

The Fate of Host and Graft Cells in Early Healing of Bone Tunnel After Tendon Graft

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Background: The behavior of host and graft cells during the healing process after autologous tendon graft has not been elucidated.

Hypothesis: Host cells will integrate into the bone-tendon interface and contribute to cellular repopulation of the graft.

Study Design: Controlled laboratory study.

Methods: Twelve-week-old, genetically identical, female green fluorescent protein transgenic rats (n = 20) and wild-type rats (n = 20) were used. The rats were divided into 2 experimental groups. In group A, the Achilles tendons of wild-type rats were harvested and transplanted into the transcondylar femoral bone tunnels of green fluorescent protein rats. In group B, the Achilles tendons of green fluorescent protein rats were transplanted into a transcondylar femoral bone tunnel of wild-type rats. Immediately after transplantation (time zero) and at 1, 2, and 4 weeks after the transplantation, distal femoral epiphyses were harvested and cut into 14- μ m serial sagittal frozen sections. The sections were examined with a confocal laser-scanning microscope to quantify green fluorescent protein-positive cell survival.

Results: At time zero, only host cells in group A and only graft cells in group B demonstrated green fluorescent protein signals. At 1 week in group A, many green fluorescent protein-positive cells were found in the graft. In group B, a few green fluorescent protein-positive cells were found in the graft. At 2 and 4 weeks in group A, many green fluorescent protein-positive cells were detected in the graft, but green fluorescent protein-positive cells had disappeared completely in group B.

Conclusion: Host cells, rather than graft cells, contribute to repair of the bone-tendon interface and the remodeling of grafts after simulated autologous tendon graft.

Keywords: ligament; transplantation; anterior cruciate ligament (ACL); transgenic animal; green fluorescent protein (GFP)

Fixed into bone tunnels, autologous hamstring tendon grafts, which are reported to be superior to bone-patellar tendon-bone grafts in terms of donor site morbidity,^{4,6} have been widely used for ACL reconstruction.^{13,29} One of the keys to successful reconstruction using autologous hamstring tendon grafts is the solid integration of grafted tendon at the bone tunnel and remodeling of the graft.^{5,8} The interface strength of bone and tendon graft, however, is inferior to that of bone and bone-patellar tendon-bone graft.²³ Furthermore, regeneration of the tendon insertion to the bone requires long time periods, and normal biomechanical properties are not achieved.^{10,25} Therefore, a

strategy to enhance integration of tendon at the bone tunnel is necessary. After transplantation, either host or graft-derived cells, or both host and graft-derived cells, intermix and enhance healing with cellular interaction²¹; however, the behavior of the cells during the healing process after a tendon graft has not been elucidated. The understanding of the cellular fates of both host and graft cells after autologous tendon transplantation should be the basis for engineering the healing process and regenerating the normal ACL properties.

It has been demonstrated that the remodeling process involves formation of fibrous tissue at the bone-tendon interface, the central necrosis of the graft, followed by incorporation of cells.²⁶ Grafted tendons contact the cancellous bone tunnel where bone marrow cells exist. The potential of bone marrow cells to differentiate and form mesenchymal tissues, such as tendon and ligament, has been demonstrated,³² and thus we hypothesized that host bone marrow cells will integrate into the bone-tendon interface and contribute to the cellular repopulation of the graft.

