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Bioengineering and Characterization of Physeal Transplant with Physeal Reconstruction Potential*

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ABSTRACT

Damage to physes is of serious consequences in children. Currently available treatments are limited and results are unpredictable. In this study, we attempted to treat proximal tibial physeal damage in rabbits with transplanted bioengineered physeal tissue. Resting chondrocytes from the reserve zone of costal cartilages of 6-week-old rabbits were pellet cultured in centrifuge tubes. The pellets were characterized histologically and biochemically with reference to the normal physis. The bioengineered tissue was then transplanted into partially damaged proximal tibial physis. Histological changes and proteoglycan metabolism of the transplants were monitored until 7 weeks posttransplantation. Our results showed that chondrocytes cultured by three-dimensional pellet exhibited cell division and the derived cells arranged in short columns similar to normal physis. They synthesized and deposited cartilaginous matrix and differentiated into hypertrophic chondrocytes marked by increases in cell size and alkaline phosphatase activity. The transplant incorporated well in host tissue with no sign of rejection for up to 7 weeks posttransplantation. A further 3-fold increase in thickness of the transplant within the host was observed. Endochondral ossification was demonstrated at 7 weeks posttransplantation. These results show that the bioengineered physeal tissue may have great potential in clinical management of physeal damage in children.

INTRODUCTION

PROLIFERATION AND DIFFERENTIATION of chondrocytes in the growth plate and subsequently calcification of the extracellular matrix are critical for longitudinal bone growth. Physeal destruction due to trauma or infection leads to arrested growth and limb deformities. Physis reconstruction is therefore an important clinical problem in orthopedic surgery. Physeal injuries are currently managed in many ways, including the treatment of partial physeal injuries with the resection of bony bridge and transplantation of biological or inert fillers.[1,2] However, these fillers lack growth potential and there is no reformation or repair of physeal cartilage.[3]

Transplantation of physis with growth potential is a logical approach to repair the entire physis. However, results have been unpredictable. Some studies reported continued function, whereas others described unsatisfactory growth or complete failure.[1,4,5] Advances in microsurgery have made it possible to preserve viability by

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transplanting a vascularized physeal plate.[5,6] Although some results showed continued and longitudinal growth with less angular deformity, complications in operation procedures, donor availability, and the risks of immunological rejection have made this method unacceptable in common clinical practice.[6,7]

Transplantation of cartilage and cultured chondrocytes has also been used for physeal reconstruction.[3,8–11] The loose structure of the matrix permitted diffusion of tissue fluid and thus the allograft survived even without vascularization. The graft was not immunologically rejected, probably owing to protection by the extracellular matrix.[3,8,9,11] Longitudinal growth was observed after transplantation of the high-density culture.[3,8–11] However, there was no histological architecture resembling the normal growth plate and the activity was short lived and unpredictable.

In this study, we synthesized bioengineered physis by the three-dimensional (3-D) chondrocyte pellet culture technique. Its *in vitro* development was monitored extensively and the bioengineered tissue was fully characterized with reference to the characteristics of a normal physis. The bioengineered tissue was then transplanted into a partial physis defect in the proximal tibial physis of rabbits and its *in vivo* development was assessed.

MATERIALS AND METHODS

Engineering of physis and its in vitro *assessments*

The project was approved by the Animal Ethics Committee of the Chinese University of Hong Kong, and the method for the isolation of growth plate chondrocytes is well established in our laboratory.[12,13] Chondrocytes were isolated from 6-week-old New Zealand White rabbits. After euthanasia, the rib cage was dissected aseptically. The resting zone of physis cartilage was taken out near the osteochondral junctions of all the ribs and temporarily stored in modified Ham's F-12 medium (magnesium free, 0.5 mM $CaCl_2$). After weighing, the cartilage was cut into small pieces and digested sequentially with 0.1% trypsin (10 mL/g tissue in modified, type III, 30 min), 0.1% hyaluronidase (10 mL/g tissue, type I-S, 1 h), and 0.1% collagenase (20 mL/g tissue, type IIA, overnight) in modified Ham's F-12 medium at 37°C (all from Sigma, St. Louis, MO). The cell suspension was then filtered through a glass wool filter and washed twice with modified F-12 medium to remove digested matrix debris.

A total of 5×10^5 released chondrocytes in 1 mL of Dulbecco's modified Eagle's medium (DMEM) with ascorbate at 50 μg/mL (both from Sigma), 10% fetal bovine serum (FBS), and 0.8% (v/v) penicillin–streptomycin (both from GIBCO Laboratories, Grand Island, NY) was centrifuged at $350 \times g$ at room temperature for 5 min in a 15-mL conical polypropylene tube (Falcon; BD Biosciences Discovery Labware, Lincoln Park, NJ). Each cell pellet was then cultured at 37°C, 5% CO2 and 100% humidity within the same tube (loosely capped), standing vertically. The medium was changed every 2 days with daily centrifugation. The culture was harvested after 2, 7, 14, and 21 days of development to carry out the histomorphological study and alkaline phosphatase histochemistry.

