Tissue engineered cartilage using human articular chondrocytes immortalized by HPV-16 E6 and E7 genes

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Abstract: Chondrocytes are useful as a cell culture system for studying arthritic degeneration in tissue engineered cartilage. However, primary chondrocytes have short in vitro lifespan and rapid shift of collagen phenotype. In this study, we used a high dosage of retroviral vector LXSN16E6E7 to transduce human primary chondrocytes and obtained an actively proliferating cell line, designated hPi, which expresses HPV-16 E6/E7 mRNA in early passages. Parental primary chondrocytes cease to grow after five passages, whereas hPi could be propagated beyond 100 passages without requiring additional cell elements in defined medium. After 48 passages, hPi can also give many profiles similar to those of parental primary chondrocyte, including type II collagen in mRNA and protein level, aggrecan in mRNA level, lacunae in type I collagen matrices, and morphology with GAG-specific Alcian blue staining. hPi has shown neoplastic transformation, as examined by NOD-SCID mice tumorgenicity assays for 3 months. Our results indicated that human primary chondrocytes could be immortalized by transduction with HPV-16 *E6/E7*, preserving stable cartilage-specific differentiation markers. The established chondrocyte cell line could provide a novel model to engineer cartilage in vitro and in vivo for cartilage repair research and clinical application. © 2005 Wiley Periodicals, Inc. J Biomed Mater Res 76A: 512–520, 2006

Key words: articular cartilage; extracellular matrix; cell differentiation; HPV-16 E6/E7; immortalization

INTRODUCTION

Hyaline cartilage, an avascular but deformable tissue, covers articular surface and prevents articular attrition from direct friction between condyles.[1] However, hyaline cartilage has little capacity for self-repair.[2] Thus, injured joints are susceptible to continual arthritic degeneration, resulting in arthritis. Current therapies for cartilage regeneration include placement of periosteum, perichondrium, carbon plugs, and subchondral drilling, in which recovery rates vary and have been seriously hindered by chemical use.[3,4]

Three-dimensional scaffold tissue engineered cartilage was later introduced to improve treatment options for patients with cartilage injuries.[5–8] Autologous chondrocytes for engineering cartilage have the advantage of reducing the risk of immunological rejection and transmittable diseases. However, decreased multiplication and shortage of human chondorcytes limit autologous chondrocytes transplantation.[9] Chondrocytes can be isolated from enzyme-digested cartilage and then proliferated in monolayer culture.[10] Previous studies have shown the limitations of monolayer culture mainly due to the short life span of chondrocytes and the rapid shift of their collagen phenotype, resulting in a decrease of type II collagen, proteoglycan, and an increase of type I collagen.[11,12] Many attempts to preserve chondrocyte-specific phenotype in monolayer culture have been reported, including suspension on or within agarose,[13] three-dimensional culture model in alginate bead,[14] serum free define medium,[15,16] polymers such as HYAFF®, PGLA, PLLA, PGA,[17]and alginate,[18,19] and collagen.[11,20–22]

One of the most challenging approaches to preserving chondrocyte-specific phenotype in mono-

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layer culture is to establish cell lines displaying both infinite proliferation capacity and stable phenotype, for example the immortalized chondrocyte model, to provide an unlimited resource of chondrocytes for engineering cartilage. Various immortalized chondrocyte cell lines from animal and human origin have been established through viral transduction with a selected single or multiple oncogenes[20,23–26]; however, the conservation of all differentiated cell functions and modulation is still equivocal and conflicting.

SV 40 large T antigen (SV40-Tag) was reported to immortalize chondrocytes, at either sensitive[20] or permissive temperature.[11] However, SV40-Tag inactivates *RB* and *p53* genes, resulting in the loss of normal RB protein function and the induction of rapid degradation. Recently, human papillomavirus (HPV), containing two early functional genes *E6* and *E7*, was also reported to immortalize primary chondrocytes by lipofection.[15] However, the immortalized chondrocytes could re-express Col II-A1 mRNA only under defined medium with additional elements such as Nutridoma, or in hyaluranon-based biomaterial culture. In addition, lipofection showed limitations, including transient gene expression, low transduction levels, and dose-dependent toxicity.[27]

A highly efficient model of immortalized chondrocytes is still in demand for both basic and clinical application. Our previous study has indicated that human mesenchymal stem cells (MSCs) could be immortalized with HPV-16 E6/E7 retroviral vector, preserving the potential to differentiate and proliferate, without neoplastic transformation.[28] In this study, the HPV-16 E6/E7 retroviral vector was also employed to immortalize human chondrocytes. The results showed that immortalized human articular chondrocytes still preserve differentiation characterizations without neoplastic transformation. Cells were also successfully applied in tissue engineered cartilage with collagen scaffold *in vitro* and *in vivo*. These results indicated that this cell line could be useful in engineered cartilage applications.