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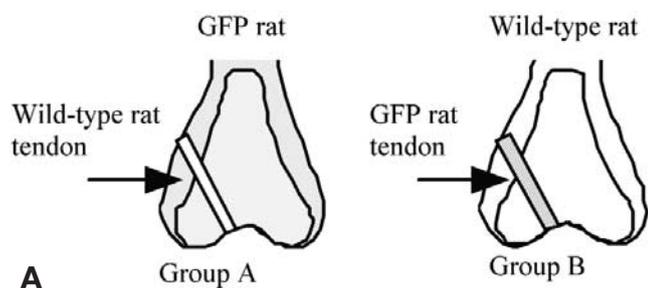
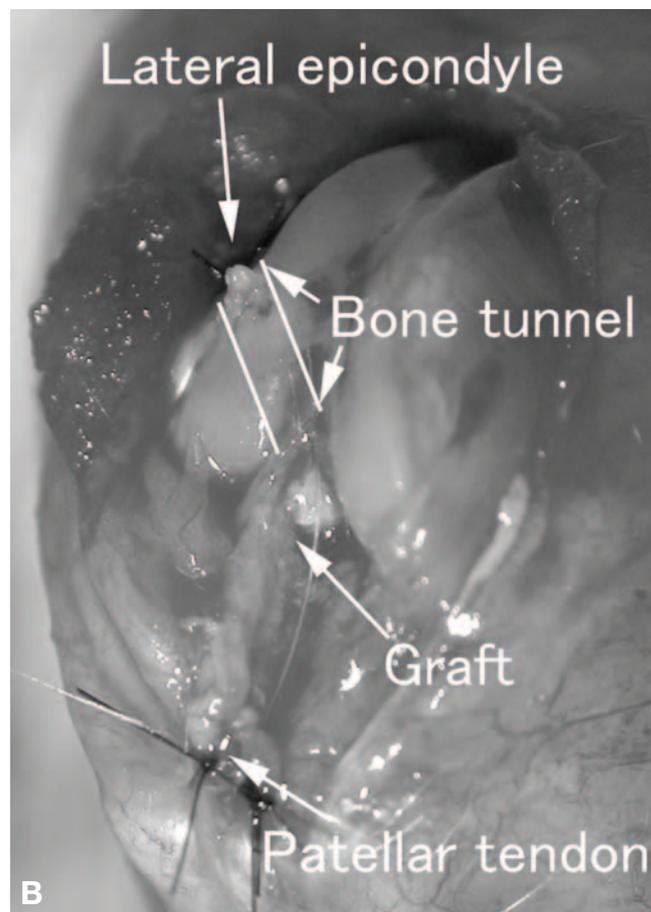


Figure 1. A, transplantation model. In group A, bilateral Achilles tendons of wild-type rats were transplanted into the bone tunnel of the GFP rats, which was created by drilling from the intercondylar notch to the lateral epicondyle of the femur. In group B, Achilles tendons of GFP rats were transplanted into the bone tunnels of the wild-type rats in the same manner as with group A. B, macroscopic view of rat model after transplantation. The proximal end of the tendon was sutured with periosteum at the epicondyle outside the joint, and the opposite, distal side was sutured to the patellar tendon. GFP, green fluorescent protein.



To simulate autologous transplantation, we used a transplantation model with Sprague-Dawley (SD) wild-type rats and green fluorescent protein (GFP) transgenic rats that were made from the SD wild-type rats. The GFP is a 27-kD protein originally discovered in the jellyfish *Aequorea victoria*. It has been demonstrated to have no immunological rejection *in vivo*.^{11,15} Transplantation among the same strain of inbred rats has been adopted as an autograft model in previous reports^{12,16,18} and is sometimes referred to as an isograft. Thus, this transplantation model simulates autologous transplantation. The purpose of this study was to examine the fate of host and graft cells at the bone-tendon interface and in the grafted tendon during the healing process after autologous tendon transplantation with the GFP rat model.

MATERIALS AND METHODS

Transgenic Animals

Twelve-week-old female GFP transgenic rats (Japan SLC Inc, Hamamatsu, Japan) and SD wild-type rats, genetically identical to each other except for the GFP transgene, were used. In the GFP transgenic rats, all cells including ligaments, tendons, bone, and bone marrow

cells express GFP signals in their cytoplasm and nuclei, and cells divided from GFP-positive cells also express GFP signals. The distribution of cell bodies was visualized through a confocal laser-scanning microscope, although no GFP signals were detected in sections of wild-type rats.

