

**METHODS ARTICLE**

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# Assessment of the Safety of Chondrocyte Sheet Implantation for Cartilage Regeneration

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We have previously studied the effects of chondrocyte sheets on the repair and regeneration of articular cartilage by using temperature-responsive culture inserts. On the basis of this work, we succeeded in rapid fabrication of chondrocyte sheets with the use of a coculture method in which inserts were placed between synoviocytes and chondrocytes. Treatment of cartilage defects using layered chondrocyte sheets promotes repair and regeneration; this method is compatible with *in vivo* osteoarthritis models that reproduce partial-thickness defects. In human stem cell clinical research guidelines, the Ministry of Health, Labour and Welfare (MHLW) approved several applications related to this technology. Indeed, its translation to a clinical setting is already yielding favorable results. In this study, we evaluated the risk of tumorigenesis associated with this treatment and characterized the dynamics of biological processes associated with the posttransplantation cell sheets *in vivo*. Furthermore, we also confirmed the safety of the procedure by using array comparative genomic hybridization (array CGH) and G-band staining to screen for deleterious genetic aberrations during prolonged subculture of cells. The safety of chondrocytes that were cultured for longer than normal was confirmed by the array CGH and G-band staining results. In addition, tumorigenicity testing confirmed that culture chondrocyte sheets are not tumorigenic. Furthermore, from the evaluation of bioluminescence imaging following implantation of the cell sheets, it was confirmed that the transplanted chondrocytes and synoviocytes remained in the knee joint and did not transfer elsewhere over time. We believe that the technique used in this study is a highly useful method for evaluating the safety of not only chondrocytes but also extensive subculturing in general.

## Introduction

**W**E HAVE ALSO previously analyzed the effects of articular cartilage on the tissue repair process by fabricating chondrocyte sheets using the UpCell Insert developed by Okano *et al.*<sup>1,2</sup> and studied their repair effects.<sup>3–8</sup> Cartilage defects can be covered by these chondrocyte sheets, which then provide a continuous supply of growth factors. Concomitantly, the cell sheets protect articular cartilage from catabolic factors that are present in the synovial fluid, thereby also promoting the recipient's self-healing.<sup>3–6</sup> Mitani *et al.*<sup>8</sup> used UpCell Inserts to fabricate layered chondrocyte sheets and found that they reduced the level of catabolic factors

present; we also identified genes that were important for cartilage maintenance.<sup>9–12</sup> Because of the demand for rapid fabrication of chondrocyte sheets in clinical applications, we have focused on the synovial tissue,<sup>13,14</sup> which functions as a nutrient source for cartilage. In this regard, UpCell Inserts have been used to coculture chondrocytes and synoviocytes. This method obviates the need for the addition of proliferative factors to the culture. We have previously utilized these techniques<sup>15</sup> to produce layered chondrocyte sheets for clinical use in the treatment of articular cartilage defects and have confirmed their efficacy in animal experiments.<sup>6</sup>

First, in human research approved by the Ministry of Health, Labour and Welfare (MHLW) in Japan, safety is

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considered the most important issue. In the prolonged culture method, it is especially important to study whether, for example, the process induces chromosomal or phenotypic changes in the cultured cells. Typically, tumorigenicity tests are performed on the cells being studied to determine whether their prolonged culture might lead to transformation. Other properties, such as distribution, absorption, migration, and engraftment, are also studied by using appropriate animal models to confirm the posttransplantation dynamics.

Based on the above premises, the current study was designed to evaluate the safety of chondrocyte sheets (Fig. 1) by using array comparative genomic hybridization (array CGH) and G-band staining of *in vitro* samples, as well as by studying the tumorigenesis evaluation and dynamics of posttransplantation cell sheets by bioluminescence imaging *in vivo*.

We used array CGH with the aim of confirming that the genome remains unchanged, even when chondrocytes are cultured for extended periods. Array CGH has the advantage of allowing high-resolution detection of any mutations that may occur in genome copies. Furthermore, in contrast to results obtained through fluorescence *in situ* hybridization (FISH) and karyotype analysis, parsing the results of array CGH is a relatively simple process, and with the appropriate software, reliable results can be readily obtained.

The final and most important aspect of this study is that the array CGH allowed us to detect abnormalities in the over-subcultured cells by using the P2 subculture as a reference, unlike traditional methods that use the HapMap DNA database.

We further performed G-band staining as additional testing to evaluate the safety of over-subcultured cells. In G-band

staining, proteases and Giemsa stain solution are used to express bands to identify nuclear chromosomes and analyze abnormalities. This is the most widely used chromosomal test, in which the specimens are semipermanently preserved. To examine the chondrocytes, Stumm *et al.* performed molecular karyotype analysis to determine the stability of the autosome and deletions on the Y-chromosome using array CGH.<sup>16</sup>

However, chondrocytes have not previously been used in array CGH and G-band staining for assessing safety with respect to over-subculturing; our data indicate that the robustness of this approach renders it suitable for this application.

Moreover, we isolated chondrocytes and synoviocytes from cartilage and synovial tissue of ROSA/Luciferase (Luc) transgenic (TG) Lewis rats, performed an enzymatic treatment, and fabricated light-emitting Luc-positive chondrocyte/synoviocyte sheets using the UpCell six-well Cell Culture Insert (hereafter “UpCell Inserts,” provided by CellSeed, Inc.). The duration of this bioluminescence within the joints allowed us to study the dynamics of cell sheet behavior following their transplantation.<sup>17</sup>

We report the above *in vivo* and *in vitro* methods used to evaluate the safety of performing treatment with chondrocyte sheets.