MATERIALS AND METHODS

Isolation of adult human chondrocytes

Articular cartilage was harvested aseptically from a 40-year-old male donor with joint injury but without history of arthritic or related pathologies. The volunteer gave informed consent for the use of the articular cartilage, as required by the institutional review board of Taipei Municipal Chung-Hsin Hospital, Taipei. The cartilage was minced into pieces in HBSS (Gibco BRL, Grand Island, NY) with antibiotics. Chondrocytes were then isolated from these slices with enzyme solution (0.4% hyaluranidase, 0.4% collagenase, and 0.25% trypsin; Sigma, St. Louis, MO) for 5 h at 37°C. The cell suspension in enzyme solution was filtered through a 40-μm nylon mesh. The filtered cells were centrifuged at 1800 rpm for 10 min, resuspended in Dulbecco's modified Eagle's medium (DMEM/F-12) (Gibco BRL) with 10% FBS. Chondrocytes were seeded into 10-cm tissue culture dishes and incubated at 37°C, 5% CO_2 before subsequent experiments.

Transfection with HPV-16 *E6/E7* gene

The retroviral vector transduction was conducted as previously described.[28,29] In brief, the HPV-16 E6/E7 retroviral vector (LXSN16E6E7)[28,30] produced by PA317 cell line, a gift from Dr. D. A. Galloway, was expanded in MEM medium. The ectotropic packaging cell line Psi-2 was transfected with plasmid DNA containing the HPV-16 E6 and E7 genes. Virus produced by the Psi-2 cells was used to infect the amphotropic packaging cell line PA317, from which virus containing *E6/E7* genes was used to infect primary cell culture. The isolated primary chondrocytes were recovered by trypsinization, seeded on 6-welled plates at a density of 10^5 cells per well, and fed with culture medium containing 4 μg/mL polybrene. Irradiated PA317 cells were added to the dish for 24 h for seeding. The cells were then passaged at 1:3 on the next day. Three weeks after infection, when obvious clones of actively dividing cells developed, these cells were harvested. The infected cells, hPi 0 cells, in passage 0 (P0), were fed with the same medium of primary culture twice a week, and were passaged at 1:5 ratio when the cells appeared subconfluently.

Proliferation assay

Cell proliferation was determined by cell proliferation reagent tetrazolium salt WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate; ROCHE, Mannheim, Germany) assay. The cleavage of WST-1 to formazan by metabolically active cells was quantified by scanning the plate at 450 and 690 nm. Aliquots of 200 μL of cell suspension (10^5 cells/mL) were seeded into each well of a 96-welled plate. WST-1 reagent was added into each well at days 1, 3, 5, and 7. OD values (OD 450–690 nm) were analyzed 4 h after WST-1 reaction by using Multiskan PC (Thermo Labsystem) and plotted against time.

The population doubling time was calculated using the following function, as previously described[31]:

$$\text{Doubling Time} = (T - T_0) \log 2/(\log N - \log N_0)$$

where $T - T_0$ indicates the length of time between two measurements and N_0 and N denote the cell number at two points of measurement. Three independent sets of experiments performed in triplicate were evaluated.

TABLE I
Primers Utilized for RT-PCR Amplification

Gene	Primer Sequence (5′–3′)	Annealing Temperature (°C)	PCR product size (bp)
HPV-16 E6/E7	ATG CAT AGT ATA TAG AGA TGG GAA T CTG CAG GAT CAG CCA TGG TAG A	55	628
COL2A1	CAC GCA GAA GTT CAC CAA GAA CCT TGC TCC AGG GCC AGC	53	501
COL1A1	AGC GCT GGT TTC GAC TTC AGC TTC C CAT CGG CAG GGT CGG AGC CCT	58	466
AGGRECAN	TGA GGA GGG CTG GAA CAA GTA CC GGA GGT GGT AAT TGC AGG GAA CA	64	350
GAPDH	GCT CTC CAG AAC ATC ATC CCT GCC CGT TGT CAT ACC AGG AAA TGA GCT T	55	346

Tumorigenicity assay

The animal experiment protocol was approved by the Institutional Animal Care and Use Committee of Taipei Medical University. The immortalized cells were trypsinized from confluent monolayer and suspended with 8×10^6 cells/mL (collected at different passages). The suspended mixture was injected subcutaneously into the dorsa of SCID/NOD mice (National Taiwan University Laboratory Animal Center, Taipei, Taiwan). Mice were kept in sterilized pathogen-free cages, and observed for tumor formation daily over 3 months. HeLa cells were injected as positive control, while nontransfected chondrocytes were used as negative control.