Surgical Technique

Twelve-week-old female GFP transgenic rats ($n = 20$) and SD wild-type rats ($n = 20$) were anesthetized with intraperitoneal injections of sodium pentobarbital. In group A, bilateral Achilles tendons of wild-type rats were approached with a longitudinal posterior incision of the lower leg using a sterile technique. Each tendon was cut 10 mm in length and 2 mm in diameter. Tendons were transplanted into the GFP rat bone tunnel, which was created by drilling from the intercondylar notch to the lateral epicondyle of the femur at a diameter of 2 mm. The proximal end of the tendon was sutured with periosteum at the lateral epicondyle, and the distal side was sutured to the patellar tendon with 7-0 nylon to add mechanical stimuli to the tendon graft. In group B, Achilles tendons of GFP rats were transplanted into the bone tunnels of the wild-type rats in the same manner as with group A (Figures 1 A and B).

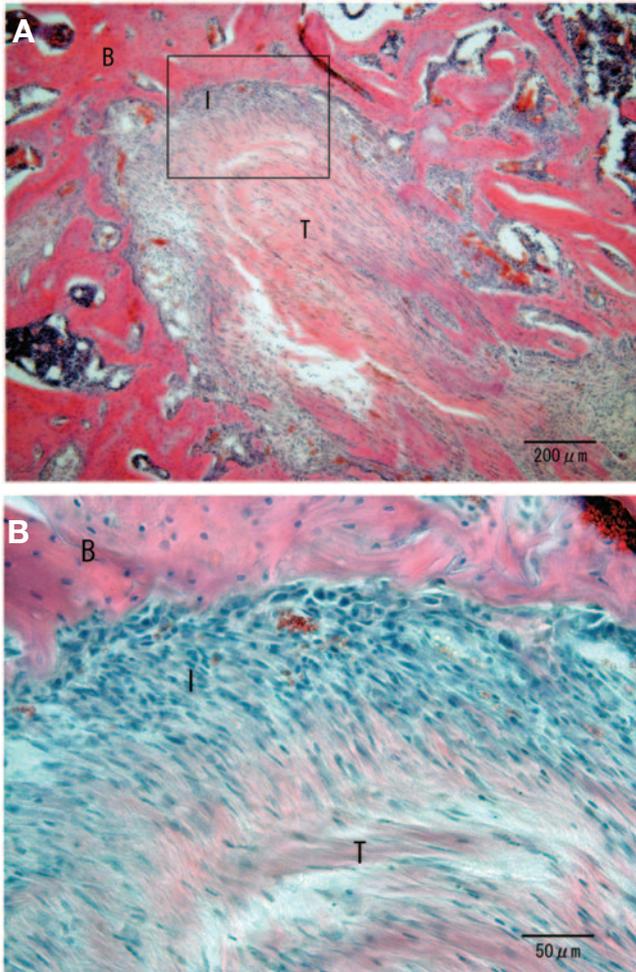


Figure 2. A, at 2 weeks, a number of mesenchymal cells were found in the bone marrow as well as at the bone-tendon interface, which also showed vascular formation. B, the bone-tendon interface was filled with connective tissue consisting of numerous mesenchymal cells, with a transition from an ovoid to spindle shape toward the graft. Hematoxylin and eosin staining. B, bone; I, bone-tendon interface; T, grafted tendon.

Tissue Preparation and Observation

Immediately after transplantation (time zero) and at 1, 2, and 4 weeks after transplantation, 4 rats from each group were anesthetized with sodium pentobarbital and then perfused with physiological saline and fixed with 4% paraformaldehyde in 0.1 M phosphate buffer. The distal epiphyses of the femurs were harvested and fixed with 4% paraformaldehyde in 0.1 M phosphate buffer for 24 hours at 4°C. They were then decalcified with 0.5 M ethylene diamine tetra-acetic acid (pH 7.5) for 2 weeks at room temperature, followed by gradient replacement with 20% sucrose for 24 hours at 4°C. Tissue blocks were quick-frozen with optimal cutting temperature compound, and sagittal serial frozen sections were cut at a