## Materials and Methods

### Analysis of over-subcultured cells: array CGH and G-band staining

Human genomic DNA from passage 4, passage 6, and passage 12 (P4, P6, and P12) chondrocytes was analyzed,

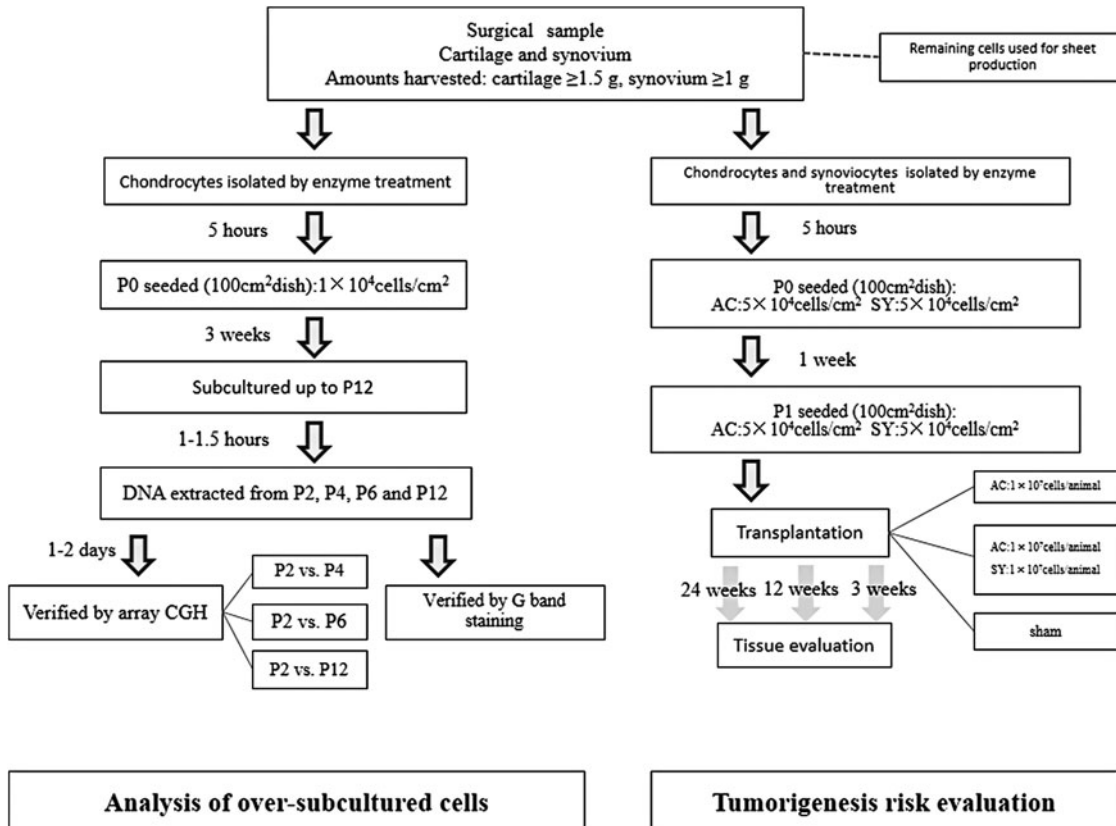


FIG. 1. Flowchart of safety assessment process.

and P2 human genomic DNA was used as the normal control sample. These samples were used to run array CGH and to check for any possible aberrations in P2 versus P4, P2 versus P6, and P2 versus P12 samples in the moving average (i.e., whether there was any substantial shift in the number of copies from  $\text{Log}_2 \text{ ratio} = 0$ ).

#### *Isolation of chondrocytes*

Antibiotic-antimycotic (AA; Gibco) was adjusted to 1% final concentration, and fetal bovine serum (FBS; Gibco) was adjusted to 20% concentration in Dulbecco's modified Eagle medium/nutrient mixture F-12 (DMEM/F-12; Gibco) in the initial culture medium (AB medium). A medium for subsequent initial exchanges was obtained by adding 0.01% ascorbic acid injection solution (Nissin Pharmaceutical) to the AB/AA medium.

We took specimens from 11 patients, 4 patients who underwent anterior cruciate ligament (ACL) reconstruction (mean age 33.5 years) and 7 patients who underwent total knee arthroplasty (TKA; mean age 73 years). The ACL cartilage was collected from the minimal weight-bearing periphery of the femoral condyles at the level of the patellofemoral, and sufficient TKA cartilage was collected from the remaining load-bearing and nonload-bearing parts of the ablated femur resected during surgery. Chondrocytes were collected and used after obtaining informed consent and with the approval of the Clinical Research Review Committee of our hospital.

The cartilage tissue collected was washed with phosphate-buffered saline (PBS; Amresco), and the wet weight of the tissue was measured; thereafter, the specimens were cut into 5 mm squares with scissors over a Petri dish, and the tissue pieces were transferred to 50-mL tubes. DMEM/F-12/20% FBS/AB was added to collagenase type 1 (Worthington Biochemical) and DMEM/F12 (Gibco), adjusted to 5 mg/mL, sterilized by filtration, and added to tubes. The mixture was incubated at 37°C with 5% CO<sub>2</sub> for 4 h with stirring for proteolysis, passed through a cell strainer (100 µm pore size; BD Falcon), and then separated by centrifugation at 1500 g for 5 min. The supernatant was removed, 30 mL of PBS was added, and then separation was performed by centrifugation at 1500 g for 5 min.

#### *Culturing of chondrocytes*

Isolated P0 chondrocytes were seeded at a density of  $1 \times 10^4$  cells/cm<sup>2</sup> using the initial medium of DMEM/F-12/20% FBS/AB. The medium was then exchanged twice per week with DMEM/F-12/20% FBS/AB/AA medium. All cultures were incubated at 37°C with 5% CO<sub>2</sub> and 95% air.

After culturing to subconfluence, P1 cells were trypsinized and recovered. P1 cells were seeded in a similar manner at  $1 \times 10^4$  cells/mm<sup>2</sup> and cultured to subconfluence. For articular chondrocytes derived from ACL, culturing proceeded to P12. The P2, P4, P6, and P12 cells were treated with trypsin-EDTA (Gibco) and recovered. After 5 min of centrifugation at 1500 g, the supernatant was removed and the cells were stored frozen at -80°C. The frozen pellets were used for DNA extraction.

