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Human articular chondrocytes immortalized by HPV-16 E6 and E7 genes: Maintenance of differentiated phenotype under defined culture conditions

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Summary

Objective: To establish an immortalized normal human articular chondrocyte line which could be useful for a better understanding of cell molecular mechanisms relevant for the development of new therapeutic approaches in rheumatic diseases.

Design: Chondrocytes from human adult articular healthy cartilage were transfected in primary culture with a plasmid containing two human papilloma virus type 16 (HPV-16) early function genes: E6 and E7, using the highly efficient cationic liposome-mediated (lipofection) procedure. The transfection was verified by reverse transcriptase-polymerase chain reaction analysis of E7 mRNA and by immunofluorence localization of the E7 protein in the cell cytoplasm. The established chondrocyte cell line was examined in monolayer and in two culture conditions that were described to re-induce differentiated characteristics: culturing in a serum-free defined medium supplemented with an insulin-containing serum substitute and seeding on a hyaluronan-based non-woven structured biomaterial. The expression of markers characteristic of cartilage was shown in the mRNA by reverse transcriptase-polymerase chain reaction. Immunohistological staining and Western blotting analysis were performed to evaluate type II collagen synthesis. Proteoglycans deposition was detected by Alcian Blue staining. A Field Emission In Lens Scanning Microscopy was used to look at the morphology of the immortalized cells at very high magnification.

Results: Normal human articular chondrocytes were efficiently transfected leading to the establishment of an immortalized cell line as confirmed by HPV-16 E7 mRNA and protein detection. These cells were able to re-express type II collagen both at mRNA and protein levels under the two defined cultured conditions we used, still maintaining type I collagen expression. Collagen IX mRNA was present only in early primary culture while collagen type X and aggrecan transcripts were always detected. Alcian Blue staining showed a proteoglycan-rich matrix production. The ultrastructural analysis of the immortalized cells revealed that their morphology strictly resembled that of normal chondrocytes.

Conclusions: The cell line that we obtained may be a useful tool for increasing our knowledge of the genetic and biochemical events involved in the processes of cartilage growth and differentiation. Moreover, it appears to be a suitable model for pharmacological and toxicological studies related to rheumatic diseases relevant to humans. © 2002 OsteoArthritis Research Society. Published by Elsevier Science Ltd. All rights reserved.

Key words: Human chondrocytes, Immortalization, HPV-16 E6-E7, Collagen II.

Introduction

Hyaline articular cartilage is a highly specialized tissue derived from mesenchyme during embryonic development[1]. Chondrocytes, the only cell type of mature cartilage, secrete and deposit around themselves a characteristic extracellular matrix composed primarily of collagens, proteoglycans and other non-collagenous proteins[2]. The collagen architecture contributes to the tensile strength and stiffness of cartilage while proteoglycans provide resistance to compression. Type II collagen comprises about 90–95% of total hyaline cartilage collagens and for that reason is generally used as a differentiation marker for chondrocytes[3]. Smaller amounts of types V, VI, IX, XI are also present[4,5], together with type X collagen which has been demonstrated to be restricted not only to the hypertrophic chondrocytes within the zone of calcified cartilage[6]. The predominant proteoglycan of articular cartilage is a hydrodynamically large, aggregating molecule termed aggrecan, representing about 90% of the proteoglycans in the tissue[7].

Chondrocytes can be easily isolated through enzymatic removal of the matrix and then amplified in cell number by serial subculture such as a monolayer[8,9]. The chondrocyte

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culture system represents a model for cartilage metabolism investigation, which is essential for identifying the pathways of both normal development and pathological degeneration and inflammation of the tissue.

However, most studies using the monolayer culture method have been seriously hindered. This was due mainly to the cultured chondrocytes limited life span and to the rapid modulation of their collagen phenotype that occurs following the first subculture and that results principally in a decrease in the synthesis of type II collagen and aggrecan and in the up-regulation of type I collagen expressed in prechondrogenic mesenchymal cells[10].

Several attempts at stabilizing the chondrocyte phenotype in culture have been reported, for example, high densities culture[11], suspension culture on or within agarose[12], three-dimensional culture system in alginate beads[13], serum free defined medium[14] and the use of bioresorbable polymer carriers for the engineering of cartilage tissue implants[15].

One more interesting approach is the generation of cell lines displaying both infinite proliferation capacity and stable phenotype. To date an immortalized chondrocyte line seems to be the ideal model system for cartilage research since it represents a permanent and abundant source of cells expressing differentiated characteristics. Until now, several immortalized chondrocyte lines of animal or human origin have been successfully developed through viral infection or transfection with selected single or multiple oncogenes[16–22], but the conservation of all differentiated cell functions and their modulation has been often problematic. Moreover, in the case of the use of material of human origin, a series of problems is encountered due to the difficulty related to the availability of this material and to ethical problems.

Goldring *et al.*[21] established and characterized two stable cell lines (C-20/A4 and T/C-28) by transfection of primary cultures of human juvenile costal chondrocytes with two different vectors both containing DNA encoding simian virus 40 large T antigen (SV40-TAg). The immortalized chondrocytes were able to express mRNAs encoding the cartilage specific type II, IX and XI collagens and proteoglycans in an insulin-containing serum substitute[21].

Recently, to overcome potential problems associated with continuous expression of SV40-Tag, Robbins *et al.*[22] immortalized primary human articular chondrocytes with a retrovirus expressing a temperature-sensitive mutant of SV40-large T antigen (tsTAg). The established tsT/AC62 cell line expressed, at the permissive temperature, the differentiated type II collagen-producing phenotype when maintained in monolayer culture at high density or in alginate culture in serum-containing medium[22]. Despite the previous study, they used, as target cells for immortalization, articular chondrocytes isolated from human knee cartilage after surgery for joint replacement for osteoarthritis due to injury, since articular cartilage represents the primary joint tissue involved in arthritic diseases[22].

The use of normal human articular cartilage obviously could be more advantageous compared to osteoarthritic or trauma-damaged cartilage, since in the latter cases some inflammatory and/or degenerative events could alter the physiologic characteristics of the original tissue.

In order to obtain a model system for both fundamental research and clinical approach in the orthopaedic field, we sought to develop a stable line of immortalized chondrocytes isolated from human adult articular healthy cartilage. To achieve this, we stably transfected the cells in primary culture with a plasmid containing two human papilloma virus type 16 (HPV16) early function genes[23], using the highly efficient cationic liposome-mediated (lipofection) procedure[24]. These DNA viral oncogenes have been already demonstrated to immortalize human keratinocytes and human embryonic and myocardial fibroblasts by the inactivation of host proteins involved in cell cycle control[23,25,26].

Since the dedifferentiated phenotype obtained in chondrocytes by serial subculture is reversible[12,13], we investigated the ability of the immortalized chondrocytes to re-express their differentiated functions in two culture conditions that have been described to re-induce differentiated characteristics: culturing in a serum-free defined medium supplemented with an insulin-containing serum[21] substitute termed Nutridoma-SP and seeding on a hyaluronan-based non-woven structured biomaterial referred as to HYAFF®11.

Materials and methods

SOURCE OF CARTILAGE

Macroscopically normal human articular cartilage was harvested aseptically within 6 h of death from the femoral condyles of a 45-year-old male multiorgan donor who did not have a known history of arthritis or related pathologies.

CHONDROCYTE ISOLATION

Articular cartilage was minced into small pieces with a scalpel and the slices were submitted to sequential digestion: 30 min with 0.1% hyaluronidase (Sigma, St. Louis, MO, U.S.A.), 1 h with 0.5% pronase (Sigma) and 45 min with 0.2% collagenase (Sigma) at 37°C in Dulbecco's modified Eagle's medium (DMEM) (GIBCO BRL, Grand Island, NY, U.S.A.) with 25 mM HEPES (Sigma), 100 units/ml penicillin (Biological Industries, Israel), 100 μg/ml streptomycin (Biological Industries), 50 μg/ml gentamicin (Flow Laboratories, Biaggio, Switzerland). The isolated chondrocytes were filtered through 100 μm and 70 μm nylon meshes, washed and centrifuged. The pellet was resuspended in DMEM supplemented with 20% fetal calf serum (FCS) and then seeded at high density (2×10^5 cells/cm^2) in flasks (Costar®, Cambridge, MA, U.S.A.), at 37°C in a humidified atmosphere of 5% CO_2.