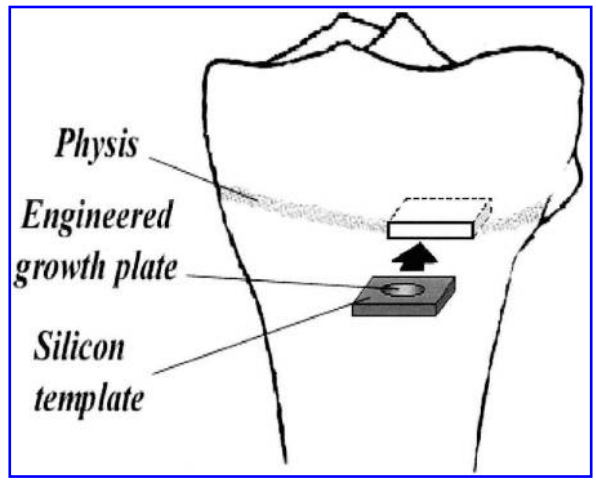

FIG. 1. Diagram illustrating the transplantation of bioengineered growth plate into the partial physeal defect model in the rabbit proximal tibia.

Each of the following *in vitro* characterization experiments was performed five times ($n = 5$), in triplicate, for the taking of average values.

Histomorphology. At different time points, the pellets were fixed in 10% neutral buffered formalin and then embedded in paraffin. Five-micrometer sections perpendicular to the short axis of pellets were cut and stained with hematoxylin and eosin (H&E) or safranin O (for proteoglycan staining) for light microscopy. Cross-sectional area of chondrocytes was measured with a Leica Q500

TABLE 1. MEAN CELL CROSS-SECTIONAL AREA AND THICKNESS OF THREE-DIMENSIONAL PELLET AFTER 2, 7, 14, 21 AND DAYS OF DEVELOPMENT *IN VITRO*[a]

Day	*Mean cross-sectional area of chondrocytes ± SD (μm^2)*	*Thickness (mm)*
2	450 ± 42	0.36
7	560 ± 40 (medium-size cells) 870 ± 56 (large cells)	0.42
14	490 ± 190 (proliferative chondrocytes) 1590 ± 59 (hypertrophic chondrocytes)	0.53
21	1630 ± 41 (hypertrophic chondrocytes)	0.70

[a] $n = 5$.

MC image analysis system (Leica Microsystems, Bensheim, Germany). An average was taken among five serial slides of a sample.

Alkaline phosphatase histochemistry. The pellets were fixed in formal calcium (3.64% formaldehyde, 0.91% calcium chloride) at 4°C for 16 h. Eight-micrometer sections were obtained with a cryostat at −12°C, and alkaline phosphatase (ALP) activity was localized by the ProtoBlot nitroblue tetrazolium (NBT)/5-bromo-4-chloro-3-indolyl-phosphate (BCIP) color development system (Promega, Madison, WI) as described by the manufacturer. After color development, the sections were counterstained with 2% methyl green and mounted in glycerol. Frozen sections of freshly prepared costal physis were used as positive controls.

Thymidine incorporation assay. The pellets were labeled with [5-^{3}H]thymidine (1 μCi/mL, 14.7 Ci/mmol; Amersham Biosciences, Piscataway, NJ) in DMEM containing 5 μM unlabeled thymidine, 10% FBS, ascorbate (50 μg/mL), and 0.8% (v/v) penicillin–streptomycin for 4 h at 37°C. The medium was aspirated and the culture was washed twice with 0.15 N NaCl. The culture was then homogenized with a 2-mL glass homogenizer (Wheaton, Millville, NJ) with 0.6 mL of 0.25 N NaOH. The cell lysate was neutralized by adding 0.6 mL of 0.25 N HCl and 1.2 mL of 0.25 mM HEPES (Mg^{2+}, Ca^{2+} free); bovine serum albumin (BSA, 2.5 mg/mL), which acted as protein carrier, was also added to each tube. DNA was precipitated with 0.6 mL of 10 N perchloric acid at 4°C for 1 h. Precipitate was spun at 15,000 rpm at 4°C (Hermle H-401; Kontron, Milan, Italy) for 30 min. After aspirating the supernatant, 0.5 mL of 0.25 N NaOH was added to resuspend the pellet. The solution was then transferred to counting vials and 4 mL of scintillation fluid was added. Radioactivity was measured with a liquid scintillation spectrometer (LS3801; Beckman, Fullerton, CA).

