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A Tissue Engineering Approach for Prenatal Closure of Myelomeningocele with Gelatin Sponges Incorporating Basic Fibroblast Growth Factor

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Myelomeningocele (MMC) is a common and devastating malformation. Although fetal surgical closure may improve outcome, a less invasive approach that can be applied earlier in gestation is desirable. The objective of this study was to evaluate the therapeutic feasibility of a tissue engineering approach for prenatal coverage of MMC. A gelatin hydrogel composite combining a gelatin sheet and gelatin sponge was prepared with or without basic fibroblast growth factor incorporation, and applied prenatally to retinoic-acid-induced fetal MMC in the rat model. Most of the composites were adherent to the MMC within the amniotic fluid environment with the help of cyanoacrylate adhesive. Histological examination revealed cells layered over the composites with associated extracellular matrix as well as cellular ingrowth into the sponges. The layer over the composite was composed of mixed nonepithelial and epithelial cells with the extracellular matrix consisting of collagen type I and hyaluronic acid. The tissue inside the sponge consisted of nonepithelial cells and hyaluronic acid. Epidermal ingrowth underneath the sponges and neovascularization into the sponges occurred and were significantly increased by the incorporation of basic fibroblast growth factor. Although further development is needed, this study supports the therapeutic potential of a tissue engineering approach for prenatal coverage of MMC.

Introduction

Myelomeningocele (MMC) is one of the most common congenital malformations and represents the most severe form of spina bifida. MMC results from failure of closure of the neural tube with secondary exposure of the neural elements. A "two-hit" hypothesis has been proposed to explain the neurologic damage observed in children with MMC. The first "hit" is the primary failure of neurulation and the second "hit" is due to the chemical and mechanical injury of the exposed neural elements that occurs throughout the remainder of gestation.[1] The compelling rationale for prenatal closure of the defect is to prevent the second hit, that is, damage to the spinal cord from exposure to the intrauterine environment. To test this rationale, an National Institutes of Health–sponsored clinical trial comparing prenatal closure of the defect with standard postnatal treatment is currently underway (Management of Myelomeningocele study). Before initiation of this trial, nonrandomized or controlled preliminary studies documented a reversal of hindbrain herniation, a reduced incidence of shunt-dependent hydrocephalus, and better than anticipated lower extremity neuromotor function in a subset of patients. However, many children after fetal surgery have significant residual deficit.[2–9] In many cases, the procedure is performed relatively late in gestation, after damage to the neural elements has already occurred. It is also possible that the surgery itself, either directly or indirectly, through scarring and tethering of the cord contributes to the neural injury. Finally, open fetal surgery involves a major operative procedure placing the mother at significant risk. Therefore, a less invasive approach that could be applied earlier in gestation would theoretically increase the benefit and reduce the risk of fetal treatment.

As the MMC defect can be viewed as a tissue defect, we hypothesized that it may be possible to apply tissue engineering techniques to achieve tissue coverage of the defect and thereby prevent the progressive destruction of the exposed neural elements.[10–13] If a tissue-engineered construct could be applied using fetoscopy or ultrasound-guided injection, the treatment would be much less invasive than current treatments and could be applied earlier in gestation to maximize preservation of neural elements.[14]

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The objective of this study was to demonstrate proof of principle that a gelatin-hydrogel-based scaffold containing basic fibroblast growth factor (bFGF) can adhere to the MMC defect in the rat model of retinoic-acid (RA)-induced fetal MMC, and subsequently promote tissue coverage over the defect.

Materials and Methods

Biomaterials

Gelatin with an isoelectric point of 5.0 (molecular weight = 99,000) was prepared through alkaline processing of bovine bone (Nitta Gelatin, Osaka, Japan). The bFGF was supplied by Kaken Pharmaceutical (Tokyo, Japan).

Preparation of gelatin hydrogels and sponge

Gelatin sponges and sheets were prepared by dehydrothermal crosslinking according to the method previously reported.[15,16] Briefly, the gelatin sponges were created by mixing a 3 w/v% solution of aqueous gelatin at 5000 rpm for 3 min with a homogenizer (Auto Cell Master CM-2000; As One, Osaka, Japan) at room temperature, and then casting the mixture in a polypropylene dish of $60 \times 60\,cm^2$. The casts were frozen in liquid nitrogen for 20 s, and then transferred to a $-20^{\circ}C$ freezer for 12 h, followed by freeze-drying (TF5-85ATANN; Takara, Tokyo, Japan). The casts were then put into a vacuum drying oven (DN-30S; Sato Vac, Tokyo, Japan) at $160^{\circ}C$ for 24 h. The dehydrothermal crosslinked gelatin sponges were punched out to obtain 1-mm-thick discs with a 3.5 mm diameter and then sterilized with ethylene oxide gas (EOG; EOG sterilizer SA-160; Elk, Osaka, Japan) (Fig. 1A). A 3 w/v% solution was chosen after comparison with 1, 5, and 7 w/v% solutions because it provided an optimal pore size for proliferation and migration of mesenchymal stromal cells and fetal fibroblasts *in vitro* as determined by colorimetric 3,[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. Pore size was determined by examination of sectioned sponges under scanning electron microscopy.

Preparation of the gelatin sheet

A bicomponent mold was constructed as follows. A 3.5 mm biopsy punch (Biopsy Punch; Kai Industries, Gifu, Japan) was used to punch out 36 holes in a Teflon sheet (PTFE; As One) in a 4×9 grid. A second Teflon sheet of the same size was glued underneath the first one representing the bottom mold. The top mold was constructed by gluing the 36 holes that were punched out from the Teflon sheet onto a plastic sheet of the same size, in the exact 4×9 grid locations matching the holes in the bottom mold (Fig. 1B). A 5 w/v% solution of gelatin solution was then poured into the bottom mold and the top mold was placed upside-down on top of it to create circular gelatin sheets designed to match the shape of the MMC defect with a central inset to accommodate insertion of the gelatin sponge. The filled molds were

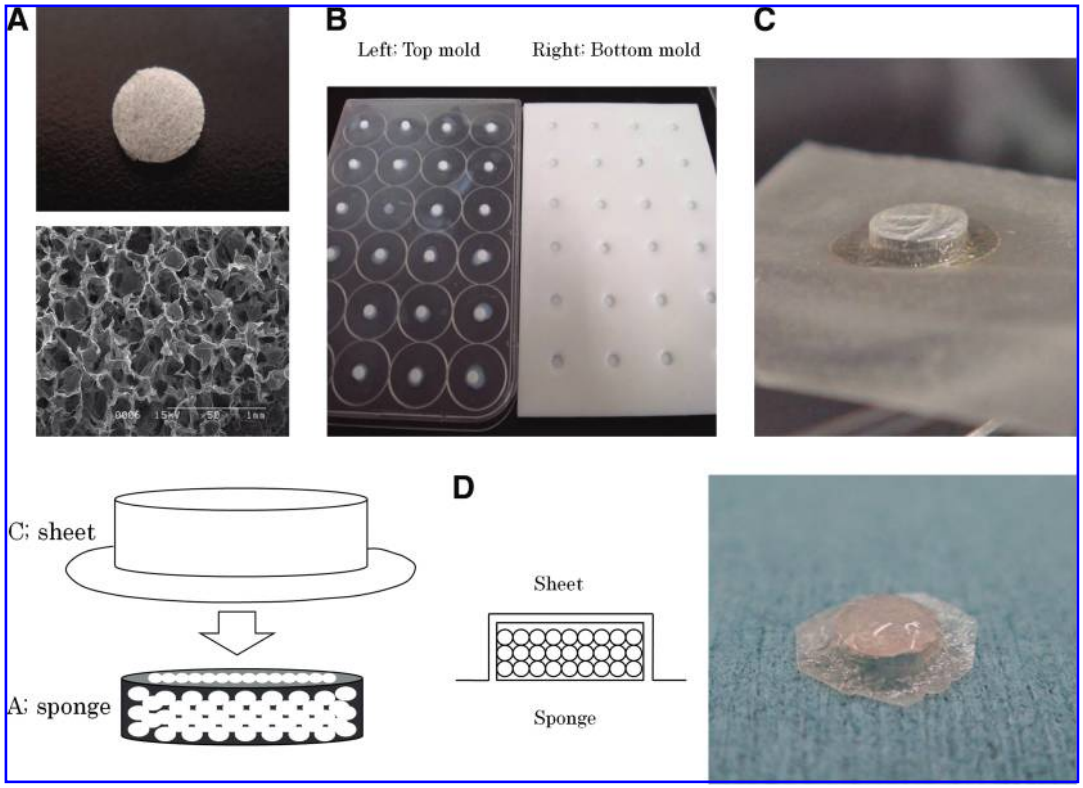

FIG. 1. Preparation of the gelatin composite. (**A**) The 3.5-mm-diameter gelatin sponge and the cross-sectional view by scanning electron microscopy. The pores range from 250 to 400 μm. (**B**) A bicomponent mold consisting of fitted internal and external components to construct the gelatin sheets. (**C**) The appearance of the dehydrothermal crosslinked gelatin sheet formed with the bicomponent mold. (**D**) Schematic diagrams of the gelatin composite composed of a gelatin sheet and a gelatin sponge and the assembled composite. Color images available online at www.liebertonline.com/ten.

