between two rhBMP2-loaded microporous collagen sponges, indicating a fibrous tissue interface between mineralized bones. The adjacent coronal suture is labeled as 'c'. **(C′)** Corresponding microscopic image showing *de novo* formation of a fibrous tissue interface (f) between two mineralized bone segments (b) in the calvarial defect filled with fibroblast-seeded gelatin scaffold sandwiched between two rhBMP-loaded collagen sponges.

RESULTS

Radiographic examination revealed a lack of radiolucency in tissue grafts with two rhBMP2-loaded microporous collagen sponges but without intervening autologous fibroblasts seeded in a gelatin scaffold (Fig. 3A), indicating ossification of the

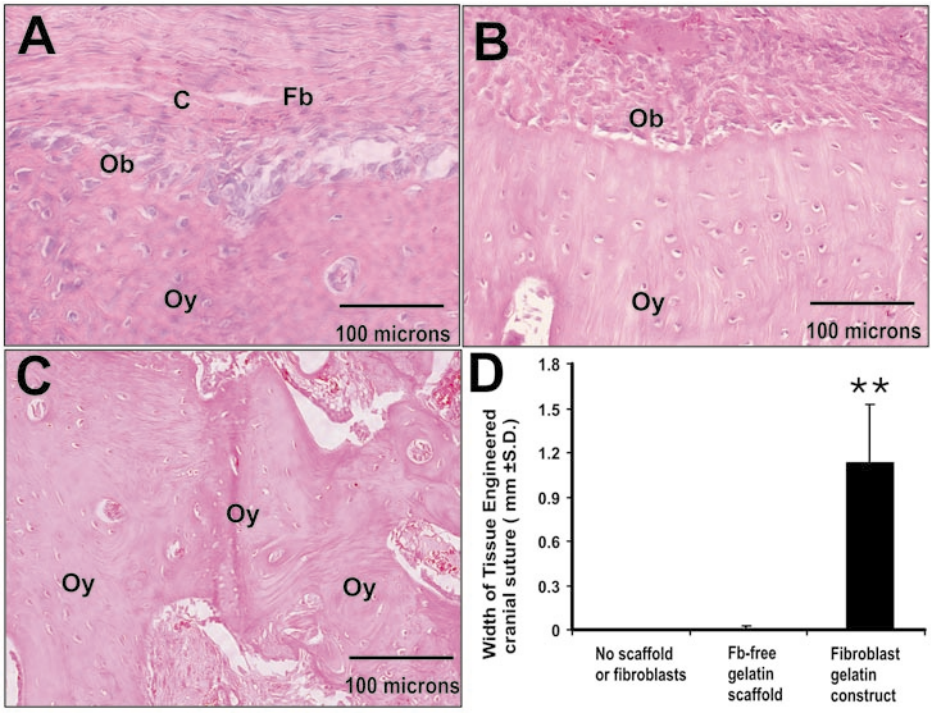

Figure 4. High-power examination of tissue-engineered cranial suture and controls. **(A)** The tissue-engineered cranial suture consisted of collagen-fiber-like structures (c), fibroblast-like cells (Fb), osteoblast-like cells (Ob), and osteocyte-like cells (Oy) in apparently mineralized bone. Osteoblast-like cells formed an approximate layer on the surface of an apparent bone formation front. **(B)** The adjacent natural sagittal suture showed fibroblast-like cells in suture mesenchyme, osteoblasts lining the sutural bone formation front, and osteocytes (Oy) in the mineralized bone. **(C)** Complete bony fusion occurred in the surgically created calvarial defect filled with tissue grafts consisting of two rhBMP-loaded collagen sponges, but without the intervening fibroblast-seeded gelatin scaffold. **(D)** Histomorphometric analysis and statistical comparison of the widths of the fibrous tissue interface between two mineralized bone formation fronts. The average width of tissue-engineered sutures consisting of autologous fibroblast-populated gelatin scaffold sandwiched between two rhBMP2-loaded microporous collagen sponges was 1.13 ± 0.39 mm (SD), significantly greater than the average widths of either tissue grafts without intervening autologous fibroblast-gelatin scaffolds (0.006 ± 0.004 mm) or an intervening fibroblast-free gelatin scaffold (0 ± 0 mm; N = 3) **P < 0.01.

