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# Articular Cartilage Cells Immortalized by a Temperature Sensitive Mutant of SV40 Large T Antigen Survive and Form Cartilage Tissue in Articular Cartilage Environment

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**Abstract** A chondrogenic cell line, TC6, was established by using cells derived from articular cartilage of transgenic mice harboring a temperature-sensitive mutant simian virus (SV) 40 large T-antigen gene. TC6 cells express genes encoding proteins related to cartilage phenotypes such as type II collagen. To examine the in vivo behavior of the TC6 cells, these cells were implanted into cavity-shaped full-thickness defects made in the articular cartilage of the central part of the patellar grooves of mouse femora. One week after implantation, the morphology of the cells was still fibroblastic but these cells were just about to start to form a cartilage-like matrix. By 6 weeks after implantation, the cells had produced abundant cartilaginous matrix and their morphology became closer to that of authentic chondrocytes. This was in sharp contrast to the fibroblastic morphology of these cells in an in vitro environment even after long-term culture. These observations indicate that a cartilage-matrix environment provides a scaffold for the TC6 cells to form cartilage tissues. Our data show that the genetically engineered chondrocytic cell line, TC6, can form a cartilage-like matrix in vivo. J. Cell. Biochem. 75:338–345, 1999. 

**Key words:** articular cartilage cells; TC6; simian virus; T-antigen; implantation

Chondrocytes are derived from mesenchymal cells during embryonic development and produce cartilage matrix in various skeletal structures. Cartilage matrix contains specific extracellular macromolecules including collagens type II, IX, and XI, aggrecan and link protein [Upholt and Olsen, 1991]. Chondrocyte phenotype expression is sensitive to a variety of environmental factors including the matrix macromolecules and local regulatory cytokines which are also contained in these matrices [Hammermann, 1993]. Articular cartilage is the site of degradation in patients with joint diseases such as osteoarthritis and rheumatoid arthritis and decreased chondrocyte function in these and other articular disorders could be, at least in part, due to dedifferentiation of chondrocytes induced by altered regulatory signals from ECM [Steinberg and Sledge, 1991] which is subject to destruction.

Molecular studies on the physiology of chondrocytes require stable chondrocyte cell models [Benya and Schaffer, 1982]. Although several chondrogenic cell lines have been established, the in vivo behavior of these cells has not yet been clearly shown, and therefore, it is not certain whether these cell lines could behave as chondrogenic cells in an in vivo environment such as in the cognate cartilage matrix.

We have established a chondrogenic cell line, TC6, using cells isolated from articular cartilage of the knee of transgenic mice which harbor temperature sensitive mutant of SV40 (ts-SV40) large T antigen. These cells express chondrocytic phenotypes including type II collagen, aggrecan, and link protein. The cells also respond to hormones or cytokines such as vita-

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min $D_3$ and FGF [Mataga et al., 1996]. At a permissive temperature, these cells grow well even at low serum condition (0.5%) in vitro, however, the ts-SV40 large T antigen is inactive at the body temperature and, therefore, these cells are expected to be not tumorigenic in vivo. In order to examine the behavior of these cells in the in vivo environment, we transplanted these cells into defects made in the knee articular cartilage and investigated the activity of the cartilage matrix environment to support, differentiation, and expression of cartilage phenotypes of these TC6 cells.

## MATERIALS AND METHODS

### Cultures of the Chondrocytic Cell Line (TC6)

TC6 cells were established using the cells derived from the articular cartilage of the distal ends of femora of the 2-month-old transgenic mice (C57BL/6J mouse) harboring a tsSV40 large T-antigen gene kindly provided by Dr. Obinata (Tohoku University) as described previously [Mataga et al., 1996]. For maintenance, the cells were cultured at 33°C to allow expression of active SV40 large T antigen in alpha-minimum essential medium (α-MEM; Sigma Chemical Co., St. Louis, MO) supplemented with 10% FBS (FBS; GIBCO BRL, Gaithersburg, MD) in a humidified atmosphere of 5% $CO_2$. For experiments, TC6 cells were plated at $1.8 \times 10^4$ cells/cm$^2$ (Costar, Cambridge, MA) and were cultured in α-MEM supplemented with 0.5% FBS at 33°C. After reaching about 80% confluency, the cells were harvested and about $2 \times 10^5$ TC6 cells were used for implantation into each of the full thickness defect made in the articular cartilage in the intercondylar region of the knee. About $10^7$ TC6 cells were used separately for implantation into the subcutaneous tissues in the back of the syngenic C57BL/6J mice. As implantation control, ROS17/2.8 cells ($10^7$ cells per pellet) were implanted into the subcutaneous tissues of the syngenic ACI rats.

