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CARTILAGE

Generation of human immortalized chondrocytes from osteoarthritic and healthy cartilage

A NEW TOOL FOR CARTILAGE PATHOPHYSIOLOGY STUDIES

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Aims

After a few passages of in vitro culture, primary human articular chondrocytes undergo senescence and loss of their phenotype. Most of the available chondrocyte cell lines have been obtained from cartilage tissues different from diarthrodial joints, and their utility for osteoarthritis (OA) research is reduced. Thus, the goal of this research was the development of immortalized chondrocyte cell lines proceeded from the articular cartilage of patients with and without OA.

Methods

Using telomerase reverse transcriptase (hTERT) and SV40 large T antigen (SV40LT), we transduced primary OA articular chondrocytes. Proliferative capacity, degree of senescence, and chondrocyte surface antigen expression in transduced chondrocytes were evaluated. In addition, the capacity of transduced chondrocytes to synthesize a tissue similar to cartilage and to respond to interleukin (IL)-1β was assessed.

Results

Coexpression of both transgenes (SV40 and hTERT) were observed in the nuclei of transduced chondrocytes. Generated chondrocyte cell lines showed a high proliferation capacity and less than 2% of senescent cells. These cell lines were able to form 3D aggregates analogous to those generated by primary articular chondrocytes, but were unsuccessful in synthesizing cartilage-like tissue when seeded on type I collagen sponges. However, generated chondrocyte cell lines maintained the potential to respond to IL-1β stimulation.

Conclusion

Through SV40LT and hTERT transduction, we successfully immortalized chondrocytes. These immortalized chondrocytes were able to overcome senescence in vitro, but were incapable of synthesizing cartilage-like tissue under the experimental conditions. Nonetheless, these chondrocyte cell lines could be advantageous for OA investigation since, similarly to primary articular chondrocytes, they showed capacity to upregulate inflammatory mediators in response to the IL-1β cytokine.

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Article focus

- Primary human articular chondrocytes tend to senesce and lose their phenotype when cultured in vitro.
- Available chondrocyte cell lines that have been generated from cartilage sources different from synovial joints have limited use for osteoarthritis (OA) research.
- For these reasons, we aimed to generate immortalized chondrocyte cell lines

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derived from the articular cartilage of patients with and without OA.

Key messages

- Transduction of articular chondrocytes with SV40 large T antigen and telomerase reverse transcriptase allows them to overcome senescence in vitro.
- Immortalized articular chondrocytes upregulate inflammatory mediators interleukin (IL)-6, IL-8, and cyclooxygenase-2 when stimulated with IL-1β in a similar way to primary articular chondrocytes.
- However, immortalized articular chondrocytes were unable to form cartilage-like tissue under the tested conditions.

Strengths and limitations

- The chondrocyte cell lines we generated are derived from articular cartilage, show high proliferation rates, and do not have special in vitro culture requirements. Most importantly, these immortalized chondrocytes can activate inflammation in response to IL-1β.
- Nevertheless, the principal limitation of this study is that immortalized chondrocytes did not show the anabolic capacities that are characteristic of primary articular chondrocytes.

Introduction

Articular chondrocytes and the cells they arise from, the chondroprogenitor cells (CPCs),[1] are the only cell types present in articular cartilage.[2,3] These cells account for only 1% to 2% of the volume of this tissue, and their functions are to maintain homeostasis and produce the extracellular matrix (ECM). Articular cartilage ECM is mainly composed of collagen and proteoglycans, and is responsible for the biomechanical properties of this tissue.[4,5] Given its lack of vascularization, its low cellularity, and the thickness of its ECM, the self-repair capacity of articular cartilage is very limited. Cartilage defects, like those derived from trauma, may progress to further deterioration, causing joint pain and disability.[6,7] Due to the increase in life expectancy of the global population, the incidence of cartilage diseases such as osteoarthritis (OA) is rapidly rising.[8]

OA is a chronic degenerative and inflammatory joint disease, involving mainly the weightbearing joints, caused by the degradation of cartilage matrix, the death of chondrocytes, and the formation of osteophytes.[9,10] These processes are initiated by injuries that activate maladaptive repair responses, including pro-inflammatory pathways of innate immunity. These pro-inflammatory stimuli induce a homeostasis imbalance in the cartilage, with predominant catabolic activity. Pro-inflammatory cytokines such as interleukin (IL)-1β are overexpressed in early osteoarthritic cartilage, and stimulate the catabolic activities of synovial cells and chondrocytes. IL-1β induces the expression of genes such as IL-6, IL-8, and prostaglandin E2, an enzymatic product of cyclooxygenase 2 (COX2) which represses ECM production and promotes cartilage degradation.[11]

Table I. Characteristics of the donors from which articular cartilage samples were obtained.

Donor number	Sex	Age, yrs	Pathology
1	Male	88	Hip fracture, OA (grade III)
2	Male	81	Hip fracture (healthy cartilage)
3	Female	88	Hip fracture, OA (grade I)
4	Male	89	Hip fracture, OA (grade I)
5	Female	69	Hip fracture (healthy cartilage)
6	Female	82	Hip fracture, OA (grade I)

OA, osteoarthritis.

In terms of its cellular component, osteoarthritic cartilage is also characterized by a higher proportion of CPCs than normal cartilage,[2,12] but these CPCs show reduced proliferation rates and increased telomere erosion in diseased tissue.[12] Senescent chondrocytes and CPCs secrete more inflammatory cytokines and metalloproteinases (MMPs), contributing to the progressive degradation of the osteoarthritic joint.[12,13] CPCs can be distinguished from mature chondrocytes by the expression of several cell surface antigens such as CD90, CD105, and CD166. In addition, CPCs from OA cartilage express CD9 along with CD90 and CD106, and these $CD9^+/CD90^+/CD166^+$ cells have certain differentiation plasticity.[14]