PLASMID

The p16HHMo plasmid, a generous gift from Dr John T. Schiller (Laboratory of Cellular Oncology, National Cancer Institute NIH Bethesda, MD 20892), contains subgenomic HPV-16 fragments cloned in pML2: the E6 and E7 genes as intact open reading frames (ORFs) and a small part of the E1 ORF, under the promotional control of the Moloney long terminal repeats (MoLTR)[23]. Plasmid purification was obtained using a plasmid-DNA isolation kit (Quiagen GmbH, Hilden, Germany) according to the manufacturer's instructions.

CATIONIC LIPOSOME-MEDIATED TRANSFECTION (LIPOFECTION)

Chondrocytes were transfected in early primary culture at passage 1 (P=1), using the DOTAP Liposomal

Transfection Reagent (Boehringer Mannheim Biochemicals, Indianapolis, IN, U.S.A.). The day before transfection, cells were subcultured according to standard protocol and plated in flasks at a density yielding approximately 60–80% confluency at the time of the transfection. The DOTAP/plasmid transfection mixture was prepared using polystyrene tubes with a ratio of 1 μg of DNA per 7 μl DOTAP. At the time of transfection, culture medium was removed and chondrocytes were incubated with medium without serum containing the transfection mixture for 3 h at 37°C in a humidified atmosphere of 5% CO_2. The medium was then replaced with fresh DMEM containing 20% FCS.

We tested three different plasmid concentrations: 5 μg, 10 μg and 15 μg per flask, using chondrocytes from the same multiorgan donor as target cells for all transfection experiments. Non-transfected cells were used as a comparison control. Transfected and non-transfected chondrocytes were fed twice weekly with DMEM containing 20% FCS. At confluency, which usually occurred after 7–10 days, they were passaged at a split ratio of 1:2.

The experiments with non-trasfected cells (control) were performed at P=0 and/or P=1 for RT-PCR, immunohistochemical, histochemical, Western blotting and ultrastructural analyses. At these early passages the cells are still differentiated expressing the cartilage-specific molecules. The experiments with transfected cells in monolayer culture were performed after >40 passages from the transfection for RT-PCR, immunohistochemical, histochemical, Western blotting and ultrastructural analyses. A further period of culture of 14 days with 1% Nutridoma-SP and 30 days on HYAFF®11 scaffold was needed to permit the re-expression of the naive phenotype. These two different time culture periods were chosen on the basis of previous experiments performed using normal human chondrocytes.

SERUM-FREE DEFINED MEDIUM CONDITION CULTURES

Confluent immortalized chondrocytes were trypsinized at P=42 (~1 year and 1 month from the transfection) plated in DMEM with 10% FCS and incubated at 37°C for 1 day. The monolayer was rinsed with Dulbecco's Ca^{2+}- and Mg^{2+} free phosohate-buffered saline (PBS) and incubated with culture medium containing 1% Nutridoma-SP (Boehringer Indianapolis). Medium was changed twice a week and the cells were splitted one time. After 14 days cells were harvested and pellets processed for RT-PCR, immunohistochemical, histochemical and Western blotting analyses.

SEEDING ON BIOMATERIAL

The biomaterial used as a scaffold for three-dimensional cell growth was a hyaluronan-based biopolymer, HYAFF®11, obtained from F.A.B. S.r.l. (FIDIA Advanced Biopolymers, Abano Terme, Italy), which has been shown to be a suitable support that allows chondrocytes to produce hyaline cartilage-specific matrix molecules[27]. Immortalized chondrocytes cultured under conventional monolayer culture were trypsinized at P=42 (~1 year and 1 month from the transfection) and seeded onto 1×1 cm HYAFF®11 non-woven meshes at a density of 1×10^6 in 150 μl of culture medium in Petri dishes (Becton Dickinson, Plymouth, U.K.). The cells were allowed to adhere for 8 h at 37°C and then 2 ml of medium was added. 50 μg/ml ascorbic acid was added daily only to the cultures that underwent to the histological staining.

The medium was changed twice a week**.** After 30 days the scaffolds were collected and processed for RT-PCR, immunohistochemical, histochemical and ultrastructural analyses.

RNA EXTRACTION AND REVERSE TRANSCRIPTASE PCR

Total RNA was extracted using RNAzol B reagent (Biotecx Laboratories, Houston, TX, U.S.A.). 1×10^6 non-transfected (P=0) and transfected chondrocytes (P=42) cultured on a monolayer with DMEM supplemented with FCS and in DMEM containing 1% Nutridoma-SP for 14 days were trypsinized, pelleted and lysed in 0.5 ml of RNAzol B reagent. HYAFF®11 scaffolds with seeded transfected chondrocytes were collected after 30 days, placed in microcon 100 filtration devices and centrifuged at 1500×***g*** for 5 min in order to remove the liquid medium. Cells were lysed directly in the culture scaffold by addition of 0.5 ml of RNAzol B reagent. Total RNA was subsequently isolated according to the manufacturer's instructions. Complementary DNA was synthesized from 1 μg of total RNA per sample using Murine Leukemia Virus (MuLV) reverse transcriptase (Perkin Elmer, Norwalk, CT, U.S.A.). The reaction was made in a final volume of 20 μl with 5 mM $MgCl_2$, 1 mM each deoxynucleotide, 2.5 μM oligo-(dT), 1 U/ μl RNase inhibitor and 2.5 U/ μl MuLV reverse transcriptase in 50 mM KCl, 10 mM Tris–HCl, pH 8.3. The mixture was incubated at 22°C for 10 min, 42°C for 45 min, heated to 99°C for 5 min and flash-cooled to 4°C. PCR amplifications for type I, II, IX, X collagens (Col I-A1, Col II-A1, Col IX-A1, Col X-A1), Aggrecan and HPV-16 E7 were performed for 35 cycles of 30 s/95°C denaturation, 30 s annealing and 30 s/72°C extension, using recombinant Taq DNA Polimerase (Perkin Elmer). Specific primer pairs and annealing temperatures are reported in Table I. Samples were also amplified for the housekeeping gene β_2-microglobulin used as internal quality standard. Positive controls used in RT-PCR reactions were: non-transfected chondrocyte (P=0) RNA for Col II-A1, Col IX-A1, Col X-A1 and aggrecan transcripts; human tonsillar stromal cells (P=4) RNA for Col I-A1 transcript and purified p16HHMo plasmid for HPV-16 E7 mRNA. In each reaction a negative control (sample without template) was included.

PCR products were visualized on a 2% agarose gel stained with ethidium bromide with a 100 bp DNA ladder (Promega Corporation) as molecular weight marker. Images were acquired under UV-light using the Kodak Digital Science Electrophoresis Documentation and Analysis System (EDAS) 120 (Kodak, Rochester, NY, U.S.A.).

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Transfected chondrocytes were seeded directly onto glass coverslips placed in six-well culture plates (Becton Dickinson, Franklin Lakes, NJ, U.S.A.), at a density of 1×10^5 cells/coverslip. At confluence, the cells were rinsed twice in PBS, fixed with 100% ethanol (The Warner-Graham Company, Cockeisville, MD, U.S.A.) for 5 min and incubated with the anti-HPV-16-E7 mouse monoclonal antibody, diluted 1:2 in PBS, (Zymed, San Francisco, CA, U.S.A.) for 1 h at room temperature. Then, after two washes in PBS, cells were incubated with the secondary antibody: fluorescein (FITC)-conjugated goat antimouse IgG (Jackson Immunoresearch, West Grove,

Table I
Primer sequences used for reverse transcriptase-polymerase chain reaction

RNA template	Primer sequences	Annealing temperature (°C)	Length (base pairs)	References*
Type I collagen	5′-AAC GGC AAG GTG TTG TGC GAT G 3′-AGC TGG GGA GCA AAG TTT CCT C	61	296	28
Type II collagen	5′-AAC TGG CAA GCA AGG AGA CA 3′-AGT TTC AGG TCT CTG CAG GT	55	621	13
Type IX collagen	5′-GTG TTG CTG GTG AAA AGG GT 3′-GGG ATC CCA CTG GTC CTA AT	55	159	45
Type X collagen	5′-AGC CAG GGT TGC CAG GAC CA 3′-TTT TCC CAC TCC AGG AGG GC	59	387	13
Aggrecan	5′-ATG CCC AAG ACT ACC AGT GG 3′-TCC TGG AAG CTC TTC TCA GT	55	501	13
HPV-16 E7	5′-AAG CAG AAC CGG ACA GAG CC 3′-TTC CTG TGC AGT AAA CAA CGC	57	398	
β_2-microglobulin	5′-CCT TGA GGC TAT CCA GCG TA 3′-TAT CTG AGC AGG TTG CTC CA	55	517	

Note. *Primer sequences were obtained from published references where indicated, or designed using the PRIMER3 analysis program.

