OsteoArthritis and Cartilage (2005) **13,** 1012–1024

doi:10.1016/j.joca.2005.06.003

Osteoarthritis and Cartilage

International Cartilage Repair Society

OARSI OSTEOARTHRITIS RESEARCH SOCIETY INTERNATIONAL

Low calcium levels in serum-free media maintain chondrocyte phenotype in monolayer culture and reduce chondrocyte aggregation in suspension culture

A. Gigout M.Sc.†, M. Jolicoeur Ph.D.† and M. D. Buschmann Ph.D.†‡*

† *Department of Chemical Engineering, Ecole Polytechnique, Montreal, Quebec, Canada*

‡ *Institute of Biomedical Engineering, Ecole Polytechnique, Montreal, Quebec, Canada*

Summary

Objective: Extracellular calcium influences chondrocyte differentiation and synthesis of extracellular matrix. Previously, calcium concentrations ranging from 0.1 mM to 2 mM have been used *in vitro* and these studies indicated that low calcium concentrations were generally favorable for chondrocyte culture. Our objective was to extend these findings to yet lower calcium concentrations and to comprehensively examine effects on morphology and phenotype in two culture systems.

Methods: Serum-free media containing 1 mM, 50 μM or 15 μM of calcium and a serum-containing medium were used to culture chondrocytes in suspension and in monolayer, at high and low inoculation density.

Results: In monolayer, at low and high density, removing serum and decreasing calcium concentration decreased cell spreading and lowered collagen type I expression whereas collagen type II expression remained stable. In suspension, cells aggregated for all media tested; however, aggregates were smaller and looser in the absence of serum.

Conclusion: The serum-free 50 μM and 1 mM calcium media provide good alternatives to classical media for monolayer culture since both growth and chondrocyte phenotype were maintained. In suspension culture, the serum-free 1 mM calcium medium also possesses the beneficial properties of limiting aggregate size while maintaining growth and phenotype.

Key words: Chondrocyte, Calcium, Cell aggregation, Chondrocyte phenotype, Suspension culture.

Introduction

In monolayer culture, primary articular chondrocytes dedifferentiate to a fibroblastic phenotype expressing collagen type I in preference to collagen type II[1,2]. In contrast, culturing chondrocytes in three dimensional (3D) gels effectively maintains the chondrocyte phenotype, although at the expense of lowering cell division[3–6]. These latter phenotypically stable systems are, however, spatially inhomogeneous since cells are exposed to different microenvironments depending on their position within these 3D systems. Chondrocytes have also been cultured in suspension[7,8] where chondrocyte phenotype was promoted, however large cell aggregates formed quickly, also leading to the loss of culture homogeneity. Thus, the challenge remains to develop homogeneous culture conditions for primary chondrocytes which allow growth, phenotypic stability and minimize cell aggregation.

Extracellular calcium has strong potentiating effects on cell adhesion and cell aggregation for diverse cell types *in vitro*. In monolayer culture, low calcium content reduces cell spreading in muscle[9], epithelial[10] and endothelial cells[11]. In suspension culture, lowering calcium to 100 μM reduces aggregation of HEK 293 cells[12]. Similarly, calcium removal by chelation with 5 mM ethylene glycol-bis(β-aminoethyl-ether)-N,N,N′,N′-tetraacetic acid (EGTA) inhibited the condensation of limb bud cells, when calcium-dependent aggregation processes were active[13].

Extracellular calcium regulates matrix synthesis of chondrocytic cells. RNA levels for aggrecan and type II collagen in a chondrocyte cell line decreased with increasing initial medium Ca^{2+} concentration (ID_{50} ~2 mM for aggrecan and 4.1 mM for type II collagen)[14]. Changes in calcium concentration in the range of 1–4 mM are also sensed by Ca^{2+} receptors (CaR), that belong to the G protein-coupled receptor superfamily[15]. These CaR are involved in the influence of extracellular calcium on differentiation where high calcium increased expression of osteopontin, osteonectin and osteocalcin in chondrogenic cells[16], and collagen type X in chondrocytes[17]. On the contrary, calcium concentrations below 0.5 mM promoted the production of articular collagens, types II and XI[18]. It has also been shown that using a medium without added calcium (but with 10% serum that contains calcium) has prevented rabbit articular chondrocytes from switching to a collagen type I-producing fibroblastic phenotype when grown in suspension culture[19,20]. Finally, elemental analysis by electron microscopy revealed low extracellular calcium levels (1/4 of the cytosolic concentration that ranges from 0.1 to 10 μM) in the proliferating zone of the cartilage growth plate[21]. Taken together, the above data from the literature strongly suggest that articular chondrocytes exist in a low calcium environment that may be necessary for their physiological function and phenotypic stability.

*Address correspondence and reprint requests to: Michael D. Buschmann, Chemical and Biomedical Engineering, Ecole Polytechnique, PO Box 6079 Station Centre-ville, Montreal, Quebec, Canada, H3C 3A7. Tel: 1-514-340-4711x4931; Fax: 1-514-340-2980; E-mail: michael.buschmann@polymtl.ca
Received 13 March 2005; revision accepted 5 June 2005.

Although existing data clearly indicate that calcium has a regulatory role on chondrocytes and that low extracellular calcium may be beneficial to chondrocytes, the lowest calcium concentration reported to date in chondrocyte culture was 0.1 mM. We believed that yet lower extracellular calcium concentrations could bear certain advantages for the culture of primary chondrocytes. We specifically hypothesized that low calcium levels in medium would: (1) promote collagen type II expression over collagen type I expression; (2) promote a chondrocytic (round) cell morphology in monolayer culture and (3) reduce cell aggregation in suspension. Since serum contains about 4 mM of calcium[22], a cell culture medium with calcium concentrations below 0.1 mM required the use of a serum-free medium (SFM). Therefore, an SFM with calcium concentrations of 1 mM, 50 μM, and 15 μM was compared to a standard serum-containing (10%) formulation that had 2 mM calcium. The influence of these different calcium levels, as well as the presence or absence of serum, was investigated using two culture systems: standard monolayer culture as well as suspension culture each at low and high cell-seeding densities.

Materials and methods

CULTURE MEDIA

All reagents were from Sigma–Aldrich Canada, Oakville, Ontario, Canada, unless indicated. Cells were cultured in either a serum-containing medium (SCM) or an SFM. The SCM was chosen to represent a commonly used SCM composed of DMEM low glucose (Life technologies, Burlington, Ontario, Canada) supplemented with 0.4 mM proline, non-essential amino acids 1× (containing 8.9 mg/L alanine, 15 mg/L asparagine, 13.3 mg/L aspartic acid, 14.5 mg/L glutamic acid, 7.5 mg/L glycine, 11.5 mg/L proline and 10.5 mg/L serine), 22 mM sodium bicarbonate, 12.5 mM N-[2-hydroxyethyl]piperazine-N′-[2-ethanesulfonic acid] (HEPES), penicillin/streptomycin 1× (containing 100 U/mL penicillin and 0.1 mg/mL streptomycin)[23]. Medium pH was adjusted to 7.2 and sterile filtered prior to addition of 10% fetal bovine serum (FBS) and 30 μg/mL ascorbate, the latter added fresh just prior to medium change. The SFM was chosen based on commonly used SFM compositions using a 1:1 (v/v) mix of calcium-free HAM's F12 (US Biological, Swampscott, MO, USA) and calcium-free DMEM low glucose (US Biological, Swampscott, MO, USA) supplemented with 0.4 mM proline, 1.5 mM glutamine, 22 mM sodium bicarbonate, non-essential amino acids 1×, 12.5 mM HEPES, penicillin/streptomycin 1×, ITS + (10 μg/mL insulin, 5.5 μg/mL transferrin, 0.05% w/v bovine serum albumin (BSA), 1.7 mM linoleic acid, 0.5 μg/mL sodium selenite), 5×10^{-5} M β-mercaptoethanol and 10^{-8} M dexamethasone[24]. Medium pH was adjusted to 7.2 and sterile filtered. The following recombinant growth factors (from R&D Systems, MN, USA) were added to SFM each at 2 ng/mL: epidermal growth factor (in sterile 0.1% BSA in 10 mM acetic acid), platelet derived growth factor-BB (in sterile 0.1% BSA in 4 mM HCl), fibroblast growth factor-2 (in sterile 0.1% BSA, 1 mM DTT in PBS without Mg^{2+} and Ca^{2+})[24]. Ascorbate, 30 μg/mL, was added fresh just prior to changing SFM. To this SFM was added a supplemental amount of $CaCl_2$ corresponding to 1 mM, 50 μM, 5 μM, 1 μM, or 0 μM. Calcium content in media was then verified by atomic absorption spectrometry with an Analyst 200 from PerkinElmer (Boston, MA, USA) with $CaCO_3$ as a standard (from PerkinElmer). Lanthane chloride (1000 ppm) was added to samples before analysis. This analysis revealed that these media contained in fact, 0.95 mM, 50.25 μM, 18.25 μM, 14.5 μM and 13.75 μM of calcium, respectively. Thus, calcium levels below 10 μM could not be achieved since calcium was present in the additives. Since our results obtained with cultures in 18.25 μM, 14.5 μM and 13.75 μM of calcium were indistinguishable, we only present results obtained with 0.95 mM, 50 μM and 14.5 μM of calcium, indicated by the rounded concentrations of 1 mM, 50 μM and 15 μM.