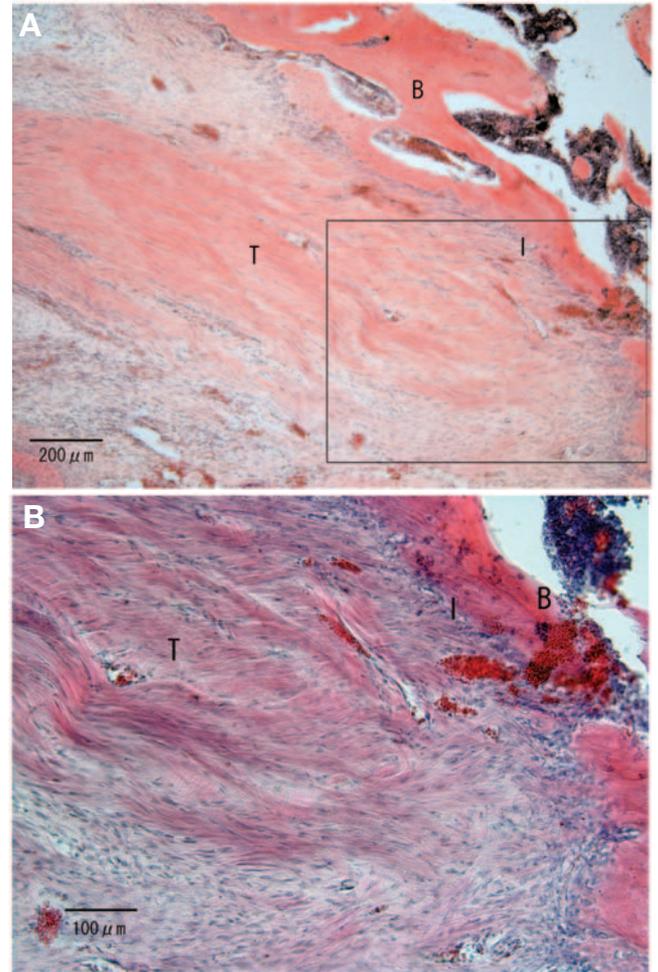


Figure 3. A, at 4 weeks, dense fibrous tissue developed at the interface, and Sharpey fibers were found at the border between the graft and the bone. B, a number of spindle-shaped cells were aligned longitudinally in the graft. Hematoxylin and eosin staining. B, bone; I, bone-tendon interface; T, grafted tendon.

thickness of 14 μm on a cryostat (Jung CM3000, Leica, Wetzlar, Germany). Sections were stained with hematoxylin and eosin, and serial sections were observed with a confocal laser-scanning microscope (LSM 510, Zeiss, Oberkochen, Germany) to examine the survival of GFP-positive cells. We counted GFP-positive cells and performed the nuclei staining using propidium iodide to determine the cell number in a randomly selected area at a magnification of $\times 200$ (0.2×0.2 mm). Then, the proportion of GFP-positive cells was assessed at each time period.

Animals were kept in the animal facility of our institute in accordance with the policies and procedures detailed in the "Guide for the Care and Use of Laboratory Animals" of the National Institutes of Health. The research protocol was reviewed and approved by the ethics committee of our institute.