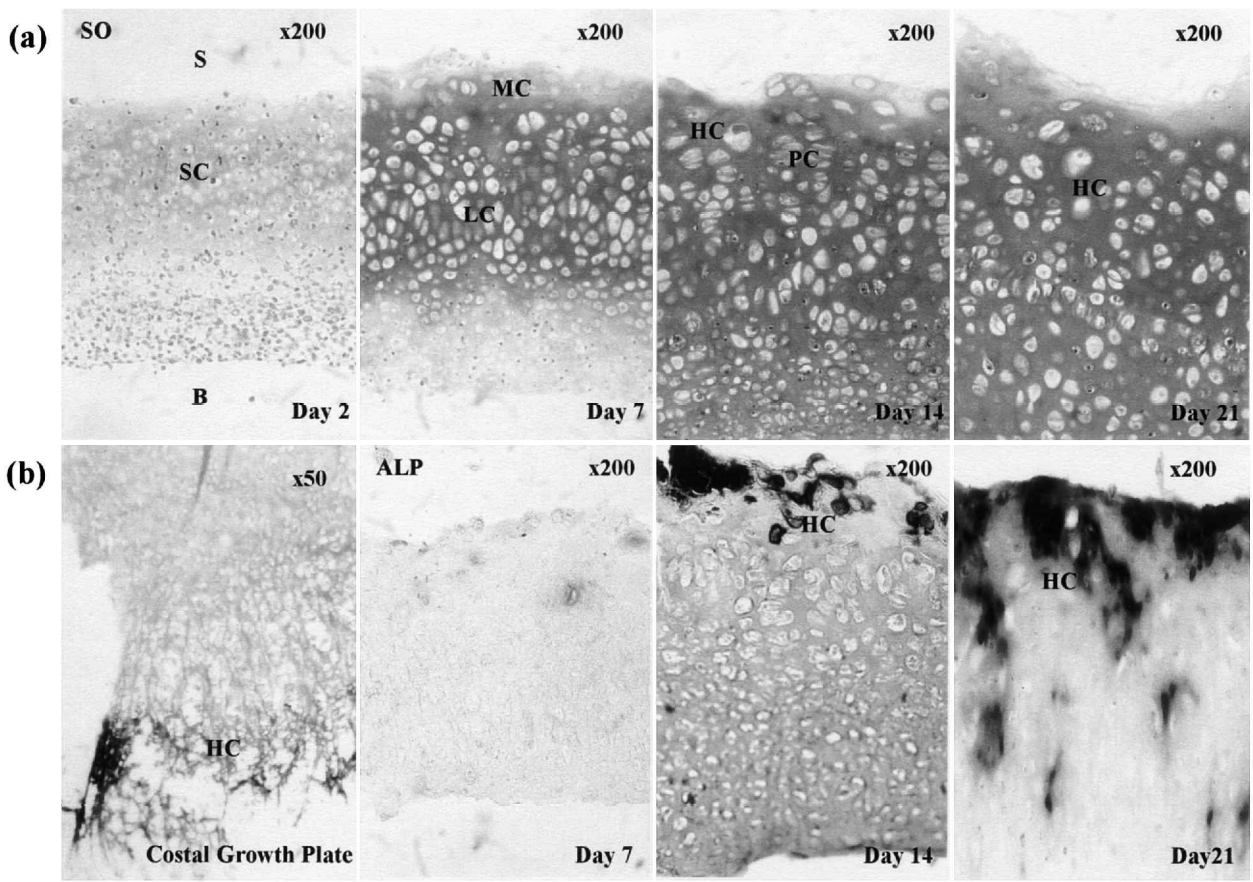

FIG. 2. (**a**) Longitudinal sections of 3-D pellet culture after 2, 7, 14, and 21 days of development *in vitro*. The histological sections were stained with safranin O (SO). Red indicates SO staining. Note the gradual increase in proteoglycan synthesis with time. B, Bottom of pellet attached on the bottom of culture tube; S, surface of pellet exposed to growth medium; SC, small spherical chondrocytes; MC, medium-sized chondrocytes; LC, large chondrocytes; PC, proliferative chondrocytes; HC, hypertrophic chondrocytes. (**b**) ALP histochemistry by NBT and BCIP assay at different times of culture. Brown indicates the localization of ALP activities in the hypertrophic zone; green represents the methyl green counterstain. Original magnifications are indicated in the upper right-hand corners.