CELL ISOLATION

Cells were isolated from the femoropatellar groove of a 1–2-month-old calf knee, obtained from a local butcher within 24 h of slaughter. Briefly, cartilage was sequentially digested, first for 90 min by protease Type XIV (Sigma–Aldrich Canada, Oakville, Ontario) 56 U/mL at 37°C in DMEM high glucose supplemented with 22 mM sodium bicarbonate and 1× penicillin/streptomycin, and then for 3 h by collagenase CLS2 (Worthington, Freehold, NJ, USA) 752 U/mL at 37°C in DMEM high glucose supplemented with 22 mM sodium bicarbonate, penicillin/streptomycin and 5% FBS. Released cells were then filtered through a 200 μm mesh (using an autoclaved 200 μm screen mounted on a screen cup, Sigma–Aldrich Canada, Oakville, Ontario), centrifuged (190 *g*, 10 min at 4°C) and filtered again through two serial 20 μm filters using a Swinnex filter holder (Fisher Scientific, Town of Mount-Royal, Quebec, Canada) containing a 20 μm Spectra/Mesh Nylon Macroporous Filter (Spectrum Laboratories, Rancho Dominguez, CA, USA). Cells were then washed three times in SFM without calcium, counted using a hemocytometer and seeded at low or high density in the different culture media and systems described below.

CELL CULTURE

Cells were cultured either in monolayer or in suspension. In monolayer, cells were suspended in 5 mL of culture media and seeded in 60 mm Petri dishes (internal diameter 54 mm, area 23 cm^2). For suspension culture, Petri dishes were previously coated with 2% SeaPlaque low-melting-temperature agarose (Mandel, St. Laurent, Quebec, Canada) in a 1:1 (v/v) mixture of calcium-free HAM's F12 and calcium-free DMEM low glucose or in DMEM low glucose, supplemented with 22 mM sodium bicarbonate, depending on which medium was to be subsequently used. In the SCM, and in 15 μM and 1 mM calcium-containing SFM, cells were seeded at both low density (10^4 cells/cm^2 hence 2.3×10^5 cells/dish) and high density (10^5 cells/cm^2 hence 2.3×10^6 cells/dish) for both monolayer and suspension cultures. To reduce the number of conditions to a manageable level, only low density cultures included the 50 μM calcium-containing SFM condition, in both monolayer and suspension cultures. Half of the media volume was changed every 2 days. In suspension culture, this was achieved by centrifuging cells (190 *g*, 10 min at 4°C) and removing 2.5 mL of the supernatant followed by addition of 2.5 mL of fresh media, that was then resuspended and transferred to the former Petri dish. Each culture condition was done in triplicate and for each result shown, at least one additional preliminary experiment confirmed the general trends of these results, supporting their reproducibility. At day 10, for two of the triplicates, cells were harvested with 1 or 0.5 mL Trizol® (Life technologies, Burlington, Ontario, Canada), depending on cell density,

and flash-frozen in liquid nitrogen before storage at −80°C. The remaining sample of each triplicate was fixed and permeabilized for immunocytochemistry as described below.

MICROSCOPIC OBSERVATIONS

Microscopic observation was performed each day with an inverted microscope (Axiovert S100TV, Carl Zeiss Canada, North York, Ontario, Canada), in Kohler transmission mode. Images were acquired with a digital camera and Northern Eclipse Software (Empix Imaging, Mississauga, Ontario, Canada).

AGGREGATION PERCENTAGE AND AGGREGATE SIZE

To assess cell aggregation in suspension culture, 15 digital photos were taken using random sampling with a 4× objective on days 1, 2, 5, 7 and 9. The fraction of cells in aggregates (% aggregation) and the number of cells per aggregate (aggregate size) were evaluated following the methods described by Martin *et al.*[25]. Briefly, images were calibrated with a hemocytometer, then thresholded and objects were analysed. Each object was fit to an ellipse and evaluated for its size and then identified as a single cell or as a cell aggregate. The number of cells in each aggregate (called aggregate size) was then evaluated by comparing the volume to the volume of a single cell. The percentage of cells in aggregates and single cells was then calculated. Aggregation percentage and average aggregate size were calculated as follows:

$$\text{Aggregation}\ (\%) = \frac{\text{Aggregated cells}}{\text{Total cells}} \times 100 \quad (1)$$

$$\text{Average aggregate size} = \frac{\text{Aggregated cells}}{\text{Number of aggregates}} \quad (2)$$

Since aggregates rapidly grew with time and became difficult to analyse when their size exceeded the frame of the digital photo, only results from day 1 are presented.

As medium was replaced every 2 days by centrifugation, pipetting and centrifugation of aggregates could have disturbed aggregation phenomena. Centrifugation did not seem to increase aggregation since aggregates were in the bottom 2.5 mL (out of 5 mL total) of the tube that was retained, but were still in solution and did not form a pellet. Pipetting did slightly disrupt aggregates, however, all samples were submitted to the same treatment and are comparable.

CELL VIABILITY

Cell viability was determined with 0.5 μM calcein AM and 1 μM ethidium homodimer-1 (LIVE/DEAD viability/cytoxicity kit, Molecular Probes, Eugene, OR, USA) in phosphate buffer saline (PBS). Samples were incubated for 30 min at 37°C after which green (live) or red (dead) cells were visualized with an inverted fluorescence microscope for cells in monolayer or with a confocal microscope for cells in suspension (Axiovert S100TV or Axioplan LSM 510 META, Carl Zeiss Canada, North York, Ontario, Canada).

CELL PROLIFERATION

Cell number was estimated at day 10 by DNA quantitation with PicoGreen® (Molecular Probes, Eugene, OR, USA). Aliquots (10 μL) of Trizol® extracts were diluted 1:10 in TE buffer (10 mM Tris−HCl, 1 mM EDTA, pH 7.5) to obtain a 100 μL final volume. PicoGreen® reagent (200 μL), previously diluted 200 times in TE buffer, was added to each 100 μL sample in a 96-well microplate immediately prior to reading with a Molecular Devices Gemini II fluorescence plate reader (Sunnyvale, CA, USA) at 480 nm excitation and 520 nm emission, with a 515 nm cutoff filter. Results were compared to a standard curve made with known cell numbers, with cells treated in an identical manner as the above samples. Fold doubling was calculated via

$$\text{Fold doubling} = \frac{\ln(X_t/X_0)}{\ln 2} \quad (3)$$

With X_0 = initial cell (DNA) content and X_t = final cell (DNA) content at 10 days.

GLYCOSAMINOGLYCAN RELEASE TO MEDIA

The dimethylmethylene blue (DMMB) assay was used[26] to quantify glycosaminoglycan (GAG) in the culture media. Media of 10 μL or 50 μL were mixed with 40 μL or 10 μL PBE (100 mM Na_2HPO_4, 5 mM EDTA pH 6.5), respectively. DMMB reagent (200 μL of 46 μM DMMB from Polyscience, Warrington, PA, USA, in 40 mM NaCl, 40 mM Glycine, pH 3.0) was then added and absorbance at 525 nm was read and compared to that of chondroitin sulfate C standards (Sigma−Aldrich Canada, Oakville, Ontario) containing the same amount of PBE and culture medium as the samples.

mRNA ISOLATION, REVERSE TRANSCRIPTION AND REAL-TIME PCR

Total RNA was isolated in 1 or 0.5 mL Trizol®, following the manufacturer's protocol (Life technologies, Burlington, Ontario, Canada). RNA was then quantified with the Quant-IT® RNA assay kit (Molecular Probes, Eugene, OR, USA) also according to the manufacturer's recommendations. Reverse transcription was performed with 0.05 μg/μL oligodT and 20 U/μL moloney murine leukemia virus reverse transcriptase, in the presence of 500 μM dNTP, 0.625 U/μL RNAse inhibitor and buffer 5× supplied with the enzyme (all reagents from Pharmacia-Amersham, Baie d'Urfée, Quebec, Canada). First, 5 μL of RNA was denatured for 15 min at 75°C, then the oligodT was added and allowed to anneal to RNA for 3 min on ice. The other reagents (details above) were then added and the reaction proceeded for 1 h at 37°C. Reverse transcriptase was inhibited by heating for 5 min at 94°C, and the reverse transcription products diluted 5× in water.