Reverse transcriptase polymerase chain reaction

Total RNA harvested from subconfluent monolayer cultures (~10^6 cells) was extracted using TRIzol Reagent (Invitrogen Life Technologies, Carlsbad, CA). Extracted RNA was dissolved in sterilized ddH_2O and stored at −80°C. Reverse transcription was performed with SuperScriptTM III (Invitrogen Life Technologies) and Oligo d(T)15 primer. Four micrograms of RNA was added into a final solution volume of 21 μL containing 10 mM dNTP mix, 10× RT buffer, 25 mM $MgCl_2$, 0.1M DTT, RNase Inhibitor, and RNase H. Six micrograms of RT product was used in PCR amplification in a final volume of 50 μL containing 2.5 mM dNTP, 25 mM $MgCl_2$, upstream/downstream primers (see Table I) and Taq DNA polymerase (Invitrogen Life Technologies). Following an initial denaturation at 95°C for 5 min, the DNA was amplified in the Touchgene Gradient (Techne, Cambridge, UK) using 35 cycles of 1 min at 94°C for denaturation and extension at 72°C for 1 min. This was followed by a final extension at 72°C for 5 min. Annealing temperature was different for specific genes (See Table I). PCRs were analyzed by 1% agarose gel (Agarose I, Amersco, OH) and visualized with EtBr staining. Images were analyzed using FloGel-I (Fluorescent Gel Image System, Top BioCom, Taiwan). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control.

Western blotting

Cells were trypsinized and dissolved in RIPA buffer (50 mM Tris pH 7.4, 150 mM NaCl, 0.5% DOC, 1% NP-40, 0.1% SDS), and then centrifuged for 15 min at 12,000 rpm at 4°C. The upper fluid, containing total protein, was extracted. The extracted protein, mixed with 6× sample buffer, was denatured for 5 min at 95°C and loaded on a 7% SDS-PADE gel. The membrane was blocked overnight in blocking-buffer [5% milk powder (w/v) in PBS-T (1× PBS, 0.1% Tween 20, pH 7.5)] and incubated with anti-collagen-type II antibody (type II collagen [Ab-1], Oncogene, CA) for 1 h, followed by PBS-T wash. Secondary antibody (peroxidase-conjugated AffinPure goat anti-mouse IgG, Jackson ImmunoResearch, West Grove, PA) was added (diluted 1:10,000 in PBS-T) for 1 h. Bands were made visible on film (Hyperfilm ECL, Amersham Pharmacia, Braunschweig, Germany) using the ECL plus-Kit (Amersham Pharmacia).

Alcian blue staining

Nontransfected chondrocytes and hPi cells were seeded at a concentration of 1.5×10^5 cells on Lab-Tak chamber slides (Nalge Nunc International, Rochester, NY) with DMEM/F-12 containing 10% FBS and cultured at 37°C, 5% CO2 for 3 days. Cells were then washed twice with PBS and fixed in 10% formalin for 1 h at room temperature. After washing with distilled water, cells were stained in 1% Alcian blue 8GX (Sigma) in 3% glacial acetic acid (pH 2.5) for 30 min at room temperature. They were then dehydrated through a series of alcohol concentrations and cleared in xylene. The slides were mounted in aqueous mounting medium (Faramount, DAKO, Carpinteria, CA) and observed under a microscope.

Type I collagen purification

Rat type I collagen was extracted and purified as described previously.[5] Collagen type was determined using a

5% SDS-PAGE, and collagen concentration was analyzed using the Lowry method. Collagen was dissolved in AcOH and diluted to a 2× stock solution of 2 mg/mL for three-dimensional cultures.

Three-dimensional cell-matrix construct in vitro and in vivo

Three-dimensional cultures were employed using type I collagen matrix as previously described.[5] Nontransfected chondrocytes and hPi cells were respectively collected in a concentration of 1.6×10^7 cells/mL, and mixed with an equal volume of type I collagen stock solution to give a final concentration of 8×10^6 cells/mL and 1 mg/mL type I collagen. Cell-Matrix construct was subcutaneously injected into the dorsa of three 5-week adult female nude mice. The mice were killed, and the injected samples were sliced for histology evaluation 1 month after cell-matrix implantation.