One of the hindrances for OA research employing in vitro models with patient-derived cells is that human articular chondrocytes have limited proliferative capacity and tend to undergo dedifferentiation during in vitro expansion. Dedifferentiated chondrocytes acquire fibroblast-like morphology and reduce the expression of articular cartilage markers, such as type II collagen and aggrecan.[15,16] Several immortalized chondrocyte cell lines (such as TC28a2, C28/I2, C-20/A4, SW-1353, and CHON-001) have been developed in an attempt to overcome this problem. However, these cell lines have not been generated from articular cartilage,[17] and are thus not ideal for articular cartilage pathophysiology research. In addition, these cell lines are known to be less sensitive than primary articular chondrocytes to inflammatory stimuli,[18] which is relevant when investigating OA pathology. Although a few chondrocyte cell lines were created from articular cartilage, they have associated drawbacks such as the requirement of unusual cell culture temperature for immortalization transgene expression,[19,20] lack of telomerase activity,[19-21] and/or low proliferation rates due to incomplete immortalization.[22,23] For these reasons, the aim of this study was the generation of two cell lines derived from articular chondrocytes from one patient with OA and one donor without OA.

Table II. Isotype controls and antibodies conjugated with fluorescein isothiocyanate, phycoerythrin, or phycoerythrin/Cy5 used for flow cytometry.

Antibody	Specificity	Clone	Source	Dilution
FITC mouse IgG1 isotype control	N/A	MOPC-21	BD Pharmingen	1:50
PE mouse IgG1 isotype control	N/A	MOPC-21	BD Pharmingen	1:50
PECy5 mouse IgG1 isotype control	N/A	1F8	Abcam	2:25
FITC mouse anti-human CD9	Human TSPAN-29	M-L13	BD Pharmingen	1:10
FITC mouse anti-human CD44	HCAM	IM7	BD Pharmingen	1:50
PE mouse anti-human CD54	ICAM1	15.2	Sigma-Aldrich	3:25
PECy5 mouse anti-human CD90	Thy-1	5E10	Immunostep	1:50
FITC mouse anti-human CD105	Human ENG	SN6	AbD Serotec	1:25
PE mouse anti-human CD106	VCAM	STA	AbD Serotec	2:25
PE mouse anti-human CD146	MCAM	P1H12	BD Pharmingen	1:10
PE mouse anti-human CD166	ALCAM	3A6	Immunostep	3:50

ALCAM, activated leucocyte cell adhesion molecule; ENG, endoglin; FITC, fluorescein isothiocyanate; HCAM, homing cellular adhesion molecule; ICAM1, intracellular adhesion molecule 1; MCAM, melanoma cell adhesion molecule; N/A, not applicable; PE, phycoerythrin; Thy-1, thymocyte differentiation antigen 1; TSPAN-29, tetraspanin-29; VCAM, vascular cell adhesion molecule 1.

Methods

The present study was reviewed and approved by the Research Ethics Committee of A Coruña-Ferrol, Spain (2016/588). Articular cartilage samples were obtained from the Collection for Research on Rheumatic Diseases, authorized by the Galician Research Ethics Committee (2013/107), and inscribed in the National Biobanks Registry with reference number C.0000424. All donors gave written informed consent. We have previously reported most of the methods described below in the "Materials and Methods" section from the Doctoral Thesis of Piñeiro-Ramil M.[24]

Isolation and culture of articular chondrocytes. Samples of articular cartilage were collected from six patients who underwent orthopaedic surgery and gave written informed consent (aged 69 to 89 years old, three males and three females) (Table I). Articular cartilage was sliced into small pieces and subjected to enzymatic digestion with 0.25% trypsin (Gibco, Thermo Fisher Scientific, USA) in Dulbecco's Modified Eagle's medium (DMEM; Lonza, Spain) with 1% penicillin/streptomycin (P/S, Gibco) for ten minutes and with 2 mg/ml type IV collagenase (Sigma-Aldrich, USA) in DMEM with 5% foetal bovine serum (FBS, Gibco) overnight at 37°C in agitation.[24] The isolated chondrocytes were filtered through a 100 µm-pore filter (Costar Corning, USA), centrifuged at 430× g for ten minutes, resuspended in DMEM with 10% FBS and 1% P/S (10% FBS/DMEM), and plated in adherent culture dishes (Costar Corning). Cell subculture was performed with 0.1% trypsin-ethylenediaminetetraacetic acid (EDTA) (Gibco).

Retrovirus production and cell transduction. Primary articular chondrocytes (first–second passage) from donors 1 and 2 were transduced by spinoculation with retrovirus produced by Phoenix Amphotropic (ATCC CRL-3213) cells (φNX-A)[25] as described by Piñeiro-Ramil et al.[26] For retrovirus production, φNX-A cells were transfected using two plasmids obtained from Addgene: pBABE-puro-SV40LT (Addgene plasmid #13970), deposited by Thomas Roberts,[27] and pBABE-hygro-eGFP-hTERT (Addgene plasmid #28169), deposited by Kathleen Collins.[28] Primary chondrocytes were transduced firstly with SV40 large T antigen (SV40LT), and SV40LT-transduced chondrocytes were subsequently transduced with telomerase reverse transcriptase (hTERT).

Briefly, plasmid DNA was diluted in with Opti-MEM (Gibco) with X-tremeGENE HP DNA Transfection Reagent (Roche, Sigma-Aldrich) and added to φNX-A cells. After incubation at 32°C for 48 hours, retroviral supernatants were collected, filtered using a 0.45 µm filter (Millipore, USA), combined with hexadimethrine bromide (Sigma-Aldrich), and added to chondrocytes previously seeded on six-well culture dishes (Costar Corning). Chondrocytes were then subjected to spinoculation at 800× g and 32°C for 45 minutes, and 2 mM valproic acid (Cayman Chemical Company, USA) was added to induce transgene expression. Selection of transduced chondrocytes was performed with 2.5 µg/ml puromycin (Thermo Fisher Scientific) and 75 µg/ml hygromycin (AMRESCO; VWR International).

