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Facultad de ciencias - Biotecnología - 2016

Mechanical stimulation of SW1353 chondrocyte-like cells with physiological and pathological levels of shear

Estimulación mecánica de células condrocíticas SW1353 con niveles fisiológicos y patológicos de esfuerzo cortante

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CERITFY:

Mr. Yago Juste Lanas has accomplished, under our direction, his corresponding Final Degree Project entitled "Mechanical stimulation of SW1353 chondrocyte-like cells with physiological and pathological levels of shear", fulfilling the Biotechnology statutory objectives.

Dña. Carmen Huesa, Investigadora Posdoctoral Asociada de la University of the West of Scotland (UWS) y D. John C. Lockhart, Director del Institute of Biomedical & Environmental Health Research (IBEHR), Principal del Centre for Musculoskeletal Science (CMS), Catedrático del School of Science & Sports de la UWS y Catedrático de la Division of Immunology, Inflammation & Infection de la University of Glasgow,

CERITFICAN:

Que D. Yago Juste Lanas ha realizado bajo nuestra dirección el trabajo correspondiente a su Trabajo de Fin de Grado, titulado "Estimulación mecánica de células condrocíticas SW1353 con niveles fisiológicos y patológicos de esfuerzo cortante", y que cumple los objetivos recogidos en las directrices TFG en Biotecnología.

24th of June, 2016

Carmen Huesa

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ABSTRACT

Osteoarthritis is a global musculoskeletal disease, with no disease-modifying drugs, leaving only analgesia and ultimately surgery to reduce pain and disability, affecting up to 80% of people older than 65 years old. There are strong functional interactions among the cartilage, synovium, and subchondral bone, leading to cartilage damage when mechanical loading is excessive chronic or repetitive, by inducing the expression of proinflammatory cytokines, and matrix metalloproteinases. PAR2 has recently been identified as a novel upstream mediator of catabolic events in the osteoarthritic chondrocytes, where its expression is enhanced, but the mechanism by which it promotes osteoarthritis has yet to be clarified. This protein is activated through the cleavage of the N-terminal domain, which can be mimicked by addition of a SLIGKV external peptide and also inhibited by using antibodies that avoid the cleavage. Fluid shear stress onto cell culture has been previously reported as good model of osteoarthritic *in vitro* studies, inducing the expression of *COX2* which through PGE2 produces an increase of proinflammatory cytokines and matrix metalloproteinases, and also the inhibition of PAR2 signal transduction by decreasing PAR2 surface expression by a still unknown mechanism.

To gain a deeper understanding of the role of PAR2 as one of the important factors in osteoarthritis, PAR2 protein levels and its location, as well as its gene expression and other important markers were studied in SW1353 chondrocyte-like cells in response to the different types and intensities of fluid shear stress. Results could suggest a direct relation between PAR2 and osteoarthritis, whose protein levels might be dependent on the type and intensity of flow. It was also found that PAR2 was internalized after FSS, suggesting that it could be in response to PGE2. Furthermore, the effect of PAR2 activation and inhibition on the gene expression of other important pathways was also investigated.

RESUMEN

La osteoartritis (o artrosis) es una enfermedad musculoesquelética, que afecta hasta el 80% de la población mayor de 65 años, para la cual no hay ningún tratamiento curativo basado en fármacos, utilizándose únicamente analgésicos para aliviar el dolor, y la cirugía como última opción. Existen fuertes interacciones entre el cartílago, el liquido sinovial, y el hueso subcondral, que hacen que cuando la carga mecánica es excesiva o crónica, se induzca la expresión de citoquinas proinflamatorias y metaloproteasas de la matriz, que producen daño en el cartílago. *PAR2*, gen cuya expresión aumenta en la osteoartritis, ha sido identificado recientemente como un nuevo mediador de los eventos catalíticos de la enfermedad, cuyo mecanismo de acción se desconoce. La proteína que codifica, es activada a través del corte de su dominio N-terminal, lo que puede ser imitado por la adición de un péptido externo SLIGKV, o puede ser inhibido usando anticuerpos que impidan la rotura. El uso de un esfuerzo cortante generado por fluido, sobre un cultivo celular, ha demostrado ser un buen modelo para el estudio *in vitro* de la osteoartritis, induciendo la expresión de *COX2*, que a través de PGE2 produce un incremento de citoquinas proinflamatorias y metaloproteinasas de la matriz, así como la inhibición de la señal de transducción de PAR2, reduciendo su expresión en la membrana por un mecanismo que se desconoce.

