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# High density micromass cultures of a human chondrocyte cell line: A reliable assay system to reveal the modulatory functions of pharmacological agents

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ABSTRACT

Osteoarthritis is a highly prevalent and disabling disease for which we do not have a cure. The identification of suitable molecular targets is hindered by the lack of standardized, reproducible and convenient screening assays. Following extensive comparisons of a number of chondrocytic cell lines, culture conditions, and readouts, we have optimized an assay utilizing C-28/I2, a chondrocytic cell line cultured in high-density micromasses. Utilizing molecules with known effects on cartilage (e.g. IL-1β, TGFβ1, BMP-2), we have exploited this improved protocol to (i) evoke responses characteristic of primary chondrocytes; (ii) assess the pharmacodynamics of gene over-expression using non-viral expression vectors; (iii) establish the response profiles of known pharmacological treatments; and (iv) investigate their mechanisms of action. These data indicate that we have established a *medium-throughput* methodology for studying chondrocyte-specific cellular and molecular responses (from gene expression to rapid quantitative measurement of sulfated glycosaminoglycans by Alcian blue staining) that may enable the discovery of novel therapeutics for pharmacological modulation of chondrocyte activation in osteoarthritis.



## 1. Introduction

Osteoarthritis (OA) is a leading cause of disability worldwide, affecting up to 2/3 of the population over 50 years of age. With incidence rates increasing with age and higher life expectancies, OA economic and social burden is ever increasing [1]. To date, a cure for osteoarthritis is not yet available though it is accepted that chondroprotection and cartilage regeneration would represent a successful strategy, if applied early enough, and chondrocytes present an attractive target for therapeutic intervention.

Chondrocytes are responsible for the synthesis and balance of the extracellular matrix (ECM) that confers tensile strength and flexibility to articular surfaces. ECM is formed by several specific macromolecules including collagen type II [2], aggrecan core protein coated with highly sulfated glycosaminoglycans (GAG) and several other smaller structural and non-structural proteins [3]. Chondrocytes are responsible for maintaining the homeostasis of cartilage and of the ECM through a low turnover state of equilibrium between synthetic activity and catabolic remodeling mediated by specific enzymes including aggrecanases (ADAMTS family) and matrix metalloproteinases (MMPs) [4]. A loss of this homeostatic equilibrium results in the destruction of articular cartilage, which is characteristic of OA [5]. With an ever-increasing understanding of the molecular processes involved in cartilage degradation and the availability of large collections of small biologically active compounds and/or suitable macromolecules, the development of high/medium throughput assays for the identification of therapeutic molecules for chondroprotection is a priority in modern medicine. Primary cultures of adult human articular chondrocytes (AHAC) have several shortcomings for screening assays. Firstly, sample availability often represents a problem and, furthermore, there is an issue linked to donor variability that might reflect genetic factors, co-morbidity, lifestyle and more. Secondly, within the same donor there is a large variability deriving from the donor site, and also from OA being a focal disease. Thirdly, culture expansion of primary AHACs is associated with a loss of phenotype and biological behavior introducing, on the one hand, a further element of variability and, on the other, further complexity in obtaining a sufficient number of authentic chondrocytes suitable for screening purposes [6]. Finally,

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primary AHACs are difficult to transfect with sufficient efficiency. The recent availability of immortalized human chondrocytic cell lines [7] represents a potential solution to some of these problems. Following comparison of different cell lines and culture conditions, we establish C-28/I2 cell line in micromass cultures [8] as optimal to test the ability of different modalities of therapeutic intervention (from drugs to gene delivery) to influence chondrocyte metabolism.

## 2. Material and methods

### 2.1. Monolayer and micromass cultures of chondrocytic cell lines

The chondrosarcoma cell lines: JJO12 (kindly provided by Dr. Joel Block, Rush Medical College, Rush-Presbyterian-St Luke's Medical Center, Chicago, IL) [9] and H-EM-SS (extraskeletal myeloid chondrosarcoma; European Collection of Animal Cell Cultures; Salisbury, UK); and the immortalized C-28/I2 cell line [8] were selected for study and compared using the same culture conditions.

Cells were cultured in Dulbecco's modified Eagle's medium (DMEM)/Ham's F-12 (1:1; Gibco-Invitrogen, Eggenstein, Germany) supplemented with 10% fetal calf serum (FCS; Gibco-Invitrogen), 100 units/mL penicillin, and 50 μg/mL streptomycin (Omega Scientific, Tarzana, CA) (growth medium) and maintained in the presence of 5% $CO_2$ in air. The medium was changed every 3 days. Cells were cultured in monolayer ($2 \times 10^4/cm^2$), grown to sub-confluence (around 80% confluent) and passaged at a ratio of 1:8.