Immunofluorescence assay. Transduced articular chondrocytes were cultured in eight-well chamber slides (Millipore) to test the expression of SV40LT and hTERT. Cells were washed with phosphate-buffered saline (PBS; Dako, Agilent Technologies Spain, Spain), fixed with 4% paraformaldehyde, permeabilized with 0.5% Triton X-100, and blocked with 4% bovine serum albumin (all from Sigma-Aldrich). Subsequent incubation with two primary antibodies, mouse anti-SV40LT (SV40LT clone Pab 108; 1:100; Santa Cruz Biotechnology, USA) and rabbit anti-green fluorescent protein (GFP) labelled with Alexa Fluor 488 dye (A-21311; 1:500; Invitrogen), was performed at 4°C overnight.

After incubation with primary antibodies, cells were washed three times with PBS and incubated with a goat anti-mouse secondary antibody labelled with Alexa Fluor 594 dye (A-11032; 1:1,000; Thermo Fisher Scientific) at room temperature for one hour. After three additional washes in PBS, a two-minute incubation with Hoechst (bisBenzimide H 33342 trihydrochloride, Sigma-Aldrich) was performed. Slides were mounted

Table III. Studied genes and respective primers employed for quantitative real-time polymerase chain reaction analysis.

Gene	Forward primer 5'→3'	Reverse primer 5'→3'
YWHAZ	GATCCCCAATGCTTCACAAG	TGCTTGTTGTGACTGATCGAC
Homo sapiens IL-6	AGTCCTGATCCAGTTCCTGC	CATTTGTGGTTGGGTCAGGG
Homo sapiens IL-8	CTCCAAACCTTTCCACCCCA	TTCTCCACAACCCTCTGCAC
Homo sapiens COX2	AGGAGGTCTTTGGTCTGGTG	ACTGCTCATCACCCCATTCA
MMP13	AACGCCAGACAAATGTGACC	AGGTCATGAGAAGGGTGCTC
ACAN	CGGTCTACCTCTACCCTAACCA	GAGAAGGAACCGCTGAAATG
SRY-SOX9	GTACCCGCACTTGCACAAC	TCGCTCTCGTTCAGAAGTCTC
COL2	TGGTGCTAATGGCGAGAAG	CCCAGTCTCTCCACGTTCAC

ACAN, aggrecan; COL2A1, type II collagen alpha chain 1; COX2, cyclooxygenase-2; IL, interleukin; MMP13, matrix metalloproteinase 13; SRY SOX9, sex-determining region Y-box 9; YWHAZ, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta.

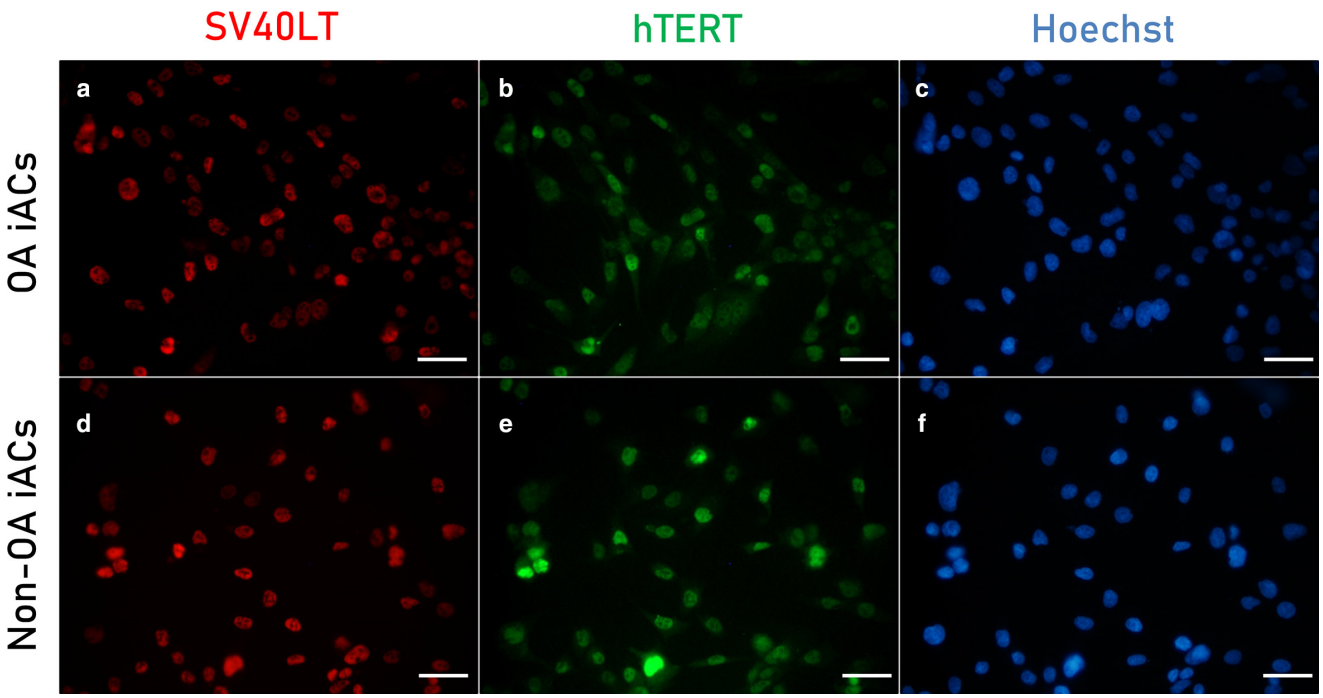

Fig. 1

SV40 large T antigen (SV40LT) and eGFP-telomerase reverse transcriptase (hTERT) immunostaining and Hoechst staining of a), b), and c) osteoarthritic (OA) and d), e), and f) non-OA immortalized articular chondrocytes (iACs). SV40LT is shown in red, eGFP-hTERT is shown in green, and Hoechst staining is shown in blue. Scale bar: 50 µm.

with Glycergel aqueous mounting medium (Dako) and observed using an Olympus BX61 fluorescence microscope (Olympus Iberia, Spain) coupled to an Olympus DP70 digital camera (Olympus Iberia). Fluorescence micrographs were obtained employing the cellSens Dimension software (Olympus Iberia).