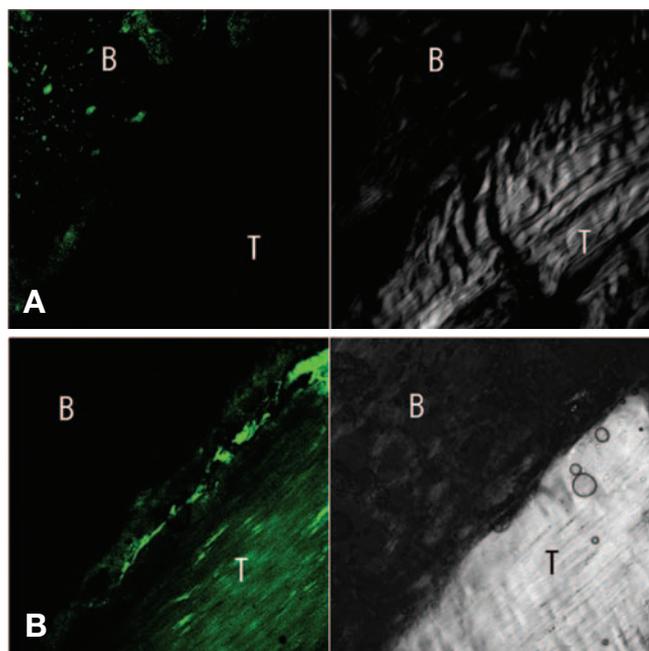


Figure 4. A, at time zero in group A, there were no GFP-positive cells in the graft. B, at time zero in group B, only graft cells showed GFP signals. Laser-scanning microscopic study. GFP, green fluorescent protein; B, bone; T, grafted tendon.

RESULTS

Micrographic Examination

In the sections at 1 and 2 weeks, with hematoxylin and eosin staining, fibrous tissue had formed at the bone-tendon interface. The border between the bone tunnel and the graft was clearly visible at these periods. An increased number of mesenchymal cells in the connective tissue was found at the bone-tendon interface and at the surface of the bone tunnel. Round to ovoid-shaped cells were aligned in layers at the surface of the bone, with a transition from an ovoid to spindle shape toward the graft. The grafted tendon revealed wavy collagen bundles, and few fibroblasts remained, especially in the center of the graft. Invasion of lymphocytes into the graft, indicating immunological rejection by the host, was not found (Figure 2). At 4 weeks, dense fibrous tissue with mesenchymal cells formed at the interface, and the border between the bone tunnel and the graft became partially unclear because of the Sharpey fibers,^{10,21} the obliquely aligned collagen bundles. Vessels were also found along the whole interface (Figure 3A). A number of spindle-shaped cells were aligned longitudinally in the graft (Figure 3B).

Laser-Scanning Microscopic Study

In sections from group A at time zero, when visualizing GFP signals of host cells with the fluorescent microscope,