PA, U.S.A.), diluted 1:50 in PBS, for 1 h at room temperature. After three washes in PBS, coverslips were mounted in glycerol 90% in PBS and observed with a Zeiss Axiophot microscope equipped with a fluorescence lamp (Oberkochen, Germany).

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Immunohistochemical staining for type II collagen was performed in non-transfected and in transfected chondrocytes at P=1 and P=42 respectively. The non-transfected cells were evaluated in monolayer culture and the transfected ones were evaluated in monolayer and once they were grown into defined culture conditions. Chondrocytes grown in monolayer were seeded in 8 Well Glass Slide (Nalge Nunc International, Naperville, IL, U.S.A.) at a density of 1.5×10^5 cells/well and after 72 h they were rinsed twice in PBS, added with filtered FCS for 10 min and then stored at –80°C. Chondrocytes grown onto HYAFF®11 scaffolds were embedded in OCT and snap-frozen in liquid nitrogen. The specimens were then sectioned into 5 μm sections, air dried and stored at −80°C. At the moment of the analysis all the slides were transferred at room temperature, air dried for 15 min and fixed in acetone at 4°C for 10 min. Air dried fixed samples were rehydrated and incubated at room temperature for 30 min with primary monoclonal antibody (MoAb) antihuman type II collagen (Chemicon International, Temecula, CA, U.S.A.), that was diluted 1:10 in 0.04 M pH 7.6 TBS containing 2% bovine serum albumin (BSA) (Sigma). An enzymatic pre-treatment with hyaluronidase 0.1% (Sigma) at 37°C for 5 min was performed. Slides were washed three times with 0.04 M pH 7.6 TBS and then incubated with goat antimouse and antirabbit immunoglobulins labelled with destran molecules-alkaline phosphatase conjugated (Envision, Dako, Carpinteria, CA, U.S.A.) at room temperature for 30 min. After three washes with 0.04 M pH 7.6 TBS, the reaction was developed using new fucsin kit (Kit New Fucsin Substrate System, Dako, Carpinteria, CA, U.S.A.) in the presence of 5 mM Levamisole (Sigma) to block endogenous alkaline phosphatase. Slides were counterstained with hematoxylin, mounted in glycerol gel and stored at 4°C for subsequent analysis. Negative controls were performed either omitting the primary antibody or using an isotype-matched control. Specific controls were performed using irrelevant monoclonal antibodies.

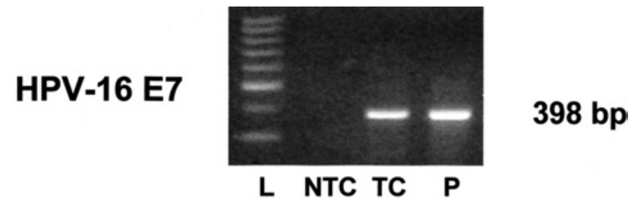

Fig. 1. Detection of HPV-16 E7 mRNA in immortalized normal human adult articular chondrocytes with RT-PCR (ethidium bromide stained agarose gel). L: DNA ladder (100 base pairs); NTC: non-transfected chondrocytes; TC: transfected chondrocytes; P: plasmid (p16HHMo).

ALCIAN BLUE STAINING

Non-transfected and transfected chondrocytes were plated at P=1 and P=42 respectively in plastic Lab-Tek 8 well chamber slides (Nalge Nunc International), at 1.5×10^5 cells/well, in DMEM and in DMEM containing 1% Nutridoma-SP. Cells were grown for three days and 50 μg/ml fresh ascorbic acid was added every day. Chondrocytes were then fixed in 10% neutral formaline 30 min at room temperature and washed in distilled water. After fixation, cells were stained in 1% Alcian Blue 8GX (Sigma, St. Louis, MO, U.S.A.) in 3% glacial acetic acid (pH 2.5) 30 min at room temperature, briefly washed in distilled water and allowed to 'blue' 20 min in running tap water. Finally chondrocytes were dehydrated through a series of alcohols (70, 80, 90 and 100%), cleared in xylene and mounted in Entellan (Merck, Darmstadt, Germany).

Alcian Blue staining was also performed onto HYAFF®11 engineered cryostat sections, which were firstly transferred at room temperature, air dried for 15 min, fixed in 10% neutral formaline 30 min at room temperature and processed as described above.

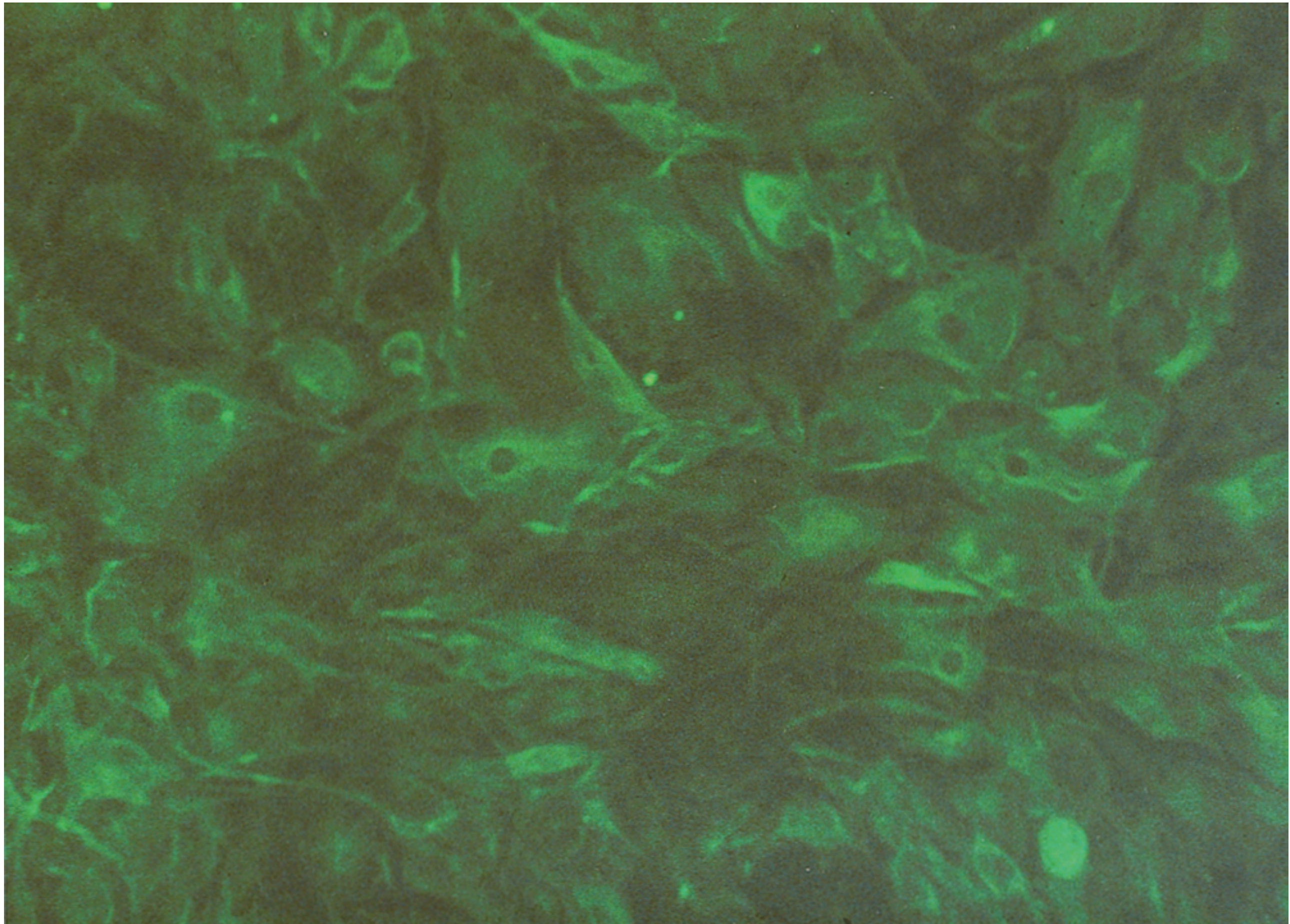

Fig. 2. HPV-16 E7 protein localization by immunofluorescence in immortalized normal human adult articular chondrocytes. A specific monoclonal antibody anti E7 protein was used.