Verification of passage cells by array CGH. DNA was extracted from frozen cells using the DNeasy Blood &

Tissue Kit (Qiagen) in accordance with the manufacturer's instructions and then subjected to array CGH.

Five hundred nanograms of genomic DNA was used for each of the P2, P4, P6, and P12 cultures; testing was carried out in accordance with the existing protocol using Agilent SurePrint G3 Human CGH Microarrays at 8×60 K (i.e., equipped with 60,000 probes; Agilent Technologies). The analysis software Agilent Genomic Workbench 6.5 Light, quantification protocol CGH\_107\_Sep09, quantification software Feature Extraction Software 10.7.3.1, and scanner G2505C were used, and the scan resolution was set to 3 µm. For the analysis conditions, extracted DNA samples were cut into short fragments using the restriction enzymes *AluI* and *RsaI*.

To label DNA, a reaction was performed using random primers and Exo-Klenow (Genomic DNA Enzymatic Labeling Kit; Agilent Technologies). This reaction enabled labeling of P4, P6, and P12 DNA with the fluorescent dye cyanine-5 (Cy5) and P2 DNA with the fluorescent dye cyanine-3 (Cy3). Competitive hybridization of the two samples was carried out on glass slides loaded with probes that were centered on coding sequences. The fluorescence intensity of each probe was scanned, and feature extraction software was used to convert the fluorescence intensities to numerical values, which were then normalized and logarithmically displayed (arranged for each chromosomal position).

Aberration Detection Method-2 (ADM-2) algorithm<sup>18</sup> and Threshold = 10 were used,<sup>19</sup> and the moving averages were set to 10 pt. A dye swap experiment (in which samples were reciprocally labeled with Cy3 and Cy5) was performed. Swapping corrects for differences in the uptake and rate of degeneration during labeling due to slight differences in the molecular structures of Cy3 and Cy5 that lead to errors in the detection of gene expression levels.

Verification of passage cells by G-band staining. We repeatedly subcultured chondrocytes by using the method described above and performed G-band staining of P2, P4, P6, and P12 chondrocytes during the metaphase. A sample of metaphase cells was developed for each passage, and Giemsa staining was carried out following trypsin treatment. Each chromosome was identified based on the shade of its chromosomal band pattern and analyzed for the presence of polyploidy, aneuploidy, and translocation.

The samples used were the same as those used for array CGH.

#### *Tumorigenesis evaluation*

Preparation of cells for transplantation. The cartilage and synovial tissue collected was washed with PBS, and the wet weight of the tissue was measured; subsequently, the tissue was cut into 5 mm squares with scissors on a Petri dish, and the tissue pieces were transferred to 50-mL tubes.

AB medium was added to collagenase type 1, adjusted to 5 mg/mL, sterilized by filtration, and added to the tubes. The chondrocytes were incubated at 37°C in 5% CO<sub>2</sub> for 2 h with stirring, passed through a cell strainer (100 µm pore size), and separated by centrifugation at 1500 g for 5 min. Synoviocytes were treated similarly, although an additional 2 h of stirring were performed. The supernatant was removed, 30 mL of PBS was added, and cells were resuspended before centrifugation at 1500 g for 5 min.

Primary chondrocytes were maintained in the AB medium until culture day 4. From 5th day, cells were then maintained in a medium to AB/AA medium. Synoviocytes were continuously maintained in the AB/AA medium. P0 cells were seeded in 100 mm dishes at  $5 \times 10^5$  cells/dish for AC and  $5 \times 10^5$  cells/dish for SY, and P0 → P1 → P2 subcultures were seeded in 500 cm<sup>2</sup> dishes at  $5 \times 10^6$  cells/dish for AC and  $5 \times 10^6$  cells/dish for SY. All cultures were incubated at 37°C with 5% CO<sub>2</sub> and 95% air. After the cells were washed twice with PBS, they were collected with a scraper, transferred to a tube, and centrifuged at 1500 g. The supernatant was aspirated to obtain pellets of the transplanted cells.

**Transplantation of the cells and tissue assessment.** In accordance with World Health Organization (WHO) standards,<sup>20</sup> we used 25 male severe combined immunodeficiency (SCID) mice (strain C.B-17/Icr-SCID/SCID jcl; age, 5 weeks) with a mean body weight of 20.7 g. After anesthesia using isoflurane, an incision of ~1 cm was made in the right lumbar area and pellets of cell masses were subcutaneously transplanted. The skin of the transplanted portion was held together with a clip. For transplantation conditions, a cell count of  $1 \times 10^7$  cells/animal was used as a reference. The transplantation conditions for the combination of chondrocytes and synoviocytes were  $1 \times 10^7$  chondrocytes/animal and  $1 \times 10^7$  synoviocytes/animal, respectively.

A control group of 25 animals were also used; incisions were made in the skin of these mice but no cells were transplanted. Each week following transplantation, the sites were visually inspected and then imaged, and body weight was measured. Mice were sacrificed and autopsied at 3, 12, or 24 weeks after transplantation. Following sacrifice, subcutaneous structure of the transplanted region, blood, lymph nodes, and organs was collected.

The blood collected was centrifuged to obtain serum, which was then stored at -20°C. After reflux, the organs and skin obtained from the transplant site were stored in 10% formalin. Paraffin sections were prepared from the skin samples, which were subjected to toluidine blue, safranin O, and hematoxylin and eosin (H&E) staining for pathological analysis. The organs collected were the lymph nodes (axillary and inguinal), lungs (full lobe), spleen, kidneys, liver, and brain.

#### *Assessment of the posttransplantation dynamics of the cell sheets*

The studies were performed according to the Takaku's Method.<sup>17</sup>

**Preparation of the Luc<sup>+</sup> cell sheets.** Sixteen-week-old ROSA/Luciferase TG Lewis rats (Fig. 6A) were sacrificed, and the hip joints were exposed to collect the synovial tissue. Cartilage and synovial tissue was then collected from the femoral head with a scalpel. The cells from these tissues were isolated using the method described above for the array CGH experiments. Chondrocytes were seeded at  $5 \times 10^4$  cells/mm<sup>2</sup> and synoviocytes were seeded at  $1 \times 10^4$  cells/mm<sup>2</sup> in the UpCell Inserts.