Proliferation analysis. The formula in Equation 1 was used to calculate the proliferation of the transduced cells as cumulative population doublings (PDs), where Nf is the final cell number, Ni is the initial cell number, and log is the natural logarithm.[29] Generation time was calculated for each cell line at each passage as the number of PDs per day. Proliferation rates of both cell lines were analyzed by regression.

$$PD = \frac{\log Nf - \log Ni}{\log 2}$$

Senescence associated β-galactosidase assay. Cytochemical staining for senescence associated β-galactosidase activity (SA-β-Gal) was performed for each cell line at three different passages, using the Senescence Cells Histochemical Staining kit (Sigma-Aldrich). After 16 hours of incubation, cells were observed and photographed with a Nikon Eclipse TS100 inverted microscope (Nikon Instruments Europe, Netherlands) coupled to a XM Full HD digital camera (Hangzhou Xiongmai Technologies, China). SA-β-Gal-positive and negative cells were counted on ten random microscope fields, and percentage of senescent cells was calculated. Results were provided as mean percentage of senescent cells and standard error (SE). Primary articular chondrocytes (from donors 4 and 5)

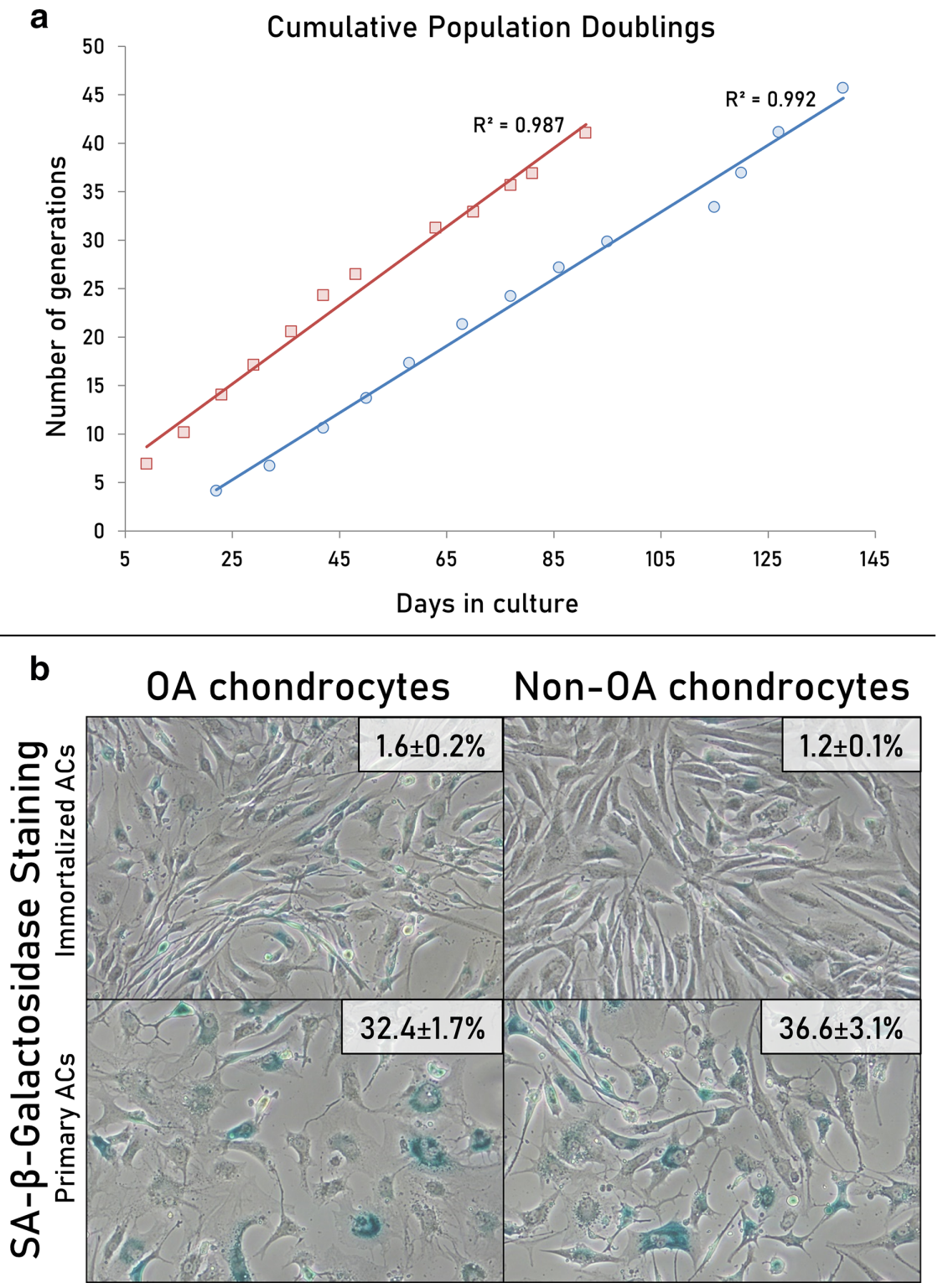

Fig. 2

a) Chart showing the number of population doublings (PDs) accumulated by osteoarthritic (OA) (blue line) and non-OA (red line) immortalized articular chondrocytes (iACs) against days in culture. PDs were calculated as (log Nf – log Ni)/log 2 (where Nf is the final cell population, Ni is the number of cells in the inoculum, and log is the natural logarithm). b) Phase contrast microscopic images of the senescence-associated β-galactosidase (SA-β-Gal) stained primary and immortalized articular chondrocytes. Percentage of SA-β-Gal stained cells for each sample is shown in panels. Magnification: 10×.

Table IV. Percentage of positive cells for the surface markers CD9, CD44, CD54, CD90, CD105, CD106, CD146, and CD166 in osteoarthritic and non-osteoarthritic immortalized articular chondrocyte populations.

Surface marker	Percentage of positive cells	
	OA iACs	Non-OA iACs
CD9	94.2	32.5
CD44	95.6	96.8
CD54	98.0	96.0
CD90	96.4	98.6
CD105	45.5	32.9
CD106	41.5	45.8
CD146	31.4	60.7
CD166	94.0	95.4

iAC, immortalized articular chondrocyte; OA, osteoarthritis.

were employed as a control and compared with transduced articular chondrocytes.