Real-Time polymerase chain reaction (PCR) occurred in the Rotor-Gene 3000 (Corbett Research, Mortlake, Australia) using SybrGreen to quantify cDNAs produced from collagen type I and collagen type II mRNA. Primers (purchased from BioCorp Montreal, Quebec, Canada) for bovine type II collagen (PUBMED accession number X02420) were 5′-GAA CCC AGA ACC AAC ACA ATC C-3′ (forward) and 5′-TCT GCC CAG TTC AGG TCT CTT AGA GA-3′ (reverse) while those for bovine type I collagen (PUBMED accession number S64596) were 5′-TGG CCC AGA AGA ACT GGT-3′ (forward) and 5′-AGG AAG GTC AGC TGG ATG-3′ (reverse). The optimized PCR mix consisted of 2 or 4 μL of cDNA, 1× reaction buffer, 200 μM dNTP, 200 nM of each primer, 2.5 mM $MgCl_2$, 0.3 × SybrGreen (from 10,000×, Molecular Probes, Eugene,

OR, USA) and 0.05 U/μL JumpStart Taq Polymerase (Sigma–Aldrich Canada, Oakville, Ontario). The PCR began with a 5 min denaturation step at 94°C, followed by 7 cycles with a touchdown between 60°C and 53°C (20 s at 95°C; 20 s at 60°C with touchdown in 1°C intervals to 53°C; 30 s at 72°C, 10 s at 82°C) and then 40 cycles of classic PCR (20 s at 95°C; 20 s at 57°C; 30 s at 72°C; 10 s at 82°C). A fluorescence reading was performed for each cycle at 72°C and at 82°C, where the latter was chosen to eliminate signal from primer-dimers[27]. The run ended by an increase in temperature from 72°C to 99°C to obtain the melting curve.

Absolute mRNA abundance was found using PCR kinetics described by

$$\mathrm{Nf} = \mathrm{Ni}(1+E)^{n} \tag{4}$$

where Nf = final copy number ∝ final fluorescence, Ni = initial copy number ∝ initial fluorescence, E = Efficiency and n = cycle number. By setting the cycle number equal to the threshold cycle number, $n = C_T$, we find

$$\mathrm{Ni} \propto \text{Theoretical initial fluorescence} = \frac{\text{Threshold fluorescence}}{(E+1)^{C_T}} \tag{5}$$

The Threshold fluorescence was set to 0.01 fluorescence units (FU) and the Efficiency (E) was calculated by the software from Eq. (4) and amplicon specific fluorescence readings at 82°C[27]. We then calculated mRNA abundance relative to total RNA found for each sample from Quant-IT (in μg/mL) according to:

$$\text{Abundance}(\mathrm{FU}/(\mu\mathrm{g}/\mathrm{ml})) = \frac{\text{Theoretical initial fluorescence}}{\text{total RNA}} \tag{6}$$

IMMUNOSTAINING AND CONFOCAL LASER SCANNING MICROSCOPY

All steps were performed at room temperature unless otherwise mentioned. Cells in monolayer or in suspension were first washed in modified hank's balanced salt solution (mHBSS) (HBSS from Life technologies, Burlington, Ontario, Canada, supplemented with 2 mM $MgCl_2$, 2 mM EGTA, 5 mM MES (2-[N-morpholino]ethanesulfonic acid), 4 mM $NaHCO_3$, pH 6.5), and then fixed/permeabilized in 0.5% w/v glutaraldehyde and 0.3% w/v triton X100 for 10 min, treated against autofluorescence with $NaBH_4$ (2.5 mg/mL, 2 × 10 min on ice) and digested with 200 U/mL chondroitinase ABC and 400 U/mL keratanase (Seikagaku America Inc., East Falmouth, MA, USA) in tris buffered saline-BSA (100 mM Tris, 100 mM NaCl, 0.01% BSA, pH 7.4) for 16 h at 37°C. For monolayers, the above incubations took place directly in the Petri dish while cells in suspension (mostly aggregates) were transferred onto a nylon membrane filter (0.45 μm pore size Whatman, Clifton, NJ, USA) mounted in a centrifuge tube and the above solutions were added onto the filter retaining the cells, and then removed by centrifugation (800 *g*, 1 min). Samples were then stained for actin with Alexa-488 phalloidin (Molecular Probes, Eugene, OR, USA), diluted 1:1000 in mHBSS for 1 h and for the nucleus with 0.5 μg/μL Hoechst 33258 (Molecular Probes, Eugene, OR, USA) in mHBSS for 10 min. Each step was followed by three washes of 5 min in mHBSS. Finally, samples were treated against quenching with 11,700 U/mL beef catalase and 133.3 U/mL glucose oxydase (both from Fluka, Buchs, Switzerland) in mHBSS, 30 min and mounted in 16.67% (w/v) Mowiol 4–88 (Fluka, Buchs, Switzerland), 33.3% (v/v) glycerol, 0.75% (w/v) *n*-propyl gallat (Sigma–Aldrich Canada, Oakville, Ontario) in mHBSS. Confocal-imaging was performed using an Apochromat 40×/NA 1.2 water immersion objective mounted on an Axioplan 2 microscope equipped with an LSM 510 META confocal laser scanning module (all from Carl Zeiss). Alexa 488 was excited with the 488 nm line from an argon laser and Hoechst 33258 was illuminated with two-photon excitation wavelength using a mode-locked pulsed Ti-saphire laser (VerdiV10/Mira 900 from Coherent Inc., Santa Clara, CA, USA) operating at 780 nm. Images were recorded using a BP 510/520 IR bandpass filter for Alexa 488 a BP 435–485 IR bandpass filter for Hoechst 33258.

STATISTICAL ANALYSIS

Statistical analysis was performed with STATISTICA 6.1 (StatSoft Inc., Tulsa, OK, USA). The effect of calcium and serum was determined by the general linear model (GLM), with culture system (suspension or monolayer) and/or inoculation density (high or low) and/or serum (absence or presence) as categorical predictors and with calcium as a continuous predictor.

Results

SERUM IN THE MEDIUM PROMOTES FIBROBLASTIC MORPHOLOGY, PHENOTYPE LOSS AND CELL AGGREGATION

SCM showed similar cell growth compared to SFM

During a 10-day culture in SCM in monolayer with half of the medium volume changed every 2 days, chondrocytes divided 5.3 times and 2.2 times at low and high density, respectively [Fig. 1(B and D)] while in SFM–1 mM Ca^{2+} chondrocytes divided 4.2 and 1.8 times at low and high density, respectively. In suspension the tendency was similar as growth was slightly better in the SCM [Fig. 1(A and C)]. Growth differences between monolayer and suspension culture were not statistically different, while growth was significantly higher at low density compared to high density cultures ($P < 0.05$).

SCM promoted a fibroblastic morphology

In the SCM, cells cultured at low density were elongated and spindle-shaped [Fig. 2(A)] with only a few round cells remaining. In the SFM–1 mM Ca^{2+}, cells were less elongated, and exhibited a "cobblestone"-morphology [Fig. 2(B)]. On the contrary, when cultured at high density, more cells were round, whether serum was present or absent [Fig. 2(E–G)]. Staining of the actin cytoskeleton revealed that, at low density with or without serum numerous stress fibers appeared [Fig. 3(A and B)] whereas at high density, stress fibers were mainly present with serum [Fig. 3(E and F)].

SCM promoted cell aggregation

In suspension, chondrocytes rapidly aggregated. After 9 days, aggregates contained several thousand cells (Fig. 4).