**Layering of the cell sheets.** Once the cells reached confluence, they were equilibrated for 30 min at 25°C. The

medium was removed, and tweezers were used to place a CellShifter (CellSeed, Inc.) on top of the sheets; care was taken to prevent bubbles from becoming trapped between the CellShifter and the layer of cells. The cells were allowed to sit for 5–6 min at 20–25°C; during this time, the next culture dish was removed from the incubator and the medium was removed.

We collected the cell sheets from the UpCell Inserts by slowly peeling them from the end of the CellShifter. Each CellShifter was placed on another prepared culture dish with the cell surface upside down to prepare groups composed of combinations of two Luc<sup>+</sup> chondrocyte (AC) sheets and two Luc<sup>+</sup> synoviocyte (SY) sheets. Three groups ( $n=4$  in each) were prepared: an Luc<sup>+</sup> chondrocyte sheet-only group (AC-AC group), a Luc<sup>+</sup> synoviocyte (SY-SY group) sheet-only group, and a combined sheets group (AC-SY group). The layered cell sheets were laid on top of 100-mm dishes and weighed with a cell strainer with a pore size of 100 μm; 10 mL of AB/AA medium was added, and the cultures were maintained for 1 week.

Three milliliters of medium was added directly to the cell strainer, which was then held in place by adding the remaining 7 mL to the outside of the vessel.

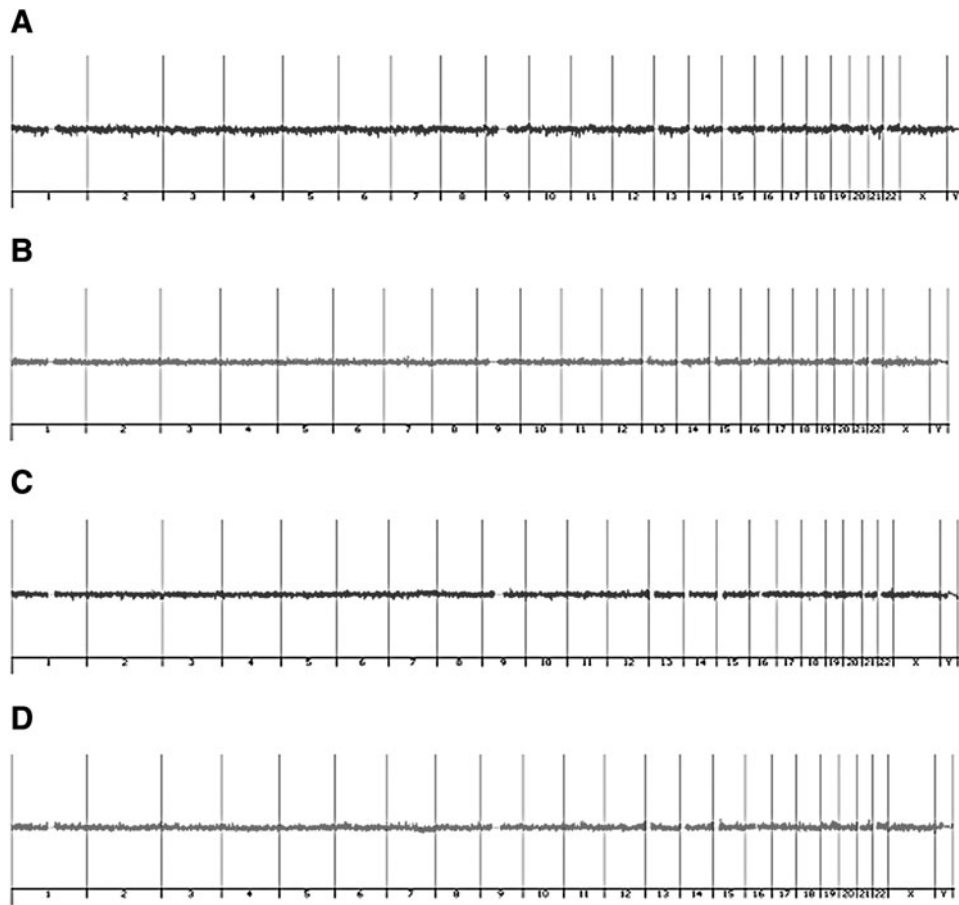
**Intra-articular transplantation of the Luc<sup>+</sup> cell sheets.** The cultured UpCell Inserts were removed from the incubator, and the medium was discarded; this was followed by 5–6 min of equilibration at 20–25°C. We recovered the layered cell sheets from the UpCell Inserts by slowly inverting the CellShifter with tweezers and then bisected them with a scalpel. An 18-gauge needle was used to create osteochondral defects (3 mm diameter) in the femoral patellar surface of the right knee in wild-type Lewis rats of the same age. The bisected layered cell sheets were transplanted onto the defect sites with tweezers and a pipette, and then sutures were made at 1 mm intervals.

**Kinetic imaging using the *in vivo* imaging system.** To compare the effects of cell sheet composition on the duration of the engraftment in the knee joint, 150 mg/kg of D-luciferin (potassium salt; Biosynth) was subcutaneously administered below the scapula and then an IVIS imaging system (Xenogen Corp.) equipped with a charge-coupled device (CCD) camera was used to repeatedly capture images over time. Ordinary photographs of the mice were also captured at the same time. Then, the luminescence images were overlapped with the digital images, and the duration of the Luc<sup>+</sup> cell sheets in the joints was measured by obtaining the distribution of luciferase expression. During imaging, the highest luminescence intensity was measured.