WESTERN BLOTTING ANALYSIS

Western blotting for type II collagen was performed for non-transfected and transfected chondrocytes using the NuPAGE® (Novex high-performance pre-cast gels) Tris-Acetate Electrophoresis System (Invitrogen™, Carlsbad, CA, U.S.A.), following the manufacturer's instructions. Non-transfected cells were evaluated in early primary culture (P=1). Transfected chondrocytes were evaluated in monolayer (P=42) in serum-free culture conditions (Nutridoma-SP). Briefly, 50 000 adherent cells were trypsinized and the pellets lysed and reduced with NuPAGE® LDS Sample buffer (4×SDS) and NuPAGE® Reducing Agent (0.5 M DTT) respectively, and then heated for 10 min at 70°C. Whole cell lysates were electrophoretically separated on Tris-Acetate buffered 3–8% polyacrylamide gel and blotted onto a nitrocellulose membrane using a continuous buffer system (NuPAGE® Transfer Buffer with 10% methanol). Protein standards were used as molecular weight markers (SeeBlue Plus2 Pre-stained standard, Invitrogen). The blots were probed with the same antihuman type II collagen MoAb used for immunohistochemistry diluted 1:200 (Chemicon), followed by ECL detection (Amersham Pharmacia Biotech, U.K.) with a horseradish peroxidase-conjugated secondary antibody, diluted 1:1000. After an exposure time of 30 s on Hyperfilm-ECL, the image was acquired using the Kodak Electrophoresis Documentation and Analysis System (EDAS) 120.

FIELD EMISSION IN LENS SCANNING ELECTRON MICROSCOPY (FEISEM)

Sterilized silicon wafer chips of 3×5 mm used as FEISEM specimen holders were coiled up with a thin layer of sterile HYAFF®11 and on each device 2×10^5 of non-transfected and transfected chondrocytes (at P=1 and P=42 respectively) were deposited. The samples were then cultivated for 3 and 7 days in DMEM supplemented with 20% FCS at 37°C and 5% CO_2. At the end of the growth periods, the specimens were fixed with 1% glutaraldehyde in 0.1 M phosphate buffer pH 7.2 for 45 min, post-fixed in 1% osmium tetroxide in Veronal buffer for 30 min, dehydrated in an increasing ethanol series and critical point dried (Critical point dryer CPD 030, Bal-Tec AG, Lichtenstein). Before the FEISEM analysis, all the samples were coated with a 1.5 nm thick platinum–Carbon film (Pt 80%; C 20%) by means of a multievaporation device Balzers MED 010 (Bal-Tec). The instrument used for observation was the FEISEM Jeol JSM 890 (Jeol Ltd, Tokyo, Japan) at 7 kV accelerating voltage and 1×10^{-11} A probe current.

Results

TRANSFECTION OF CHONDROCYTES

Transfected chondrocytes were maintained in culture in DMEM supplemented with 20% FCS at 37°C in a humidified atmosphere of 5% CO_2 until the setting time for the experiments. At confluency, which usually occurred after 7–10 days, they were passaged at a split ratio of 1:2.

Non-transfected cells, used as a comparison control for all the experiments, naturally senesced after 38 culture passages in the monolayer as previously reported[28]. Chondrocytes transfected with 15 μg of the p16HHMo plasmid entered a crisis just after the transfection procedure and did not recover. Chondrocytes transfected with 10 μg and 5 μg of the p16HHMo plasmid overcame the crisis and were propagated further in monolayer culture. For about 20 passages the two cell populations were morphologically indistinguishable and the reached cell density joined at confluence was similar (35 000± 40 000/cm^2). However, the p16HHMo 10 μg-transfected cells started decreasing their proliferation rate and stopped dividing at P=35 (data not shown), while cultures transfected with 5 μg of the plasmid were able to escape senescence and have been propagated for more than 50 passages so far. At present the p16HHMo 5 μg-transfected chondrocytes are still growing in culture. Given the length of time these cells have been in culture and the number of passages since the control cells senesced, it is reasonable to think that they will continue to grow indefinitely.

EXPRESSION OF HPV-16 E7 mRNA

In general, RT-PCR and Western blotting analysis on both transformed cervical and oral epithelial cells demonstrated that the mRNA level of HPV-16 E7 corresponded to the E7 protein level, while it is difficult to correlate E6 mRNA with protein expression[29]. The reason is that only a single form of HPV-16 E7 mRNA has been identified in cervical carcinoma, while the E6 mRNA exists in three different forms, with the ratios among them varying among samples. Thus RT-PCR analysis of E7 mRNA is more straightforward than analysis of E6 mRNA[29]. In our experiments, HPV-16 E7 mRNA expression was detected in the chondrocytes transfected with 5 μg of the p16HHMo plasmid, starting from few passages after transfection (Fig. 1), but not in the ones transfected with 15 μg and 10 μg (data not shown).

EXPRESSION OF HPV-16 E7 PROTEIN

The HPV-16 E7 protein was detected by indirect immunofluorescence in the cytoplasm of the 5 μg-p16HHMo-transfected chondrocytes, starting from the early passages (Fig. 2). In chondrocytes transfected with 15 and 10 μg no fluorescence was observed as for non-transfected cells.

RE-EXPRESSION OF DIFFERENTIATED FUNCTIONS

Freshly isolated articular chondrocytes have been shown to produce a matrix of collagens and proteoglycans that is characteristic of cartilage. Our RT-PCR analyses confirmed that, at the time of isolation (P=0), the normal human adult articular chondrocytes that we used for our experiments were fully differentiated, expressing Col II-A1, Col IX-A1, Col X-A1 and aggrecan mRNAs, but not Col I-A1 mRNA (Fig. 3). The cells kept the above-mentioned differentiated characteristics just for the very first culture passages from isolation (i.e. at the time of the transfection). Starting from the 5th–6th subculture, transfected chondrocytes drastically switched from Col II-A1 to Col I-A1 mRNA expression, as expected in dedifferentiated cells[10]. The cartilage-specific messengers for Col X-A1 and aggrecan were still observed, while Col IX-A1 mRNA was not detectable anymore (Fig. 3).