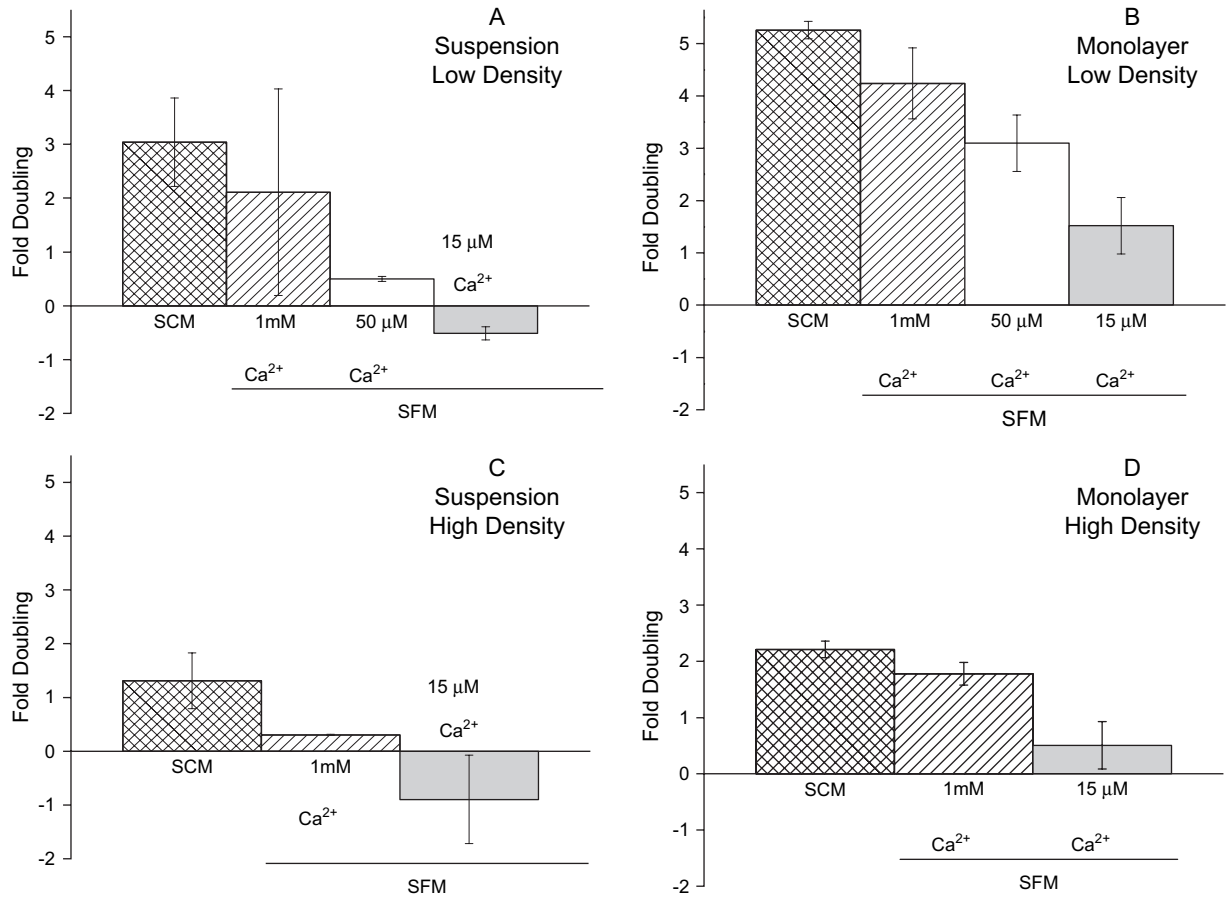

Fig. 1. Fold cell doubling after 10 days, determined by comparing inoculation density and cell density after 10 days of culture, by DNA quantitation. Cells were cultured at low density in suspension (A) or in monolayer (B) and at high density in suspension (C) or in monolayer (D). Culture media used included an SCM or an SFM supplemented with different calcium contents indicated on the abscissa. Negative results indicate net cell mortality. Results are mean for $n = 2$ with error bars representing duplicates.

In the SCM, at low or high density, aggregates were large and dense exhibiting a smooth well-defined contour. On the contrary, aggregates in the SFM−1 mM Ca^{2+} were looser with a more granulated contour [Fig. 4(B−D vs A)]. At low density after 9 days in SCM, 99.4% of the cells were in aggregates as compared to 93.7% in the SFM−1 mM Ca^{2+} (Table I). Average aggregate size was of 462 vs 44 cells, respectively, in SCM and SFM−1 mM Ca^{2+} at low density (Table I). Analysis of the aggregates by confocal microscopy showed that, in SCM, at low or high density, the cells seemed to be well-separated [Fig. 5(A and E)] with few cells in direct contact. Also, cells that were peripherally located in aggregates appeared elongated in SCM ([Fig. 5(A)], arrow). In the SFM, on the contrary, cells were tightly packed, showed evidence of cell−cell contacts and were without elongated cells at the periphery [Fig. 5(B and F)].

SCM increased collagen type I mRNA expression ($P < 0.05$)

When cultured in monolayer, at high or low density in the SCM, collagen type I and type II mRNA abundance were similar [Fig. 6(B and D)]. In low density monolayers collagen types II and I were 1.01 and 1.38, respectively (arbitrary FU normalized to total RNA), and at high density collagen types II and I were 1.23 and 0.87, respectively. In the SFM−1 mM Ca^{2+}, the collagen type I expression was greatly inhibited and fell to 0.04 and 0.15 at low and high density vs 1.01 and 1.38 in SCM whereas collagen type II expression remained almost identical. Thus, SFM promoted maintenance of the chondrocyte phenotype by suppressing collagen I expression. In suspension at both high and low density a similar preferential inhibition of collagen type I expression vs type II by SFM was seen [Fig. 6(A and C)]. It can also be noted that collagen type I expression was in general lower in suspension vs monolayer.

Collagen type II and type I expression were also assessed in freshly isolated, non-expanded chondrocytes and were found to be 8.45 and 0.018, respectively. This type II expression level was far greater than after 10 days in culture in any of our culture conditions, probably due to the response of chondrocytes to synthesize a pericellular matrix just post-isolation. Nonetheless the collagen type I to type II expression ratio can be used to compare expanded to non-expanded chondrocytes. By comparing this ratio it was found that samples cultured in suspension in the SFM−1 mM Ca^{2+} have the lowest type I to type II ratio of 0.02 and 0.004 for low and high density, respectively, that compares favorably with 0.0022 for non-expanded chondrocytes.

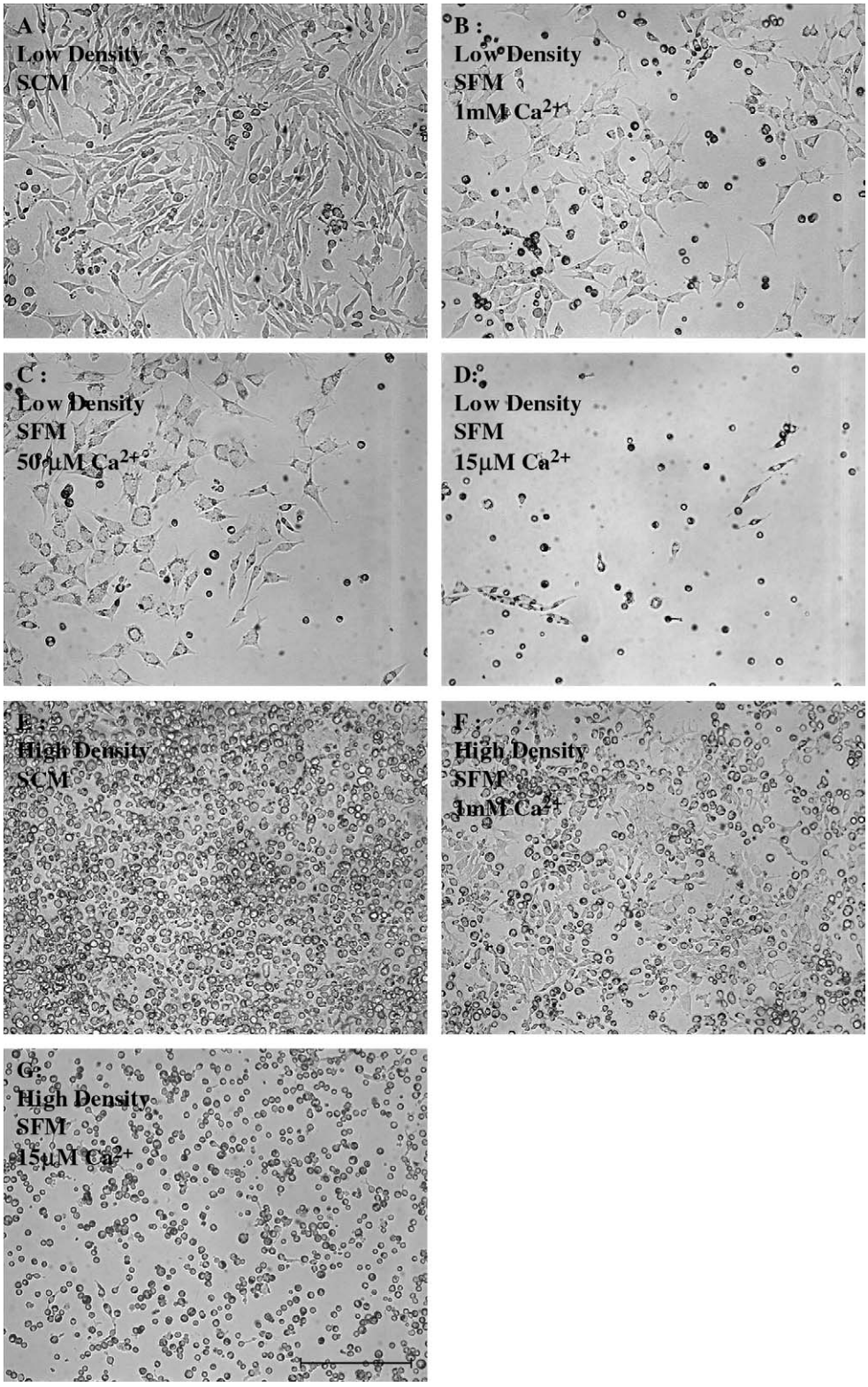

Fig. 2. Morphology of chondrocytes in monolayer at day 7. Cells were inoculated at low density (A, B, C, D) or high density (E, F, G) either in an SCM (A, E) or in an SFM containing 1 mM (B, F), 50 μM (C), 15 μM (D, G) of calcium. Plating density, presence of serum and calcium content affect cell morphology. Scale bar = 250 μm.