kept in a refrigerator at 4°C until the gelatin solution was completely air-dried. The molded gelatin was peeled off of the cast and put into a vacuum drying oven at 160°C for 24 h for crosslinking. The finished product was harvested by cutting around each of the grooves and bumps to form 36 individual gelatin sheets that fit the shape of the MMC defect (Fig. 1C). The sheets were sterilized with EOG.

Preparation of bFGF-incorporated gelatin sponges and gelatin composites

Aqueous solution containing 10 μg/μL of bFGF (10 μL) was applied to a freeze-dried gelatin sponge and left overnight at 4°C. The prepared bFGF-incorporated gelatin sponges were used without washing. Each gelatin sponge was fitted within the recessed area of the gelatin sheet to make a gelatin composite just before use (Fig. 1D).

Animal experiments

All experimental protocols were approved by the Institutional Animal Care and Use Committee at The Children's Hospital of Philadelphia or the Committee on Animal Care and Use at Kyoto University and followed guidelines set forth in the National Institutes of Health Guide for Care and Use of Laboratory Animals.

Animal preparation and RA exposure

The procedures for creating MMC defects in fetal rats have been described by us previously.[14] Briefly, time-dated Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA, and Shimizu Laboratory Supplies, Kyoto, Japan) were used for this study. After a brief exposure to isoflurane (Abbott Lab, North Chicago, IL), pregnant rats were fed 50 mg/kg of *all-trans* RA (Sigma-Aldrich, St. Louis, MO) dissolved in olive oil (10 mg/mL) at embryonic day 10 (E10).

Surgical procedure

Sprague-Dawley pregnant rats exposed to RA on E10 underwent surgery at E18.[17] General anesthesia was induced and maintained by inhaled isoflurane. After sterile preparation, the uterine horns were exposed through a maternal midline laparotomy. A 6-0 polypropylene suture (PDS-II; Ethicon, Somerville, NJ) was passed through the uterine wall and the amniotic membranes. The back of each fetus was exposed through a small hysterotomy, and a gelatin sheet or composite was applied on the MMC area for each of the groups as follows (Fig. 2A): group A—gelatin composite (gelatin sponge and sheet) containing bFGF; group B—gelatin composite without bFGF; group C—gelatin sheet alone; and group D—untreated. The edge of the gelatin sheet was spot glued with a cyanoacrylate adhesive (Instant Krazy Glue; Krazy Glue, Columbus, OH) for better adhesion. After the gelatin was securely adherent, the fetuses were returned into the uterus. The amniotic fluid (AF) was replaced with sterile normal saline (NS) and the hysterotomy was closed with a purse-string suture. The uterus was returned to the abdomen and the incision closed in two layers with a 5-0 Vicryl running suture (Ethicon). Three to four fetuses with MMC per pregnant rat were manipulated, and the rest of the littermates served as controls.

Estimation of degradation of gelatin sponges in utero

Degradation of the gelatin sponges *in utero* was evaluated in a separate experiment by loss of fluorescence activity. Gelatin sponges were prelabeled with fluorescein isothiocyanate

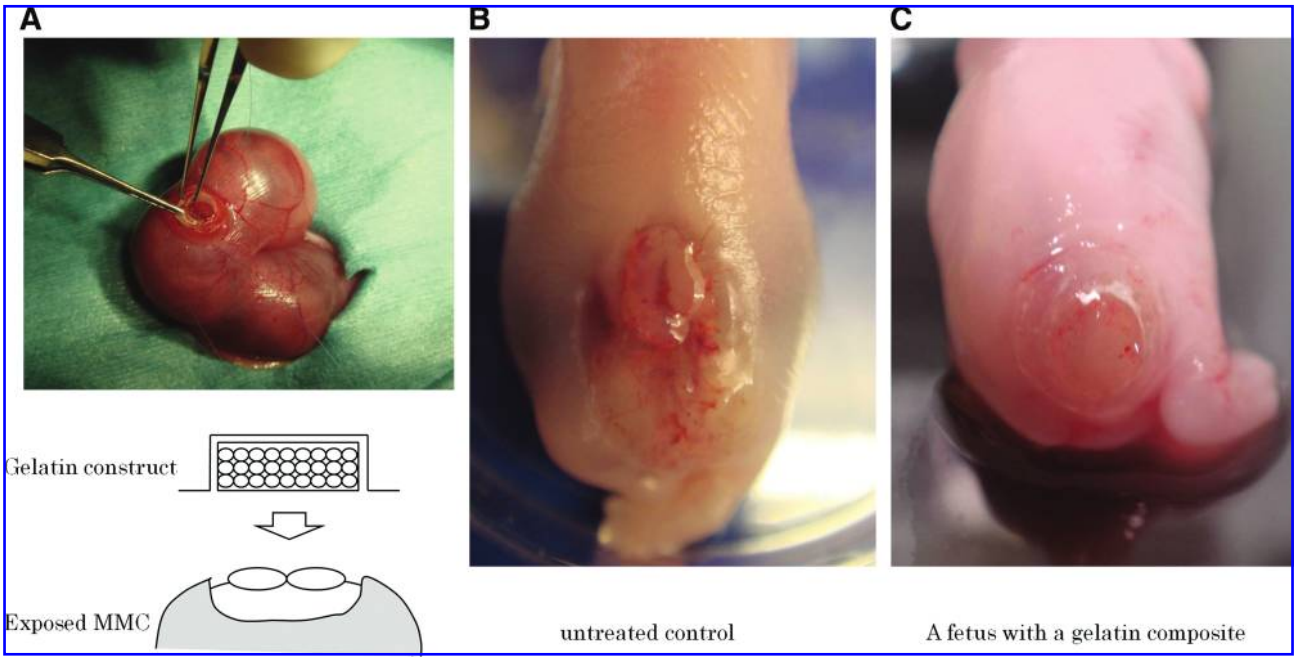

FIG. 2. The rat model of MMC with application of the gelatin composite. (**A**) Application of a gelatin composite to the MMC defect through a hysterotomy incision and its schematic. (**B**) Representative photograph of an untreated fetal rat with MMC at E21. (**C**) Representative photograph of a fetal rat with MMC at E21 after gelatin construct application at E18. MMC, myelomeningocele; E21, embryonic day 21. Color images available online at www.liebertonline.com/ten.

(FITC) by adding 1500 μL of FITC (Sigma-Aldrich) solution (1 μg/μL) to twenty 3.5 mm gelatin sponges (0.6 mg wt/per sponge) that were expanded with 0.2 M sodium bicarbonate buffer (pH 9.7) in 2 mL tubes. The sponges were incubated for 8 h at room temperature and then centrifuged at 8000 rpm for 10 min. The supernatant was removed and the sponges were washed in distilled water until the supernatant was clear. After assembly with the gelatin sheet, the FITC-labeled gelatin composites were applied to fetuses as described above. They were harvested on E21, and sponges and AF were taken for fluorescence analysis. Sponges were autoclaved with 1 mL NS in a glass tube for complete thermal dissolution. The fluorescence of the autoclaved solution was measured in a microplate spectrofluorometer (Ex. 494 nm, Em 518 nm; Gemini EM; MDS, Toronto, Canada). Each of the experimental groups and controls was as follows: group I—sponges implanted on fetuses *in utero* from E18 to E21; group II—sponges immersed in AF at 36°C *in vitro* for 3 days; group III—sponges immersed in NS at 36°C *in vitro* for 3 days; and group IV—unmanipulated sponges. Five samples in each group were analyzed.