Con el objetivo de conocer mejor el papel que juega PAR2, como uno de los factores importantes, en la osteoartritis, sus niveles de expresión proteica y localización, así como su expresión génica y la de otros marcadores importantes, fueron estudiados en células condrocíticas SW1353 en respuesta a diferentes tipos e intensidades de esfuerzos de corte generados por fluido. Los resultados sugieren que puede tratarse de una relación directa entre PAR2 y osteoartritis, siendo la cantidad de proteína PAR2 dependiente del tipo e intensidad del flujo. También se observó que PAR2 aparecía internalizado después del esfuerzo de corte, sugiriendo que podría ser en respuesta a PGE2. Además, también se investigó el efecto de la activación y la inhibición de PAR2 sobre la expresión génica en otras rutas importantes para la osteoartritis.

INTRODUCTION

Osteoarthritis (OA), also known as osteoarthrosis, or degenerative joint disease, is a global musculoskeletal disease, with no disease-modifying OA drugs (DMOADs), leaving only analgesia and ultimately surgery to reduce pain and disability [1]. OA is the most common form of arthritis, and it can damage any joint in the body, although the disorder most commonly affects joints in the hands, knees, hips and spine. The surfaces within the joints become damaged so it doesn't move as smoothly as it should [2], producing pain and stiffness, which are the most common symptoms [3]. Osteoarthritis aetiology is multifactorial, with injury being the main influence on the onset and severity of osteoarthritis. Age and genetic influences also play a large role in the severity of osteoarthritis, affecting up to 80% of people older than 65 years old [4], as well as weight, which is playing a more significant role for modern populations. Furthermore sex differences, as a consequence of hormones, body size, anatomy, and intense activity starting at a young age still may also influence osteoarthritis [5].

OA is a disease that includes pathologic changes in all tissues of the joint, including articular cartilage degradation, subchondral bone thickening, osteophyte formation, synovial inflammation, and degeneration of ligaments (and the menisci in the knee). These strong functional interactions among the cartilage, synovium, and subchondral bone, produces an impact on cartilage function that is difficult to know where and when the pathological changes begin [6]. However, C. Huesa *et al*. [7] recently showed that the earliest changes appear in bone, driving osteophyte formation and other subchondral bone alterations. The temporal characterization of the early stages in OA demonstrates that although bone changes precede, they do not necessarily lead to, cartilage damage, which seems to occur independently [4, 7]. Then, if the damaged cartilage is not able to regenerate, it could steer to development of OA.

Mechanical loading, despite its physiological effects, when excessive chronic or repetitive has been proposed as the critical signal for the initiation and progression of OA, being chondrocytes the target of these abnormal biomechanical factors [8, 9, 10], which have been found to mediate the above-mentioned earliest bone changes [4, 7], and cartilage damage by inducing the expression of proinflammatory cytokines (PICs), matrix metalloproteinases (MMPs), and apoptosis signals in late OA [9, 11].

Protease activated receptor 2 (PAR2), a G protein–coupled receptor (GPCRs), has recently been identified as a novel upstream mediator of catabolic events in the osteoarthritic chondrocytes, where its expression is enhanced [4, 7, 11, 12]. This GPCRs is activated through the cleavage of the PAR2 N-terminal domain by specific serine proteases [4], unmasking a new N-terminal sequence (SLIGKV), which acts as a tethered ligand, binding to the extracellular loop 2, and activating the receptor itself, transducing the signal via the G-proteins at the C-terminal domain, and upregulating its expression. This activation is irreversible, and the cleaved receptor activated, is internalized, and degraded. Then cell membrane PARs are restored from the intracellular pools [11].