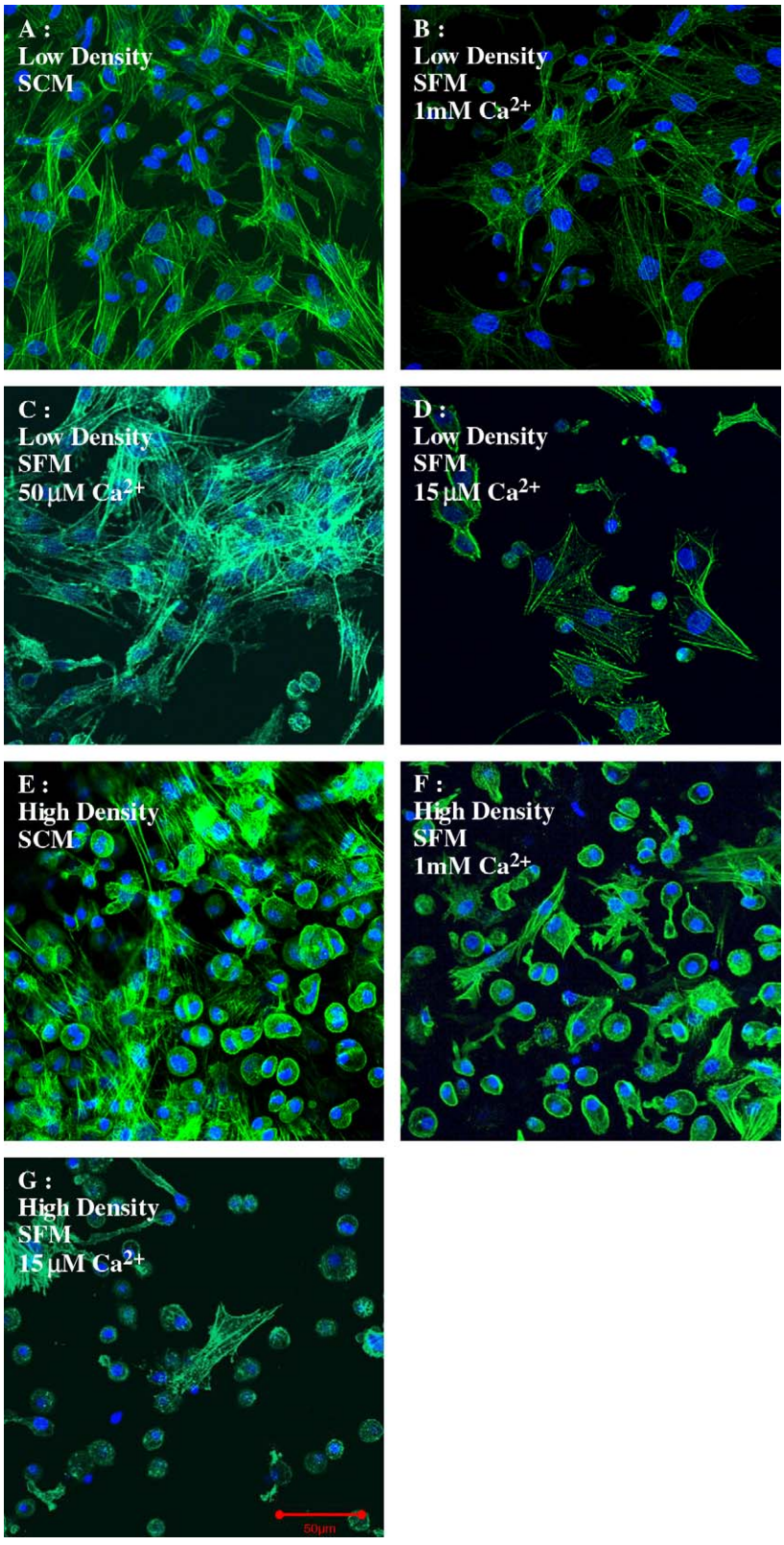

Fig. 3. Chondrocytes in monolayer inoculated at low or high density were stained for actin (green) and the nucleus (blue) at day 10. With SCM, SFM−1 mM and 50 μM Ca^{2+}, most cells displayed stress fibers. In contrast, at 15 μM Ca^{2+}, cells presented far fewer stress fibers. Scale bar = 50 μm.

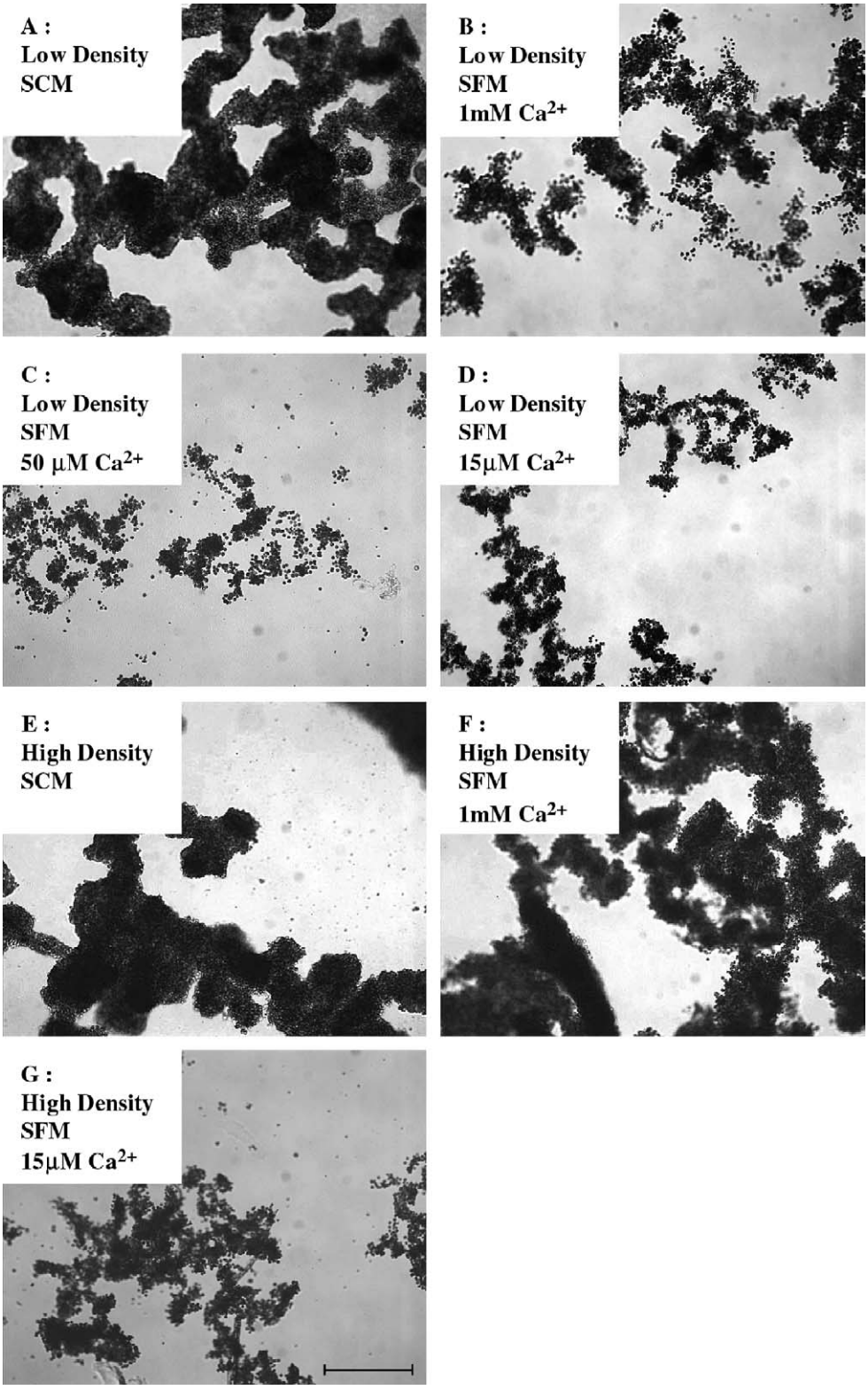

Fig. 4. Cell aggregation in suspension after 9 days. Cells were inoculated at low density (A, B, C, D) or at high density (E, F, G) in SCM (A, E) or in an SFM containing 1 mM (B, F), 50 μM (C) or 15 μM (D, G) of calcium. In the SCM, large and tightly bound aggregates were present. In the SFM, aggregates were smaller and more loosely bound. Scale bar = 500 μm.

Table I

Aggregation and average aggregate size at low inoculation density. Aggregation percentage, average aggregate size, after 1 day of culture at low density in suspension. Aggregation is greater in the SCM, as the aggregation percentage is slightly higher and the average aggregate size is 10 times greater than in SFMs. On the contrary, aggregation percentage and average aggregate size do not change for different calcium concentrations in SFM. Results are mean ± standard deviation, $n = 3$

	SFM−15 μM Ca^{2+}	SFM−50 μM	SFM−1 mM Ca^{2+}	SCM
Aggregation (%)	96.3 ± 3.1	96.4 ± 2.0	93.7 ± 1.2*	99.4 ± 0.4*
Average aggregate size (cells)	41.7 ± 17.9	43.8 ± 26.4	44.0 ± 20.9	462 ± 287

*Aggregation (%) was significantly different in the presence and absence of serum ($P < 0.05$) using GLM (see Material and methods) with serum as categorical predictor.

LOW EXTRACELLULAR CALCIUM REDUCED CHONDROCYTE PROLIFERATION AND GAG PRODUCTION

In monolayer at low and high density, growth was about three times lower in the SFM−15 μM Ca^{2+} than in the 1 mM Ca^{2+} [Fig. 1(B and D)]. This effect was even stronger in suspension, where at low density growth fell from 2.1 doublings/10 days in SFM−1 mM Ca^{2+} to 0.5 in the SFM−50 μM Ca^{2+} [Fig. 1(A)]. At yet lower calcium concentration (15 μM) in suspension there was no growth and even cell death [Fig. 1(A)]. A similar inhibition of cell division was observed at high density in suspension as cells grew poorly in the SFM−1 mM Ca^{2+} (0.3 doubling/10 days) and died in the SFM−15 μM Ca^{2+} [Fig. 1(C)]. At both low and high density proliferation was found to be statistically dependent on calcium concentration ($P < 0.05$).