Histological analysis

Pregnant rats were euthanized by CO_2 inhalation followed by cervical dislocation at E21. The fetuses were harvested by cesarean section. Whole fetuses were fixed in 10% neutral buffered formalin (Sigma-Aldrich) and decalcified using Immunocal (Decal Chemical, Tallman, NY) for 7–10 days. After histoprocessing, they were embedded in paraffin wax and sectioned at 4 μm thickness. Sections were dewaxed, serially rehydrated, and stained with hematoxylin and eosin and Trichrome. Sections assigned for immunohistochemistry were air-dried overnight in a 50°C incubator, deparaffinized, and rehydrated in distilled water. To block endogenous peroxidase, slides were incubated for 30 min with 3% hydrogen peroxide solution (Sigma-Aldrich). The sections were then immersed in antigen unmasking solution pH 6.0 (Vector Laboratories, Burlingame, CA) and microwaved (Ted Pella, Redding, CA) for 4 min on high power, cooled at room temperature for 30 min, washed in distilled water, and transferred to phosphate-buffered saline (PBS) containing 0.1% Triton X-100. The following primary antibodies were used: anti-vimentin (Invitrogen, Camarillo, CA; predilute antibody); anti-pancytokeratin (1:100; Abcam, Cambridge, MA); anti-alpha-smooth muscle actin (α-SMA; 1:200; Abcam); anti-collagen I (1:500; Abcam); anti-collagen III (1:400; Abcam); hyaluronan binding protein 2 (HBP2; 1:50; LifeSpan BioSciences, Seattle, WA); anti-glial fibrillary acidic protein (GFAP; 1:100; DakoCytomation Denmark A/S, Glostrup, Denmark); and anti-neuron-specific beta III Tubulin (1:100; Abcam).

Primary antibodies were diluted using ready-to-use antibody diluent (DakoCytomation Denmark A/S), and applied over the slides and then incubated over night at 4°C. On the following day, the sections were washed three times with PBS containing 0.1% Triton X-100 and then incubated with horseradish peroxidase polymer conjugate broad spectrum (diaminobenzidine [DAB]; SuperPicture™; Zymed Laboratories, Carlsbad, CA) for 30 min at room temperature. Finally, the sections were washed one time with PBS containing Triton X-100, observed using Peroxidase Substrate Kit (DAB; Vector Laboratories), counterstained with hematoxylin, dehydrated, and mounted using Permount (Fisher Scientific, Pittsburgh, PA). If necessary, the images were merged into a single image, using an Apple Macintosh computer (Mackintosh, Cupertino, CA) with Adobe Photoshop Elements 2.0. (Adobe, San Jose, CA).

Evaluation of healing by assessment of epidermal ingrowth and neovascularization in the gelatin sponge

The distance between the original keratinized epidermis and the edge of epidermis, where the gelatin sponges were applied, was measured to assess epidermal ingrowth using a Windows XP computer (Microsoft, Redmond, WA) with Axio Vision 4.5. (Carl Zeiss MicroImaging, Thornwood, NY). Also, the area of the gelatin sponges occupied by red blood cells (RBCs) relative to the whole area of the gelatin sponge was assessed using the same software as an indicator of neovascularization.[18,19]

Statistical data analysis

All continuous data are presented as mean ± standard deviation. Parametric data were analyzed by Student's *t*-test for comparison of two groups, and by analysis of variance for more than two groups. Pairwise comparison after analysis of variance was adjusted with the Bonferroni procedure. Categorical data were analyzed with Fisher's exact test. Statistical significance was defined as $p \leq 0.05$. STATA version 10 (College Station, TX) was used for analysis.

Results

Operative results and gross appearance

A total of 29 pregnant rats underwent operation. Four died intraoperatively due to anesthetic complications, but no rats delivered before scheduled harvest. The cesarean section was performed on the remaining 25 pregnant rats, and 85 operated and 5 control fetuses underwent analysis. Of the 85 operated fetuses, there were 32 survivors (37% survival). There was no significant difference in the survival rate among groups (group A, 23%; group B, 22%; and group C, 31%). Since the strength of adherence of the molded gelatin sheet to fetal skin in water was only between 0.51 and 0.89 N by *in vitro* strength testing in our preliminary studies (data not shown), we secured the gelatin composite with cyanoacrylate adhesive. Of the 32 survivors, the gelatin composite remained adherent on 21 fetuses and had detached from 11 fetuses. Sixteen of the 21 fetuses with complete adherence of the gelatin composite underwent analysis (group A, $n = 6$; group B, $n = 5$; and group C, $n = 5$) and were compared to sham operated controls (group D, $n = 5$).

Figure 2B and C shows the gross appearance of an untreated control fetus with MMC and a fetus with MMC who had an adherent gelatin composite, respectively. There were no signs of inflammation around the gelatin composite.

In vivo *degradation of the gelatin sponge*

Figure 3 shows the fluorescence of gelatin sponges in different conditions. The fluorescence was significantly lower in I (2077 ± 432, $p \leq 0.005$) than other groups, which indicates the degradation of sponge by the *in utero* environment. No significant differences in the fluorescence intensity between groups II (32835 ± 4483), III (28705 ± 8666), and IV (38104 ± 2237) were observed.

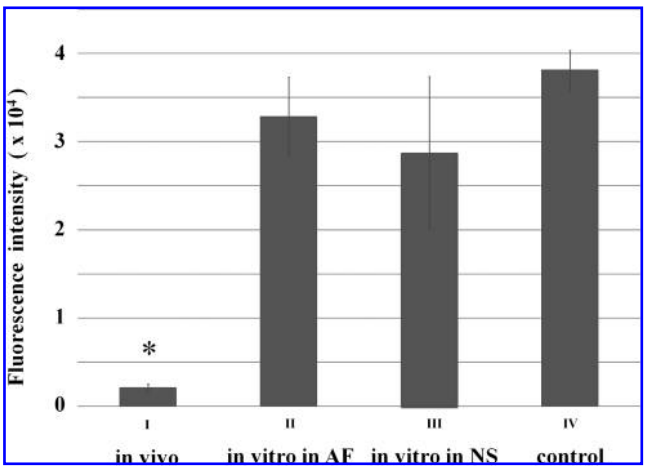

FIG. 3. *In vivo* and *in vitro* degradation of the gelatin sponge. Change in the fluorescence intensity of fluorescein-isothiocyanate-labeled gelatin sponges 3 days after the exposure to various environments. The gelatin sponges were (I) applied on the MMC at E18; (II) immersed in AF *in vitro*; (III) immersed in NS *in vitro*; or (IV) nonimmersed control. $^*p < 0.05$; significant against the fluorescence intensity of fluorescein-isothiocyanate-labeled gelatin hydrogel sponges in other samples. AF, amniotic fluid; NS, normal saline.

Histological evaluation

Cellular adherence and migration to the gelatin composite. Despite the evidence of degradation in the AF, the gelatin composites were grossly and microscopically adherent to fetuses with MMC for 3 days (Figs. 2C and 4). At higher magnification, cells and extracellular matrix (ECM) could be seen as an adherent layer along the outside of the gelatin sheet in all groups (Fig. 5). Also, cells and ECM could be seen between the fetal surface and gelatin construct. The cells stained positively for vimentin and pancytokeratin, but not α-SMA, suggesting that these cells were comprised of epithelial and nonepithelial types. The ECM stained positively for hyaluronan binding protein 2 (HBP2) and collagen type I.

In addition to the cellular layer above the gelatin sheet, cells and their associated ECM could be seen within the sponges in groups where a sponge was present (Fig. 6). Cells inside the sponges appeared to be streaming from the fetal tissue and stained positively only for vimentin. The ECM stained positively only for HBP2. These results were consistent irrespective of the presence of bFGF.

A very minimal inflammatory response was observed with only a few neutrophils and monocytes seen within the sponges and in the surrounding fetal tissues. Granulation tissue was observed underneath the gelatin sponges and interdigitated within the margin of the sponges. However, there were no significant fibrotic changes.