After activation, PAR2 is downregulated through C-terminal phosphorylation by GPCR kinase, followed by association with an adaptor protein like β-arrestin, recruited to clathrin-coated pits and internalized from the cell surface. Internalized PARs are modified with ubiquitin, which facilitates lysosomal degradation [13, 14]. The redistribution of uncleaved PARs from intracellular pools to the cell surface as well as de novo receptor synthesis permits rapid recovery of protease signaling, being critical for cellular re-sensitization [13, 15, 16].

The mechanism by which PAR2 promotes osteoarthritis has yet to be clarified, but PAR2 provides for sensing by cells of the protease environment and allows them to respond subsequently [12]. Extracellular signal-regulated kinase 1/2 (Erk1/2) and p38 pathways, but not those of JNK or NF-κB, are activated very early in response to a specific PAR2 stimulation, as well as widely implicated in the ongoing catabolic events in cartilage degradation, like production of MMP-1, MMP-13 by ERK1/2 and activation of cyclooxygenase 2 (*COX2,* also known as Prostaglandin-Endoperoxide Synthase 2, *PTGS2)* by p38 [4, 11, 14].

Furthermore, PAR2 has also been proposed as a potential therapeutic target not only to slow the disease progression, but also likely to reduce the symptoms [4, 7, 11]. Chronic joint pain is one of the major symptoms of arthritis, and PAR2 activation appears to lead to hyperalgesia, rather than analgesia. Thus, in addition to any potential disease modifying effects like anticatabolic and anti-inflammatory, PAR2 antagonism may also have the added benefit in reducing joint pain [4, 7, 11].

Then, it was discovered that administration of an exogenous agonist peptide that mimics the tethered ligand sequence (SLIGKV) can also lead to activation of the receptor [17]. In contrast, there are a number of potential approaches to prevent PAR-2 activation. It can be obtained by gene silencing using PAR-2 small interfering RNA, or extracellularly by prevention of proteolytic activation, employing serine protease inhibitors, administering antibodies or antagonist that target the serine protease cleavage site, as shown in the Figure 1B [17].

Other studies revealed that fluid shear stress (FSS) caused by mechanical loading, induces the synthesis of COX-2 in chondrocytes via a Rac/MEKK1/MKK7/JNK2/c-Jun-C/EBP-dependent pathway [18, 19], and that its derived prostaglandin (PG) E2 has anabolic effects at picomolar concentrations, and catabolic effects at nano and micromolar concentrations, producing PICs and MMPs by a mechanism that has yet to be elucidated [9].

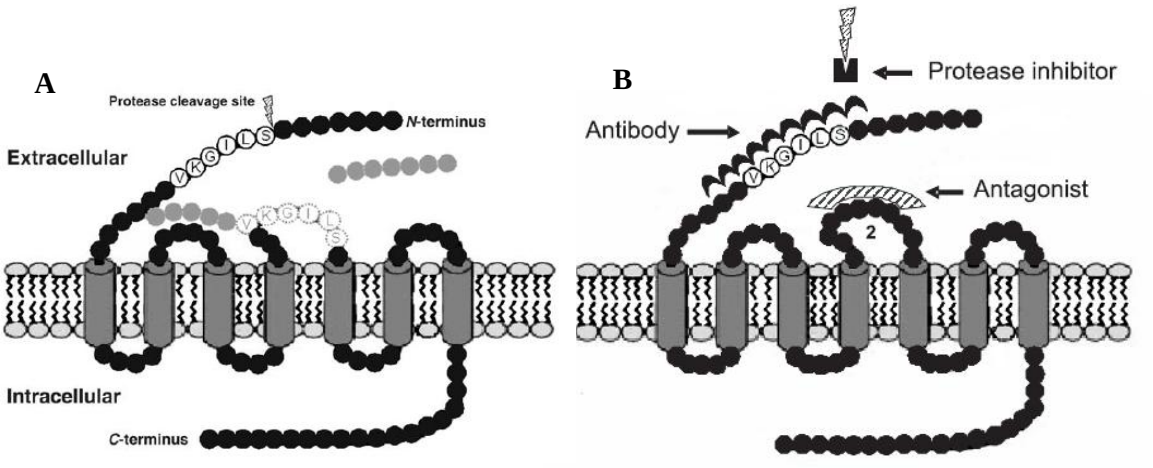

Figure 1. A) PAR2 secondary structure and its activation. *This representation of PAR2 secondary structure shows how PAR2 is activated by proteolysis of the N-terminus (in black), allowing the SLIG peptide (in white) to tether the second extracellular loop. Softened colors represent PAR2 structure after its proteolysis.* ***B) External inhibition of PAR2 activation.*** *PAR2 activation can be inhibited by many different mechanisms, like protease inhibitors, antibodies, agonists or siRNAs. Extracted from Ref. 17.*