surgically created calvarial defect without delivery of autologous fibroblasts, as corroborated by the corresponding microscopic image (Fig. 3A′). This was further confirmed by ossification of defects upon delivery of two rhBMP2-loaded microporous collagen sponges with an intervening fibroblast-free gelatin scaffold (Fig. 3B) and corresponding microscopic image (Fig. 3B′). In contrast, a band of radiolucency (between opposing white arrows in Fig. 3C) was present in the center of each of the tissue-engineered composite constructs, consisting of a fibroblast-seeded gelatin scaffold sandwiched between two rhBMP2-loaded microporous collagen sponges. The corresponding microscopic section (Fig. 3C′) corroborated the radiographic image (Fig. 3C) by demonstrating a fibrous tissue interface (f in Fig. 3C′) between the two osteogenic bone formation fronts (b in Fig. 3C′), indicating that autologous fibroblasts are essential for *de novo* formation of a tissue-engineered cranial-suture-like structure.

High-power microscopic examination of the tissue-engineered cranial suture indicated *de novo* formation of a non-mineralized, fibrous interface between two apparently new bone formation fronts in the surgically created calvarial defects devoid of natural cranial sutures (Fig. 4A). Fibroblast-like cells resided among apparent collagen fibers, with areas of angiogenesis in the tissue-engineered cranial suture (Fig. 4A). Columnar osteoblast-like cells lined up along the surface of the bone formation front with osteocyte-like cells embedded in mineralized bone that possessed distinct lacunae-like structures (Fig. 4A). These microscopic characteristics of the tissue-engineered cranial suture were similar to those of the adjacent natural sagittal suture, in which osteogenic cells lined the surface of the sutural bone formation front with embedded osteocytes also possessing lacunae (Fig. 4B). In comparison with the tissue-engineered cranial suture in Fig. 4A, tissue grafts consisting of two rhBMP2-loaded collagen sponges lacking an intervening fibroblast-free gelatin scaffold led to complete ossification of the surgically created defect (Fig. 4C), similar to complete ossification by tissue grafts consisting of two rhBMP2-loaded collagen sponges with an intervening fibroblast-free, gelatin scaffold (data not shown but *cf.* Fig. 3B′). Histomorphometric data measured from multiple microscopic sections demonstrated that the average width of tissue-engineered cranial sutures consisting of an autologous fibroblast-populated gelatin scaffold sandwiched between two rhBMP2-loaded microporous collagen sponges was 1.13 ± 0.39 mm (SD), significantly greater than the average widths of tissue grafts either without an intervening autologous fibroblast-gelatin scaffold (0.006 ± 0.004 mm) or with an intervening fibroblast-free gelatin scaffold (0 ± 0 mm; N = 3) (Fig. 4D).

DISCUSSION

The present findings represent the first report of *de novo* formation of tissue-engineered cranial suture from autologous cells. A cranial-suture-like structure is generated in the surgically created calvarial defect only in the presence of a tissue-engineered composite construct including fibroblast-seeded gelatin scaffold intervening between two rhBMP2-loaded collagen sponges. This indicates that autologous fibroblasts are essential for *de novo* formation of a tissue-engineered cranial suture in the current calvarial model. The microscopic characteristics of the tissue-engineered cranial suture are similar to those of the adjacent natural suture. Osteoblast-like cells line the bone formation fronts, indicating that the tissue-engineered cranial suture is not fibrous non-union. Up to 4 wks of *in vivo* implantation, the tissue-engineered cranial suture remains patent without complete

ossification. In contrast, calvarial defects filled with tissue grafts consisting of two rhBMP2-loaded collagen sponges, with or without an intervening fibroblast-gelatin scaffold, readily ossify.