## **Results**

### *Analysis of over-subcultured cells*

**Array CGH.** Array CGH of genomic DNA in chondrocytes and comparisons among P2, P4, P6, and P12 cells did not reveal any copy number aberrations for articular chondrocytes derived from ACL (Fig. 2A–D). In articular chondrocytes derived from TKA, there were no large aberrations in copy number (Fig. 3A–F), although slight genomic alterations were found, and these presented most frequently in



**FIG. 2.** Results of array comparative genomic hybridization (array CGH) in articular chondrocytes derived from anterior cruciate ligament (ACL). (A) P2 versus P4, (B) P4 versus P2 swap with color further switched with articular chondrocytes derived from ACL, (C) P2 versus P6 in articular chondrocytes derived from ACL, and (D) P6 versus P2 swap with color further switched. A dye swap experiment was performed to confirm that the results we obtained were not due to labeling artifacts. No deviation from the moving average baseline was observed in any instance; there were no evident aberrations in copy numbers.

chromosome 7. Samples that showed a deviation in P2 versus P4 in chromosome 7 also had a deviation from the baseline in P2 versus P6 and P2 versus P12. However, this aberration has already been reported in osteoarthritis,<sup>21</sup> which was the primary clinical condition in the patients enrolled in the current study (Fig. 3G–L). Within the range of our analyses, no aberrations in copy number were evident apart from the chromosome 7 alteration. This was true for all 11 samples for which we ran array CGH.

**G-band staining.** G-band staining did not reveal any abnormalities in articular chondrocytes derived from ACL. However, in articular chondrocytes derived from TKA, abnormalities in chromosome 7 were evident in two rows (Fig. 4). Specifically, chromosome 7 trisomy was evident at P6 in one row and at P4, P6, and P12 in the other row. Their frequencies also increased in successive cultures. No chromosomal abnormalities that were continuously observed in the culture process were evident in any other cells.

#### *Tumorigenesis evaluation*

The transplantation sites of the control, AC, and AC-SY groups were evaluated at 3, 12, and 24 weeks (Fig. 5A–I). At 12 weeks, the transplantation sites were observed in all groups, although they showed reduction. At 24 weeks, the transplantation site could not be identified by visual inspection in any of the individuals. No difference in the ex-

ternal appearance was evident between animals of the three groups at any time point.

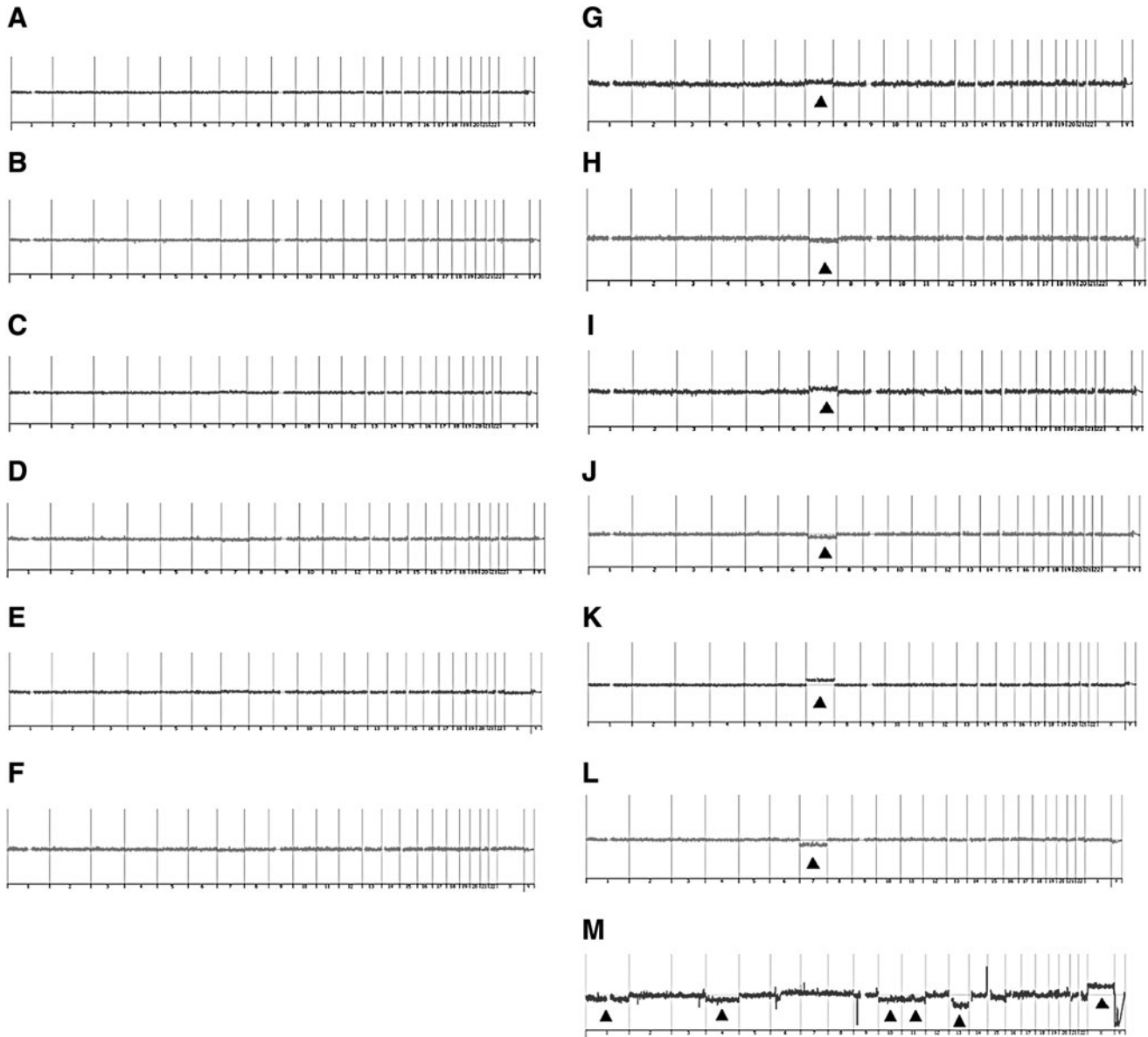
Transplanted cells persisted in some animals at 3 and 12 weeks posttransplantation. Histopathological analysis revealed that the persistent cells were chondrocytes. At 24 weeks posttransplantation, lingering of the transplanted chondrocytes could not be confirmed. Chondrocytes were found in some animals at both 3 and 12 weeks posttransplantation, but were completely absent from all mice by 24 weeks posttransplantation.