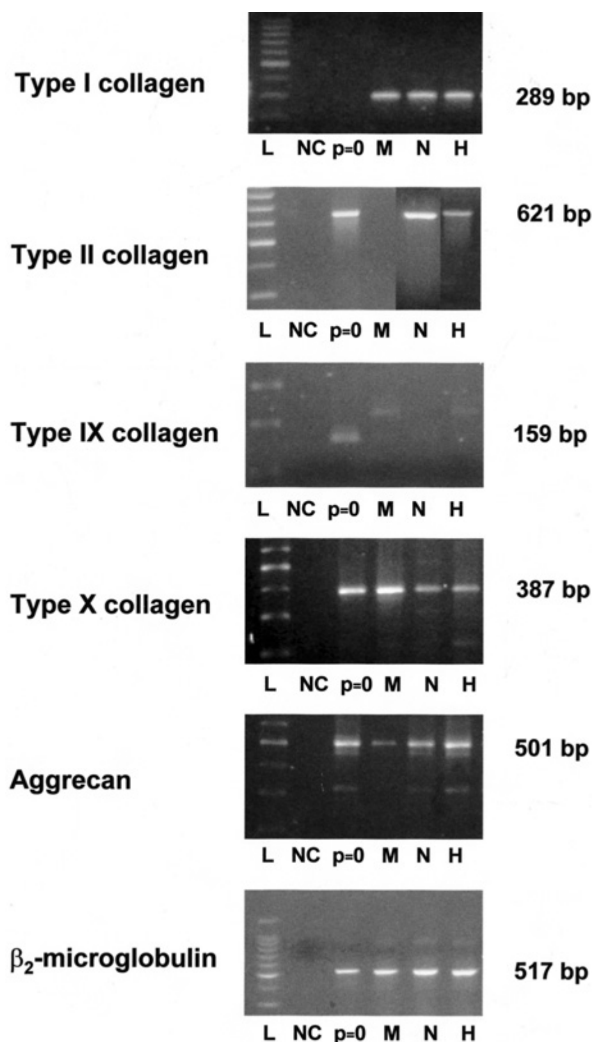

Fig. 3. RT-PCR analysis of extra-cellular matrix protein mRNAs expressed by HPV-16-immortalized normal human adult articular chondrocytes (ethidium bromide stained agarose gel). L: DNA ladder (100 base pairs); N: negative control (sample without template); P=0: freshly isolated non-transfected chondrocytes; M: monolayer culture of transfected chondrocytes at P=42; N: Nutridoma-SP culture of transfected chondrocytes at P=42; H: hyaluronan-based biomaterial (HYAFF®11) culture of transfected chondrocytes at P=42.

The stable proliferating chondrocyte line re-expressed (at P=42) Col II-A1 mRNA under the two defined culture conditions that we tested i.e. serum-free defined medium containing 1% Nutridoma-SP and HYAFF®11 cultures, despite retaining expression of Col I-A1 mRNA. The cartilage-specific Col X-A1 and aggrecan mRNAs, but not

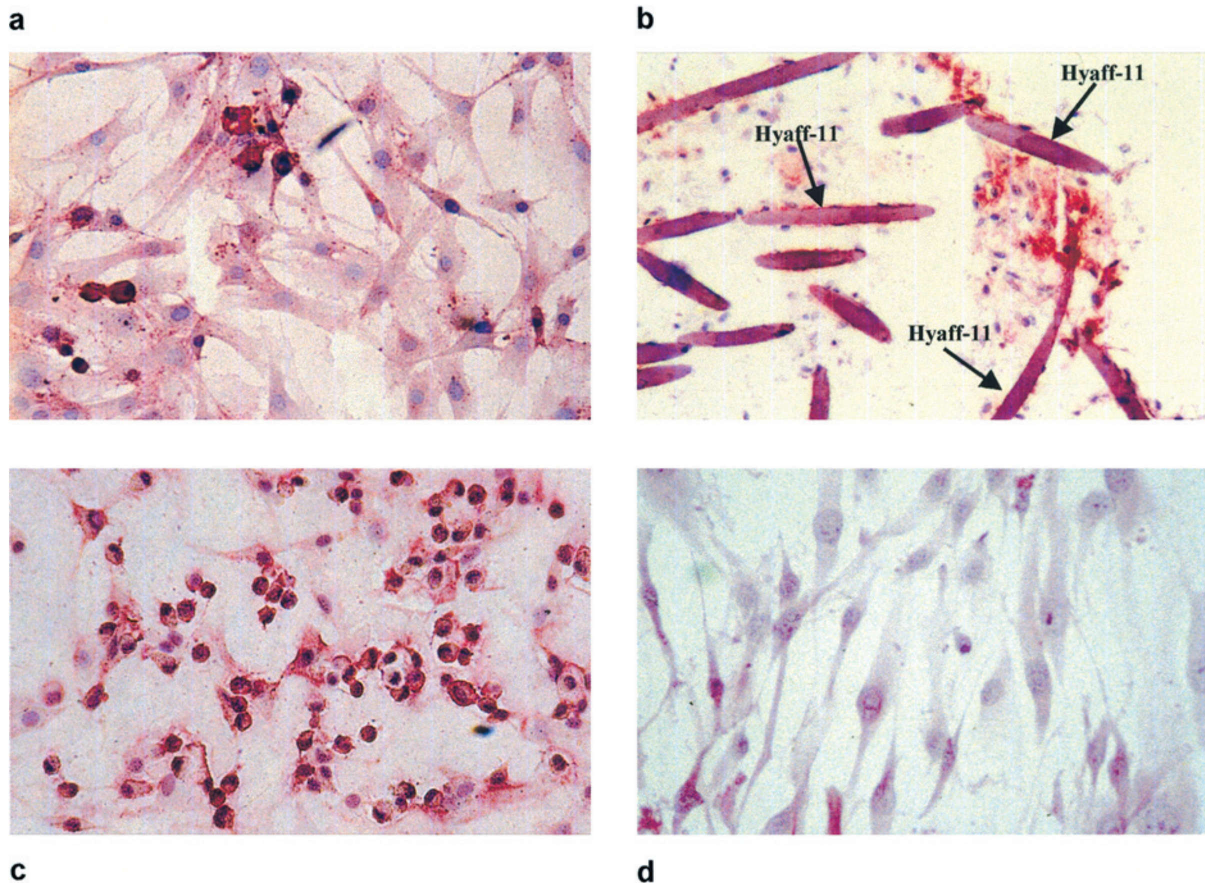

Fig. 4. Collagen II immunostaining of the immortalized cells at P=42 grown in serum-free medium (Nutridoma-SP) (a) and seeded on HYAFF®11 (b) (the HYAFF®11 fibers are indicated with arrows). As controls are reported the non-transfected chondrocytes in the first culture passage from isolation (c) and the transfected cells further propagated in monolayer (P=42) (d). Collagen II was developed using new fucsin (red color is positive stain).

Col IX-A1 mRNA, were also detected under these culture conditions (Fig. 3).

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The re-acquisition of the chondrocyte phenotype was confirmed by the presence of type II collagen positive cells in the two defined culture conditions studied [Fig. 4(a), (b)] indicating the occurrence of a re-differentiation process. Figure 4(c) represents fully differentiated chondrocytes in an early culture passage: the totality of the cells show a positive staining. The transfected chondrocytes further propagated in monolayer (P=42) showed very low positivity for the protein, indicating a state of phenotipic dedifferentiation [Fig. 4(d)].

ALCIAN BLUE STAINING

Sulfated proteoglycans in the extra-cellular matrix, detected by selective staining with Alcian Blue, can be one of the markers of differentiated chondrocyte function[30]. Transfected chondrocytes at P=42 grown in DMEM with 1% Nutridoma-SP were observed to deposit a proteoglycan-rich matrix after 72 h of culture [Fig. 5(a)]. The same cells grown onto HYAFF®11 scaffold for a month were able to synthesize an Alcian Blue-stainable extra-cellular matrix [Fig. 5(b)]. As controls, Fig. 5(c) shows strongly positive stainable material in the pericellular regions of the non-transfected chondrocytes in early primary culture (P=1), while Fig. 5(d) represents the weakly stainable transfected cells in late monolayer culture passages.

WESTERN BLOTTING ANALYSIS

Western blotting analysis confirmed that transfected chondrocytes cultured in serum-free defined conditions synthesize type II collagen compared to the non-transfected cells in primary culture (Fig. 6), further demonstrating the re-acquisition of the differentiated phenotype by this cell line.