Transplantation of bioengineered physeal tissue and its in vivo *assessments*

Sixteen New Zealand White rabbits, 13 weeks of age, were used. The partial physis defect model had been established in our laboratory (Fig. 1).[14] Briefly, an incision was made on the medial side of the proximal tibia, exposing the proximal physis. A series of holes were drilled along the physis with a 1.6-mm-diameter drill bit to create a partial defect. A 1.6-mm burr tip was used to create a 1.6 mm (w) $\times$ 5 mm (l) $\times$ 5 mm (d) rectangular defect. A silicone template with dimensions identical to those of the physeal defect created was prepared so that it could be inserted into the defect like a tight-fit drawer. A 3-mm-diameter hole from top to bottom was made at the center of such a template with a 3-mm plunge. The hole was then filled with the bioengineered physeal tissue or left empty as a control. Before transplantation, the 14-day pellet culture was made 3 mm in diameter with the same 3-mm plunge. The pellet was put into the template with the upper surface of the pellet, which was in contact with the culture medium in the centrifuge tube, facing the epiphyseal side of the tibia. The silicone template was then gently inserted into the physeal defect. The control was the contralateral leg of the same animal, in which an empty template was inserted in the same manner. The wound was closed by suture. Temgesic was given intramuscularly, 0.1 mL/kg twice per day, for 3 days. The rabbits were allowed free cage movement and were killed 1, 3, 5, and 7 weeks after operation for *in vivo* assessment ($n = 4$ for each time point).

Histology. Proximal tibiae with the silicone template left *in situ* were fixed in 4% paraformaldehyde and decalcified with formic acid–formalin. The template was bisected and removed carefully. The tissue was then embedded in paraffin. Five-micrometer sections were cut and stained with H&E or safranin O. The maximum thickness of the transplants was measured with the Leica Q500 MC image analysis system.

Metabolism of bioengineered physeal tissue in vivo: *radiosulfate autoradiography.* Eight rabbits were injected with a 0.5-mCi/kg concentration of $Na_2{}^{35}SO_4$ (specific activity, 250–1000 mCi/mmol; PerkinElmer Life Sciences, Boston, MA) 1, 3, 5, and 7 weeks after operation. The animals were killed 24 h later, and their proximal tibiae were removed. Specimens were fixed, processed, embedded, and sectioned as described above.

Liquid emulsion was prepared according to Kopriwa and Leblond.[15] In brief, 5-μm unstained sections were dried after deparaffinization and rehydrated by treatment with high to low concentrations of alcohol. The slides were dipped into NTB-2 emulsion (Eastman Kodak, Rochester, NY), dried, and exposed in the dark at 4°C for 3 weeks. Emulsion was developed with Kodak D-19 developer and fixed with Kodak fixer. The slides were then washed in distilled water and stained with safranin O before mounting.

Statistical analysis

Data were analyzed with SPSS version 10.0 software (SPSS, Chicago, IL). Data from different time points in the thymidine incorporation assay were compared by one-way analysis of variance (ANOVA) ($\alpha = 0.05$). p Values less than 0.05 were considered significant.

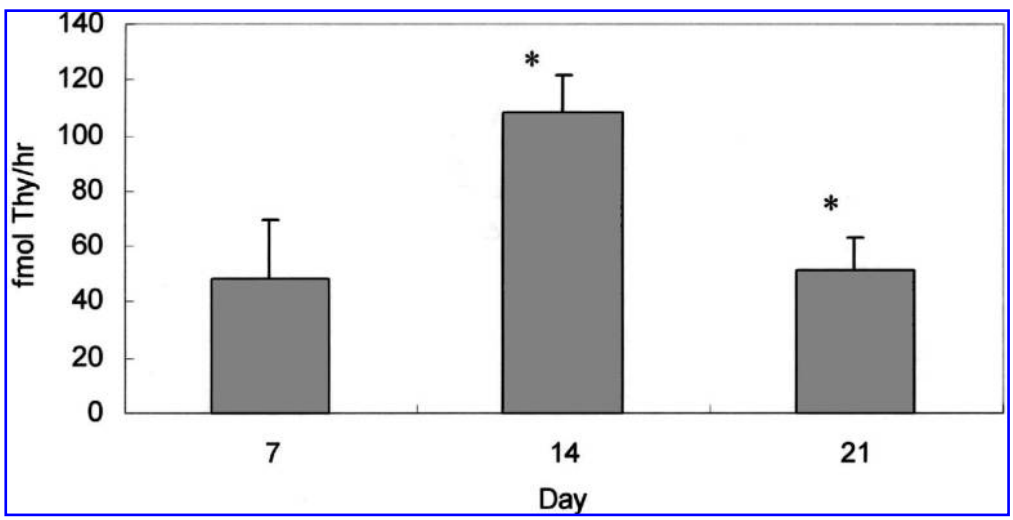

FIG. 3. Thymidine incorporation rates of chondrocytes in 3-D pellet culture at various times of culture ($n = 5$). Uptake of [^{3}H]thymidine increased significantly from week 1 to week 2 ($^*p < 0.05$) and then decreased. The rate was significantly lower ($^*p < 0.05$) in week 3.