This finding suggests that when cultured chondrocytes are transplanted *in vivo*, although some individual differences do exist, the transplanted cells disappear. In summary, these results confirm that cultured chondrocyte sheets are not tumorigenic.

In addition, there were no clear abnormalities or malignant transformations found in the major organs harvested at the same time.

#### *Assessing the posttransplantation dynamics of the cell sheets*

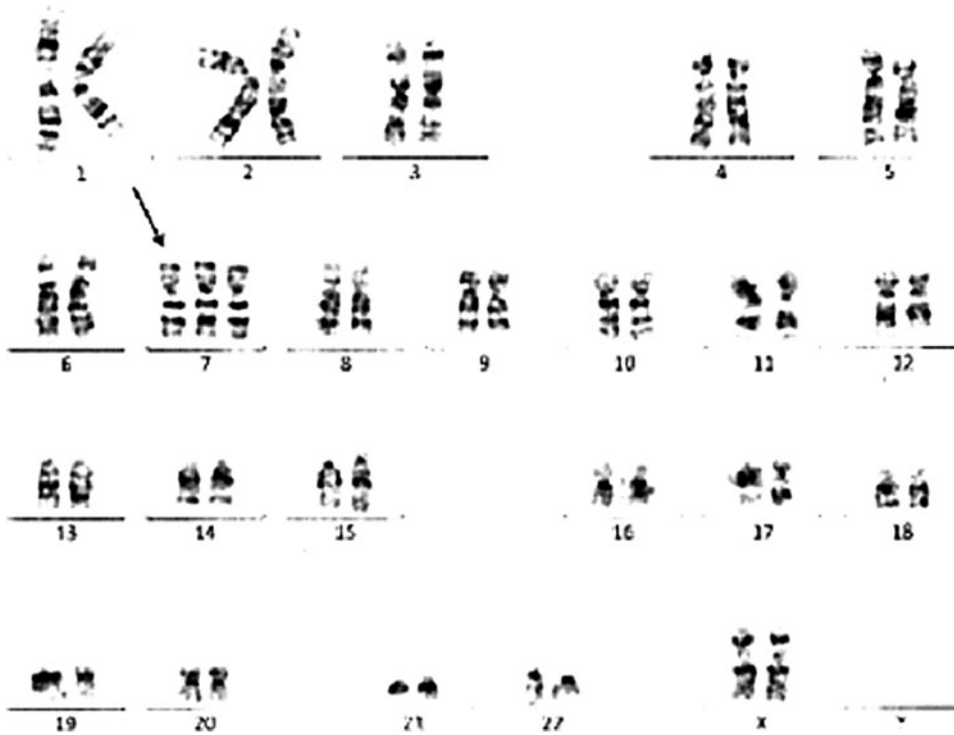
Luc<sup>+</sup>-transplanted cells could be tracked by imaging for a year or longer and were detected only at the transplanted right knee joint, but not at any other site (Fig. 6B). Luminescence was more intense in the AC-AC group than in the AC-SY group, with the SY-SY group displaying the lowest intensity. This pattern was established by 1 month after



**FIG. 3.** Results of array CGH in articular chondrocytes derived from total knee arthroplasty (TKA). (A) P2 versus P4, (B) P4 versus P2 swap with color further switched with articular chondrocytes derived from TKA, (C) P2 versus P6 in articular chondrocytes derived from TKA, and (D) P6 versus P2 swap with color further switched. (E) P2 versus P12 in articular chondrocytes derived from TKA, and (F) P12 versus P2 swap with color further switched. No deviation from the moving average baseline was observed; there was no evidence of copy number aberrations. (G–J) Samples with deviations from the baseline noted in chromosome 7. The *triangles* indicate deviations from the baseline, suggesting that chromosomal abnormalities may have occurred at those locations. (G) P2 versus P4, (H) P4 versus P2 swap with color further switched with articular chondrocytes derived from TKA, (I) P2 versus P6 in articular chondrocytes derived from TKA, and (J) P6 versus P2 swap with color further switched. (K) P2 versus P12 in articular chondrocytes derived from TKA, and (L) P12 versus P2 swap with color further switched. Deviation from the baseline was noted in a location corresponding to chromosome 7, showing an aberration in the copy number. The aberration in chromosome 7 in P2 versus P4 was also observed in P2 versus P6 and P2 versus P12. However, this was previously reported as a genomic change associated with osteoarthritis, which was the primary disease of the patients enrolled in the current study. (M) Analysis of cases of osteosarcoma by array CGH (positive control). Using the results from HAPMAP DNA NA19000 (Japanese male) versus osteosarcoma array CGH, aberrations in the copy numbers of genomic DNA, including those for cancer-related genes, are assessed visually as deviations from the moving average baseline.

transplantation and was stable for at least 21 months. We observed cartilage regeneration following cell sheet transplantation.<sup>17</sup> This finding demonstrates that covering physical osteochondral defects using the adhesive cell sheets can protect against catabolic factors present in the synovial fluid.