MORPHOLOGICAL CHARACTERIZATION

The ultrastructural analysis performed by FEISEM revealed a precocious tendency of the cell to adhere to the HYAFF®11. In fact, after 3 days in several foci the wires of the substratus were covered in cells. Mainly, they appear flattened and tightly adherent to the HYAFF®11, even characterized by a smooth surface and a tight overlapping. Nevertheless, few elements were more globular and less adherent to the substratus, with a surface covered in heterogeneous microvilli and a border rich in spoke-like

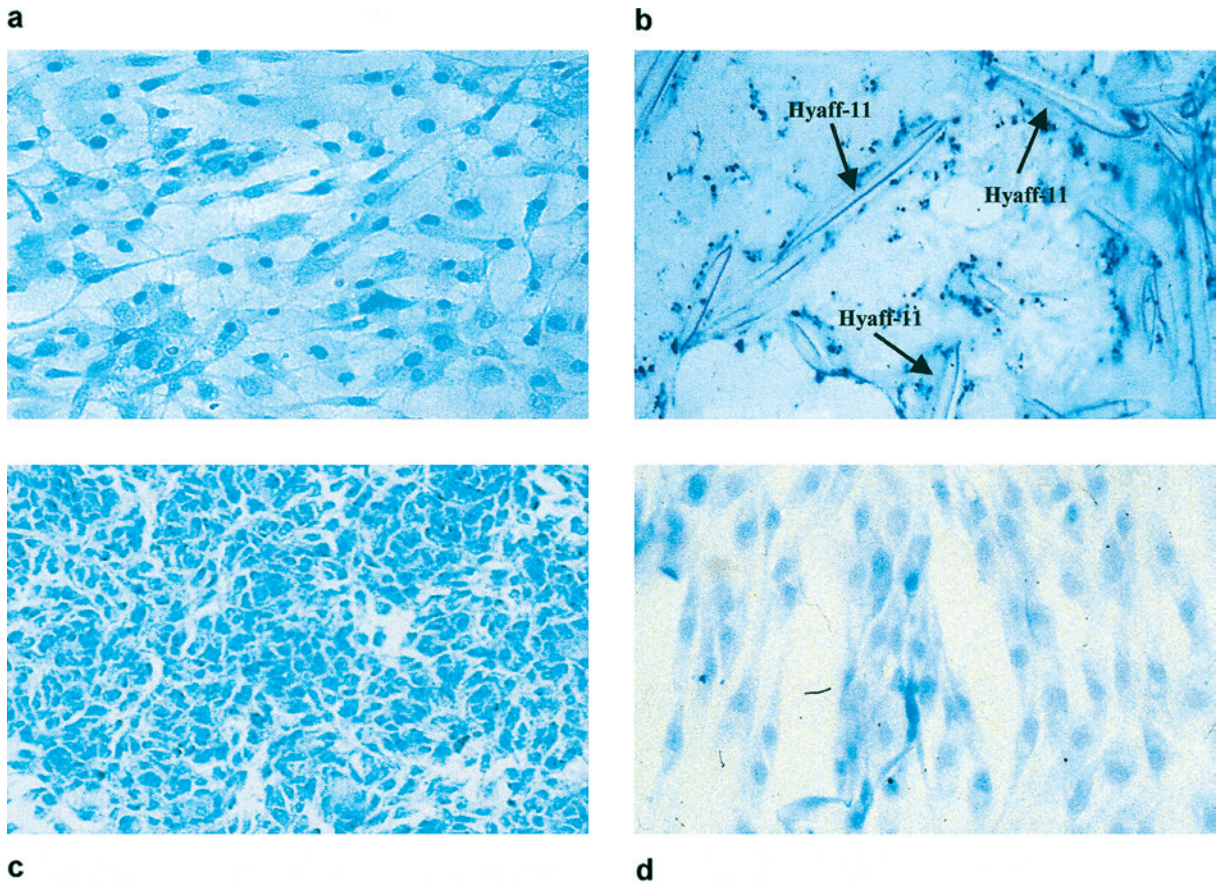


Fig. 5. Alcian Blue staining of immortalized human chondrocytes grown in DMEM with 1% Nutridoma-SP (a) and onto HYAFF®11 scaffold (b) (the HYAFF®11 fibers are indicated with arrows). As controls are reported the non-transfected chondrocytes in the first culture passage from isolation (c) and the transfected cells further propagated in monolayer (P=42) (d). Alcian Blue-stainable material (referred to the presence of proteoglycans) is evident in the pericellular regions of most of the cells in the cultures.

plasmalemmal evaginations [Fig. 7(a)]. At higher magnifications, the surface of the more globular cells appeared to be covered in a filamentous secretion where a regular period can be detected on the fibers [Fig. 7(b)]. After 7 days of growing, almost all the globular cells appeared to be replaced by flattened and adherent elements. Generally, we didn't observe in transfected chondrocytes relevant morphological differences compared to the non-transfected ones [Fig. 7(c)].

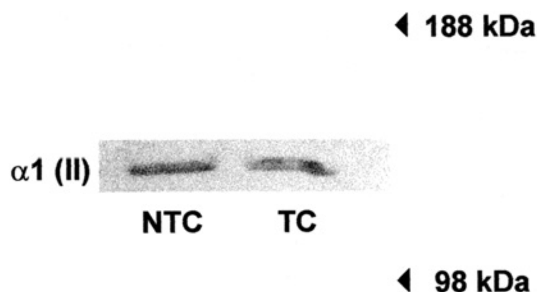


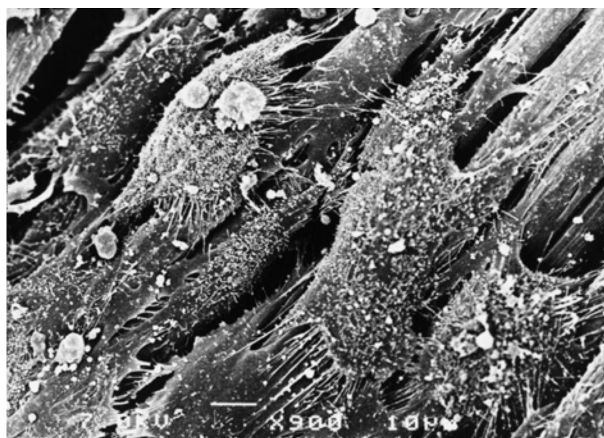
Fig. 6. SDS-polyacrylamide (3–8% w/v) gel electrophoresis of collagen type II. NTC: non-transfected chondrocytes at P=1; TC: transfected chondrocytes at P=42 in Nutridoma-SP culture. An antihuman type II collagen monoclonal antibody diluted 1:200 was used. The arrows indicate the protein standard weight markers.

Discussion

Articular chondrocytes in primary cultures are generally considered to be a well-differentiated cell population: they synthesize high levels of cartilage proteoglycans and type II collagen[2]. When serially cultured in monolayers they cease to synthesize cartilage-specific macromolecules, replacing them with molecules normally expressed by mesenchymal cells, like type I collagen[3,10,13].

In this study, adult normal human articular chondrocytes were transfected in primary culture with a plasmid containing the two HPV-16 early function genes E6 and E7 (p16HHMo). This led to immortalization of these cells and to an apparently irreversible suppression of their type II collagen-differentiated phenotype, stable expression of type I collagen and synthesis of an extracellular matrix as shown by the Alcian Blue staining.

HPV-16 E6 and E7 expression is likely to be responsible for this immortalization since no proliferation was observed in non-transfected cells after they had reached senescence. The E6 and E7 genes products are oncoproteins, which exert their biological effects mainly by binding respectively with the products of p53 and retinoblastoma genes and thus inactivating these tumor suppressor genes[31]. This causes deregulation of the cell cycle with loss of control on crucial events, such as DNA replication, DNA repair and apoptosis[31].



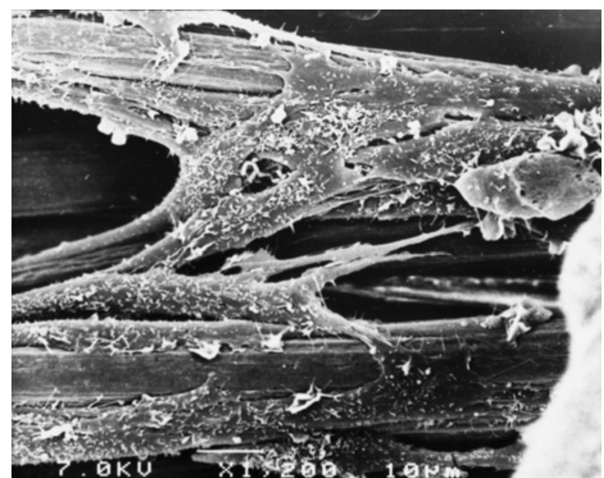


Fig. 7. Field Emission in Lens Scanning Electron Microscopy (FEISEM) images of immortalized normal human adult chondrocytes after 3 days from the seeding on the biomaterial HYAFF®11 (hyaluronan-based non-woven structured biomaterial). (a) The wires of HYAFF®11 are detectable covered by adherent and smooth cells. Rare non-adherent cells are also evident and characterized by more irregular surface and shape (origin. magn.: 900×). (b) Detail of the same field. Irregularly sized microvilli decorate the surface of the non-adherent cells. A characteristic regularly banded filamentous secretion is also evident (orig. magn.: 20 000×). (c) Non-transfected chondrocytes seeded on the biomaterial HYAFF®11 after three days of culture used as control (orig. magn.: 1200×).