To evaluate SV40 large T antigen expression in TC6 cells, we performed immunohistochemical analysis. TC6 cells were grown on Lab-Tech chamber slides (Nunc) for 3 days, rinsed with PBS, fixed in 10% formaldehyde for 10 min at room temperature, and rinsed in PBS. The fixed cells were incubated for 2 h at room temperature with mouse anti-SV40 large-T monoclonal antibody (Oncogene Science) that was diluted 500-fold with PBS. Then the cells were rinsed with PBS and incubated with biotin-conjugated goat anti-mouse IgG (ABC Kit, Vectastain, Vector Laboratories) diluted at 1:200 with PBS for 40 min at room temperature. Cells were incubated for 20 min with a solution containing avidin and biotinylated alkaline phosphatase (ABC Kit, Vectastain, Vector Laboratories) and then the slides were incubated in BM Purple AP Substrate, to visualize the signal (Boeringer Mannheim, GmbH, Germany).

### Chondrocyte Transplantation

Ten 8-week-old C57BL/6J mice weighing about 25 g were used as hosts to be implanted with TC6 cells. A cavity-shaped full-thickness defect (0.8 mm in diameter) was produced in the central part of the intercondylar region of patellar grooves of both right and left femora. The articular cartilage defect in the left femora was left untreated as a control. The depth of the cavities was set to reach the levels of subchondral bone by using a dental drill. After the operation, all animals were allowed to move freely without any splintage.

### Histological Evaluation

Animals were sacrificed at 1 and 6 weeks (five and five animals, respectively) after the implantation. The bones were fixed in 10% formaldehyde for 24 h. The bones were then decalcified in 10% formic acid for 1 week, embedded in paraffin, and were sectioned serially. Sections (10 um thick) were made and stained with hematoxylin and eosin, toluidine blue (pH 7.0) and alcian blue (pH 2.5).

### Immunohistochemistry for Detecting Mouse Type II Collagen

The sections were preincubated with 0.25% bovine hyaluronidase in PBS for 20 min at room temperature, and then were incubated in 1% BSA-PBS for 30 min to block nonspecific binding in the tissue. They were then incubated with rabbit anti-mouse monoclonal antibody to type II collagen (Hybridoma Bank, University of Iowa, Iowa City, IA) at a dilution of 1:100 for 2 h at room temperature. Specimens were subsequently incubated with HRP-goat antirabbit IgG (Zymed Laboratories Inc.). The sections were incubated with True Blue Peroxidase Substrate (KPL) to visualize the signal.

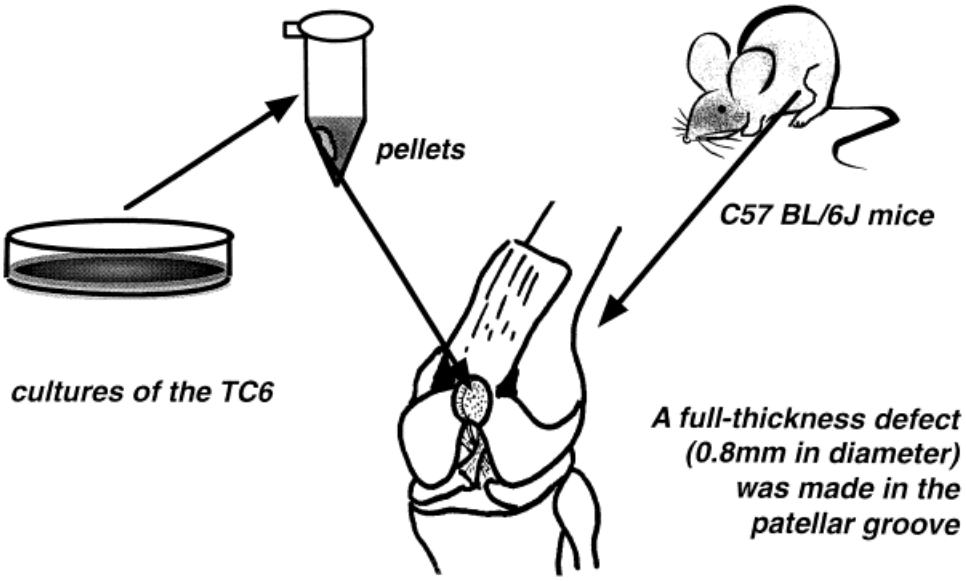


**Fig. 1.** Procedures for TC6 cell implantation. About $2 \times 10^5$ TC6 cells in a pellet were implanted into full-thickness defects in the central part of the boundary between intercondylar regions and patellar grooves of femora, just anterior to the insertion of posterior cruciate ligament.