Flow cytometric analysis. Flow cytometry was used to analyze the expression surface markers characteristic of articular chondrocytes and CPCs from OA and non-OA articular cartilage in transduced articular chondrocytes. Cells were split with 0.1% trypsin-EDTA, washed twice in fluorescence activated cell Ssrting (FACS) buffer (BD Biosciences, Spain), and incubated at 4°C for 45 minutes with fluorescent-labelled antibodies and isotype controls listed in Table II. After incubation, cells were washed, resuspended in FACS buffer, and transferred to polypropylene tubes (NUNC, VWR International). Data acquisition was made using a BD FACSCalibur flow cytometer (BD Biosciences), and the data obtained were analyzed using BD Cell-Quest Pro software (BD Biosciences). For each assay, a minimum of 10^5 cell events were acquired. Results are shown as percentage of positive cells.

3D cell culture and histological analysis. The ability of primary and immortalized articular chondrocytes to produce cartilage-like ECM was assayed in 3D cell culture. The micropellet method[30] was employed to create micromass from 2×10^5 cells of chondrocytes, OA immortalized articular chondrocytes (iACs), and non-OA iACs lines, and the immortalized chondrocyte cell line T/C28a2.[17,31] As T/C28a2 was unable to form micropellets, the hanging drop method was used to create cell aggregates from 5×10^4 cells of T/C28a2, and the OA iACs and non-OA iACs lines.[32] All these cell lines were subsequently incubated for 15 days: cell aggregates in PromoCell Mesenchymal Stem Cell Chondrogenic Differentiation Media (LabClinics, Spain), and micropellets in human mesenchymal stem cell (hMSC) Chondrogenic Differentiation Medium (Lonza, Spain) supplemented respectively. Both micropellets and cell aggregates were supplemented with 10 ng/ml of human transforming growth factor β-3 (TGF-β3) (ProSpec-Tany TechnoGene, Israel).

After 15 days, at least ten cell aggregates of each cell type were pooled and stored at -80°C for further analysis. Additionally, cells were seeded in 6 mm-diameter sponges of type I collagen (Opocrin, Italy)[30] and maintained in hMSC Chondrogenic Differentiation Medium with 10 ng/ml of TGF-β3 for 30 days. After that, cell aggregates (hanging drop), micropellets, and COL1A1 sponges were fixed with 3.7% formaldehyde (Panreac Química, Spain), embedded in paraffin (Merck Millipore, Germany), cut in a microtome, and stained with Masson's trichrome and Safranin O in order to study the presence of collagen fibres and proteoglycans in the ECM. Primary articular chondrocytes obtained from donor 3 were employed as a control.

In vitro inflammation model. The response of immortalized articular chondrocytes to the inflammatory cytokine IL-1β was investigated. Primary chondrocytes (from donors 2, 4, 5, and 6), immortalized chondrocytes, and T/C28a2 were seeded in six-well adherent culture dishes in 10% FBS/DMEM. Cells were incubated in DMEM containing 0.5% FBS for 48 hours before treatment and thereafter stimulated with IL-1β (5 ng/ml) (PeproTech, USA) for 24 hours in serum-free DMEM.[11] Non-stimulated controls were incubated in serum-free DMEM. After that, supernatants were collected and RNA was isolated from cells in order to quantify the expression of inflammation-related genes.

Quantitative real-time PCR. RNA was isolated from monolayer cells of the in vitro inflammation model and the pooled chondrogenic cell aggregates with TRIzol Reagent (Thermo Fisher Scientific) and chloroform (Sigma-Aldrich). Afterwards, the aqueous phase was recovered and precipitated with isopropanol (Sigma-Aldrich) and washed with ethanol (MilliporeSigma). Reverse transcription polymerase chain reaction (RT-PCR) was performed using the SuperScript VILO cDNA Synthesis kit, following the manufacturer's instructions (Thermo Fisher Scientific). Retrotranscription was performed with 1 µg of RNA, when available, and obtained complementary DNA (cDNA) was diluted 1:5 to 1:50; otherwise, the total amount of isolated RNA was retrotranscribed and cDNA was diluted accordingly. Quantitative real-time polymerase chain reaction (qPCR) was performed in a LightCycler 480 Instrument (Roche), employing LightCycler 480 SYBR Green I Master (Roche) in addition to gene-specific primers shown in Table III.

Data analysis was done using the LightCycler 480 Relative Quantification software (Roche), and relative gene expression levels (RELs) were calculated employing the 2-ΔCT method. YWHAZ was employed as the reference gene.

Immunoenzymatic assay of IL-6 production. The levels of IL-6 and IL-8 in culture supernatants from primary and immortalized articular chondrocytes after 24-hour treatment with IL-1β were determined with the human IL-6 and IL-8 DuoSet ELISA Kit (DY206 and DY208, respectively; Bio-Techne R&D Systems, UK), following the manufacturer's instructions. Absorbance measurements were performed using a NanoQuant Infinite M200 microplate reader (Tecan Ibérica Instrumentación, Spain). Data were expressed as released picograms per ml of