For micromass cultures, the protocol described by De Bari et al. [10] using human periosteum-derived cells was employed, with some modifications. Briefly, confluent monolayer cultures of chondrocyte cell lines were released by trypsin-EDTA, tested for viability by trypan blue exclusion, and re-suspended in growth medium at a density of $2.5 \times 10^7$ viable cells/mL. Micromasses were obtained by pipetting 20 μL of cell suspension into individual wells of 24-well plates. Following a 3-h attachment period without medium, the growth medium was gently added and cultures left resting for a further 24 h. The medium was then changed to serum-free and phenol red-free medium (Gibco BRL) for 24 h (Supplementary data Fig. 1). Differentiation was promoted by serum starvation and ITS supplementation as described [11,12]. On day 3 of the culture, fresh differentiation medium was added and treatments were performed as described in the results. After 48 h, some of the micromasses were harvested for Alcian blue matrix staining and others for quantitative reverse transcription-polymerase chain reaction (qRT-PCR) gene expression analysis of marker genes for chondrocytes: type II collagen alpha-1 (*COL2A1*), aggrecan (*ACAN*), sex determining region (SRY)-box 9 (*SOX9*), matrix metalloproteinase-1 and -13 (*MMP1*, *MMP13*), and a disintegrin and metalloproteinase with a thrombospondin type 1 motif 5 (*ADAMTS5*), as described in the section 2.6.

### 2.2. Cell proliferation assay

Cells were counted with a Neubauer hemacytometer and plated at different densities in phenol red- and serum-free medium. The top cell density was $8 \times 10^4$, and subsequent serial dilutions (1:2, 1:4 and 1:8 1:16) were made; thus, various concentrations of cells were then seeded in triplicate in a 48-well plate (400 μL $well^{-1}$) and incubated in 5% $CO_2$ at 37 °C. After an initial 4 h period to allow cell attachment, 40 μL of alamar blue solution (Alamar, Sacramento, CA) was directly added to the medium resulting in a final concentration of 10% and the cell were returned to incubation for 24 h. The absorbance, presented as mean optical density (O.D.), of test and control wells was read after 24 h at 570 and 595 nm with a standard spectrophotometer [Spectronic 2000 spectrophotometer (Bausch & Lomb, Rochester, NY)].

### 2.3. Cartilage harvest and primary adult human articular chondrocyte (AHAC) isolation

AHACs were obtained with informed consent from patients who underwent joint replacement for knee OA. Cartilage samples were provided by Mr P. Achan (Barts and the London National Health Service Trust, London, UK). All procedures were approved by the East London and The City Research Ethics Committee 3. Cartilage tissue was dissected from preserved areas of the femoral condyles and the patellar groove, as recently reported [13]. Briefly, cartilage was sliced full thickness, excluding the mineralized cartilage and the subchondral bone and initially washed twice in high-glucose DMEM (DMEM/F-12 1:1 plus GlutaMax; Invitrogen, Eggenstein, Germany) containing 10% FBS, 1 mM sodium pyruvate and 2% antibiotic antimycotic solution (Invitrogen, Eggenstein, Germany). Chondrocytes were isolated from the cartilage samples by enzymatic digestion with 1 mg/mL pronase (Roche, Welwyn, UK) for 30 min at 37 °C and then overnight at 37 °C with 1 mg/mL collagenase P (Roche, Welwyn, UK) prepared in complete medium (same composition as above, with 1% antibiotic/antimycotic solution) under agitation. The AHACs recovered from the digestion were then resuspended in complete media, assessed for cell viability using trypan blue, and seeded at a density of 10,000 cells/$cm^2$. The cells were cultured at 37 °C in a humidified atmosphere containing 5% $CO_2$. Only cells obtained from samples with a histological Mankin score <4 for features of OA were used for subsequent experiments [14,15]. All experiments were performed using freshly isolated or confluent P0 cells, cultured either in monolayer or micromasses.

### 2.4. Alcian blue (AB) staining in vitro

For semi-quantitation of cartilage-specific sulfated glycosaminoglycans, we used a protocol optimized by De Bari et al. [16], based on staining with AB 8GS at pH 0.2, which is highly specific for cartilage ECM [10,16]. Micromasses were rinsed twice with PBS, fixed with 4% glutaraldehyde solution (v/v in distilled water) for 15 min at room temperature (RT), washed with 200 μL of 0.1 N HCl (Sigma, St. Louis, MO, USA) solution in distilled water, and covered with AB dye at pH < 1 (1% Alcian blue 8 GS in 0.1 N HCl; Carl Roth, Karlsruhe, Germany) at RT. Cultures were then washed extensively with distilled water, prior to extraction with 200 μL of 6 M guanidine HCl (Sigma–Aldrich, St. Louis, Mo, USA) overnight at RT. The optical density (OD) of the extracted dye was measured at 630 nm using a Spectronic 2000 spectrophotometer (Bausch & Lomb, Rochester, NY, USA), and OD values were interpolated with an AB standard curve (from 625 to 9.76 μg/mL). Protein content for each sample was determined using the BCA Protein Assay Kit (Pierce, Rockford, IL, USA). Initially, the amount of extracted AB was normalized with protein content and expressed as AB/protein (μg/μg), such as in the experiments where cell lines and primary AHAC were compared (Supplementary data Fig. 2). In all other assays, GAG accumulation was measured by AB quantification and normalized to DNA content (μg/μg), as described below.

### 2.5. DNA quantification

DNA content was measured by fluorescence using SYBR Green I dye (Invitrogen, Paisley, UK). For each micromass sample, DNA concentration was assessed against a DNA standard curve [dsDNA for Standard Curve–Lambda DNA (Invitrogen, Paisley, UK)] after preparation in an assay solution [10 mM Tris–HCl, 1 mM EDTA, pH8, containing 1% (v/v) of SYBR Green I dye]. DNA samples were diluted (1:50), homogenized with the assay solution (1:20), measured at 485/535 nm by using a spectrophotometer