PGE2 has been also found to inhibit PAR2 signal transduction by decreasing PAR2 surface expression [14], through a mechanism involving EP2 that is not well understood. It is known that PGE2 binds to specific G-protein coupled cell surface prostaglandin EP receptors (EP2), and modulate intracellular levels of cyclic AMP [14, 18, 19], which could produce internalization of PAR2, an increase of β-arrestin protein expression, and the inhibition of ERK phosphorilation (and thus its pathway) [14]. Nevertheless, PGE2 also induces the production of proteases, which may induce PAR2 internalization too [14].

In addition, it was suggested that PAR2 internalization can be uncoupled from G-protein activation and phosphorylation, indicating that distinct determinants control the capacity of PAR2 to signal versus recruitment of β-arrestin and endocytosis [15]. Activated PAR2 phosphorylation is critical for receptor desensitization and β-arrestin recruitment, which facilitates receptor recruitment to clathrin coated pits and endocytosis. Nevertheless, it was found that PAR2 internalization can proceed independent of G-protein activation and phosphorylation [15].

Previous experiments revealed the similarities of fluid shear stress *in vivo* and *in vitro*, providing OA with a FSS experimental model *in vitro* to mimic *in vivo* fluid shear stress (Figure 2). Accumulating in vitro results support the notion that low fluid shear (<10 dyns/cm^2) is chondroprotective, whereas high shear stress (>10 dyns/cm^2) elicits the release of PICs, MMPs, etc. [9].

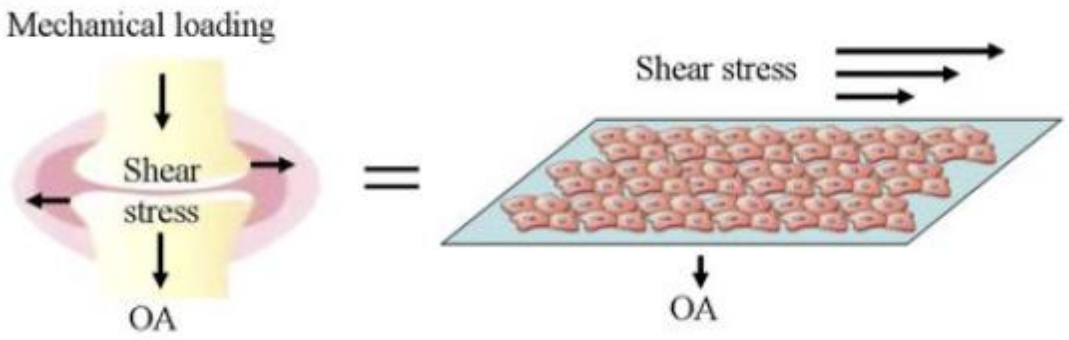

Figure 2. Similarities of mechanical stimulation in vivo and in vitro. *In vitro FSS on cell culture can work as an experimental model of the in vivo FSS occurred in osteoarthritis. Extracted from Ref. 9.*

AIM

The aim of this project is to gain understanding of the role of PAR2 as one of the important factors in osteoarthritis. First of all, PAR2 and OA relation will be tested *in vivo* with mice models, and *in vitro* with a cell line and FSS model. Secondly, we will try to adjust the optimal length of *in vitro* stimulation with FSS to study how PAR2 behaves in response to these different types and intensities of fluid shear stress, and we will also attempt to elucidate the localization of PAR2 within the cell by using immunofluorescence methods. Furthermore PAR2 gene expression as well as some key proinflammatory cytokines and matrix metalloproteinases gene expressions will be studied, firstly after 1 hour of FSS, and later after 1 hour of FSS, and 2 hours of resting time, meanwhile PAR2 protein will be rather activated or inhibited by external treatment, in order to know how its activation or inhibition could affect directly or indirectly to the downstream genes expression, thought to be involved in osteoarthritis disease.