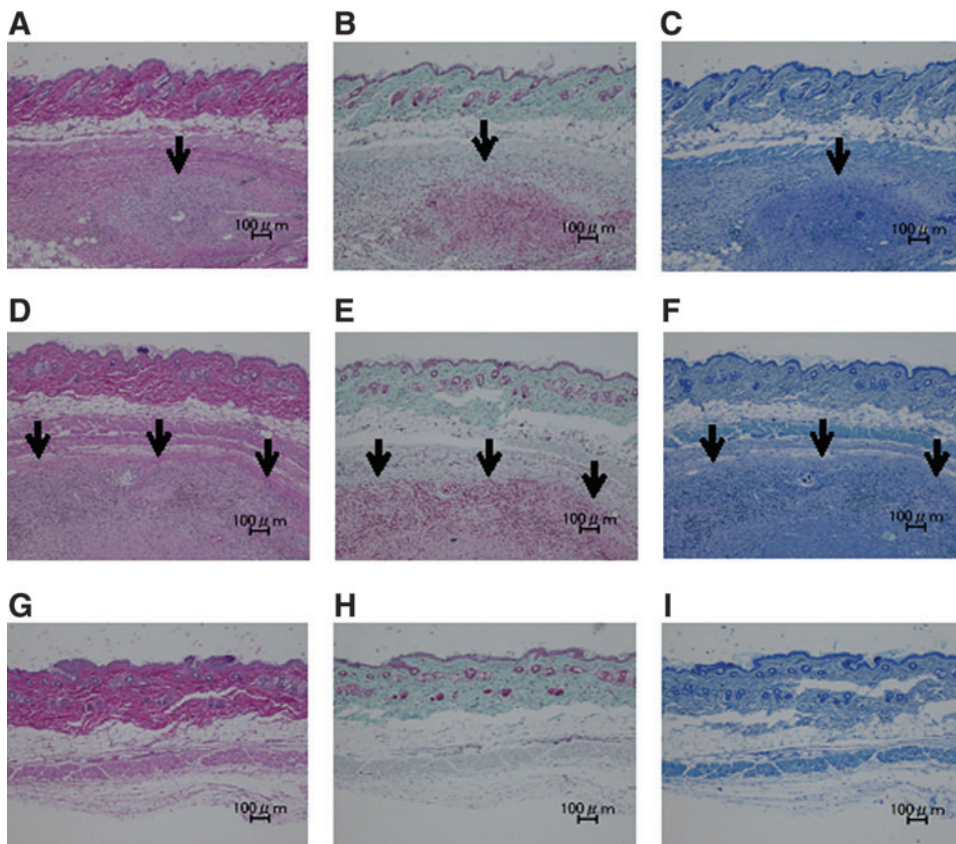
It also shows that growth factors from the cell sheets are continuously supplied, thereby promoting the recipient's self-healing. Thus, although cells derived from cell sheets seemed to disappear ~4 weeks after transplantation, engraftment in the injured cartilage does occur and actively



**FIG. 4.** Results of G-band staining. No chromosomal abnormalities other than that seen in chromosome 7 (arrow) were observed during continuous monitoring of the culture process.

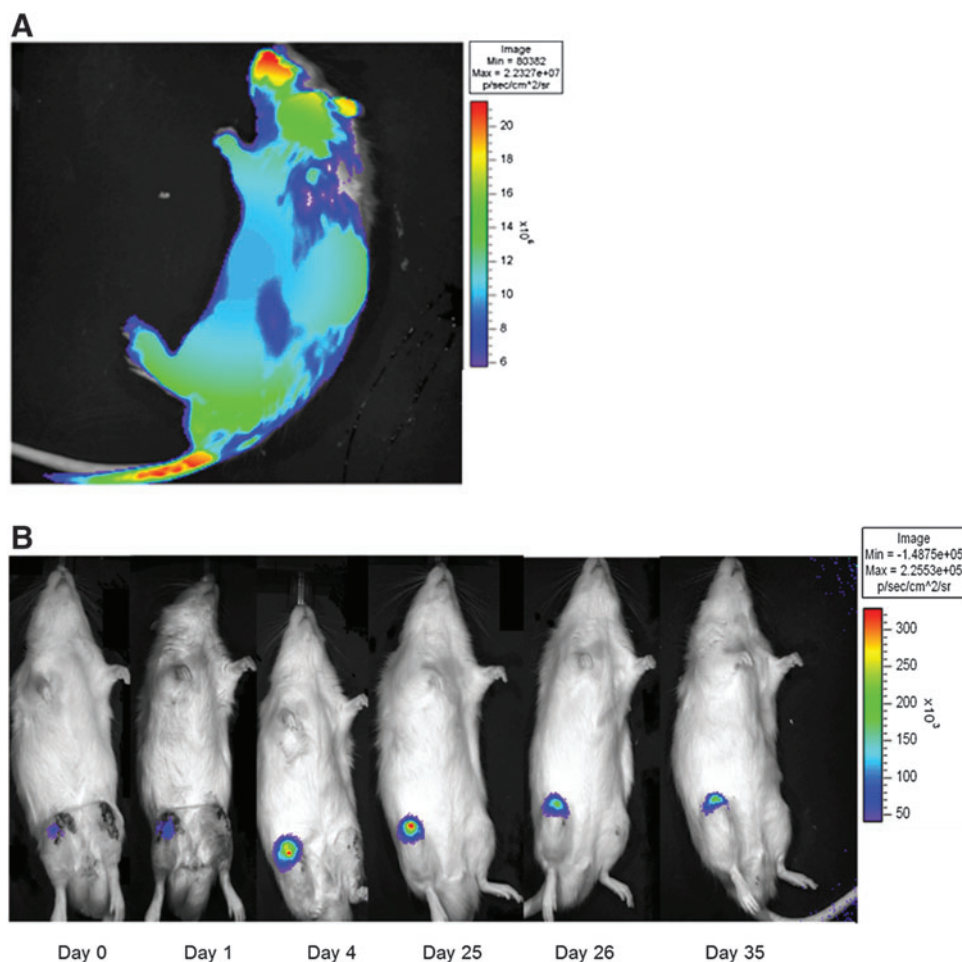
contributes to cartilage regeneration. The luminescence intensity is related to the cell count, but differences in the cellular environments of chondrocytes and synoviocytes also appeared to have an impact. The effect of combining chondrocytes and synoviocytes, which survived for a pro-

longed period in all three groups, did not translate to long-term persistence of up to 21 months.<sup>17</sup> With signals of luminescence observed over the long term, the involvement of  $\text{Luc}^+$  chondrocytes and  $\text{Luc}^+$  synoviocyte sheet-derived cells is also considered and is a subject for future research.