Lowering calcium concentration reduced GAG production. GAG production and release to media by chondrocytes was evaluated between days 8 and 10 (Table II). In the SFM−1 mM Ca^{2+}, when cells were cultured at high density GAG production was 30.8 ± 1.8 and 26.7 ± 0.4 μg/2 days/million cells, in monolayer and suspension, respectively, and was significantly higher on a per cell basis than in SCM. Lowering calcium to 15 μM reduced GAG production by 2.3 times at high density in monolayer and in suspension. At low cell density, GAG production was no longer detectable in the SFM−15 μM Ca^{2+}.

LOW EXTRACELLULAR CALCIUM DOES NOT IMPEDE CELL AGGREGATION IN SUSPENSION CULTURE

For the entire range of 1 mM down to 15 μM Ca^{2+} in SFM at low cell density, more than 90% of the cells were present in aggregates on day 1 (Table I) and average aggregate sizes were between 30 and 45 cells. Actin staining showed that in SFM−1 mM Ca^{2+} aggregated cells were round and tightly packed [Fig. 5(B and F)]. Images suggested cell−cell contacts as actin cytoskeleton of adjacent cells seemed to be interconnected. Cell aggregates displayed a similar global morphology for the entire range of 1 mM down to 15 μM Ca^{2+}, at low or high inoculation density [Fig. 5 (B−D, F and G)].

LOW EXTRACELLULAR CALCIUM INHIBITED CELL SPREADING IN MONOLAYER

In the presence of 1 mM Ca^{2+}, cells adhered to the Petri dish and spread [Fig. 2(B and F)]. Round cells were also present, and significantly more so at high density [Fig. 2(E)]. In 50 μM Ca^{2+} a similar spread morphology was observed [Fig. 2(C)], however cells were less elongated and were more rectangular than at 1 mM Ca^{2+}. In 15 μM Ca^{2+} this morphological shift was amplified, at both low and high cell density, where many cells were round, attached to the dish but for the most part did not spread [Fig. 2(D and G)]. When observing the actin cytoskeleton, in SFM−1 mM Ca^{2+}, numerous stress fibers were seen to expand in several directions [Fig. 3(B and F)]. When calcium concentration was further reduced to 15 μM, cells also exhibited stress fibers but to a lesser extent and in one direction only [Fig. 3(D and G)]. Moreover, many cells did not develop stress fibers and remained round with a diffuse actin distribution.

LOW EXTRACELLULAR CALCIUM REDUCED COLLAGEN TYPE I EXPRESSION BUT NOT COLLAGEN TYPE II EXPRESSION

Collagen type I expression depended significantly on medium calcium concentration at low density ($P < 0.05$) (Fig. 6). For example, in monolayer, lowering the calcium concentration from 1 mM to 50 μM had a drastic effect on the collagen type I expression which fell from 0.035 to 0.0013, whereas collagen type II expression remained relatively stable at low density [Fig. 6(B)]. A similar tendency was observed in suspension at low density [Fig. 6(A)] and in monolayer at high density [Fig. 6(D)]. Thus, reduced calcium levels decreased collagen type I expression to bring the type I to type II ratio closer to that obtained for non-expanded chondrocytes.

Discussion

One challenge in chondrocyte culture is to obtain high levels of growth without loss of cell phenotype, both to retain phenotype for physiological studies as well as to maximize chondrogenic potential when primary chondrocytes are used. In our study, we examined the effect of low calcium concentrations on chondrocyte growth, phenotype and morphology when cultured at low and high density, both in suspension and in monolayer culture. We hypothesized that low calcium levels in medium would promote collagen type II expression over type I and promote a chondrocytic (round) cell morphology in monolayer culture. These hypotheses were confirmed by our results (Figs. 2, 3 and 6). We also hypothesized that reduced calcium concentration would inhibit cell aggregation in suspension. This hypothesis was not supported by our data, however, removing serum from the media did have the unexpected effect of reducing aggregate size (Table I and Figs. 4 and 5).

Lowering calcium concentrations promoted chondrocyte phenotype. Collagen type I expression was decreased in the presence of low calcium concentration whereas collagen type II expression was unaffected (Fig. 6). This reduced dedifferentiation by lowering calcium could be partly due to lower growth as dedifferentiation and proliferation are related. However, our results indicate that slow growth is not the only parameter maintaining phenotype. For example,

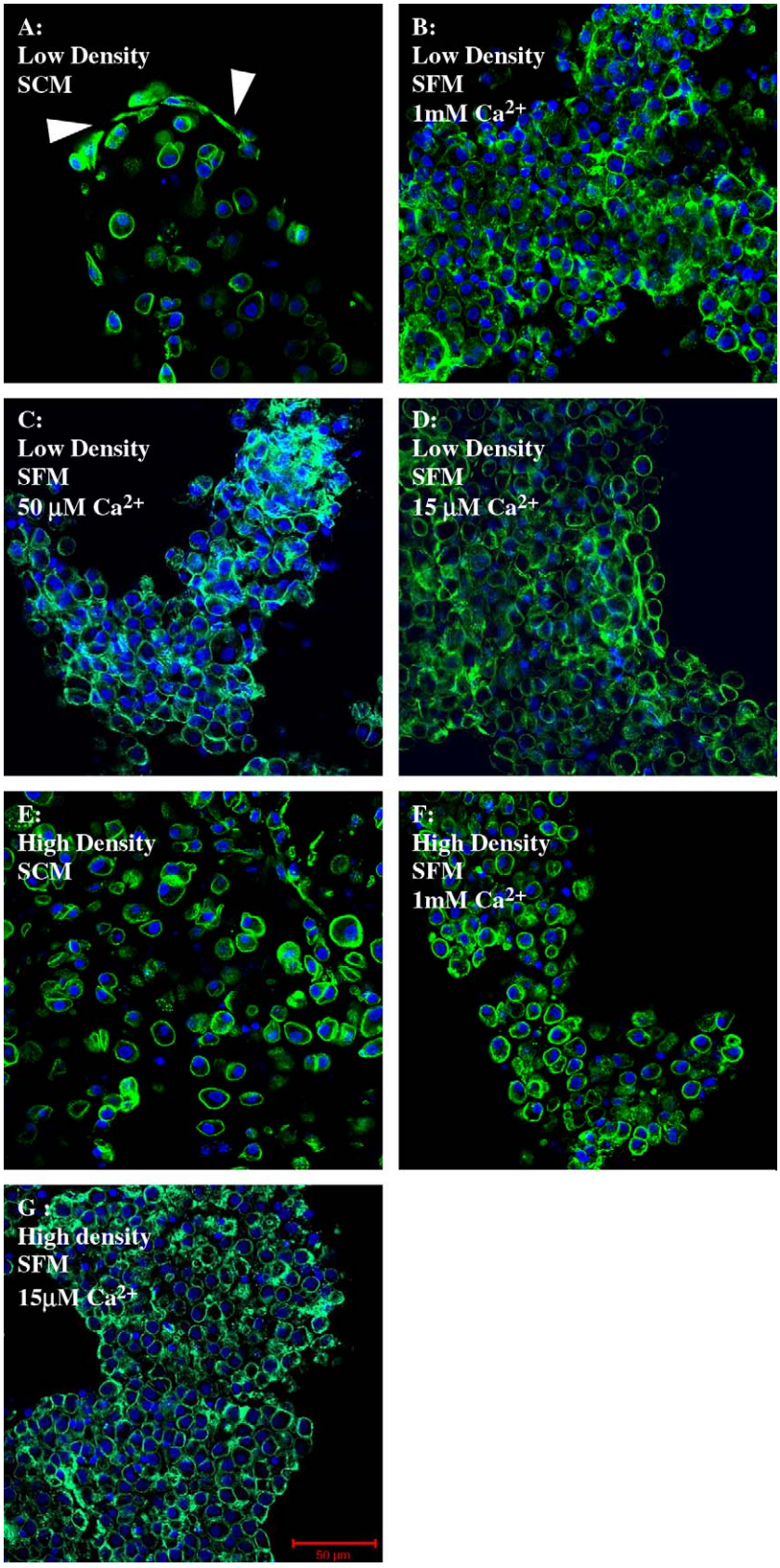

Fig. 5. Chondrocytes in suspension inoculated at low or high density on day 10 were stained for actin (green) and the nucleus (blue). In the SCM, aggregated cells were not tightly packed indicating the presence of intervening ECM. On the contrary in the SFM, cells were tightly packed most likely forming cell–cell contacts. No major differences appeared in the different SFM. Scale bar = 50 µM. Arrowheads in A show elongated cells at the aggregate periphery.