MATERIALS and METHODS

Mice joints immunostaining

Samples were obtained from knee joints 4 weeks after experimental OA induction by the destabilization of the medial meniscus (DMM) method [20]. 6 µm sections were cut and used for immunohistochemical analysis. For this we carried out dewaxing with Histo-Clear for 5 minutes at room temperature. Subsequently, samples were rehydrated with decreasing concentrations of ethanol (100%, 80% and 70%) for 2 min at room temperature. Then, sections were washed with PBS followed by antigen retrieval performed for 6 min using 10 mM sodium citrate buffer. Without washing samples, endogenous peroxidase inhibition was carried out by incubating the slides in 1% H_2O_2 in methanol for 30 minutes. Afterwards, samples were washed twice with PBS for 5 min and blocked with PBS, 0.5% BSA and 0.05% serum (where the 2nd species was raised in), for 30 min at room temperature. Sections were incubated overnight at 4ºC with SAM-11 antibody and equivalent mouse monoclonal IgG2a antibody as a negative control. Next morning, sections were washed twice for 5 min with PBS and then incubated with biotinylated universal pan-specific antibody for 30 min at room temperature. Samples were washed twice with PBS for 5 min, followed by incubation with Vectastain ABC solution for 30 min, at room temperature. Samples were washed as previously indicated. Detection was carried out using 3,3-diaminobenzidine (DAB) substrate, counterstained with haematoxylin followed by a 2 min incubation with increasing ethanol (100%, 80% and 70%). Finally, samples were then incubated for 2 min in xylene and mounted with DPX (a synthetic resin mounting media).

SW1353 Cells

SW1353 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) F12 (Lonza, Belgium) + 10% Fetal Bovine Serum +1%L-Glutamine +1%Penicilin/Streptomycin in humidified atmosphere 5% CO_2 in air at 37°C. Passages were done at 75-85% cell confluence.

Fluid Flow (FF)

Steady, oscillating and pulsatile fluid flows were used to mimic knee shear stress. 5dyns/cm^2 (0.5Pa) pressure fluid flows were used as a model for the physiological effects (rate stress 500) of a healthy knee, and 20dyns/cm^2 (2.0Pa) pressure fluid flows were employed as a model for the pathological effects (rate stress 2000) of OA within the knee. Ibidi Pump system (Ibidi, Germany), a computer-controlled air pressure pump, was used to create by positive pressure the above-mentioned fluid flows.

Chambers µ-Slide VI$^{0.4}$

Fuid flow experiments were run in collagen coated tissue culture chambers (Ibidi, Germany) that contain 6 channels of 0.4mm height, 17mm length and 3.8mm width dimensions, resulting in 30µl volume per channel, interlinked by 0.8mm and 1.6mm inner diameter tubing (Ibidi, Germany) for 5dyns/cm^2 and 20dyns/cm^2 pressure, respectively.

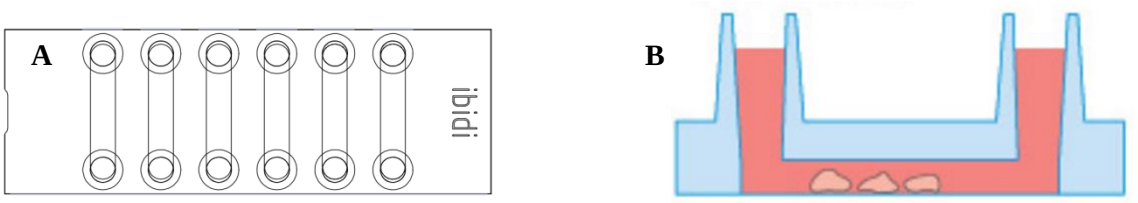

***Figure 3. A) Chamber µ-Slide VI$^{0.4}$ representation**. Top view of the 6 channels chamber µ-Slide VI$^{0.4}$ (Ibidi, Germany). **B) Cell culture in Chamber µ-Slide VI$^{0.4}$.** Lateral view of one of the channels of the chamber, full of culture media and some cells attached to the bottom.*

Fluid Flow Set up

Ibidi Pump system and chambers were connected, and Fluidic Units (Ibidi, Germany), holders for perfusion sets (10 ml each) and connected to chambers through 15cm length and 0.8mm and 1.6mm inner diameter tubing respectively. Manufacturer's instructions (Ibidi, Germany) were followed for the set up (http://ibidi.com/).