**FIG. 5.** Pathological findings at week 3 post-transplantation. Histopathological analysis of transplant site skin in individuals with confirmed persistence of cells. (A–C) After AC transplant: (A) hematoxylin and eosin (H&E) staining, (B) safranin O staining, and (C) toluidine blue staining. Lingering cells were confirmed to be chondrocytes. The arrows indicate the residual chondrocyte sheet. (D–F) AC-SY transplant: (D) H&E staining, (E) safranin O staining, and (F) toluidine blue staining. Subcutaneously transplanted cell sheets were stained; however, detection of the boundaries between cell sheets was challenging. (G–I) Control group: (G) H&E staining, (H) safranin O staining, and (I) toluidine blue staining.

**FIG. 6.** Assessment of the posttransplantation dynamics of the cell sheets. **(A)** Charge-coupled device (CCD) image of luciferase transgenic (TG) Lewis Rat: 16-week-old ROSA/Luciferase TG Lewis rats expressing luciferase in the whole body. The rats were sacrificed, and the synovial tissue was collected from the hip joint, after which the cartilage and synovial tissue was collected from the femoral head to prepare laminated cell sheets. **(B)** Changes over time in CCD images of the same individual after cell sheet transplantation. The *label* shows luminescence intensity, which is observed only in the right knee portion. Similar images were obtained for all individuals in the AC-AC group, AC-SY group, and SY-SY group.



## Discussion

The CGH method was published by Kallioniemi *et al.* in 1992<sup>22</sup> and utilizes FISH<sup>23</sup> for full-chromosome study of regions in which genomic DNA segments have been amplified or lost.

Array CGH can be implemented in fewer days and at a lower cost than conventional karyotype analysis and testing. This technique also facilitates identification of minute genomic aberrations, many of which offer clues for the elucidation of disease pathogenesis. It has also been applied to the diagnosis of already known chromosomal disorders and genomic alterations associated with cancer (Fig. 3M).

Array CGH that we used enables high-resolution detection of changes in copy number over the entire genome. The results of array CGH are also easier to interpret than the results of FISH or karyotype analysis, and the mutation calls can be made in an automated manner using the associated software, which allows the tests to be run even without the availability of a highly skilled technician. Chromosomal abnormalities evident on G-band staining were also detected on single analysis of the entire genome by array CGH. The use of both these tests in combination would enable analysis of both chromosomal abnormalities derived from the cell source and changes engendered during culturing. In this regard, the combined use of array CGH and G-band staining

may provide an extremely useful method for safety evaluation during transplantation.

The tests used in this study to rule out tumorigenicity were performed in accordance with WHO TRS 878,<sup>20</sup> which sets out internationally accepted guidelines.

WHO TRS 878 quantifies the methods for transplantation when nude mice are used to detect tumorigenic cells. The SCID mice used in this study are more sensitive than nude mice, in which some cell lineages derived from pancreatic cancer, breast cancer, glioma, lymphoma, and leukemia cells do not form tumors, and are therefore better at detecting very small numbers of tumorigenic cells.

In this study, we transplanted chondrocytes into SCID mice as an immunodeficient animal model and did not observe any abnormal proliferation of the transplanted cells or tumor cells. We infer that the cells were broken down at some stage within the body and, therefore, suggest that the final product may be safe to transplant.

The chondrocytes and synoviocytes used in this study were human somatic cells and human somatic stem cells, which are mesenchymal stem cells and thus terminally differentiated. From this perspective, the use of human stem cells may entail a lower risk of tumorigenicity than those of embryonic stem cells and induced pluripotent stem cells. Our experiments also confirmed that the cells did not acquire tumorigenicity in the artificial manipulation involved



in creating the final product, including isolation, enzyme treatment, frozen storage, and culture. Added to the fact that we used array CGH and G-band staining as *in vitro* tests to check for chromosomal abnormalities and genetic aberrations, the results of our *in vivo* experiment are highly significant.

The tests used in this study to rule out tumorigenicity have the disadvantage of requiring several weeks to several months to perform; despite this limitation, our results are extremely beneficial for future clinical applications.

TG rats were used to measure the remaining cell sheets so that we could continuously assess the dynamics of transplanted cell sheets without sacrificing the animals. The use of luciferase TG cells is now a standard approach in medical research, as exemplified by the tracking of transplanted and immunocompetent cells.<sup>24</sup> Bioluminescent imaging has also been used to track transplanted cells *in vivo*. Firefly luciferase, which catalyzes the production of light in a chemical reaction, is most frequently used for this purpose. The use of a high-sensitivity CCD to capture images of photons released by the reaction of the luminescent substrate, luciferin, has made it possible to noninvasively, repeatedly, and continuously track the same experimental animal for a longer period without the need for animal sacrifice.

Bioluminescence refers to a phenomenon whereby excited molecules are produced *in vivo* and their energy causes emission of light. Photon generation using this method is more efficient than other forms of chemiluminescent reaction, and the process is called “cold light,” as it is not associated with concomitant generation of heat. Luciferase catalyzes a reaction between luminescence substrate luciferin, ATP, and Mg<sup>2+</sup> ions, leading to the production of light.<sup>25,26</sup>

One limitation of this study is that it is impossible to directly study the posttransplantation dynamics of the cell sheet in humans. Furthermore, in studies that use array CGH and G-band staining methods for the analysis of overcultured cells or tumorigenesis evaluation, more than 1.5 g of cartilage and at least 1.0 g synovial membrane must be collected, and when sufficient amounts cannot be obtained, these safety evaluations may become quite difficult.

After having conducted these investigations and confirmed the safety of chondrocyte sheets we obtained approval from the university Ethics Committee to conduct clinical research. A plan to conduct human stem cell clinical research was then submitted to the MHLW. In accordance with the “Guidelines on Clinical Research Using Human Stem Cells,” an application was filed on March 3, 2011 with MHLW and a notification from the Ministry “On the plan for implementing clinical research with human stem cells” (October 3, 2011, 1003 No. 3 issued by MHLW) approved implementation of clinical research in the hospital of the Tokai University School of Medicine. We have implanted layered chondrocyte sheets in eight patients thus far.

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### Disclosure Statement

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