The cell-matrix construct was cultured at 37°C in a T-25 flask for 1 month, and was evaluated by histology.

Histology

The cell-matrix constructs, which were cultured in media and implanted in mice, were removed and fixed in 10% formalin for 24 h at 4°C, then embedded in paraffin and cross-sectioned (5–10-μm thick). Serial sections were stained with hematoxylin–eosin (H&E).

RESULTS

Detection of HPV-16 E6/E7 mRNA

To transduce human chondrocytes within three in vitro passages of primary cultures, we used multiplicity of infection of more than 10, on the basis of replication-defective amphotropic retroviral vector LXSN16E6E7[29,30] infectious units for NIH3T3 cells,[28] and total mRNA was extracted from cultured cells and detected for the presence of HPV-16 E6/E7 mRNA. Transfected chondrocytes, designated as hPi cells,

Figure 1. Expression of HPV-16 E6/E7 mRNA. Total mRNA was extracted and detected for the presence of HPV-16 *E6/E7* from immortalized human articular chondrocytes at passage 0 (Lane 1, hPi 0); at passage 4 (Lane 2, hPi 4); at passage 5 (Lane 3, hPi 5); at passage 48 (Lane 4, hPi 48). PA317 cells containing retrovirus vector as positive control (Lane 5). Lane 6, nontransfected chondrocytes shown as negative control.

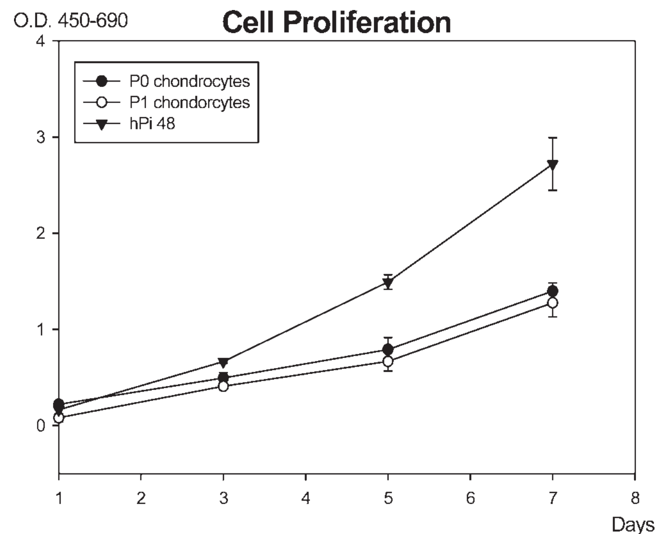

Figure 2. Population doubling time of transfected and nontransfected chondrocytes. Cell Proliferation capacity of nontransfected chondrocytes at passage 0 (●), passage 1 (○), and immortalized chondrocyte at passage 48 (▼) were determined. Cells were seeded into 96-welled plate at a density of 2000 cells/well and maintained in DMEM/F-12 medium containing 10% FBS. All results shown are means and SEM of triplicate determinations.

were cultured in 10% FBS containing DMEM/F-12 medium, and hPi cells at passage 0 were denoted hPi 0. PA317 cells containing the retrovirus vector were used as positive control in RT-PCR assay, while nontransfected chondrocytes constitutively negative with HPV-16 *E6/E7* genes were used as negative control. The RT-PCR products of *E6/E7* in PA317, hPi 0 and hPi 4 cells showed a distinct band at 628 bp; in contrast, the band was not observed in hPi 5, hPi 48, and nontransfected chondrocytes (Fig. 1).

hPi cells increase proliferation without inducing neoplastic formation

Nontransfected chondrocytes at passage 0 and 1 (designated as P0 and P1) were modestly proliferative, while hPi cells appeared highly proliferative (Fig. 2). Population doubling times of P0 chondrocytes, P1 chondrocytes, and hPi 48 were 27.089 ± 1.105 h, 32.976 ± 2.316 h, and 17.901 ± 2.151 h respectively. The parental chondrocytes used in our study entered the crisis of replication at about the fifth passage, when subcultivation became unavailable, while the E6/E7 transduced hPi culture continued to proliferate beyond 100 passages (data not shown).

To determine whether the oncogene-transduced chondrocytes were tumoriginic, 8×10^6 cells/mL of human articular chondrocytes, hPi 48, and HeLa cells (a malignant cancer cell line) were injected subcutaneously into the dorsa of SCID/NOD mice, respectively.