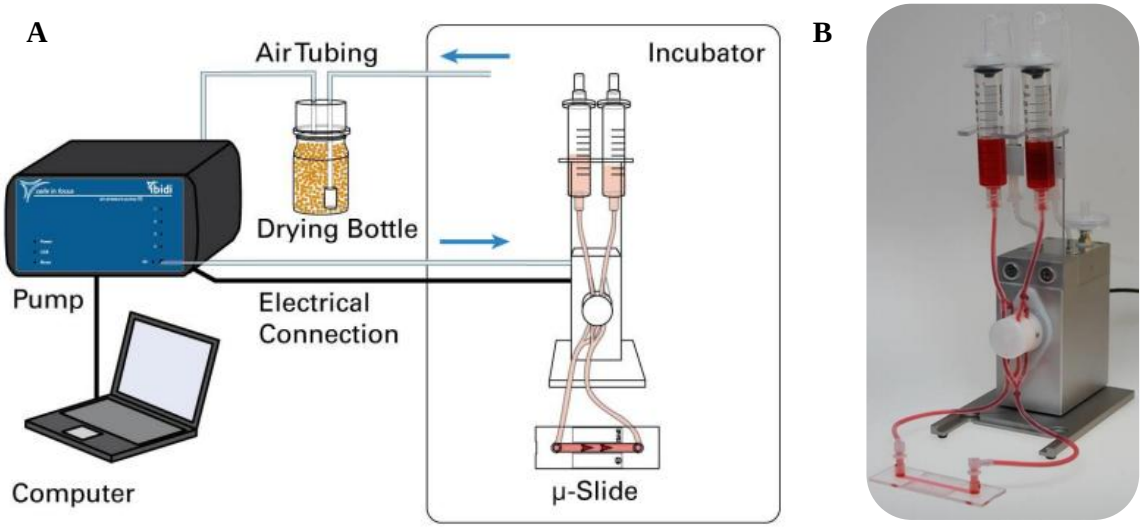

***Figure 4. A) Representation of Fluid Flow set up.** Representation of the air pump connection to the Fluidic Unit(s), controlled by specific computer software. **B) Fluidic Unit.** Image of one fluidic unit working on one channel chamber.*

Fluid flow experiment

30µl of 10^6cells/ml of the SW1353 chondrocytes cell line with DMEM F12 (+10%FBS +1%L-Glutamine +1%Penicilin/Streptomycin) were added into each channel of the µ-Slide and after cells fixation to the bottom of the channel, 150µl of the same medium were added, and incubated in humidified atmosphere with 5% CO_2 and 37°C over night. Half an hour before the flow experiment, the medium was changed for DMEM F12 (+1%FBS +1%L-Glutamine +1%Penicilin/Streptomycin) and cells were rested to achieve a steady-state level.

Every chamber was subject to 1 hour of experiment with 11ml of total working volume (and tubing dead volume 0.5ml) under humidified atmosphere with 5% CO_2 and 37°C. Static state (control) underwent no fluid flow. Steady Flow 5dyns/cm^2 and Steady Flow 20dyns/cm^2 experienced a unidirectional flow. Oscillating Flow 5dyns/cm^2 and Oscillating Flow 20dyns/cm^2 underwent a bidirectional flow switching every 0.5s. And finally, Pulsatie Flow 5dyns/cm^2 and Pulsatile Flow 20dyns/cm^2 a unidirectional flow stopped every 0.5s, which equates to a frequency of 1Hz.