RESULTS

Bioengineered physeal tissue created by 3-D pellet culture showed characteristics of a normal growth plate. The 3-D pellet was thin on day 2 and increased in thickness with time, indicating that the chondrocytes in the 3-D pellet exhibited growth potential. Table 1 shows the thickness of the pellet after 2, 7, 14, and 21 days of development.

Figure 2a shows the histology of pellets cultured for different amounts of time. On day 2, small spherical chondrocytes were closely packed at the bottom of the pellet. Those on the upper portion became larger and a few layers of medium-size chondrocytes appeared on the uppermost surface on day 7. On day 14, these medium-size cells were replaced by flattened proliferative chondrocytes arranged in columns of four or five cells, forming an active cell proliferation zone resembling the normal physis. The proliferative chondrocytes enlarged

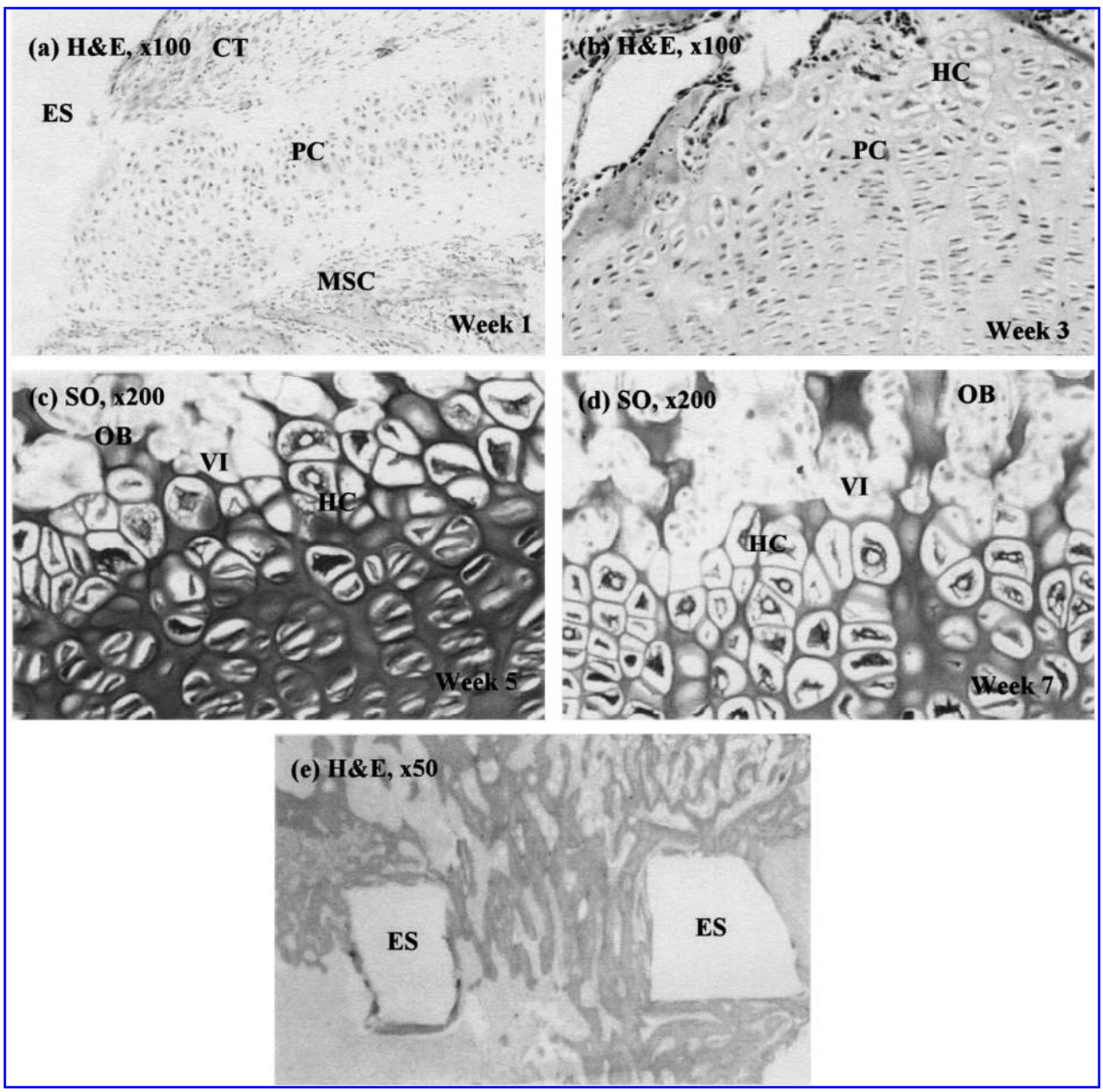

FIG. 4. Histology showing *in vivo* development of bioengineered physeal tissue, with hypertrophic chondrocytes near the host epiphysis at various time after transplantation. (**a**) week 1; (**b**) week 3; (**c**) week 5; (**d**) week 7; (**e**) control at week 5 (without bioengineered growth plate). The stains used and the magnifications are indicated at the top of each panel. H&E, Hematoxylin and eosin; SO, safranin O and fast green; ES, empty space left after silicone template removal; CT, connective tissue; PC, proliferative chondrocytes; MSC, mesenchymal stem cells; HC, hypertrophic chondrocytes; OB, osteoblasts; VI, vascular invasion.

and finally all differentiated into hypertrophic chondrocytes on day 21. The proportion of cells active in proteoglycan synthesis increased with time as indicated by safranin O staining.