Oncogenic HPV-16 is strongly associated with cervical cancer and its precursor, cervical intraepithelial neoplasia[32]. It has been shown that the HPV-16 genome has *in vitro* activity in its natural host cells: when transfected onto normal human foreskin keratinocytes or human cervical epithelial cells it was able to indefinitely extend cell proliferative capacity[33]. Further analyses were undertaken in order to define the early HPV-16 genes that directly participate in the *in vitro* transformation process. Different studies using respectively primary human keratinocytes[34], primary human embryonic fibroblasts (WI38)[25] and human bronchial epithelial cells[35], as target cells for transfection, demonstrated that both full-length E6 and E7 genes are required for the induction of immortalization. In fact, mutations in either the E6 or E7 ORF completely abrogated transformation of these cells. Mutations in E1, E2 and E2–E4 ORFs, on the other hand, had no effect[36]. Hawley-Nelson *et al*. demonstrated that transfection with the p16HHMo plasmid, in which E6 and E7 were the only intact ORFs, induced an indefinite lifespan in human genital keratinocytes, with an efficiency similar to that of the entire early region of the viral DNA[23]. Moreover, it has been demonstrated that human smooth muscle, bone marrow and myocardial immortalized cells expressing HPV-16 E6 and E7 oncogenes display a quite well preserved phenotype[26,37,38].

In several reports, the SV40T-Ag and the myc oncogene have been transfected into chondrocyte cultures and immortalized chondrocyte cell lines have been obtained, with varying results regarding the maintenance of the differentiated phenotype[16–22]. Although cartilage-specific proteoglycan synthesis has been demonstrated in several immortalized chondrocyte lines, the expression of type II collagen seems to be particularly difficult to maintain. While several authors reported either the complete loss or a marked decrease in type II collagen in immortalized chondrocytes[17,19,20], some cell lines of v-myc- or T-Ag-immortalized chondrocytes maintained the expression of this marker[16,18,21,22]. Given the chondrocyte phenotype *in vitro* lability and the dissociation of proliferative and phenotypic stabilities found in some immortalized chondrocyte systems, we investigated which kind of molecules the articular normal human E6/E7-immortalized chondrocyte line that we obtained was able to express. Like normal articular chondrocytes grown in conventional culture conditions, our immortalized chondrocytes expanded in monolayer did not express type II and IX collagen mRNAs while retaining the expression of aggrecan and type X collagen already evidentiated at the early culture passages. Moreover, they started to express type I collagen, a classically described marker of dedifferentiated chondrocytes. This dedifferentiation process was also demonstrated by immunohistochemical and histochemical analyses that showed the lack of production of collagene type II and a weak synthesis of proteoglycans. When we evaluated our immortalized cells in two culture conditions that have been shown to successfully induce re-expression of chondrocyte original characteristics (culturing in a serum-free defined medium and seeding on a three-dimensional support) we detected the re-expression of the messenger for type II collagen, the main differentiation marker for chondrocytes, and the maintenance of the expression of collagen type X which is probably a characteristic molecule of this cell line. These data were also confirmed at protein level by the detection of collagen type II protein by immunohistochemistry and Western blotting analysis and by the detection of

a proteoglycans active synthesis revealed by Alcian blue staining.

Most animal and human cell lines have been established in serum-supplemented medium[39]. However, a number of them required hormonally defined serum-free medium culture conditions in order to allow cell survival, growth and/or maintenance or re-expression of differentiated characteristics. The stable proliferating lines of immortalized human chondrocytes (C-20/A4 and T/C-28) established by Goldring *et al.* had an optimal 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[21] and 50 μg/ml ascorbic acid (which stimulates the neosynthesis of collagen molecules)[40]. Insulin may serve as a substitute for insulin-like growth factor-I (IGF-I), a major stimulator of matrix synthesis in cartilage[41]. FCS replacement with 1% Nutridoma-SP was permissive for type II collagen mRNA re-expression and, together with ascorbic acid, for protein production by our immortalized chondrocytes. Otherwise, type I collagen mRNA was still expressed even if it was not detected in the C-20/A4 and T/C-28 lines, under these same culture conditions[21].

The use of polymeric bioresorbable scaffolds as devices for chondrocyte growth and differentiation in order to produce a three-dimensional cartilage tissue is well documented[15,42–44]. Our previous experiments demonstrated that human articular dedifferentiated chondrocytes grown onto HYAFF®11 non-woven meshes re-express type II collagen[27]. When we investigated the re-differentiation capability of the HPV-16-immortalized chondrocytes in the same culture conditions, we detected type II collagen mRNA expression and protein production after 30 days of culture. However, type I collagen mRNA was still present. Thus, as observed for dedifferentiated chondrocytes, HYAFF®11 represents a suitable cell carrier that enhances the HPV-16-immortalized chondrocytes to revert to their original function of producing type II collagen. Moreover, the ultrastructural analysis reveals how similar the behavior of this cell line can be compared to the non-immortalized chondrocytes. In fact, both the time necessary for the full adhesion to the HYAFF®11 and the three-dimensional pattern of the cells after the adhesion appears the same. Also the typical cell orientation in the secretion of collagen microfibrills appears strictly related to the adhesion in this model as well as in the non-immortalized cells[27].

In summary, HPV-16 early genes were able to immortalize normal human adult articular chondrocytes, allowing the establishment of a new cell line expressing specific cartilage molecules, even if further studies are needed to better characterize it.

This line could be a useful model system for studying the metabolism of chondrocyte cells and their responsiveness to treatments which are known to modulate their phenotype. These data will shed important light on our understanding of the biology of these cells and may have therapeutic implications in combating some of the most important rheumatic diseases.

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References

1. Kuettner KE. Cartilage integrity and homeostasis. In: Klippel J, Dieppe PA, Eds. Rheumatology. London: Mosby International 1998:6.1–6.16.
2. Buckwalter JA, Rosenberg LC, Hunzinker EB. Articular cartilage: composition, structure, response to injury and methods of repair. In: Ewing JR, Ed. Articular Cartilage and Knee Joint Function: Basic Science and Arthroscopy. New York: Raven Press 1990: 19–56.
3. Aulthouse AL, Beck M, Griffey E, Sanford J, Arden K, Machado MA, *et al.* Expression of the human chondrocyte phenotype in vitro. In Vitro Cell Dev Biol 1989;25:659–68.
4. Eyre DR, Wu JJ, Woods P. Cartilage-specific collagens. In: Kuettner K, Schleyerbach R, Peyron JG, Hascall VC, Eds. Articular Cartilage and Osteoarthritis. New York: Raven Press 1992:119–30.
5. Sandell LJ, Aigner T. Articular cartilage and changes in arthritis. An introduction: cell biology of osteoarthritis. Arthritis Res 2001;3:107–13.
6. Rucklidge GJ, Milne G, Robins SP. Collagen type X: a component of the surface of normal human, pig, and rat articular cartilage. Biochem Biophys Res Commun 1996;224:297–302.
7. Sweet MBE, Grisillo A, Coehlo A, Schnitzler CM. Proteoglycan synthesis by articular chondrocytes in agarose culture. S Afr J Sci 1987;83:153–6.
8. Schwartz R. Cartilage cells and organ culture. In: Simmon DJ, Kunin AR, Eds. Skeletal Research. An Experimental Approach. New York: Academic Press 1979:61–78.
9. Green WT. Behavior of articular chondrocytes in cell culture. Clin Orthop Rel Res 1971;75:248–60.
10. Archer C, McDowell J, Bayliss M, Stephens M, Bentley G. Phenotypic modulation of sub-populations of human articular chondrocytes in vitro. J Cell Sci 1990;97:361–71.
11. Watt F. Effect of seeding density on stability of the differentiated phenotype of pig articular chondrocytes in culture. J Cell Sci 1988;89:373–8.
12. Benya PD, Shaffer JD. De-differentiated chondrocytes re-express the differentiated collagen phenotype when cultured in agarose gels. Cell 1982;30:373–84.
13. Bonaventure J, Kadhom N, Cohen-Solal L, Ng KH, Bourguignon J, Lasselin C, *et al.* Reexpression of cartilage-specific genes by dedifferentiated human articular chondrocytes cultured in alginate beads. Exp Cell Res 1994;212:97–104.
14. Adolphe M, Froger B, Ronot X, Corvol MT, Forest N. Cell multiplication and type II collagen production by rabbit articular chondrocytes cultivated in a defined medium. Exp Cell Res 1984;155:527–36.
15. Freed L, Marquis JC, Nohria A, Emmanual J, Mikos AG, Langer R. Neocartilage formation in vitro and in vivo using cells cultured on synthetic biodegradable polymers. J Biomed Mat Res 1993;27:11–23.
16. Horton WE, Cleveland J, Rapp U, Nemuth G, Bolander M, Doege K, *et al.* An established rat cell line expressing chondrocyte properties. Exp Cell Res 1988;178:457–68.