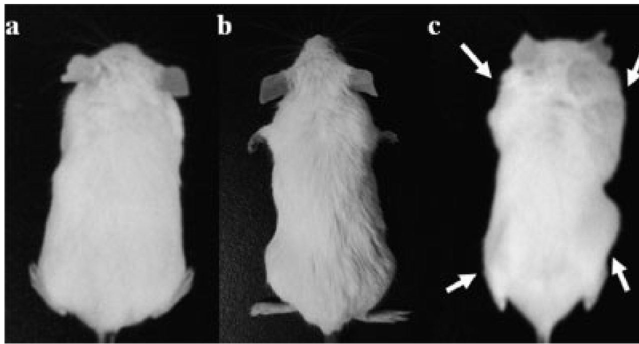

Figure 3. Tumoriginecity assay. NOD/SCID mice were subcutaneously injected with nontransfected human articular chondrocytes (a), immortalized chondrocyte at passage 48 (b), and HeLa cells (c) for 3 months. A mass (white arrow) was found on the dorsa of mice with HeLa cells injection only 3 days after injection (c).

No tumor formation was found after 3 months of observation in mice injected with nontransfected chondrocytes [Fig. 3(a)] and hPi 48 cells [Fig. 3(b)]. In contrast, a mass was found on the dorsa of mice 3 days after injection with HeLa cells [Fig. 3(c)]. Results suggest that hPi cells, like the nontransfected chondrocytes, are nontumorigenic.

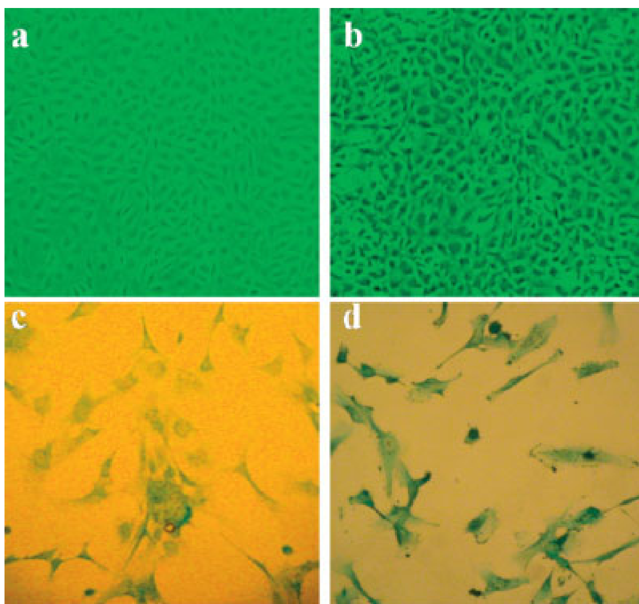

Figure 4. Morphology of transfected and nontransfected human articular chondrocytes. Parental primary chondrocyte (a) and immortalized human articular chondrocytes at passage 48 (hPi 48) (b) under phase-contrast microscopy (×100). Nontransfected human articular chondrocytes and hPi cells both express a differential, rounded morphology on monolayer culture. Alcian blue staining of parental primary chondrocytes (c) and hPi 48 cells (d) on monolayer cultures. hPi cells could maintain a uniform polygonal morphology as primary cells through long term in vitro cultures. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

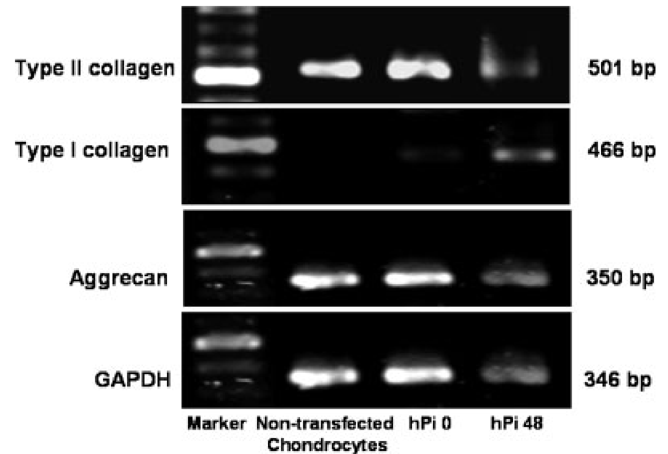

Figure 5. RT-PCR analyses of cartilage-specific gene expressions, including type II collagen, type I collagen and aggrecan. Lane 1, Marker, Bio-100TM DNA Ladder (100 bp DNA Ladder). Lane 2, nontransfected chondrocytes. Lane 3, hPi 0, immortalized human articular chondrocytes at passage 0 while transduced with HPV-16 E6/E7 genes. Lane 4, hPi 48, immortalized human articular chondrocytes at passage 48.