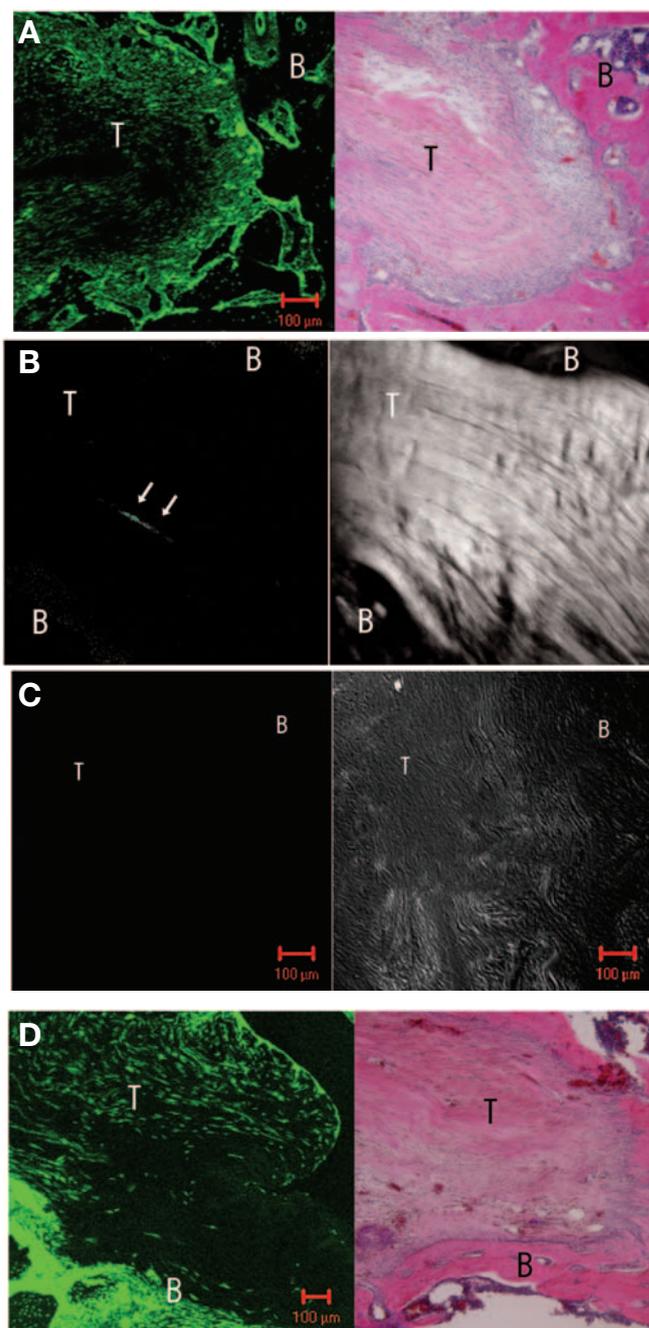


Figure 5. A, at 2 weeks in group A, a number of ovoid- to spindle-shaped GFP-positive host mesenchymal cells occurred at the bone-tendon interface. The graft showed a small number of host mesenchymal cells. B, at 1 week in group B, a small number of spindle-shaped GFP-positive cells were found in the graft. C, at 2 weeks in group B, no GFP-positive cells, indicating graft cells, were observed at the bone-tendon interface or in the graft. D, at 4 weeks in group A, a number of host cells were detected at the interface, and numerous host fibroblasts aligned in the graft longitudinally to its axis. Laser-scanning microscopic study. GFP, green fluorescent protein; B, bone; T, grafted tendon.

host cells demonstrated GFP signals, whereas the graft showed no signals (Figure 4A). In sections from group B, only graft cells showed GFP signals at this period (Figure 4B).

At 1 and 2 weeks in group A, a number of ovoid- to spindle-shaped GFP-positive cells, indicating host mesenchymal cells, were found at the bone-tendon interface. The graft showed a small number of spindle-shaped GFP-positive cells, also indicating host mesenchymal cells (Figure 5A). At 1 week in group A, the proportion of GFP-positive cells in the bone-tendon interface was approximately 100% and that in the grafted tendon was 70% to 90%. At 1 week in group B, a small number of spindle-shaped GFP-positive cells were found in the graft (Figure 5B). The proportion of GFP-positive cells in the bone-tendon interface was 0% and that in the grafted tendon was 0% to 10%. At 2 and 4 weeks in group B, GFP-positive cells, which would indicate graft cells, were not observed at the bone-tendon interface or in the graft (Figure 5C), indicating no graft cells survived 2 weeks after transplantation.

At 4 weeks in group A, a number of GFP-positive host cells were detected at the interface, and numerous host fibroblasts were aligned longitudinally to the axis of the graft (Figure 5D). Cellular shape and cellularity found in the sections manifesting GFP coincided with those of hematoxylin and eosin staining.

DISCUSSION

Solid integration of grafted tendon at the bone tunnel is indispensable for successful ACL reconstruction with tendons.^{5,8} There are 2 different types of tendon and ligament insertion to bone. One is chondral ligament or tendon insertion, referred to as direct-type insertion, and the other is fibrous insertion, referred to as indirect-type insertion.^{3,20,30} The insertion of normal ACL to bone is a direct-type insertion.