Epidermal ingrowth is increased by bFGF

Epidermal ingrowth, as determined by measuring the distance between the original keratinized epidermis and the edge of epidermis, was measured in the groups A and B (Fig. 7). The epidermis appeared to elongate and thicken underneath the gelatin sponges in both groups, but group A (with bFGF) showed significantly greater epidermal ingrowth than group B (without bFGF) ($120.67 \pm 58.45\,\mu m$ vs. $42.84 \pm 26.51\,\mu m$, $p \leq 0.05$).

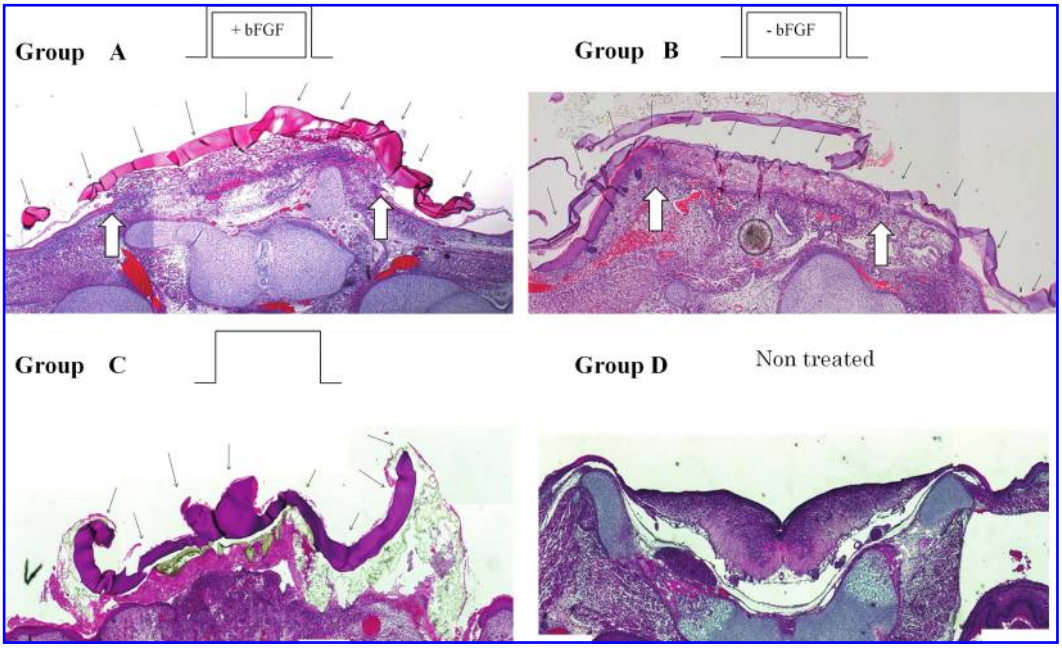

FIG. 4. Transverse sections through the MMC defect 3 days after application of the gelatin composite. Group A, gelatin composite incorporating bFGF; group B, gelatin composite without bFGF; group C, gelatin sheet alone; and group D, untreated MMC. An adherent gelatin sheet that resembles a pink-purple membrane can be seen adhering to the fetus (arrows). The contained gelatin sponge demonstrates a porous structure between the fetal body and gelatin sheet (the edge of sponge is indicated with white arrows). H&E staining; magnification, ×5. bFGF, basic fibroblast growth factor; H&E, hematoxylin and eosin. Color images available online at www.liebertonline.com/ten.

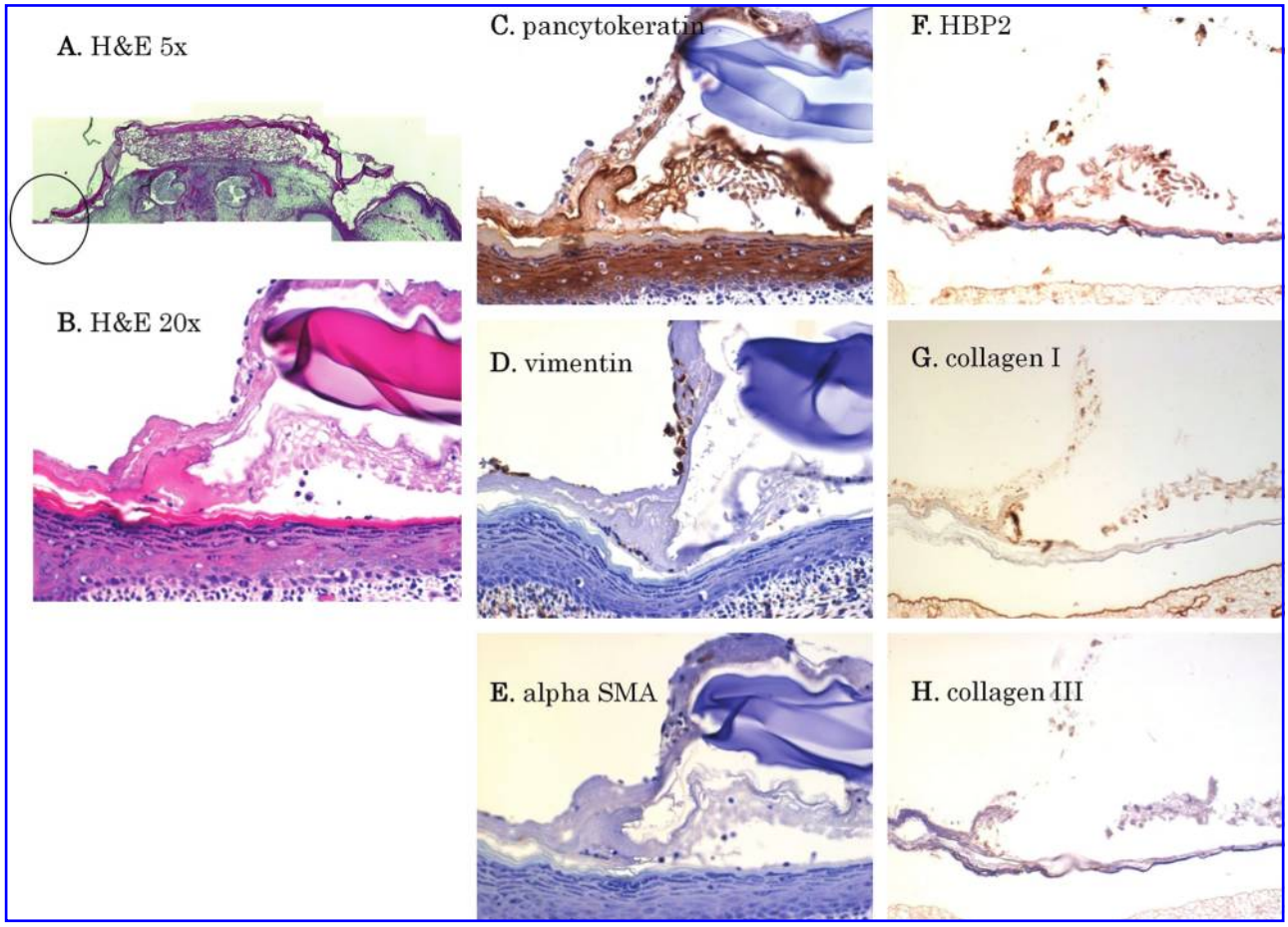

FIG. 5. (**A**) Representative H&E-stained section of the MMC defect with an adherent gelatin composite. The circle shows the area of focus for images (**B**) through (**H**) at the edge of the fetal epidermis and zone of adherence of the gelatin sheet. Magnification, ×5. (**B**) H&E staining, and immunohistochemistry for (**C**) pancytokeratin, (**D**) vimentin, (**E**) α-SMA, (**F**) hyaluronan binding protein 2 (HBP2), (**G**) collagen I, and (**H**) collagen III. Magnification, ×20. α-SMA, alpha-smooth muscle actin; HBP2, hyaluronan binding protein 2. Color images available online at www.liebertonline.com/ten.