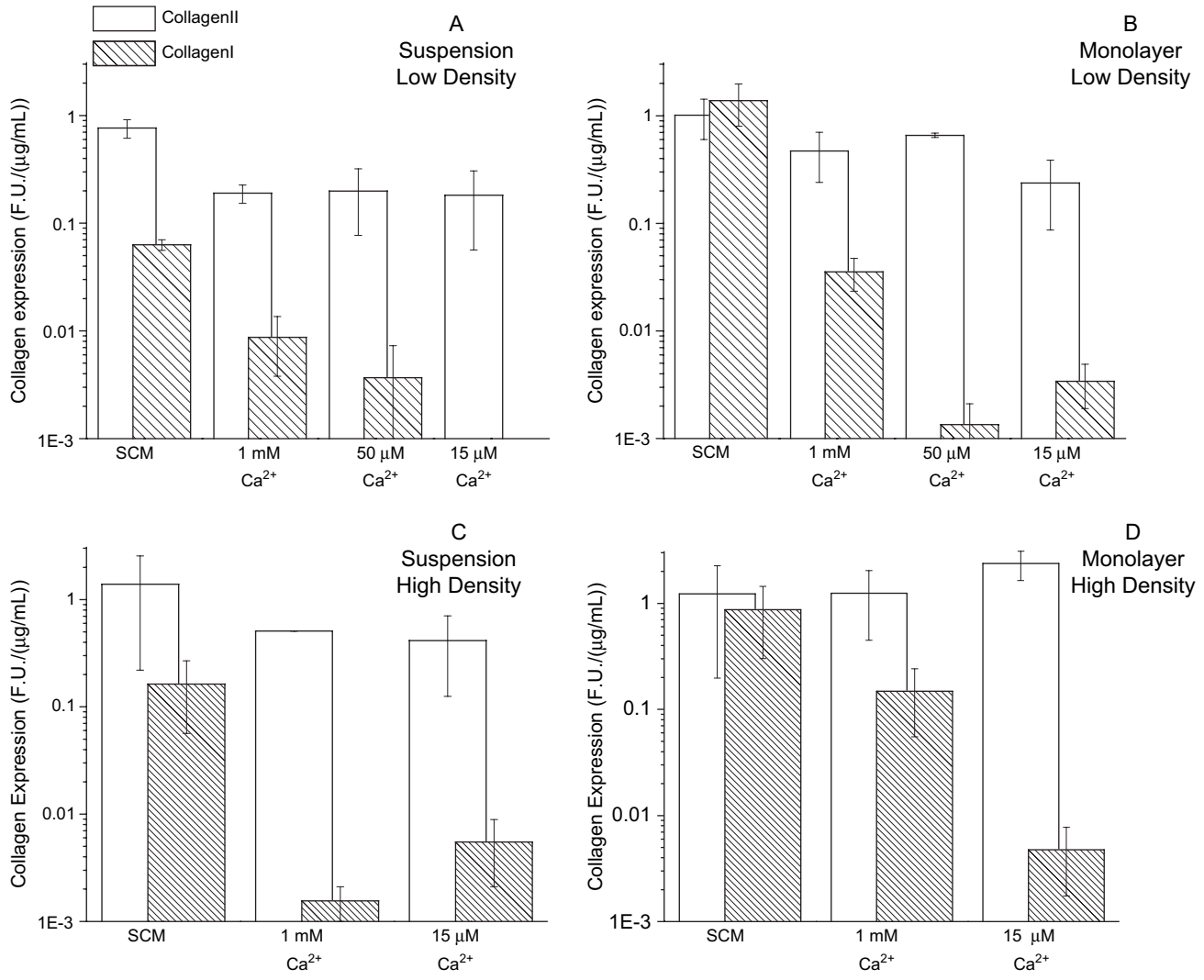

Fig. 6. mRNA abundance for collagen type II and collagen type I (Eq. (6) in Methods) were evaluated by quantitative Reverse-Transcription PCR. Cells were inoculated at low density (A, B) or high density (C, D) and cultured in suspension (A, C) or in monolayer (B, D) in SCM or in SFM supplemented with different calcium concentrations. The absence of serum greatly reduced collagen type I expression. Decreasing medium calcium also generally decreased collagen type I expression. On the contrary, collagen type II expression remained quite stable. Results are mean for $n = 2$, with error bars representing duplicates.

in the SFM−50 µM Ca^{2+} at low density, growth was higher than at high density in the SCM and SFM−1 mM Ca^{2+} but phenotype maintenance was better (ratio was 0.002 vs 1.08 and 0.11). Deshmukh *et al.*[20] also found that collagen type II became predominant over collagen type I when calcium supplementation in culture medium (DMEM with no calcium but containing 10% FCS) was decreased from 1 mM to 10 µM. However, since collagen types II and I synthesis was

Table II

GAG production at low and high inoculation density, in both monolayer and suspension culture. GAG released to medium between days 8 and 10 for cultures inoculated at high and low density. For low density cultures, GAG in medium was not detectable below 1 mM of calcium. GAG production per cell was higher at high density in SFM at 1 mM calcium compared to SCM, however lower calcium concentrations than 1 mM in SFM appeared to reduce GAG production per cell. Results are mean ± standard deviation, n = 3

GAG production (µg/(2 days × 10^6 cells))	High density			Low density		
	SFM−15 µM	SFM−1 mM	SCM	SFM−15 µM	SFM−1 mM	SCM
Monolayer	5.8 ± 2.3†	30.8 ± 1.8*,†	21.0 ± 5.6*	ND	6.7 ± 5.2*	15.1 ± 5.1*
Suspension	8.0 ± 4.8†	26.7 ± 0.4*,†	24.6 ± 9.2*	ND	4.3 ± 5.3*	28.3 ± 19.9

*GAG production was significantly different in the absence or presence of serum ($P < 0.05$, with serum, culture system and density as categorical predictor).

†GAG production was significantly different depending on calcium concentration ($P < 0.05$, with culture system as categorical predictor and calcium as continuous predictor).

expressed as a relative proportion than absolute levels, it was not clear from this study if collagen type II was stimulated or collagen type I expression was inhibited at low calcium levels. Chang *et al.*[14] saw an inhibition of collagen type II expression for concentrations higher than 4.2 mM $CaCl_2$, for concentrations ranging from 0.4 to >6 mM, but observed no effect below 0.4 mM, also in accordance with our observations. On the contrary, Koyano *et al.*[18] found that collagen type II expression was higher at low (<0.9 mM) and high (>4 mM) calcium concentrations but lower at intermediate concentration (1.8 mM) for fetal bovine chondrocytes where calcium concentrations ranged from 0.1 to 10 mM. These latter contradictory results may arise from use of fetal cells, in which case existing literature and our data concord that low calcium concentrations enhance or stabilize collagen type II expression. Our results are the first, however, to demonstrate a clear reduction in collagen type I expression due to reduced calcium concentrations.

Imaging of the chondrocyte actin network allowed us to relate reduced cell spreading in low calcium media to a rounded morphology and promotion of the chondrocyte phenotype. Previously, inhibition of spreading and promotion of a rounded morphology has been achieved by 3D culture[28,29] or by the use of dihydrocytochalasin B[30,31] or by high density culture in monolayer[32,33]. Moreover Mallein-Gerein *et al.*[31] also showed that type II collagen synthesis was coincident with faint actin architecture (i.e., non-spreading cells) and type I collagen with the presence of large actin filaments (i.e., spread cells) consistent with our observations (Fig. 3). Different calcium concentrations could also be sensed by CaR, which are known to be expressed by chondrocytes[15]. Since we found that low calcium decreased collagen type I expression in suspension as well [Fig. 6(A and C)], it would seem that both adhesion-dependent and direct sensing mechanisms could be operating. Calcium is, thus, a critical parameter that must be controlled in order to attain desired growth and differentiation properties for chondrocytes.

Aggregate size was reduced when serum was removed although the fraction of cells in aggregates was similar in SFM vs SCM (Fig. 4, Table I). In suspension, aggregates were significantly larger with serum (Table I) and also contained important intercellular matrix [Fig. 5(A and E)]. On the contrary, cells in SFM were tightly packed and established cell–cell contacts in suspension [Fig. 5(B–D, F and G)]. Related observations have been made with chick embryo dedifferentiated pre-chondrogenic cells[7], where removing serum inhibited cell aggregation seen after 6 h of suspension culture. The addition of fibronectin restored cell aggregation. These authors proposed that aggregate formation is mediated by fibronectin, and then reinforced by cell–cell contacts via N-CAM (neural-cell adhesion molecule) and N-cadherin after which synthesis of extracellular matrix (ECM) by cells fills the intercellular space[34]. In our study, the absence of fibronectin in SFM may have slowed down the aggregation process but did not inhibit it. Nonetheless smaller aggregates found in SFM are beneficial in facilitating nutrient transfer and creating a more homogeneous culture environment.

Aggregate size was not affected by extracellular calcium. This result was surprising as chondrocytes are known to express N-cadherins (calcium-dependent adhesion molecules) which play a major role in chondrocyte condensation[7,34–36]. We expected that lowering calcium would have disrupted cadherin function resulting in lower levels of aggregation. Moreover, in a preliminary experiment we found that addition of EGTA to chelate all the calcium did reduce aggregation (data not shown). However, our minimum calcium concentration of 15 μM appears to be sufficient to allow chondrocyte condensation and this process is probably not solely calcium-dependent[13].