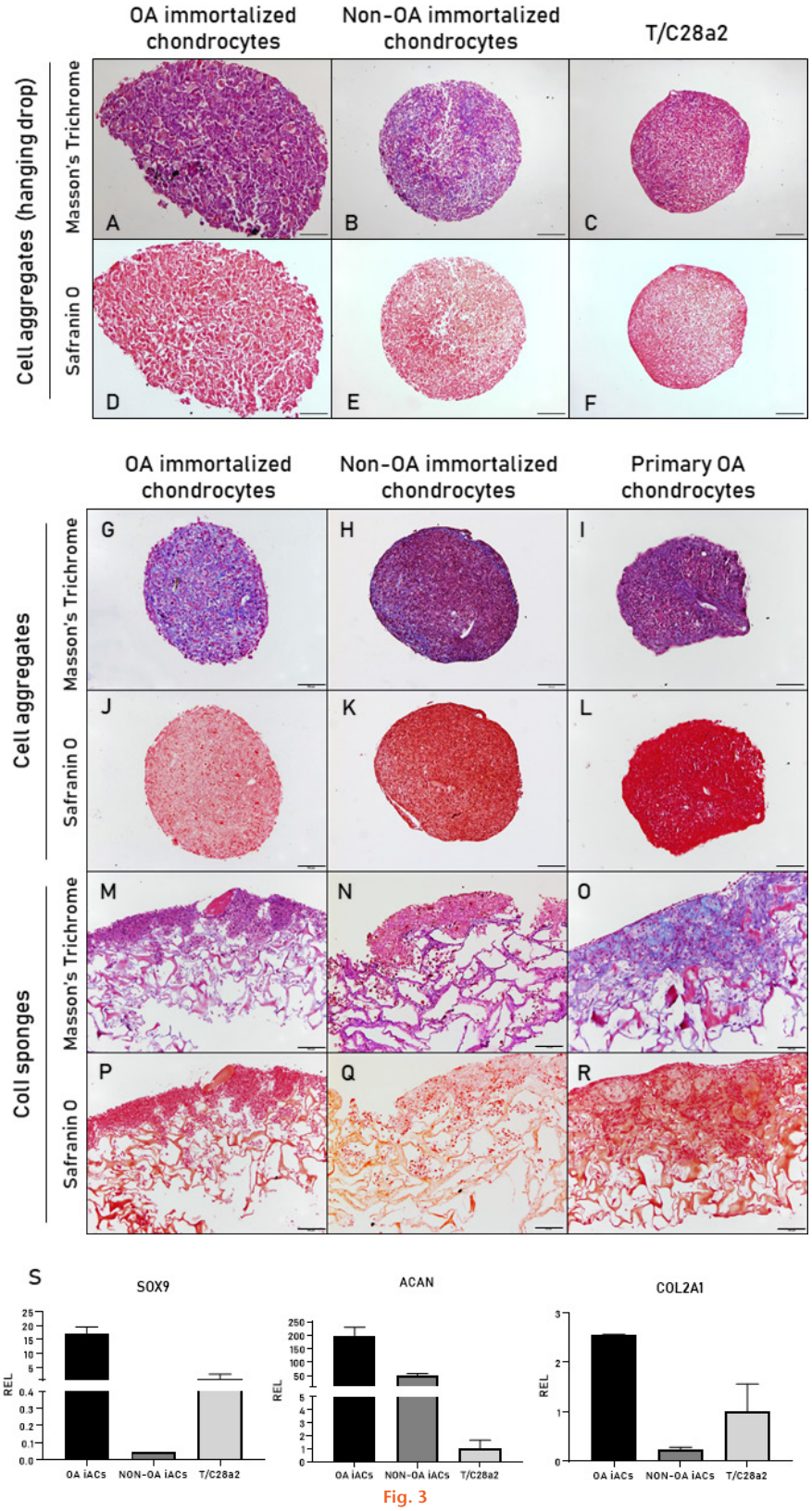

Fig. 3

a) to f) Masson's trichrome and Safranin O staining after tridimensional culture in chondrogenic medium: osteoarthritic (OA) and non-OA immortalized chondrocytes and T/C28a2 cell aggregates, g) to l) OA and non-OA immortalized chondrocytes and primary OA chondrocyte micropellets, and m) to r) the same cells seeded on collagen sponges. Scale bar: a), d) 50 µm; b) c), e), f), g) to r) 100 µm. s) Relative expression levels (RELs) of the characteristic chondrogenic markers type 2 collagen alpha chain 1 (COL2A1), aggrecan (ACAN), and sex-determining region Y (SRY)-Box Transcription Factor 9 (SOX9), analyzed on cell aggregates.