Neovascularization in the gelatin sponge is increased by bFGF

Numerous RBCs could be seen inside the gelatin sponges in the groups A and B, an indirect indicator of neovascularization. The percentage of the area occupied by RBCs to the whole area of the sponge was statistically greater in the group A than in the group B ($4.81 \pm 1.78\%$ vs. $1.05 \pm 0.73\%$, $p \leq 0.05$) (Fig. 8).

Assessment of exposed neural elements

Figure 9 shows that exposed neural elements and ganglia stained positively for neuron-specific beta III Tubulin in all groups. These results were consistent with untreated control MMC.[20,21] The neural elements in all experimental groups and in the nonoperated controls demonstrated partial staining for GFAP as well.

Discussion

This study is the first report of the use of gelatin hydrogel as a scaffold for promoting tissue coverage of fetal MMC. Gelatin is a biodegradable and natural biomaterial that is a denatured form of collagen that has several advantages. It has been proven to be cytocompatible and can be formulated to various shapes, such as sheets, sponges, and particles. The kinetics of degradation can be readily controlled by changing the extent of crosslinking. Also, it has been demonstrated that gelatin can function as a carrier for the controlled release of growth factors with biological activities.[22–24] Gelatin incorporating bFGF has been applied to wounds in adult wound healing models and has been shown to promote angiogenesis, epithelization, granulation tissue formation, and wound closure.[25–30] Based on these observations, we reasoned that the use of gelatin hydrogel containing bFGF might promote tissue coverage over the MMC defect and provide a baseline for further studies to optimize the growth factors or cellular elements required to achieve this goal. Previous investigators have applied collagen, cellulose, polytetrafluoroethylene, and polyglycolic acid to MMC in the surgically created fetal sheep MMC model, demonstrating some acceleration of skin regeneration.[10–13] However, these materials do not allow the controlled release of growth factors or promote the survival and biological function of cellular implants and may induce significant inflammatory response.

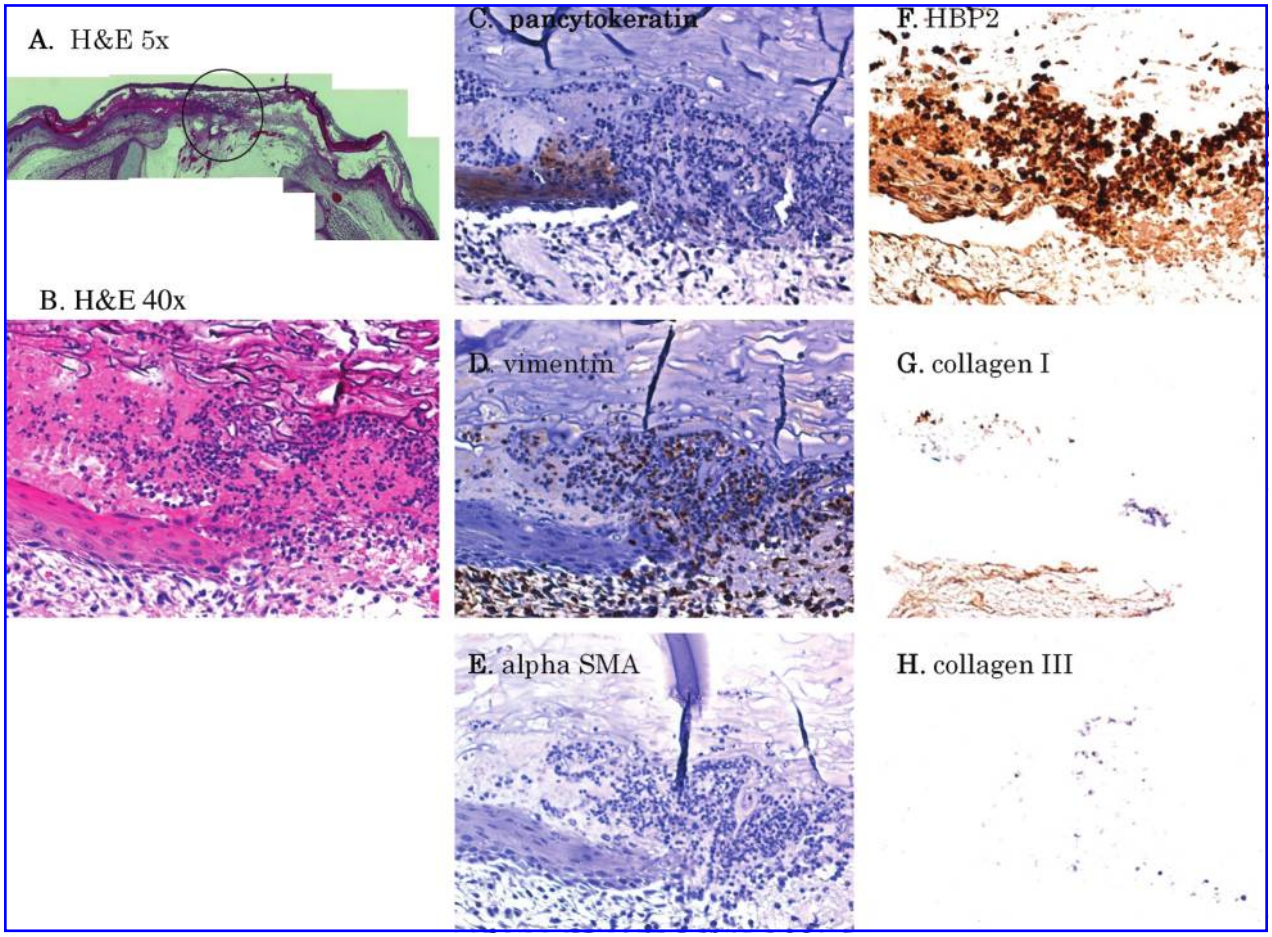

FIG. 6. (**A**) Representative H&E-stained section of the MMC defect with an adherent gelatin composite on top left. The circle shows the area of focus for images (**B**) through (**H**) at the fetal skin edge and edge of the gelatin sponge. Magnification, ×5. (**B**) H&E staining, and immunohistochemistry for (**C**) pancytokeratin, (**D**) vimentin, (**E**) α-SMA, (**F**) HBP2, (**G**) collagen I, and (**H**) collagen III. Magnification, ×20. Color images available online at www.liebertonline.com/ten.

In the present study, different formulations of gelatin hydrogel were used to create the composites to cover the MMC defect. The gelatin sheet was designed to act as an adhesive covering over the MMC that would exclude AF. The sheets were prepared with a 5% gelatin solution and 24 h dehydrothermal crosslinking. A concentration of less than 5% gelatin resulted in sheets that were very fragile, resulting in disruption of the applied sheet when the fetus was returned to the uterine cavity. A gelatin concentration of more than 5% resulted in noncompliant sheets that would not conform well to the contours of the MMC. The gelatin sponge component was designed to function as a cell scaffold that allowed the controlled release of bFGF. The pore size of the scaffold is one of the important variables controlling cell migration. The sponges used in the present study had pore sizes of 250 to 400 μm, allowing optimal cell migration into the sponge based on an *in vitro* cellular proliferation assay (data not shown).

It has been previously shown that biologically active growth factor can be released as a result of *in vivo* degradation of the hydrogel and that the release profile is determined by the water content of the hydrogel and the strength of crosslinking.[22–24] Our analysis by loss of fluorescence activity, however, demonstrated a considerable amount of degradation *in utero* within 3 days (Fig. 3). This faster *in vivo* degradation is likely due to enzymatic degradation by factors, such as MMP2 gelatinase present in AF.[31,32] Although the gelatin sponges became smaller by the time of harvest on E21, they were still present and functional as a scaffold for cellular ingrowth. In addition, the increased cellular ingrowth and neovascularization in the sponges incorporating bFGF experimentally confirmed the *in vivo* release of biologically active bFGF. Although the degradation profile of our composite appeared to be adequate in this relatively short-term rat model, it is likely that a slower degradation profile would be optimal for human MMC.

Ideally, the gelatin composite would adhere firmly to fetal skin in the AF environment after simple application. However, the strength of adherence of the molded gelatin sheet to fetal skin proved inadequate to carry out surgical manipulations by an open surgical technique. Therefore, a minimum amount of cyanoacrylate adhesive was used to allow the gelatin products to adhere firmly. Although the cyanoacrylate adhesives did not appear to add toxicity in this study, a more biocompatible adhesive would be required for clinical application.