Whiston and Walmsley²⁸ demonstrated degeneration of the graft with the formation of fibrous tissue adjacent to bone marrow tissue and osseous incorporation at the interface in a rabbit model. Forward and Cowan⁷ also showed similar findings in a rabbit model; however, the origin of the cells that formed the fibrous tissue during remodeling was not determined. Goradia et al⁹ reported that it took 12 weeks for tendon grafts to be surrounded by fibrous tissue in a sheep model. Panni et al¹⁹ showed that it takes 6 months in a rabbit model to obtain a direct-type insertion. Blickenstaff et al² showed that bony fixation occurred with indirect tendon insertion and was complete by 26 weeks in a rabbit model. Furthermore, attempts have been performed using genes or cytokines to enhance healing of bone-tendon interface and to obtain direct-type insertion.^{1,14,22,24,31} We believe that clarification of cellular origin and cellular fate after autologous tendon transplantation is necessary in engineering the healing process for regenerating normal ACL properties.

To accurately follow host and graft cells in the healing tissue after autologous transplantation where these cells are intermixed, we previously developed simulated autologous transplantation models between transgenic rats, in

which no foreign proteins from transgenes were produced, and nontransgenic wild-type rats.^{17,18,27} In these models, *in situ* hybridization was necessary for the detection of transgenes to follow the cells. In the present study, we used syngeneic transplantation with GFP rats and wild-type rats, as GFP has been demonstrated to have no immunological rejection *in vivo*,^{11,15,16} thus simulating autologous transplantation. Furthermore, because GFP signals are visible on the sections under confocal laser-scanning microscopy, it is not necessary to do any histologic staining procedure compared with *in situ* hybridization. In all sections, there was no invasion of lymphocytes, which would have indicated an immunological rejection, in the graft up to 4 weeks. Survival of GFP-positive cells in the sections could clearly be followed during the experiment. Thus, this transplantation model was effective in distinctly observing graft and host cells in the healing process after simulated autologous transplantation.

The present study demonstrated the contribution of host cells rather than graft cells to remodeling after a tendon graft. At 1 and 2 weeks after the transplantation, there was coarse fibrous tissue at the bone-tendon interface; however, there were few fibroblasts. Thus, the graft was revealed to be necrotic.

At 4 weeks, denser fibrous tissue formed at the interface, and the fibroblasts repopulated the graft. Obliquely aligned collagen bundles appeared in the interface between graft and host bone, and a number of spindle-shaped cells aligned longitudinally inside the graft. This finding would mean that the graft was placed under tension. Regarding cellular fate, only host cells were detected both in the interface and inside the graft at each period in group A, and thus the survival of host cells to 4 weeks was elucidated. On the other hand, it is possible that graft cells survived in group A because these cells did not have GFP and could not be visualized even if they had survived. Graft cells, which should have shown GFP-positive signals in group B, were detected in the sections at 1 week. However, in the sections at 2 and 4 weeks, GFP-positive cells were not found. This finding means that graft cells survived for at least 1 week but disappeared within 2 weeks. Host cells survived in the necrotic area inside the graft throughout. It is conceivable that only host cells exist at the bone-tendon interface at 2 weeks and form dense connective tissue over time. In this study, graft cells completely disappeared in the bone tunnel. However, several studies have demonstrated the survival of donor cells in the autologous osteochondral grafts and bone grafts, showing a decrease, although not completely disappearing, in the number of donor cells and their replacement by the host cells.^{18,33} Therefore, the graft generally functions as scaffolding, whereas the survival and viability of graft cells vary among tissues. Furthermore, the round to ovoid-shaped bone marrow cells aligned themselves in layers at the surface of the bone at 1 and 2 weeks and seemed to enter the interface histologically. Therefore, invasion of host bone marrow mesenchymal cells from the interface could contribute to the remodeling of the graft.

In conclusion, it is host mesenchymal cells rather than graft cells that contribute to the repair of the bone-tendon

interface and the remodeling of the graft after autologous tendon grafting. This study clearly demonstrated the survival and invasion of the host cells into the graft in the bone tunnel. Tissue engineering strategies are currently being studied to enhance healing of the bone-tendon interface and remodeling of the graft; therefore, the data in this study could be crucial for the engineering of bone-tendon healing after autologous tendon transplantation in the bone tunnel.

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