## RESULTS

We first implanted TC6 cells into the subcutaneous tissues of syngenic mice. Although $10^7$ cells per site were implanted, these cells did form neither any tumor mass nor any tissues which could be identified macroscopically at 4 and 8 weeks. As an implantation control, rat osteosarcoma ROS17/2.8 cells were implanted at $10^7$ cells per site in syngenic rats. In contrast to the TC6 cells, these osteosarcoma cells were able to form tumors with a diameter of 0.5–1 cm within 2–3 weeks and these tumors continued to grow at least up to 6–8 weeks as reported previously (data not shown).

Since ectopic (subcutaneous) implantation experiments of TC6 cells did not yield any tumors or tissues, we speculated that certain in vivo environments such as cartilaginous matrix might be required for the TC6 cells to survive in vivo. Therefore, we examined whether the TC6 cells could survive when they were implanted into the cavity-shaped defects in the cartilage (Figs. 1, 2A). Knee joint articular cartilage was chosen as the site for TC6 implantation since the TC6 cells were derived from knee cartilage when they were established.

For controls, similar drilled cavity-defects were made in the cartilage of the patellar groove in the intercondylar regions in the contralateral side knee joints, but they were left untreated and were not implanted with the cells (Fig. 2B). At 1 week post-surgery, these control cavity-shaped defects were covered with thin layers of fibroblastic cells, possibly derived from the outer-most cellular layers of the cartilage adjacent to the defects (Fig. 3A). Such cell layers covering the control lesions became slightly thicker at 6 weeks (Fig. 4A–C). The cells in the control cavity-shaped defects at 1 week were only weakly stained for alcian blue and toluidine blue (Fig. 3B,C) and the staining was similarly weak even at the 6-week time point (Fig. 4B,C).

The cavity-shaped defects made in articular cartilage that were filled by the transplantation of TC6 cells showed a similar defect size compared to the control at 1 week (Fig. 3D–F). They were filled with a mass of relatively high cellularity. By 1 week, the cells exhibited a small amount of matrix, which was lightly stained positive for alcian blue (Fig. 3E) and was slightly metachromatic when stained with toluidine blue (pH 2.5; Fig. 3F).

By 6 weeks, the cells filling the articular defects had produced extracellular matrix (Fig. 4D) that showed high stainability to alcian blue (Fig. 4E) and toluidine blue (Fig. 4F), indicating the survival and production of cartilage matrix by the TC6 cells in the context of the cartilage matrix environment. The morphology of some of the cells revealed features resembling hypertrophic chondrocytes (Fig. 4D–F).