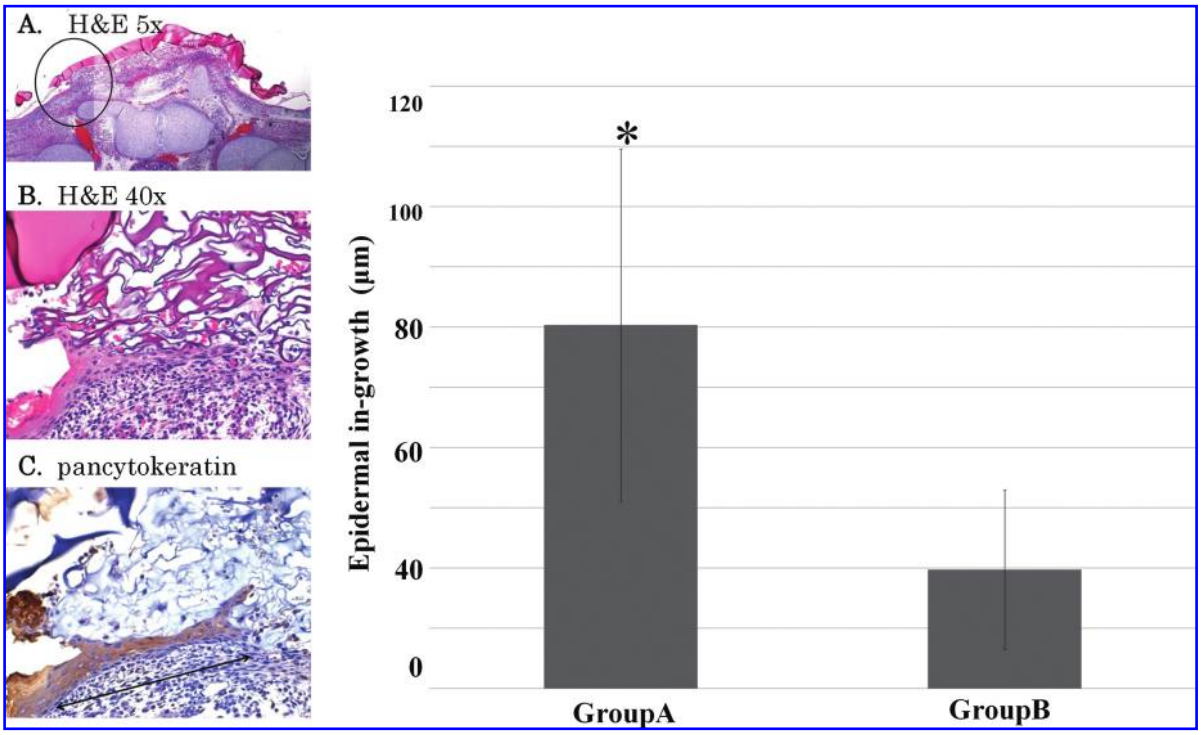

FIG. 7. Epidermal ingrowth underneath gelatin sponges 3 days after application of gelatin composites. (**A**) Representative H&E staining of MMC defect with a gelatin composite. Magnification ×5. The circle shows the area of magnification for (**B**) and (**C**). (**B**) H&E staining focused on the epidermal ingrowth from the edge of MMC defect and (**C**) its immunohistochemistry for pancytokeratin. Magnification, ×20. The arrow indicates the measured distance between the original keratinized epidermis and the edge of epidermis under a gelatin sponge. Graph of the distance of epidermal ingrowth under the composite containing bFGF (group A) and without bFGF (group B) (*$p < 0.05$). Color images available online at www.liebertonline.com/ten.

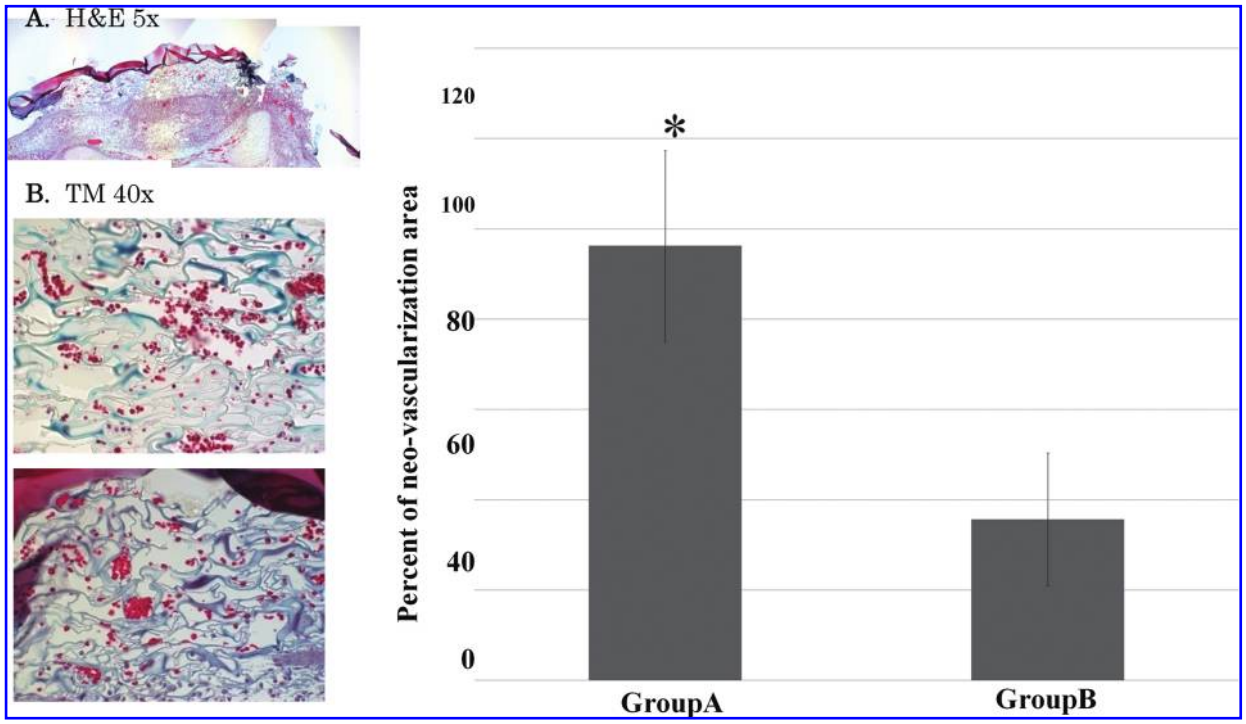

FIG. 8. Neovascularization inside the gelatin sponges 3 days after application of gelatin composites. (**A**) Representative H&E staining of the MMC defect covered with a gelatin composite. Magnification, ×5. (top) (**B**) Trichrome (TM) staining focused on the areas containing the mass of red blood cells inside a gelatin sponge. Magnification, ×20. Graph of the area of neovascularization in gelatin composite sponges containing bFGF (group A) and without bFGF (group B) (*$p < 0.05$). Color images available online at www.liebertonline.com/ten.