Morphology and Alcian blue staining

Nontransfected chondrocytes [Fig. 4(a)] are characterized by a phenotype of round-polygonal morphology when cultured at 90% confluent phase. hPi 48 cells preserved primary chondrocytes morphology after 48 passages in monolayer [Fig. 4(b)].

GAG, a marker of differentiated chondrocytes, was evaluated by selective staining with Alcian blue.[32] An abundant amount of GAG expression was noted in both primary chondrocytes [Fig. 4(c)] and hPi 48 [Fig. 4(d)] cells stained with Alcian blue. hPi 48 cells express GAG similar to nontransfected chondrocytes.

Cartilage-specific gene expression

Col II and aggrecan have been reported as major matrix proteins produced by freshly dissociated chondrocytes from cartilage, while Col I is considered as dedifferentiation marker protein.[10,33] hPi 0 cells clearly expressed Col II-A1 and aggrecan mRNAs after being transduced with retrovirus plasmid, despite a minimal expression of Col I-AI mRNA being observed (Fig. 5). In hPi 48 cells, Col II-A1 and aggregan mRNAs were preserved. Col I-A1 mRNA showed an increase in comparison with hPi 0 cells under regular cultured conditions. Cartilage-specific gene expressions of nontransfected chndrocyte were also detectable in hPi cells after 48 passages in regular cultivation.

Western blotting analysis

To determine whether immortalized human articular chondrocytes express type II collagen under regular cell culture, western blotting analysis was conducted. The results demonstrated that type II collagen, the major differentiated marker of articular chondrocytes, was apparently similar in protein level to that of parental primary chondrocytes over several passages even without any additional cell elements for culture (Fig. 6).

Type I collagen characterization and tissue engineered cartilage

To obtain construction scaffold used in tissue engineered cartilage, type I collagen was extracted from rat tail tendon by dissolving with HCl. Extracted proteins were loaded onto 7% SDS-PAGE and stained with Coomassie blue for 1 h and bands were detected [Fig. 7(A)]. Brilliant bands were observed and determined as α1, α2, β, and γ helix. The indicated bands are characteristics of type I collagen, thus demonstrating the purified collagen.

Purified type I collagen was then used to construct three-dimensional chondrocyte-matrix in vitro or implanted into nude mice in vivo [Fig. 7(B)]. After 3–4 weeks of in vitro cultivation, samples were fixed and histologically observed. Both nontransfected chondrocyte- and hPi 48 cell-type I collagen matrices showed shinning, and elastic cartilagelike appearance [Fig. 7(C(a,b))]. hPi 48-type I collagen matrices, which were implanted in vivo into nude mice dorsum for 4 weeks, showed a white firm mass encapsulated with fibrous tissue [Fig. 7(C(c))].

The histology from H&E staining showed that cells with round morphology were surrounded by cartilage-specific lacunae in nontransfected human articular chondrocyte-type I collagen matrices [Fig. 7(C(d))]. hPi cell-type I collagen matrices also appeared to have rounded cell morphology in vitro and in vivo, except that less lacunae were observed compared with nontransfected chondrocyte-collagen matrices [Fig. 7(C(e,f))].

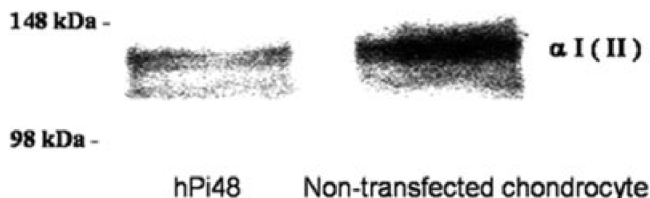

Figure 6. Western blotting for type II collagen from monolayer cultures of immortalized and nontransfected chondrocytes. Denatured proteins were electrophoresed on SDS-PAGE (7% w/v). hPi 48, immortalized human articular chondrocytes at passage 48. Nontransfected chondrocytes at passage 0.