17. Mallein-Gerin F, Olsen BR. Expression of simian virus 40 large T (tumor) oncogene in mouse chondrocytes induces cell proliferation without loss of the differentiated phenotype. Proc Natl Acad Sci USA 1993;90:3289–93.
18. Benoit B, Thenet-Gauci S, Hoffschir F, Penfornis P, Demignot S, Adolphe M. SV40 large T antigen immortalization of human articular chondrocytes. In Vitro Cell Dev Biol 1995;31:174–7.
19. Steimberg N, Viengchareun S, Biehlmann F, Guenal I, Mignotte B, Adolphe M, *et al*. SV40 large T antigen expression driven by col2a1 regulatory sequences immortalizes articular chondrocytes but does not allow stabilization of type II collagen expression. Exp Cell Res 1999;249:248–59.
20. Oyajobi BO, Frazer A, Hollander AP, Graveley RM, Xu C, Houghton A, *et al*. Expression of type X collagen and matrix calcification in three-dimensional cultures of immortalized temperature-sensitive chondrocytes derived from adult human articular cartilage. J Bone Miner Res 1998;13:432–42.
21. Goldring MB, Birkhead JR, Suen LF, Yamin R, Mizuno S, Glowacki J, *et al*. Interleukin-1β-modulated gene expression in immortalized human chondrocytes. J Clin Invest 1994;94:2307–16.
22. Robbins JR, Thomas B, Tan L, Choy B, Arbiser JL, Berenbaum F, *et al*. Immortalized human adult articular chondrocytes maintain cartilage-specific phenotype and responses to interleukin-1β. Arthritis Rheum 2000;43:2189–201.
23. Hawley-Nelson P, Vousden KH, Hubbert NL, Lowy DR, Schiller JT. HPV16 E6 and E7 protein cooperate to immortalize human foreskin keratinocytes. EMBO J 1989;8:3905–10.
24. Goomer RS, Deftos LJ, Terkeltaub R, Maris T, Lee MC, Harwood FL, *et al*. High-efficency non-viral transfection of primary chondrocytes and perichondral cells for ex-vivo gene therapy to repair articular cartilage defects. Osteoarthritis Cart 2001;9:248–56.
25. Watanabe S, Kanda T, Yoshiike K. Human papillomavirus type 16 transforming of primary human embryonic fibroblasts requires expression of open reading frames E6 and E7. J Virol 1989;63:965–9.
26. Harms W, Rothämel T, Miller K, Harste G, Grassmann M, Heim A. Characterization of human myocardial fibroblasts immortalized by HPV16 E6-E7 genes. Exp Cell Res 2001;268:252–61.
27. Grigolo B, Lisignoli G, Piacentini A, Fiorini M, Gobbi P, Mazzotti G, *et al*. Evidence for redifferentiation of human chondrocytes grown on a hyaluronan-based biomaterial (HYAFF®11): molecular, immunohistochemical and ultrastructural analysis. Biomaterials 2001;23:1187–95.
28. Kolettas E, Buluwela L, Bayliss MT, Muir HI. Expression of cartilage-specific molecules is retained on long-term culture of human articular chondrocytes. J Cell Sci 1995;108:1991–9.
29. Ke LD, Adler-Storthz K, Follen Mitchell M, Clayman GL, Chen Z. Expression of human papillomavirus E7 mRNA in human oral and cervical neoplasia and cell lines. Oral Oncol 1999;35:415–20.
30. Capasso O, Gionti E, Pontarelli G, Ambesi-Impiombato FS, Nitsch L, Tajana G, *et al*. The culture of chick embryo chondrocytes and the control of their differentiation functions. Exp Cell Res 1982;142:197–206.
31. Narayanan BA, Holladay EB, Nixon DW, Mauro CT. The effect of all-trans and 9-cis retinoic acid on the steady state level of HPV16 E6/E7 mRNA and cell cycle in cervical carcinoma cells. Life Sci 1998;63:565–73.
32. Biswas C, Kell B, Mant C, Jewers RJ, Cason J, Muir P, *et al*. Detection of human papillomavirus type 16 early-gene transcription by reverse transcription-PCR is associated with abnormal cervical cytology. J Clin Microbiol 1997;35:1560–4.
33. Pirisi L, Yasumoto S, Feller M, Doniger J, DiPaolo JA. Transformation of human fibroblast and keratinocytes with human papillomavirus type 16 DNA. J Virol 1987;61:1061–6.
34. Durst M, Dzarlieva-Petrusevska RT, Boukamp P, Fusenig NE, Gissmann L. Molecular and cytogenetic analysis of immortalized human primary keratinocytes obtained after transfection with human papillomavirus type 16 DNA. Oncogene 1987;1:251–6.
35. Viallet J, Liu C, Edmond J, Tsao MS. Characterization of human bronchial epithelial cells immortalized by the E6 and E7 genes of human papillomavirus type 16. Exp Cell Res 1994;212:36–41.
36. Munger K, Phelps WC, Bubb V, Howley PM, Schlegel R. The E6 and E7 genes of the human papillomavirus type 16 together are necessary and sufficient for transformation of primary human keratinocytes. J Virol 1989;63:4417–21.
37. Perez-Reyes N, Halbert CL, Smith PP, Beneditt EP, McDougall JK. Immortalization of primary human smooth muscle cells. Proc Natl Acad Sci USA 1992;89:1224–8.
38. Loeuillet C, Douay L, Hervè P, Chalmers DE. Preservation of the myofibroblastic phenotype of Human Papilloma Virus 16 E6/E7 immortalized human bone marrow cells using the lineage limited α-smooth muscle actin promoter. Cell Growth Differ 2001;12:233–42.
39. Taub M. The use of defined media in cell and tissue culture. Toxic in Vitro 1989;4:213–25.
40. Hering TM, Kollar J, Huynh TD, Varelas JB, Sandell LJ. Modulation of extracellular matrix gene expression in bovine high density chondrocyte cultures by ascorbic acid and enzymatic resuspension. Arch Biochem Biophys 1994;314:90–8.
41. Bohme K, Conscience-Egli M, Tschan T, Winterhalter KH, Bruckner P. Induction of proliferation of hypertrophy of chondrocytes in serum-free culture: the role of insulin-like growth factor-I, insulin, or thyroxine. J Cell Biol 1992;116:1035–42.
42. Saldanha V, Grande DA. Extracellular matrix protein gene expression of bovine chondrocytes cultured on resorbable scaffolds. Biomaterials 2000;21:2427–31.
43. Ramdi H, Legay C, Lievremont M. Influence of matricial molecules on growth and differentiation of entrapped chondrocytes. Exp Cell Res 1993;201: 449–54.
44. Schreiber RE, Dunkelman NS, Naughton G, Ratcliffe A. A method for tissue engineering of cartilage by cell seeding on bioresorbable scaffolds. Ann NY Acad Sci 1999;875:398–404.
45. Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca DJ, *et al*. Multilineage potential of adult human mesenchymal stem cells. Science 1999;284:143–7.