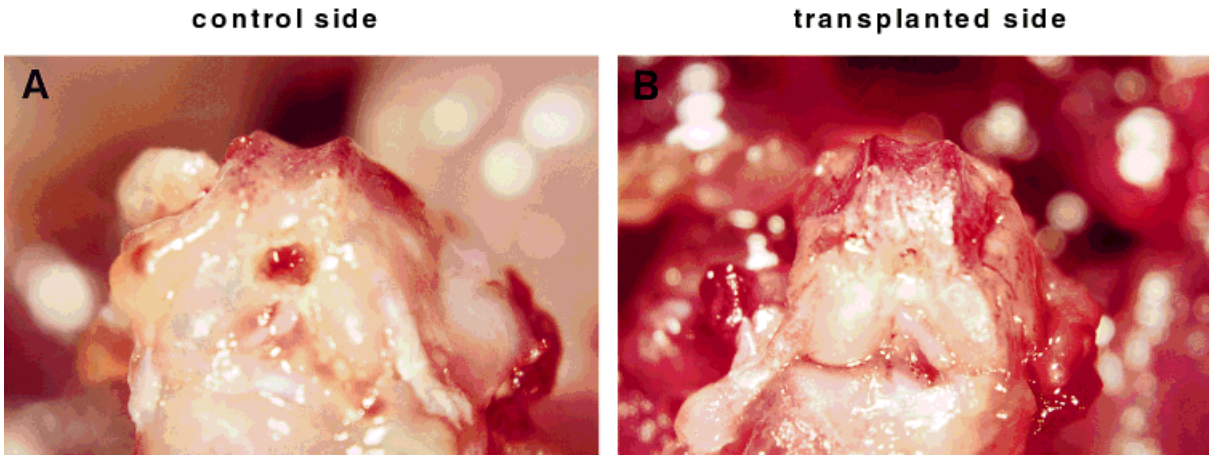


**Fig. 2.** Macroscopic view of the knee. **(A)**: Control side. The cavity-shaped defect (arrowhead) made in the right femur was left untreated and was used as a control. **(B)**: Transplanted side. TC6 cells were implanted into the cavity-shaped defect (arrowhead) of left femur.

In these knees, the transplanted TC6 cells made a large cellular mass that exceeded the size of the defect and protruded out of the surface of the articular cartilage. The large mass contained a relatively abundant matrix suggesting that the articular cartilage environment support the survival as well as matrix production of these cells.

As shown in Figure 5B, TC6 cells grown on chamber slides at 33°C were stained according to the immunohistochemical technique with anti-SV40 large T antigen. SV40 large T antigen was expressed in TC6 cells in in vitro culture, indicating that the cultures at 33°C allow the expression of active SV40 large T antigen.

We performed additional immunohistochemical tests to determine if the matrix produced by the implanted chondrocytes contains type II collagen. Immunoreactivity against anti type II collagen-antibody was observed in the mass filling the articular defects at six weeks after implantation (Fig. 6). No specific immunoreactivity was observed in a section stained with nonimmune rabbit serum (data not shown).

## DISCUSSION

We have characterized the behavior of the TC6 cell line, which was established from articular chondrocytes of transgenic mice harboring a temperature-sensitive mutant of SV40 large T antigen, when implanted into both cartilagenous and noncartilagenous environments. The TC6 cells produced cartilagenous matrix when they were implanted into the cavity-shaped defects made in articular cartilage. The implanted TC6 cells survived at least up to 6 weeks in this implantation experiment. In sharp contrast to these observations on the implantation into the cartilage defects, these TC6 cells did not survive when implanted into subcutaneous tissues. No tumor formation was observed at least by 6 weeks in either cartilagenous or subcutaneous environments. The reasons for the observation that TC6 cells could survive longer in the defects in the articular cartilage but not in the subcutaneous tissues are not clear at this point. Cartilage matrix contains structural proteins such as type II, IX, XI collagens, link protein, and proteoglycans. It also contains cytokines such as FGF [Schlessinger et al., 1995] and chondromodulin [Hiraki et al., 1991]. These environmental signals could allow the TC6 cells to survive and to produce cartilage matrix. Subcutaneous environments not only lack such structural proteins and cytokines as mentioned above but also could provide unfavorable conditions for the survival of chondrocytes, such as vascularization and/or presence of enzymes that would be unfavorable for the cells to survive and/or destructive to cartilage matrix.

The temperature in the knee could be lower than the temperature in the back of the mice. However, that could fluctuate depending on the motion of the limbs and therefore hard to measure precisely. At this point, we cannot exclude the possibility that survival of the TC6 cells in