Figure 2b shows the ALP histochemistry at different times of culture. Few brown spots were observed on day 7, indicating that ALP activity was low during this period. On day 14 and day 21, strong ALP activity was observed in cells located exclusively on the surface of the pellet, corresponding to the hypertrophic zone. In the positive control, only the hypertrophic chondrocytes and their surrounding matrix were intensively stained in the costal physis cartilage.

Figure 3 shows the thymidine incorporation rates of chondrocytes in pellets at various times of culture. The results showed that the thymidine incorporation rate was relatively low on day 7 and increased significantly on day 14. Afterward the rate dropped and was significantly lower on day 21.

Figure 4 shows the development of the bioengineered physis, with the hypertrophic chondrocytes adjacent to the host epiphysis in rabbit partial physeal defect. At week 1, connective tissue (CT), such as fibroblasts with their secreted extracellular matrix, formed a perichondrium-like structure surrounding the transplant (Fig. 4a). Erythrocytes and leukocytes were found adjacent to the transplant. Proliferative chondrocytes (PCs), in the transplant were arranged in short columns and cell division was directional, toward the epiphyseal side of the host. In contrast, chondrocytes near the metaphyseal side of the host were consistently smaller. The transition between the transplant and the host tissue was smooth, and cartilage matrix was observed around the nearby host mesenchymal stem cells (MSCs). In the first week posttransplantation, the transplant grew 1.4 times in thickness (Table 2).

The transplant continued to increase in size. At week 3, the size of the bioengineered physeal tissue was four times larger than the original pellet (Table 2). Flattened proliferative chondrocytes toward the end of the column enlarged and differentiated into hypertrophic chondrocytes (HCs). (Fig. 4b).

After 5 weeks, the matrix at the end of the hypertrophic zone was resorbed by vascular elements. Osteoblasts were present on the new bone spicules laid down on the cartilage matrix (Fig. 4c). At week 7, the transplant remained intact and produced cartilaginous matrix as shown by safranin O staining (Fig. 4d). The transplanted cartilage was three times thicker than the original pellet (Table 2). Active endochondral ossification occurred at this stage. There was no sign of rejection, as indicated by the absence of necrosis, cell debris, and infiltrated macrophages for up to 7 weeks.

Figure 4e shows the control at week 5, in which the transplanted silicone templates did not contain any bioengineered growth plate in the plunged space. Such empty space was filled with trabecular bone by week 5.

To assess the metabolic activity of the transplanted cells in the host microenvironment, radioactive $Na_2{}^{35}SO_4$ was injected intraperitoneally after weeks 1, 3, 5, and 7. After 24 h the incorporation of radiosulfate into the extracellular matrix was then examined by autoradiography. The bioengineered physeal tissue was functionally active in proteoglycan synthesis up to week 7 after transplantation (Fig. 5a). Higher magnification revealed that autoradiographic labeling was most intense in the cytoplasm and extracellular matrix of the proliferative chondrocytes (Fig. 5b). Lower activity was noted in the hypertrophic zone, and the resting zone showed the least labeling (Fig. 5b). A similar labeling pattern was observed for normal physis (Fig. 5c).

To compare the development of bioengineered growth plate between *in vivo* and *in vitro* conditions, some pellets maintained in tissue culture were harvested at times corresponding to weeks 1, 3, 5, and 7 after transplantation (Fig. 6). As demonstrated by safranin O staining, the pellets corresponding to 1 week and 3 weeks postoperation showed high proteoglycan-synthesizing activity. However, the activity decreased by 5 weeks postoperation. At 7 weeks postoperation, the proteoglycan within the pellet was greatly decreased.

DISCUSSION

In the present study, we demonstrated the biosynthesis of physis, using a 3-D chondrocyte pellet culture technique. Three-dimensional chondrocyte culture has been used in previous studies to investigate the effects of various growth factors on chondrocyte terminal differentiation, calcification, and endochondral bone formation.[16–19] We modified this *in vitro* culture method to produce bioengineered physeal tissues with cytological characteristics resembling normal physes *in vivo*.