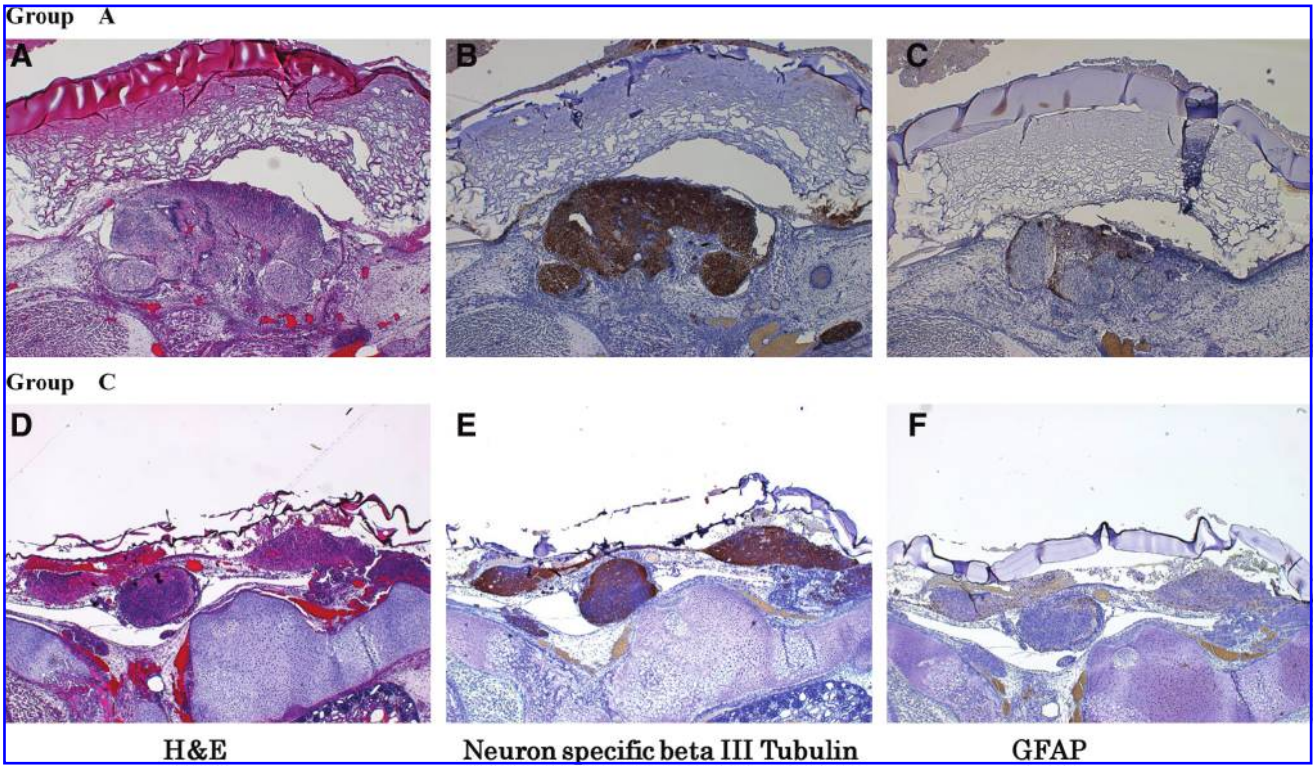

FIG. 9. (**A**) Representative H&E staining of MMC defect with a gelatin composite containing bFGF (group A) and (**D**) a gelatin sheet alone (group C). Corresponding immunohistochemistry for (**B, E**) neuron-specific beta III tubulin and (**C, F**) GFAP. Magnification, ×5. GFAP, glial fibrillary acidic protein. Color images available online at www.liebertonline.com/ten.

We observed cellular populations and associated ECMs both covering the gelatin sheet and between the gelatin construct and the fetal surface (Figs. 5 and 6). The cells adherent to the surface of gelatin sheet expressed cytokeratin and vimentin, but not α-SMA. This suggests that they were a mixed population consisting of epithelial cells and nonepithelial cells excluding smooth muscle cells, myoepithelial cells, and myofibroblast. The ECM associated with these cells was stained positively for HBP2 and collagen I. Although collagen III is the predominant type of collagen in the ECM of fetal tissues, it was not found in significant amounts in this study.[33] The expression level of hyaluronan in the matrix appeared stronger than that of collagen I. It is well known that hyaluronan is a major component of fetal tissues and is a major component of the ECM observed in fetal wound healing. Fetal fibroblasts can produce hyaluronan and hyaluronan stimulates the migration of fibroblasts.[33,34] In addition, it was found that the level of hyaluronan in the AF ranged from 16 to 72 μg/mL depending on gestational age. The AF is known to contain amniocytes that are a mixture of epithelial and mesenchymal cells. Given the rapid appearance of this cell layer and associated matrix (within 3 days), it is likely that this layer is derived from adherence of amniocytes to the gelatin rather than overgrowth from the fetal surface.

In contrast, cells between the fetal surface and the gelatin sponge and within the sponge stained positively only for vimentin and the ECM were primarily stained by HBP2. These cells were distinguishable from the cells coating the outside of the gelatin composites, suggesting that they are comprised of mesenchymal cells migrating into the sponges from the underlying fetal tissue. The amount of cellular ingrowth into the sponges appeared to be equal between group A (with bFGF) and group B (without bFGF), but these cells were absent in group C (gelatin sheet alone), supporting our precept that the gelatin sponge would provide a scaffold for cellular ingrowth.

We also observed significant differences in epidermal ingrowth underneath the gelatin sponges between gelatin sponges with and without bFGF. It is apparent that the released bFGF appeared to stimulate epidermal cells at the margin of the defect with the desirable effect of initiating dermal closure of the MMC defect. Interestingly, a dramatic difference in the area occupied by red blood cells within the sponges was observed. This indirectly suggests that the bFGF released stimulated angiogenesis, which has been reported in other systems.[35,36] However, in this study, direct assessment of endothelial ingrowth could not be done due to the lack of antiendothelial antibodies with high specificity for rat endothelial cells.

A major limitation of this study is the small size and relatively short gestation of the rat model. This prevented an adequate duration of observation to confirm that durable tissue coverage would eventually occur. In addition, we have previously demonstrated that significant neural damage has already occurred in this model by E18, making the timing of fetal surgery too late for prevention of neural damage.[21] Our findings indicate that most of the exposed neural tube elements stained positively for neuron-specific beta III and a portion of the neurons stained positively for GFAP. These

findings were consistent with the staining we observed in untreated MMC controls, suggesting that at least the gelatin constructs and bFGF had no detrimental impact on the neural elements.

Our precept for this study was that the tissue coverage achieved needs to be bidirectionally impermeable to both AF and CSF to prevent damage to neural elements and the associated Arnold-Chiari malformation, respectively. It must also have adequate strength to prevent mechanical disruption with fetal movement and delivery. Whether the cellular coating over the composites and cellular ingrowth observed beneath it in this study would have ultimately provided an adequate tissue covering is impossible to know given the limitations of the model. However, the present study supports our hypothesis that gelatin hydrogel could function as a scaffold to induce the cellular migration and the consequent proliferation required to achieve tissue coverage from endogenous cells. In addition, we anticipate further optimization of the construct by (1) adjustment of its degradation and adhesion characteristics; (2) screening for other growth factors (i.e., transforming growth factor-β, epidermal growth factor (EGF), and platelet-derived growth factor) that may further accelerate cellular ingrowth and neovascularization; and (3) incorporation of exogenous cellular components such as fibroblasts, mesenchymal stem cells, or amniocytes in the construct. Ultimately optimal constructs developed in this model can be tested in larger animal models of MMC such as the sheep. Although further material and technological development of the scaffold is needed, the present data strongly support the future potential of a tissue engineering approach for prenatal coverage of MMC.

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Disclosure Statement

No competing financial interests exist.