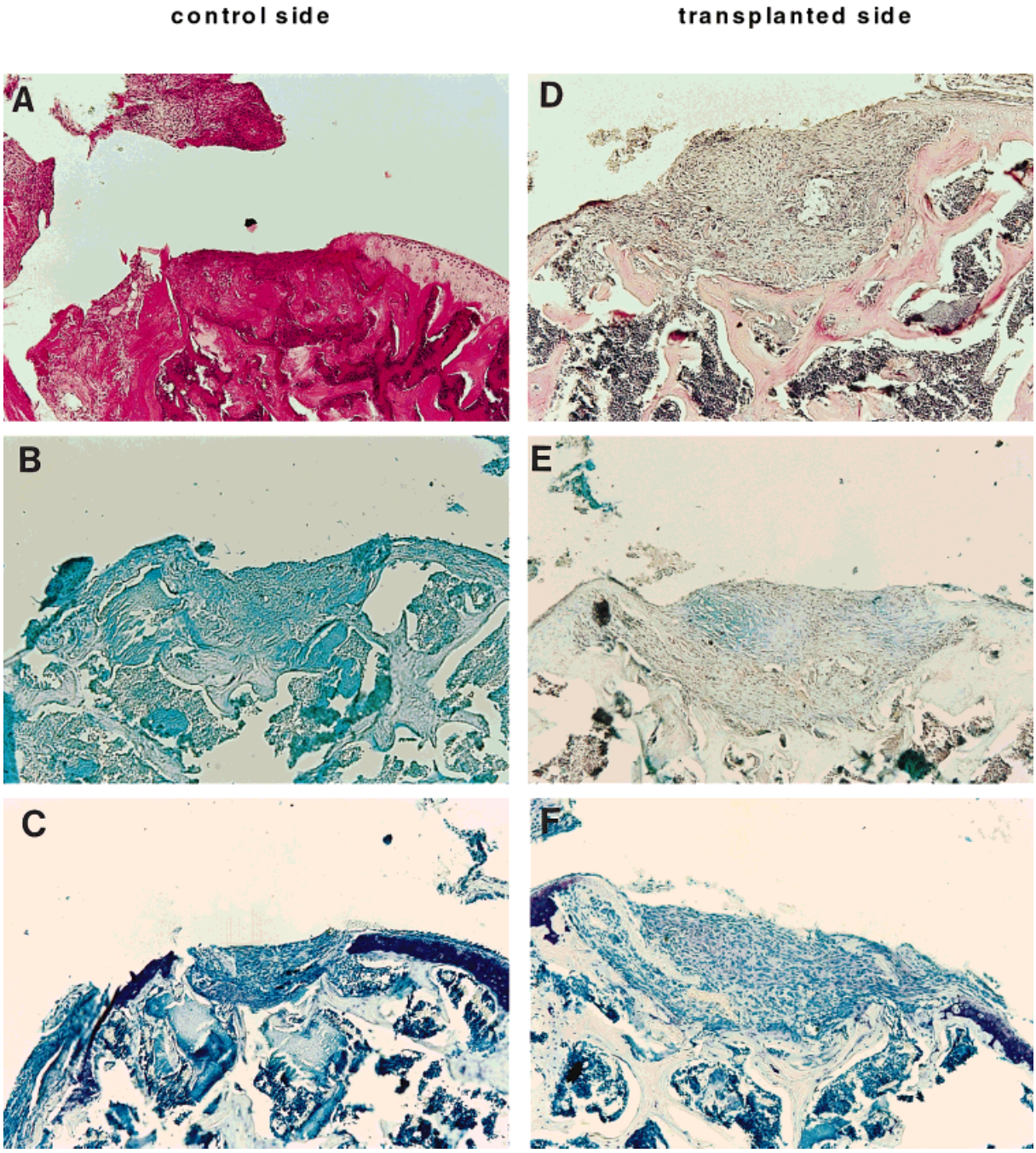


**Fig. 3.** Histology of the knee after one week of surgery. **A–C**: The surface of the cavity-shaped defects of untreated side was covered with several layers of fibroblastic cells. **D–F**: The articular cartilage cavity-shaped defects implanted with pellets of TC6 cells were filled with a mass of implanted cells. Sections were stained with hematoxylin and eosin (A,D), alcian blue (B,E), and toluidine blue (C,F). The picture shows the histology in one of five animals examined at 1 week.

the knee but not in the back of the mice could be in part influenced by the possible difference of the temperatures and hence the nature of the temperature-sensitive mutant of the large T antigen.

Previously, several chondrogenic cell lines were reported to be able to form tumors when transplanted into the subcutaneous tissues [Hiraki et al., 1991; Takigawa et al., 1989; Wang et al., 1993]. In contrast, although TC6 cells prolif-