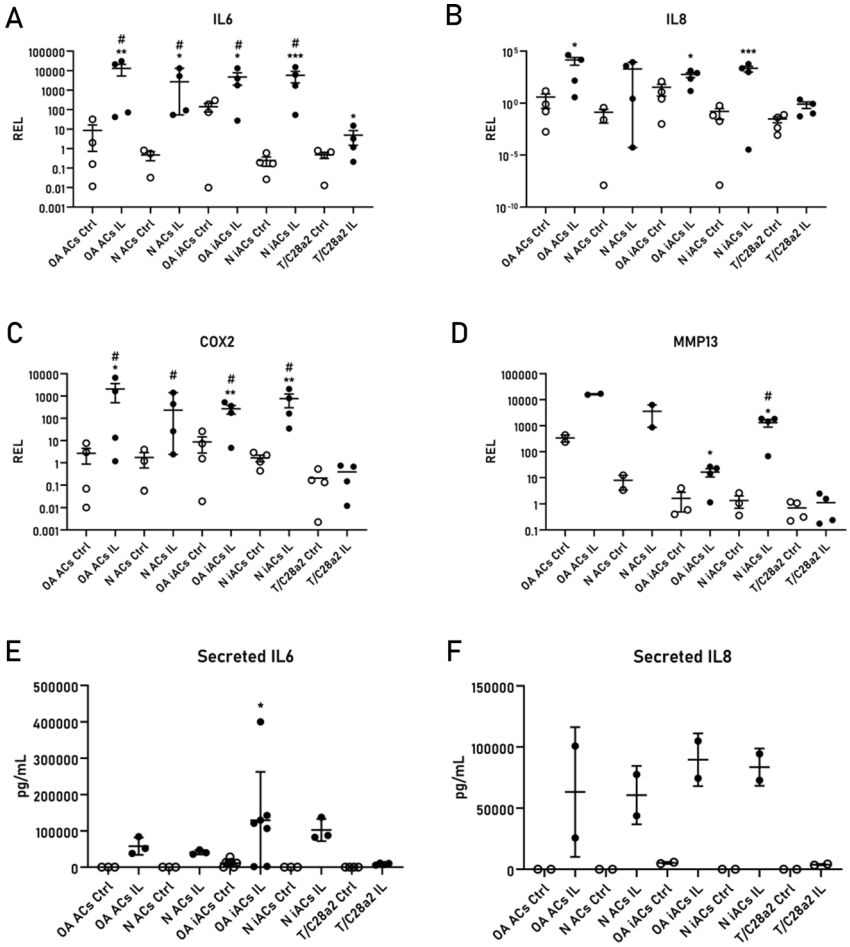

Fig. 4

Relative expression levels (RELs) of a) interleukin (IL)-6, b) IL-8, c) cyclooxygenase-2 (COX2), and d) matrix metalloproteinase 13 (MMP-13) in osteoarthritic primary articular chondrocytes (OA ACs), non-OA primary articular chondrocytes (N ACs), OA immortalized articular chondrocytes (OA iACs), non-OA immortalized articular chondrocytes (N iACs), and T/C28a2 cells after 24-hour treatment with IL-1β. Levels of e) secreted IL-6 and f) IL-8 in culture supernatants from OA ACs, N ACs, OA iACs, N iACs, and T/C28a2 cells after 24-hour treatment with IL-1β. *p ≤ 0.05, Mann-Whitney U test.

culture supernatant. Working range was between 9.38 to 600 pg/ml for IL-6 and 31.25 to 2,000 pg/ml for IL-8.

Statistical analysis. Statistical analysis was performed with GraphPad PRISM 8 software (USA). Statistically significant differences between cell lines were determined using non-parametric Mann-Whitney U test. Statistically significant differences between control and IL-1β-treated conditions for each cell line were determined by ratio paired *t*-test. A difference was considered significant with p-value ≤ 0.05.

Results

Immortalization of articular chondrocytes by transduction of SV40LT and hTERT. Spinoculation with SV40LT and hTERT was successfully employed for the transduction of primary articular chondrocytes (from donors 1 and 2). SV40LT-transduced chondrocytes were selected in puromycin within one week, and selected cells reached the confluence to be trypsinized ten days later. Subsequent hTERT transduction of SV40LT-transduced cells was also achieved. After hygromycin selection, expression of SV40LT and hTERT was detected in the nucleus of transduced chondrocytes by immunostaining (Figure 1). Both hTERT and SV40LT were located in the nucleoplasm and showed the same nucleolar exclusion expression pattern.

The mean generation time of these cell lines was 2.8 days (SE 0.8) for OA iACs and 2.4 days (SE 0.5) for non-OA iACs. There were no significant differences between OA and non-OA iACs regarding proliferation capacity. However, the generation time of both OA and non-OA iACs was significantly higher in comparison with previously immortalized MSCs (mean 2.0 days (SE 0.7))[26,33] (p = 0.001). Immortalized chondrocytes were grown over 40 PDs, and regression analysis showed a constant proliferation rate, with a multiple correlation coefficient $R > 0.95$ (Figure 2a) for both OA and non-OA iACs, and a p-value < 0.001. Moreover, the percentage of SA-β-Gal-positive cells was 1.6% (SE 0.2) for OA iACs and 1.2% (SE 0.1) for non-OA iACs (15th to 25th passages), compared with 34.5% (SE 3.2) for primary ACs (Figure 2b).

Phenotypic characterization of immortalized articular chondrocytes. The expression of several surface markers

characteristic of articular chondrocytes and CPCs was analyzed in both OA and non-OA iACs (Table IV). As expected, more than 90% of OA iACs were positive for CD9, a surface marker characteristic of CPCs from OA cartilage, whereas only 32.5% of non-OA iACs expressed it on their surface. Both OA and non-OA iACs showed high positivity for the chondrocyte markers CD44 and CD54 and for the CPC markers CD90 and CD166, and a similar positivity for the chondrocyte marker CD106. However, the expression of the CPC marker CD105 was higher in OA iACs (45.5%) than in non-OA iACs (32.9%). Conversely, the expression of the surface marker CD146, characteristic of CPCs from healthy cartilage,[2] was higher in non-OA iACs (60.7%) than in OA iACs (31.4%).

Cartilage extracellular matrix production. Similarly to primary OA chondrocytes, immortalized chondrocytes presented the ability to form 3D aggregates when subjected to both the micropellet and hanging drop methods. However, the immortalized T/C28a2 cell line can only form cell aggregates because larger micropellets tend to break up. These cell aggregates (hanging drop) showed a greater presence of collagens (Figures 3a to 3c) and proteoglycans (Figures 3d to 3f) on the OA and non-OA immortalized chondrocytes than in the T/C28a cell line. The micropellets presented an ECM containing collagen, as shown by Masson's trichrome staining (Figures 3g to 3i), but low amounts of proteoglycans detected by Safranin O (Figure 3j to l). However, immortalized chondrocytes were unable to form a cartilage-like tissue when seeded on COL1A1 sponges, unlike primary OA chondrocytes, which formed an ECM with collagen (Figures 3m to 3o) and proteoglycans (Figures 3p to 3r).

The gene expression analysis showed a trend of higher relative expression levels (REL) in OA iACs compared to TC28a2 line for the early chondrogenic marker Sex Determining Region Y (SRY)-Box 9 (SOX9) (17.33 (SE 2.21) and 1.00 (SE 1.64), respectively), and the late chondrogenic markers type 2 collagen alpha chain 1 (COL1A2) (2.55 (SE 0.01) and 1.00 (SE 0.50), respectively) and aggrecan (ACAN) (199.12 (SE 31.05) and 1.00 (SE 0.66), respectively). The non-OA immortalized cell line also showed high presence of ACAN (48.92 (SE 8.82)), as also observed in the histological analysis (Figure 3).

Response to inflammatory stimulus. Overall, immortalized articular chondrocytes showed more susceptibility to IL-1β than the cell line T/C28a2. Significant differences were found between IL-1β-stimulated immortalized articular chondrocytes (OA iACs and non-OA iACs) and T/C28a2 regarding REL of IL-6 and COX-2 ($p = 0.028$), but for IL-8 gene expression induction after exposure to IL-1β, the difference is only significant between T/C28a2 and OA iACs or OA ACs ($p < 0.050$) (Figures 4a to 4c). The expression of MMP13 tended to be higher in all the stimulated samples compared to the basal controls, and significant differences were seen between stimulated T/C28a2 and N iACs ($p < 0.050$) (Figure 4d).