One limitation of our study is the use of chondrocytes of only one age (young) and one species (bovine). For example, it has been shown that fetal chondrocytes are more responsive to calcium changes than adult chondrocytes[18]. Also, our phenotype analysis relied on absolute mRNA rather than protein levels of collagen types I and II. However, since this phenotype analysis based on mRNA levels correlated well with expected morphological changes in chondrocytes, we speculate that mRNA levels reflect well the actual phenotype.

Conclusion

Our study revealed that removing serum and controlling calcium concentration allow for better control of chondrocyte phenotype and aggregation behavior in monolayer and suspension culture. We found superior alternatives to currently used culture media, depending on the desired culture properties. If proliferation is required, monolayer culture inoculated at low density provides the highest growth rate and the use of SFM containing 1 mM down to 50 μM of calcium enables significant cell doubling (four vs five doubling in SCM over 10 days) and a greatly stabilized cell phenotype (10 times less collagen type I mRNA in SFM vs SCM). For physiological studies, suspension culture at high or low density more closely approximates the *in situ* microenvironment of chondrocytes and removal of serum allows for reasonable growth with negligible collagen I expression and smaller, more loosely bound cell aggregates that more closely *approximate* a homogeneous culture system. These results may be useful to proliferate chondrocytes with minimal loss of phenotype and to perform basic studies on chondrocytes in a homogeneous suspension culture environment.

Acknowledgments

Support for this study was provided by a grant from the Canadian Institutes of Health Research and by the Natural Sciences and Engineering Research Council of Canada. The authors wish also to acknowledge Antoine Blanc, Nicolas Tran-Khanh, Dr Caroline Hoemann and Julie Deschênes for technical assistance, Dr Sylvie Mader for allowing us to use the Rotor-Gene and Christian Dallaire for calcium analysis.

References

1. Elima K, Vuorio E. Expression of mRNAs for collagens and other matrix components in dedifferentiating and redifferentiating human chondrocytes in culture. FEBS Lett 1989;258(2):195–8.
2. Sailor LZ, Hewick RM, Morris EA. Recombinant human bone morphogenetic protein-2 maintains the articular chondrocyte phenotype in long-term culture. J Orthop Res 1996;14(6):937–45.
3. Kimura T, Yasui N, Ohsawa S, Ono K. Chondrocytes embedded in collagen gels maintain cartilage phenotype during long-term cultures. Clin Orthop Relat Res 1984;186:231–9.

4. 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.
5. Tamponnet C, Ramdi H, Guyot JB, Lievremont M. Rabbit articular chondrocytes in alginate gel: characterisation of immobilized preparations and potential applications. Appl Microbiol Biotechnol 1992;37:311–5.
6. Hauselmann HJ, Fernandes RJ, Mok SS, Schmid TM, Block JA, Aydelotte MB, *et al*. Phenotypic stability of bovine articular chondrocytes after long-term culture in alginate beads. J Cell Sci 1994;107:17–27.
7. Tavella S, Bellese G, Castagnola P, Martin I, Piccini D, Doliana R, *et al*. Regulated expression of fibronectin, laminin and related integrin receptors during the early chondrocyte differentiation. J Cell Sci 1997;110: 2261–70.
8. Tacchetti C, Quarto R, Nitsch L, Hartmann DJ, Cancedda R. *In vitro* morphogenesis of chick embryo hypertrophic cartilage. J Cell Biol 1987;105:999–1006.
9. Mermelstein CS, Rebello MI, Amaral LM, Costa ML. Changes in cell shape, cytoskeletal proteins and adhesion sites of cultured cells after extracellular Ca^{2+} chelation. Braz J Med Biol Res 2003;36: 1111–6.
10. Sigurdson SL, Lwebuga-Mukasa JS. Divalent cation-dependent regulation of rat alveolar epithelial cell adhesion and spreading. Exp Cell Res 1994;213:71–9.
11. Schwartz MA. Spreading of human endothelial cells on fibronectin or vitronectin triggers elevation of intracellular free calcium. J Cell Biol 1993;120:1003–10.
12. Peshwa M, Kyung Y-S, McClure DB, Hu W-S. Cultivation of mammalian cells as aggregates in bioreactors: effect of calcium concentration on spatial distribution of viability. Biotechnol Bioeng 1993;41:179–87.
13. Bee JA, von der Mark K. An analysis of chick limb bud intercellular adhesion underlying the establishment of cartilage aggregates in suspension culture. J Cell Sci 1990;96:527–36.
14. Chang W, Tu C, Bajra R, Komuves L, Miller S, Strewler G, *et al*. Calcium sensing in cultured chondrogenic RCJ3.1C5.18 cells. Endocrinology 1999;140:1911–9.
15. Chang W, Tu C, Chen TH, Komuves L, Oda Y, Pratt SA, *et al*. Expression and signal transduction of calcium-sensing receptors in cartilage and bone. Endocrinology 1999;140:5883–93.
16. Chang W, Tu C, Pratt S, Chen TH, Shoback D. Extracellular $Ca(^{2+})$-sensing receptors modulate matrix production and mineralization in chondrogenic RCJ3.1C5.18 cells. Endocrinology 2002;143:1467–74.
17. Bonen DK, Schmid TM. Elevated extracellular calcium concentrations induce type X collagen synthesis in chondrocyte cultures. J Cell Biol 1991;115:1171–8.
18. Koyano Y, Hejna M, Flechtenmacher J, Schmid TM, Thonar EJ, Mollenhauer J. Collagen and proteoglycan production by bovine fetal and adult chondrocytes under low levels of calcium and zinc ions. Connect Tissue Res 1996;34:213–25.
19. Deshmukh K, Kline WH. Characterization of collagen and its precursors synthesized by rabbit-articular-cartilage cells in various culture systems. Eur J Biochem 1976;69:117–23.
20. Deshmukh K, Kline WG, Sawyer BD. Role of calcium in the phenotypic expression of rabbit articular chondrocytes in culture. FEBS Lett 1976;67:48–51.
21. Hargest TE, Gay CV, Schraer H, Wasserman AJ. Vertical distribution of elements in cells and matrix of epiphyseal growth plate cartilage determined by quantitative electron probe analysis. J Histochem Cytochem 1985;33:275–86.
22. Freshney IR. Culture of Animal Cells a Manual of Basic Technique. 4th edn. New York: Wiley Liss 2000.
23. Buschmann MD, Gluzband YA, Grodzinsky AJ, Hunziker EB. Mechanical compression modulates matrix biosynthesis in chondrocyte/agarose culture. J Cell Sci 1995;108:1497–508.
24. Cancedda R, Dozin B. Inventors; Consorzio per la Gestione del Centro di Biotecnologie Avanzate, Genova, Italia, Istituto Nazionale per la Ricerca sul Cancro, Geneva, Italia, Applicants. Serum free medium for chondrocyte-like cells. International patent WO 00/27996, May 18, 2000.
25. Martin I, Dozin B, Quarto R, Cancedda R, Beltrame F. Computer-based technique for cell aggregation analysis and cell aggregation in *in vitro* chondrogenesis. Cytometry 1997;28:141–6.
26. Farndale RW, Buttle DJ, Barrett AJ. Improved quantitation and discrimination of sulphated glycosaminoglycans by use of dimethylmethylene blue. Biochim Biophys Acta 1986;883:173–7.
27. Yin JL, Shackel NA, Zekry A, McGuinness PH, Richards C, Putten KV, *et al*. Real-time reverse transcriptase-polymerase chain reaction (RT-PCR) for measurement of cytokine and growth factor mRNA expression with fluorogenic probes or SYBR Green I. Immunol Cell Biol 2001;79:213–21.
28. Benya PD, Shaffer JD. Dedifferentiated chondrocytes reexpress the differentiated collagen phenotype when cultured in agarose gels. Cell 1982;30:215–24.
29. 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.
30. Benya PD, Brown PD, Padilla SR. Microfilament modification by dihydrocytochalasin B causes retinoic acid-modulated chondrocytes to reexpress the differentiated collagen phenotype without a change in shape. J Cell Biol 1988;106:161–70.
31. Mallein-Gerin F, Garrone R, van der Rest M. Proteoglycan and collagen synthesis are correlated with actin organization in dedifferentiating chondrocytes. Eur J Cell Biol 1991;56:364–73.
32. Kuettner KE, Memoli VA, Pauli BU, Wrobel NC, Thonar EJ, Daniel JC. Synthesis of cartilage matrix by mammalian chondrocytes *in vitro*. II. Maintenance of collagen and proteoglycan phenotype. J Cell Biol 1982;93:751–7.
33. Watt FM, Dudhia J. Prolonged expression of differentiated phenotype by chondrocytes cultured at low density on a composite substrate of collagen and agarose that restricts cell spreading. Differentiation 1988;38:140–7.
34. Tavella S, Raffo P, Tacchetti C, Cancedda R, Castagnola P. N-CAM and N-cadherin expression during *in vitro* chondrogenesis. Exp Cell Res 1994; 215:354–62.
35. Oberlender SA, Tuan RS. Spatiotemporal profile of N-cadherin expression in the developing limb mesenchyme. Cell Adhes Commun 1994;2: 521–37.
36. Delise AM, Tuan RS. Analysis of N-cadherin function in limb mesenchymal chondrogenesis *in vitro*. Dev Dyn 2002;225:195–204.