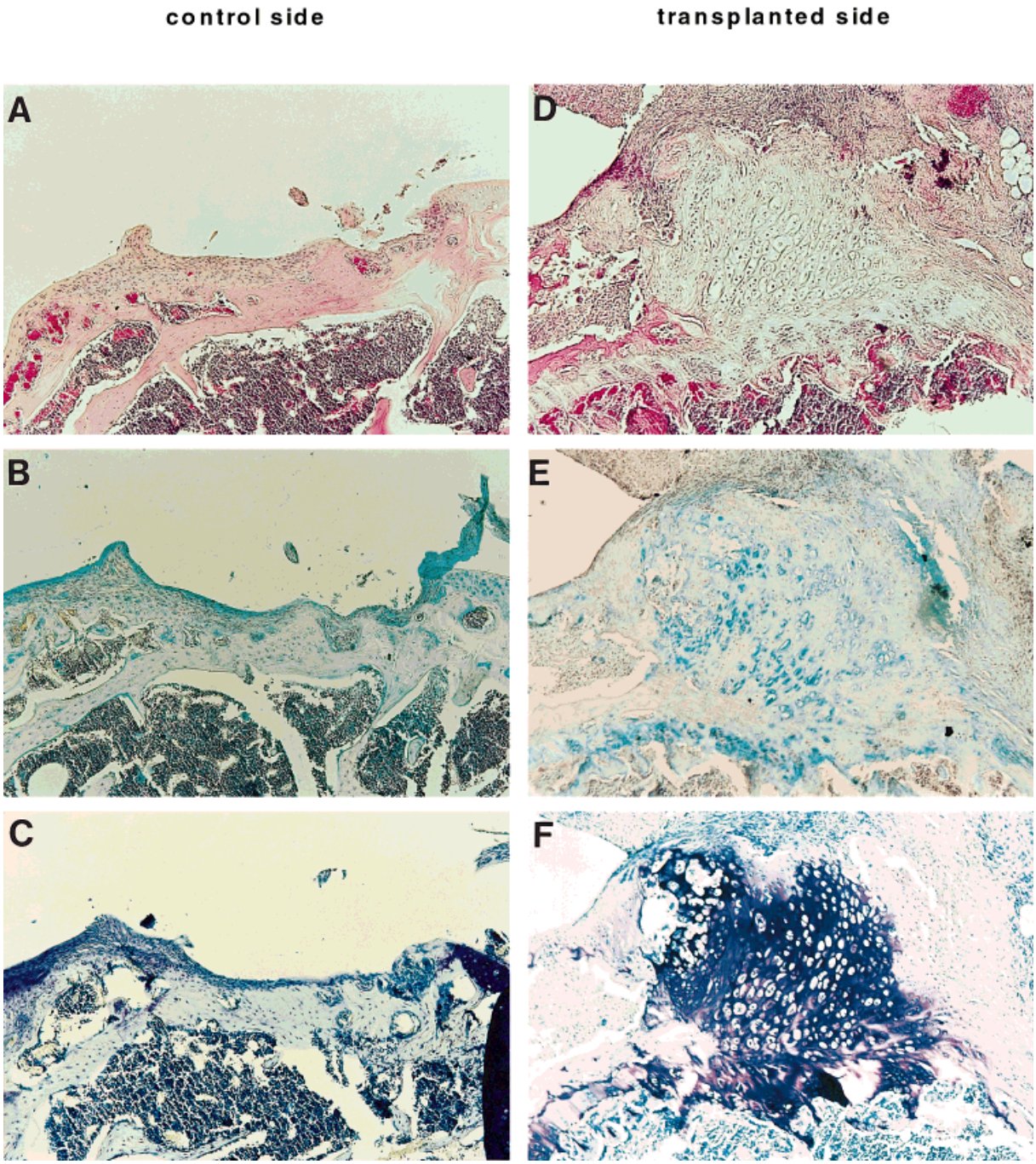


**Fig. 4.** Histology of the knee after 6 weeks of surgery. **A–C**: In the control lesions, thin layers of the cells were observed. **D–F**: The lesions were filled with TC6 cells which contained an extracellular matrix stained positive for alcian blue and toluidine blue. Some of the cells showed hypertrophic morphology (D–F). Sections were stained with hematoxylin and eosin (A,D), alcian blue (B,E), and toluidine blue (C,F). The picture shows the lesions in one of five animals examined at 6 weeks.

erate well at 33°C in culture, they do not form tumors in vivo either in subcutaneous tissues or in the articular cartilage matrix, possibly due to the nature of the temperature sensitive mutation of the SV40 large T-antigen, which is inactive at 37°C [Mataga et al., 1996]. Although the TC6 cells could produce a relatively large amount of cartilaginous matrix in a cartilaginous environment in vivo, in cell culture they produced very little extracellular matrix even