We are successfully using resting chondrocytes isolated from 6-week-old rabbits to produce the bioengineered physeal tissues. We have also performed a characteriza-

Table 2. Growth of Bioengineered Physeal Tissue at Various Time Posttransplantation[a]

	Thickness (mm) ± standard error
Day 0	0.53 ± 0.16[b]
Week 1	0.77 ± 0.20
Week 3	2.34 ± 0.58
Week 5	2.34 ± 0.78
Week 7	1.66 ± 0.31

[a] $n = 4$.

[b] Thickness of day 14 engineered physis.

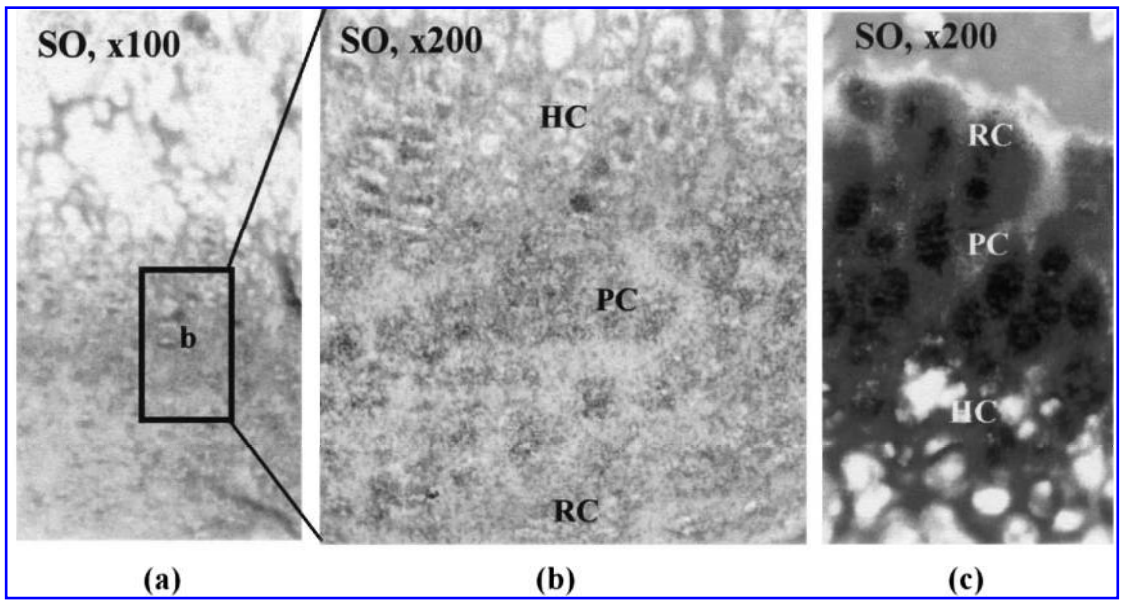

FIG. 5. Radiosulfate autoradiograph of bioengineered physeal tissue 7 weeks after transplantation. (**a**) Interface between bioengineered physeal tissue and host tissue; (**b**) bioengineered physeal tissue at higher magnification; (**c**) normal physis. Dark brown spots represent the radiosulfate activities; general red background represents safranin O stain. Original magnifications are indicated on the top of each diagram. Note that chondrocytes, especially proliferative chondrocytes, in bioengineered physeal tissue with hypertrophic chondrocytes near the host epiphysis were active in proteoglycan metabolism. RC, Resting chondrocytes; PC, proliferative chondrocytes; HC, hypertrophic chondrocytes.

tion of the bioengineered physeal tissue development in temporal sequence. Our results indicated that chondrocytes maintained as 3-D pellets in centrifuge tubes retained a differentiated phenotype, and synthesized and deposited cartilaginous matrix for a specific period of time. They resembled normal physis histologically and biochemically and exhibited cell division. We also showed that the 3-D pellets exhibited cell division as indicated by thymidine uptake assays.

In this study, we found that mechanical packing of chondrocytes in the early stage of their development was vital for their subsequent differentiation along the lineage of physeal chondrocytes. After centrifugation, the chondrocytes in the 3-D pellet were closely packed and re-