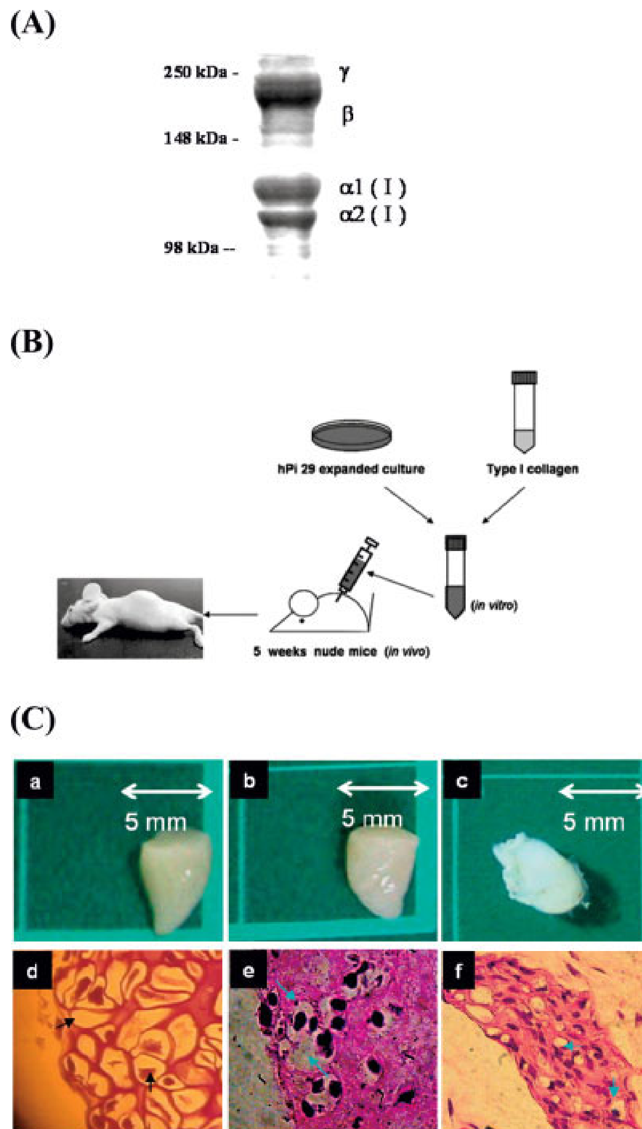

Figure 7. Type I collagen characterization and tissue engineered cartilage in vitro and in vivo. A: Sample of type I collagen extracted from RTT (rat tail tendon) were electrophoresed on 7% PAGE and monitored by Coomassie blue staining. Molecular mass markers were shown as indicated. B: Schematic diagram of in vitro three-dimensional culture and in vivo in animal model. C: Samples were fixed and observed from three-dimensional cultures of nontransfected human articular chonderocytes (a), hPi 48 (b), in type I collagen scaffold, and hPi 29 mixed with type I collagen scaffold was injected onto nude mice dorsum (c). Histology (H&E staining, ×1000) of three-dimensional cultures of nontransfected human articular chonderocytes (d); hPi 48 (e); and hPi 29 with scaffold injected into nude mice (f). Arrows indicate lacunae formed by proteoglycans. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

DISCUSSION

In this study, we applied an amphotropic retroviral vector, LXSN16E6E7, for transfer of HPV-16 *E6/E7* genes into freshly isolated human articular chondro-

cytes. This experimental approach has been previously proven successful for immortalization of human mammary epithelial cells,[34] human bone marrow stromal cells,[35] human embryonic fibroblasts,[36] and human bronchial epithelial cells.[37] LXSN16E6E7 has also been used to transduce various cells of human normal tissues in our laboratory, including rodent inner ear epithelial cells[38] and human MSCs,[28] so as to establish cell lines possessing their innate physiological properties. Transfection with HPV-16 E6/E7 DNA, E6 and E7 oncoproteins exhibited a cooperative effect in cell culture, thus maintaining long-term immortalization.[28,39,40] Transformation and immortalization functions of HPV-16 were located in the E6 and E7 regions of the viral genome. E6 proteins bind and inactivate p53 protein, while E7 proteins interact with Rb and other cellular proteins, thus inactivating these tumor suppressor genes.[40] This causes deregulation of cell cycle with loss of control on crucial events, such as DNA replication, DNA repair, and apoptosis.[41] After transfection of HPV-16 *E6/E7* into chondrocytes, the results showed that hPi cells have higher proliferation abilities than do primary chondrocytes (Fig. 3). hPi cells have been successfully cultured beyond 100 passages.