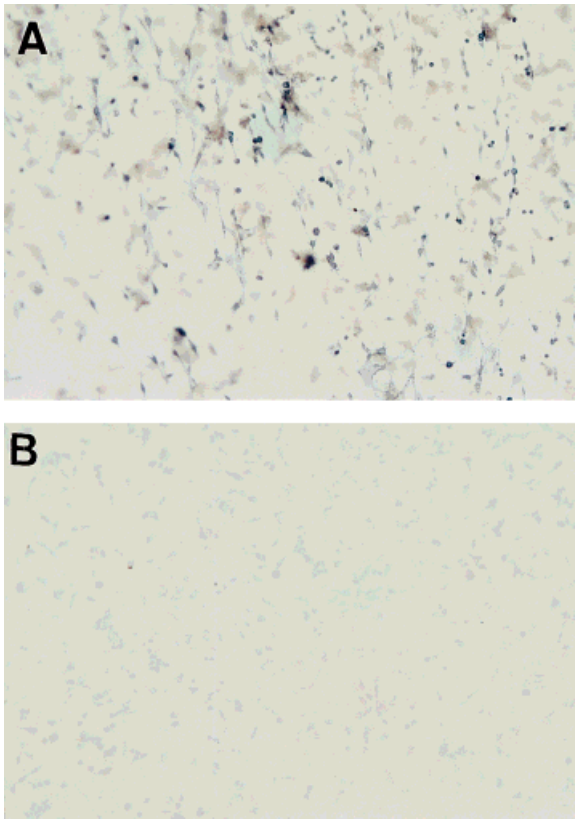


**Fig. 5.** Immunochemical staining of SV40 large T antigen in TC6 cells. TC6 cells were cultured on chamber slides for 3 days and fixed in 10% formaldehyde. The cells were incubated with mouse anti-SV40 large-T monoclonal antibody (**A**) or without antibody as a control (**B**). Magnification: ×100.

after 4 weeks at 33°C or 37°C (data not shown). The morphology of the implanted TC6 cells in lacunae in vivo resembled that of authentic chondrocytes (Fig. 4D–F). Such morphologies were never observed as long as these cells were cultured in vitro. It is likely that the presence of certain factors in the in vivo cartilage matrix environment possibly including matrix proteins and cytokines encouraged these cells to produce more cartilage matrix and to adopt a more chondrocyte-like morphology in vivo than in vitro. Such supporting activity of cartilage matrix for chondrocytes has been seen in the transplantation of primary chondrocytes [Aston and Bentley, 1986; Wakitani et al., 1989; Shapiro et al., 1993; Jingushi et al., 1994; Hunziker and Schenk, 1995; Brittberg et al., 1996].

Although the TC6 cells produced abundant cartilage matrix in vivo, the histology was quite different from the authentic articular cartilage. For instance, the hypertrophy of the implanted TC6 cells seen at six weeks time point would never occur in normal chondrocytes at the surface of the articular cartilage. In addition, the mass of newly produced cartilage matrix exceeded the level of the adjacent original articular cartilage. However, the matrix filling the cavity contains type II collagen as shown by immunohistochemistry (Fig. 6). These data indicate that the cartilage matrix environment in vivo supported differentiation of the newly established chondrocyte-like cell line, TC6, at least in our protocol of implantation.

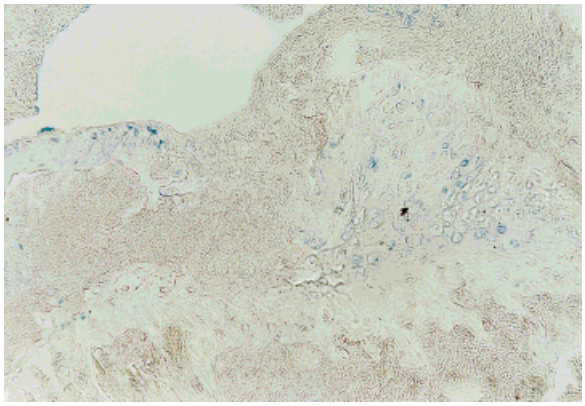

**Fig. 6.** Expression of type II collagen in the cartilaginous mass filling the cavity. The sections were incubated with rabbit anti-mouse type II collagen monoclonal antibody as described in Materials and Methods. The cavity was filled with TC6 cells that form a mass containing extracellular matrix stained positive for type II collagen. The picture shows one of the lesions in one of five animals examined at 6 weeks.

## ACKNOWLEDGMENTS

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