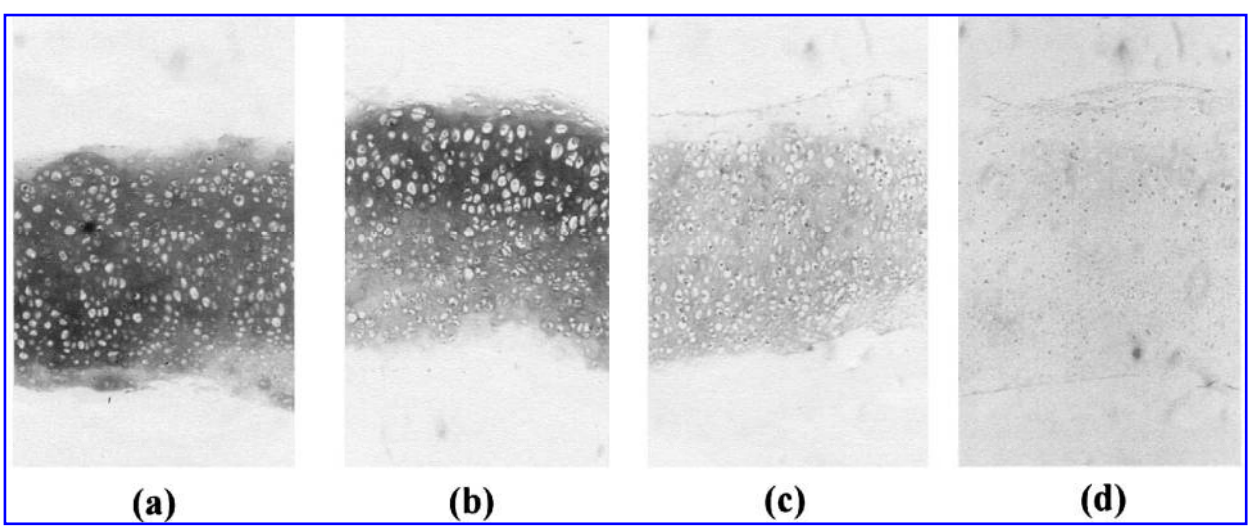

FIG. 6. Morphologic appearance of bioengineered growth plate, maintained under *in vitro* culture conditions, at various times after transplantation of specimens into animals. (**a**) One week and (**b**) 3 weeks after transplantation, proteoglycan-synthesizing activity was high. (**c**) At week 5, the activity became lower. (**d**) At week 7, the proteoglycan within the bioengineered growth plate was decreased.

mained spherical in shape. This was in contrast to chondrocytes cultured in a monolayer in the absence of mechanical compression. During the first 7 days of *in vitro* development after mechanical compression, few chondrocytes were active in cell division. Instead, they secreted and deposited cartilaginous matrix consisting of proteoglycan and collagens. These findings are consistent with previous experiments exploring the relationship between cell shape and characteristics of chondrocytes.[20,21] High cell density or reduced cell–substratum interactions have also been reported to favor chondrogenesis.[22]

The potential of the bioengineered physis and its *in vivo* development was monitored for up to 7 weeks after transplantation into the physis defect model. Three-dimensional pellet cultured for 14 days was used for transplantation as chondrocytes at this stage exhibited the highest cell division potential as indicated by thymidine uptake assay.

Our results indicate that such bioengineered physeal tissue incorporated well with host tissue from 1 week posttransplantation, and it resumed physeal characteristics after transplantation. For up to 7 weeks, there was no sign of rejection and columnar architecture of the bioengineered physeal tissue was maintained after transplantation. Further growth and cell division occurred as demonstrated by the increase in size of the proliferative zone. Toward the end of the cellular columns facing the epiphyseal side, invasion of blood vessels and osteoblasts occurred at week 5 and continued up to week 7. Radiosulfate autoradiography also provided evidence that the bioengineered physeal tissue could maintain active proteoglycan metabolism during the 7 weeks after transplantation.

Compared with conventional physis reconstruction models, the bioengineered physis developed in this study not only prevents bony bridge formation, it also acquires growth potential and resumes endochondral ossification *in vivo*. Only a 0.03-g (wet weight) resting zone of cartilaginous tissue from ribs was required to synthesize the bioengineered physis, which subsequently resumed the growth potential of a 3-mm-diameter defect. In our study, no immunorejection of the transplanted tissue occurred for up to 7 weeks posttransplantation. In clinical application, chondrocytes for implantation could be obtained from the ribs of other individuals or even from the patient. The advantages of using rib physis in repairing joint physis are its high availability and low harmfulness, as floating ribs are of limited function in an adult.

Our system holds great potential because it may avoid the donor problems of physeal transplantation. Growth potential and the mechanical properties of the donor site will not be sacrificed. In addition, in our study the bioengineered physeal tissue incorporated well. Theoretically, our system is not restricted by the shape and size of the donor tissues as artificial growth plates of different sizes and shapes can be engineered. The use of cultured chondrocytes for the treatment of articular cartilage defects in patients has been reported by Brittberg *et al.*[23] Our study represented another approach using *in vitro* tissue-engineering techniques to restore the growth activity of chondrocytes for physis reconstruction.

In summary, with suitable mechanical stimulation, resting chondrocytes from the reserve zone of costal cartilage in rabbits could be developed into growth platelike tissue and then transplanted into partial growth plate defects in rabbits to restore growth and resume endochondral ossification. It holds intriguing possibilities for the treatment of physeal injuries in human, and forms a basis for further basic scientific research and future clinical applications.

ACKNOWLEDGMENTS

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