The make-up of cells in natural suture mesenchyme is not entirely clear, although there are certainly multiple cell types, such as mesenchymal cells, fibroblast-like cells, osteoblast-like cells, and any blood-vessel-borne cells (Rice *et al.*, 2000; Wilkie and Morriss-Kay, 2001). Type I collagen is the most abundant collagen phenotype in cranial sutures (Meikle *et al.*, 1982; Yen *et al.*, 1989; Zimmerman *et al.*, 1998; Rafferty and Herring, 1999). Thus, cranial sutures likely contain cells that produce type I collagen fibrils. The lack of ossification of suture mesenchyme during normal development indicates the presence of cells in suture mesenchyme capable of producing type I collagen fibrils and a mechanism(s) preventing their mineralization. Accordingly, autologous fibroblasts have been delivered in the present tissue-engineered composite constructs to initiate the fibrous interface between mineralized bones. The presently delivered fibroblasts are autologous in that tissue-engineered cranial sutures are formed *de novo* in rabbits from which dermal fibroblasts have been isolated. This autologous tissue-engineering approach may eliminate the potential problem of immune rejection by allografts or xenografts. The osteogenic cells lining the osteogenic fronts in the tissue-engineered cranial suture can derive from the overlying periosteum or underlying dura mater, or from mesenchymal cells resident in the dermal fibroblast population that differentiated into osteogenic cells in the presence of rhBMP2. Cell labeling is necessary to differentiate among these cell sources.

The present approach appears to complement previous meritorious efforts on suture transplantation or delaying the rate of suture synostosis. Surgical replacement of synostosed rabbit suture with an allogeneic suture graft, including dura mater from the wild-type rabbit, allows post-operative sutural growth to occur (Mooney *et al.*, 2001), representing perhaps the most direct approach to the replacement of synostosed sutures. However, this allogeneic suture transplantation approach necessitates the creation of secondary bony defects, requires donor availability, and has the potential of immune rejection for human applications. Placement of e-PTFE membrane in a surgically created calvarial defect involving the rat sagittal suture led to a suture-like tissue similar to the original sagittal suture, and it was suggested that this suture-like tissue likely developed by migration of cells from the remaining portion of the sagittal suture (Mardas *et al.*, 2002). The exogenously delivered rhBMP2 in the present work, known as a potent osteo-inductive factor, may have multiple effects. The rationale for incorporating BMP2, in the present work, was to simulate the high osteogenic potential of synostosed cranial sutures (De Pollack *et al.*, 1996). The bone formation rate of synostosed sutures can be 50% higher than that of normal sutures (De Pollack *et al.*, 1996). Even in the presence of rhBMP2, dermal fibroblasts seeded in gelatin scaffold intervening between two BMP2-loaded collagen sponges are capable of maintaining the presence of the tissue-engineered cranial suture. Application of rhBMP2 may also have implications in providing osteogenic stimulation in the adult craniosynostosis patient, due to a potential shortage of bone during surgical skull reshaping. The application of certain doses of TGFβ-3 loaded in collagen gel placed over the suture delays the timed fusion of the rat interfrontal suture (Opperman *et al.*, 1999, 2002), further substantiating the potential role the TGFβ superfamily plays in regulating sutural fusion (Bradley *et al.*, 2000; Greenwald *et al.*, 2000; Moursi *et al.*, 2003). It is probable that many of the currently pursued approaches toward regeneration of cranial sutures may find their applications among heterogeneous clinical phenotypes of craniosynostosis.

Much additional work is necessary before a tissue-engineered cranial suture from autologous cells is available for clinical applications. The long-term outcome must be evaluated. The effects of mechanical stresses on its development should be explored, because sutures also function as articulations for the transmission of mechanical stresses (Rafferty and Herring, 1999; Mao, 2002; Kopher *et al.*, 2003; Kopher and Mao, 2003; Mao *et al.*, 2003; Collins *et al.*, 2004). The mechanical properties of a tissue-engineered cranial suture should be compared with those of natural cranial sutures (Radhakrishnan and Mao, 2004). The selection of biomaterials in the present study—namely, microporous collagen and gelatin scaffolds—was based on the rationale that they are biocompatible, biodegradable, and have been widely used in biomedical applications. Analysis of the present data, taken together, may serve as a 'proof of concept' for additional studies leading to tissue-engineered cranial sutures that surgeons can use to replace synostosed cranial sutures in craniosynostosis patients.

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