References

1. Heffez, D.S., Aryanpur, J., Hutchins, G.M., and Freeman, J.M. The paralysis associated with myelomeningocele: clinical and experimental data implicating a preventable spinal cord injury. Neurosurgery **26,** 987, 1990.
2. Sutton, L.N., Adzick, N.S., and Bilaniuk, L.T. Improvement in hindbrain herniation demonstrated by serial fetal magnetic resonance imaging following fetal surgery for myelomeningocele. JAMA **282,** 1826, 1999.
3. Tulipan, N., Sutton, L.N., Bruner, J.P., Cohen, B.M., Johnson, M., and Adzick, N.S. The effect of intrauterine myelomeningocele repair on the incidence of shunt-dependent hydrocephalus. Pediatr Neurosurg **38,** 27, 2003.
4. Johnson, M.P., Sutton, L.N., Rintoul, N., Crombleholme, T.M., Flake, A.W., Howell, L.J., Hedrick, H.L., Wilson, D.W., and Adzick, N.S. Fetal myelomeningocele repair: short-term clinical outcomes. Am J Obstet Gynecol **189,** 482, 2003.
5. Johnson, M.P., Gerdes, M., Rintoul, N., Pasquariello, P., Melshionni, J., Sutton, L.N., and Adzick, N.S. Maternal-fetal surgery for myelomeningocele: neurodevelopmental outcomes at 2 years of age. Am J Obstet Gynecol **194,** 1145, 2006.
6. Fichter, M.A., Dornseifer, U., Henke, J., Schneider, K.T., Kovacs, L., Biemer, E., Bruner, J., Adzick, N.S., Harrison, M.R., and Papadopulos, N.A. Fetal spina bifida repair-current trends and prospects of intrauterine neurosurgery. Fetal Diagn Ther **23,** 271, 2008.
7. Sutton, L.N. Fetal surgery for neural tube defects. Best Pract Res Clin Obstet Gynaecol **22,** 175, 2008.
8. Danzer, E., Adzick, N.S., Rintoul, N.E., Zarnow, D.M., Schwartz, E.S., Melchionni, J., Ernst, L.M., Flake, A.W., Sutton, L.N., and Johnson, M.P. Intradural inclusion cysts following *in utero* closure of myelomeningocele: clinical implications and follow-up findings. J Neurosurg Pediatr **2,** 406, 2008.
9. Danzer, E., Gerdes, M., Bebbington, M.W., Sutton, L.N., Melchionni, J., Adzick, N.S., Wilson, R.D., and Johnson, M.P. Lower extremity neuromotor function and short-term ambulatory potential following *in utero* myelomeningocele surgery. Fetal Diagn Ther **25,** 47, 2009.
10. Sanchez, R., Valente, P.R., Abou-Jamra, R.C., Araujo, A., Saldiva, P.H., and Pedreira, D.A.L. Biosynthetic cellulose induces the formation of a neoduramater following prenatal correction of meningocele in fetal sheep. Acta Cirurgica Brastletra **22,** 174, 2007.
11. Kohl, T., Hartlage, M.G., Kiehitz, D., Westphal, M., Buller, T., Achenbach, S., Aryee, S., Gembruch, U., and Brentrup, A. Percutaneous fetoscopic patch coverage of experimental lumbosacral full-thickness skin lesions in sheep. Surg Endosc **17,** 1218, 2003.
12. Eggink, A.J., Roelofs, L.A.J., Feitz, W.F.J., Wijnen, R.M.H., Mullaart, R.A., Grotenhuis, J.A., van Kuppevelt, T.H., Lammens, M.M.Y., Crevels, A.J., Hanssen, A., and van den Berg, P.P. *In utero* repair of an experimental neural tube defect in a chronic sheep model using biomatrices. Fetal Diagn Ther **20,** 335, 2005.
13. Fauza, D.O., Fishman, J., Mehegan, K., and Atala, A. Videofetoscopically assisted fetal tissue engineering; skin replacement. J Pediatr Surg **33,** 357, 1998.
14. Danzer, E., Schwarz, U., Wehrli, S., Radu, A., Adzick, N.S., and Flake, A.W. Retinoic acid induced myelomeningocele in fetal rats: characterization by histopathological analysis and magnetic resonance imaging. Exp Neurol **194,** 467, 2005.
15. Ozeki, M., and Tabata, Y. *In vivo* degradability of hydrogels prepared from different gelatins by various cross-linking methods. J Biomater Sci Polym Ed **16,** 549, 2005.
16. Takahashi, Y., Yamamoto, M., and Tabata, Y. Osteogenic differentiation of mesenchymal stem cells in biodegradable sponges composed of gelatin and β-tricalcium phosphate. Biomaterials **26,** 3587, 2005.
17. Kitano, Y., Yang, E.Y., Allmen, D.V., Quinn, T.M., Adzick, N.S., and Flake, A.W. Tracheal occlusion in the fetal rat: a new experimental model for the study of accelerated lung growth. J Pediatr Surg **33,** 1741, 1998.
18. Borges, J., Mueller, M.C., Padron, N.T., Tegtmeier, F., Lang, E.M., and Stark, G.B. Engineered adipose tissue supplied by functional microvessels. Tissue Eng **9,** 1263, 2003.
19. Day, R.M., Boccaccini, A.R., Shurey, S., Roether, J.A., Forbes, A., Hench, L.L., and Gabe, S.M. Assessment of polyglycolic acid mesh and bioactive glass for soft-tissue engineering scaffolds. Biomaterials **25,** 5857, 2004.
20. Danzer, E., Radu, A., Robinson, L.E., Volpe, M.V., Adzick, N.S., and Flake, A.W. Morphologic analysis of the neuromuscular development of the anorectal unit in fetal rats with

retinoic acid induced myelomeningocele. Neurosci Lett **430,** 157, 2008.
21. Danzer, E., Kiddoo, D.A., Redden, R.A., Robinson, L., Radu, A., Zderic, S.A., Doolin, E.J., Adzick, N.S., and Flake A.W. Structural and functional characterization of bladder smooth muscle in fetal rats with retinoic acid-induced myelomeningocele. Am J Physiol Renal Physiol **292,** 197, 2006.
22. Tabata, Y., Nagano, A., and Ikada, Y. Biodegradation of hydrogel carrier incorporating fibroblast growth factor. Tissue Eng **5,** 127, 1999.
23. Tabata, Y., and Ikada, Y. Vascularization effect of basic fibroblast growth factor released from gelatin hydrogels with different biodegradabilities. Biomaterials **20,** 2169, 1999.
24. Ozeki, M., and Tabata, Y. *In vivo* degradability of hydrogels prepared from different gelatins by various cross-linking methods. J Biomater Sci Polym Ed **16,** 549, 2005.
25. Miyoshi, M., Kawazoe, T., Igawa, H.H., Tabata, Y., Ikada, Y., and Suzuki, S. Effects of bFGF incorporated into a gelatin sheet on wound healing. J Biomater Sci Polym Ed **16,** 893, 2005.
26. Tsuji-Saso, Y., Kawazoe, T., Morimoto, N., Tabata, Y., Taira, T., Tomihata, K., Utani, A., and Suzuki, S. Incorporation of basic fibroblast growth factor into preconfluent cultured skin substitute to accelerate neovascularization and skin reconstruction after transplantation. Scand J Plast Reconstr Surg Hand Surg **41,** 228, 2007.
27. Kawai, K., Suzuki, S., Tabata, Y., and Nishimura, Y. Accelerated wound healing through the incorporation of basic fibroblast growth factor-impregnated gelatin microspheres into artificial dermis using a pressure-induced decubitus ulcer model in genetically diabetic mice. Br J Plast Surg **58,** 1115, 2005.
28. Tabata, Y. Biomaterial technology for tissue engineering applications. J R Soc Interface **6,** 311, 2009.
29. Hashimoto, T., Koyama, H., Miyata, T., Hosaka, A., Tabata, Y., Takato, T., and Nagawa, H. Selective and sustained delivery of basic fibroblast growth factor for treatment of peripheral arterial disease: results of a phase I trial. Eur J Vasc Endovasc Surg **38,** 71, 2009.
30. Esaki, J., Marui, A., Tabata, Y., and Komeda, M. Controlled release systems of angiogenic growth factors for cardiovascular disease. Expert Opin Drug Deliv **4,** 635, 2007.
31. Gremlich, S., Nquyen, D., Reymondin, D., Hohlfeld, P., Vial, Y., Witkin, S.S., and Gerber, S. Fetal MMP2/MMP9 polymorphisms and intrauterine growth restriction risk. J Reprod Immunol **74,** 143, 2007.
32. Peltier, M.R., Barney, B.M., and Brown, M.B. Effect of experimental genital mycoplasmosis on production of matrix metalloproteinases in membranes and amniotic fluid of Sprague-Dawley rats. Am J Reprod Immunol **57,** 116, 2007.
33. Bullard, K.M., Longaker, M.T., and Lorenz, H.P. Fetal wound healing: current biology. World J Surg **27,** 54, 2003.
34. Longaker, M.T., Chiu, E.S., Adzick, N.S., Stern, M., Harrison, M.R., and Stern, R. Studies in fetal wound healing. V. A prolonged presence of hyaluronic acid characterizes fetal wound fluid. Ann Surg **213,** 292, 1991.
35. Tabata, Y., Hijikata, S., Muniruzzaman, M.D., and Ikada, Y. Neovascularization effect of biodegradable gelatin microspheres incorporating basic fibroblast growth factor. J Biomater Sci Polym Ed **10,** 79, 1999.
36. Tabata, Y. Regenerative inductive therapy based on DDS technology of protein and gene. J Drug Target **14,** 483, 2006.

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