Human articular chondrocytes immortalized with SV40-Tag have been reported in several studies, with varying degrees of maintaining the differentiated phenotype.[9,20] These studies show either complete loss or a marked inhibition of cartilage-specific type II collagen and proteoglycan in immortalized chondrocytes,[24] in which only few cell lines of T-Ag-immortalized chondrocytes still express these markers.[9,18]

HPV-16 E6/E7 DNA was reported to immortalize human articular chondrocytes via cationic liposome-mediated transfection (lipofection).[15] However, dedifferentiation of the cell line in phenotype in vitro after several passages was observed. This occurrence is similar to that of mesenchymal cells.[42] Moreover, several features, such as transient gene expression, strong immune responses, and high toxicity, cause cell death and limit the clinical use of cationic liposomes.[27] To preserve the chondrocyte phenotype in vitro, we applied LXSN16E6E7 in retroviral system to immortalize primary chondrocytes, and found that the E6/E7 immortalized chondrocyte line expresses cartilage-specific type II collagen and GAG with greater stability (Fig. 5). The results showed that after five passages of immortalized chondrocyte culture, the *E6/E7* gene was not detected on the gel (Fig. 1) and might be pumped out from the viral genome, supporting the findings of Macpherson,[43] who demonstrated that virus-transformed cells might apparently lose their viral genes, thus accounting for the low immungenicity level and advantages on the therapeutic goals.[27]

Several human cell lines have been established in serum-supplemented medium,[44] in which additional cell elements were required to maintain or re-express cell terminal differentiation. Goldring et al. reported that stable proliferating chondrocyte cell lines immortalized with SV40-Tag could preserve expression of type II collagen mRNA only after one to several days of culture in serum-free defined medium supplemented with 1% Nutridoma-SP (an insulin-containing serum substitute) and 50 μg/mL ascorbic acid (a cofactor for collagen synthesis).[18,45] Results in this research showed that after 48 passages of monolayer cultures, chondrocyte-specific type II collagen and GAG were expressed without the addition of supplemented reagents. These evidences demonstrate that hPi cells maintain differentiation capacity compared to terminally differentiated human chondrocytes, thus could be used for future studies in rheumatoid-related researches or cartilage repair models.[15,20,23,46]

Three-dimensional cultures were applied in several recent studies for chondrocyte growth and differentiation mimic cartilage tissue.[16,47] The reconstituted type I collagen-matrix in three-dimensional culture made in this study showed a cytological agglomerate (Fig. 7), which was similar to the one in previous studies,[48,49] indicating that chondrocytes grown in type I collagen show a higher proliferation capacity compared with other scaffolds.[50,51] Both primary human chondrocytes and hPi cells in type I collagen matrix showed a potential for chondrogenic differentiation [Fig. 7(C(d,e))]. Lacunae surrounding hPi 48 showed that hPi cells, similar to nontransfected chondrocytes, secrete proteoglycans. Thus, type I collagen is a candidate matrix for maintaining chondrocyte differentiation. Immortalized cells transplanted and cultured in vivo for 3 weeks are more rigid and elastic than three-dimensional cultures in vitro [Fig. 7(C(f))]. Lacunae morphology varied from round to oval shape, containing single or double cells. The nuclei of the cells were round with minimal pleomorphism. Chondrocytes cultured within in vivo scaffolds showed a differentiated phenotype. These results indicate that type I collagen matrix-chondrocyte construct could further be implanted in vivo for maintaining chondrocyte differentiation. hPi cell-type I collagen matrices appeared round morphology of cells both in vitro and in vivo, except less lacunae compared to nontransfected chondrocyte-collagen matrices. From RT-PCR results, we found that cartilage-specific marker collagen type II was still expressed in hPi 48 cells (Fig. 5). It might indicate that the cells underwent dedifferentiated state in the later passages so that they expressed less lacunae in type I collagen matrices compared to nontransfected chondrocytes.

hPi 48 showed a higher rate of proliferation compared with primary chondrocytes (Fig. 2). However, no neoplastic transformation was observed by tumorigenicity test in NOD/SCID mice, even in the prolonged cultivation of immortalized chondrocytes (Fig.

3). Thus, the HPV-16 E6/E7 immortalized chondrocytes can be exploited as an unlimited cell resource for cartilage engineering.

The DNA histogram obtained by cytofluorometry with P.I. staining showed that most chondrocytes have diploid DNA content (data not shown), and that the chondrocytes are in their G0 phase, while diseased chondrocytes possess DNA content between 2n and 4n, which is comparable to results reported previously.[52] This phenomenon indicates that DNA content of hPi cells resembles that of normal primary chondrocytes.

In summary, HPV-16 *E6/E7* early genes are able to immortalize normal human adult articular chondrocytes, allowing hPi cells to preserve a stable chondrocyte phenotype. This established chondrocyte cell line provides a novel approach to engineer cartilage in vitro and aid for cartilage repair.

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