Furthermore, there are increased trends in the amount of secreted IL-6 and IL-8 in both OA iACs and non-OA iACs compared to T/C28a2 cells, as well as in the primary articular chondrocytes (Figures 4e and 4f). However, no significant differences were found.

Discussion

Research involving human articular chondrocytes is limited by the fact that these cells have reduced proliferation capacity and undergo dedifferentiation during in vitro expansion.[15,16] For this reason, immortalized chondrocyte cell lines, such as T/C28a2, have been extensively used instead of primary articular chondrocytes. However, chondrocyte cell lines like this one, not derived from articular cartilage,[17] are not equal to primary articular chondrocytes, especially in terms of their response to inflammatory stimuli.[18] The few articular cartilage-derived chondrocyte cell lines that have been generated also have associated drawbacks, some of which are likely due to incomplete immortalization with a single transgene.[19-23] Transduction with viral genes, such as SV40LT and human papillomavirus (HPV) E6/E7 genes, prevents cell growth arrest by interfering with p53 and Rb-mediated pathways,[34,35] but does not prevent replicative senescence driven by telomere shortening. On the contrary, transduction with hTERT prevents telomere shortening,[36,37] but cannot avoid stress-induced senescence. This is why somatic cells transduced with only SV40LT,[38] HPV E6/E7 genes,[39] or hTERT[40,41] can finally undergo senescence.

In this study, we performed transduction with both SV40LT and hTERT to immortalize human primary chondrocytes. In transduced cells, both SV40LT and hTERT were expressed in the nucleoplasm and excluded from the nucleoli. Although intranuclear localization of hTERT is regulated by the cell cycle phase, SV40LT transduction has been described to promote the release of telomerase from nucleoli to the nucleoplasm.[28] Immortalization with these transgenes allowed chondrocytes to bypass senescence, as evidenced by the absence of granular content related to SA-β-Gal activity, similarly to immortalized MSCs,[33,42,43] and unlike primary articular chondrocytes derived from aged donors and hTERT-transduced chondrocytes.[23] However, mean generation time of immortalized chondrocytes was higher than that previously shown for immortalized MSCs,[33] suggesting that immortalized chondrocytes retain a higher degree of differentiation than immortalized MSCs.

When comparing both cell lines generated in this study, we found a higher proportion of CPCs among OA immortalized articular chondrocytes, as evidenced by greater expression of CPC surface antigens CD9 and CD105. CPCs have been described to be present at an increased frequency in OA articular cartilage,[2,12] although they are prone to senescence and unable to restore the tissue.[12] On the contrary, non-OA immortalized articular chondrocytes showed greater expression of CD146, a surface antigen characteristic of healthy cartilage-derived

CPCs,[2] which has been described to interact with Wnt16 to inhibit chondrocyte hypertrophy.[44] As expected, both cell lines showed high positivity for the hyaluronan receptors CD44 and CD54.[45] Furthermore, both cell lines showed high positivity (> 90%) for the CPC markers CD90 and CD166, but lower positivity for CD106, which may be indicative of chondrocyte dedifferentiation.[46]

Despite this, immortalized chondrocytes generated in this study were able to form 3D micropellets similar to those formed by primary articular chondrocytes, but, unlike primary articular chondrocytes, they were unable to form a cartilage-like tissue when seeded in 3D scaffolds in the tested conditions. This could indicate either that immortalization does not prevent dedifferentiation, as has also been observed by other authors,[21,31] or that the proper stimulus to keep the differentiated phenotype was not used. Conversely, Yang et al[23] reported that gene expression of ECM cartilage markers ACAN, COL2A1, and SOX9 in hTERT-transduced chondrocytes was similar to primary chondrocytes. However, they performed their experiments using chondrocytes at the fourth passage as a control, when primary chondrocytes are known to dedifferentiate as soon as the second passage.[47] Other studies suggest that culture on 3D matrixes of alginate,[48] or use of serum-free defined medium,[17] enables the recovery of the chondrocyte phenotype. To test this question, we checked their capacity to form ECM in 3D cell aggregates (also using other commercial chondrogenic media), and the presence of collagens and proteoglycans had improved as observed by histology and gene expression. The immortalized T/C28a2 cell line was unable to form micropellets, but they could be cultured in cell aggregates. These cells expressed chondrogenic markers at gene and protein level, however in lower amounts than the OA iACs and non-OA iACs.

Nonetheless, although they did not show the 'perfect' anabolic capacities that are characteristic of primary articular chondrocytes, immortalized chondrocytes generated in this study were able to respond to the inflammatory stimulus of IL-1β similarly to primary articular chondrocytes (and differently from T/C28a2), increasing the release of ILs and the expression of ILs and metalloproteases.

IL-1 is the most potent inducer of cartilage degradation.[49] Articular chondrocytes respond to IL-1β by reducing anabolism and increasing catabolism,[50] and this cytokine is present at elevated levels in OA cartilage.[51] IL-1β-treated articular chondrocytes have been widely used as in vitro models to study OA initiation or post-traumatic OA.[10] Therefore, the ability of immortalized chondrocytes to respond to IL-1β could be an interesting characteristic for the future development of an in vitro model of OA for drug screening purposes.

In summary, we were able to immortalize articular chondrocytes by transduction of SV40LT and hTERT. Immortalized chondrocytes acquired the ability to overcome senescence in vitro, showed high expression of surface antigens characteristic of chondrocytes and CPCs, and were able to form cartilage-like tissue under some specific tested conditions. Additionally, these cell lines might be useful for OA research due to their ability to upregulate inflammatory mediators and metalloproteases in response to IL-1β similarly to primary articular chondrocytes.

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- F. J. Blanco-García: Resources, Funding acquisition, Writing – review & editing.
- I. Fuentes-Boquete: Funding acquisition, Supervision, Writing – review & editing.
- C. Vaamonde-García: Conceptualization, Formal analysis, Supervision, Writing – review & editing.
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