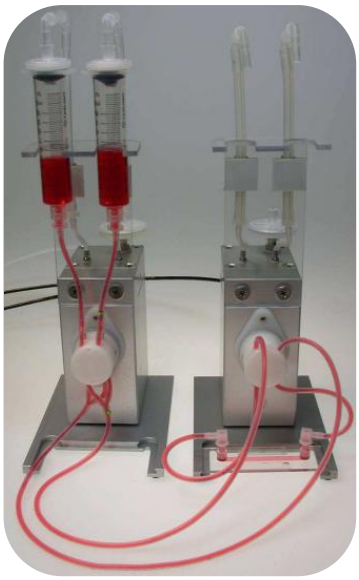

Figure 5. Several fluidic units set up. *Image of two fluidic units generating pulsatile flow on one channel chamber.*

Activation and inhibition of PAR2 after fluid flow

After the fluid flow experiment, cell cultures were incubated for 2 hours in humidified atmosphere with 5% CO_2 and 37°C. DMEM F12 (+1%FBS +1%L-Glutamine +1%Penicilin/Streptomycin) was used as the control medium, which was compared with the activation medium DMEM F12 (+1%FBS +1%L-Glutamine +1%Penicilin/Streptomycin + 10µM SLIG-KV), and the inhibitor medium DMEM F12 (+1%FBS +1%L-Glutamine +1%Penicilin/Streptomycin, + 200ng/ml SAM 11 (Santa Cruz Biotech, USA)). SAM11 (Santa Cruz Biotech, USA), is an IgG2a monoclonal antibody produced in mice immunized with the peptide SLIGKVDGTSHVTG corresponding to residues 37 to 50 of the human PAR-2 sequence.

Immunofluorescence Microscopy

After removing the medium of the wells and washing with PBS, paraformaldehyde (PBS +4% PFA) was added for 15 minutes, to fix the cells. Then, the wells were washed with PBS three times, and blocked with blocking solution (PBS +3% BSA) for 30 minutes at room temperature. The solution was changed for 30µl of primary antibody solution [blocking solution + primary antibody (mouse monoclonal IgG 200µg/ml) at 1:100] and incubated at room temperature for 1h. After washing with PBS three times, 30µl of secondary antibody solution [488nm goat anti mouse IgG, 2mg/ml at 1:200] were added, and incubated at room temperature for at least 30mins in the dark. Chamber channels were washed again three times and Vectashield was added and stored at 4°C until visualisation in fluorescent microscope. No addition of the primary antibody was used as the negative control (isotype).

Immunofluorescence quantification

Immunofluorescence was assessed by taking images of immunostained SW1353 cells with a standard LED light source immnunofluorescence microscope (Zeiss, Germany). Cell perimeter within the images was delineated and immunofluorescence brightness was quantified using ImageJ software. Areas in every image not containing cells were used to subtract background fluorescence.

Immunofluorescence localization and 3D imaging

An Inverted Spinning Disk Confocal Microscope (Zeiss, Germany) with a Evolve Delta 512 EMCCD Camera (Photometrics, USA) was used to obtain a 3D image of immunostained cells and localize PAR2 within the cells (http://www.gla.ac.uk/researchinstitutes/iii/facilities/sdconfocal/). Z stack images of 1 µm were taken and reconstructed in ZEN (Zeiss, Germany) software for visualization.

RNA extraction and purification

Chamber media was removed and stored at -20ºC, and chambers were washed with PBS. 350µl of Lysis Buffer (RLT Lysis Buffer + 1% β-Mecaptoethanol) were added to two channels and, after a minute, collected in 1.5 ml RNase free Ependorff tubes. 350µl of 70% ethanol were also added to the homogenized lysate. The 700µl of the sample was transferred to an RNeasy mini column (Qiagen, UK) placed in a 2ml collection tube, which was centrifuged for 30sec at 8000 *g*. Then, the flow-through was taken and applied to the mini column again, centrifuged for 30sec at 8000 *g* and the new flow-through discarded. After that, 700µl of Buffer RW1 were added to the RNeasy column, centrifuged for 30sec at 8000 *g* to wash the column, and the new flow-through discarded. This step was repeated now with 80µl of DNase mix (10µl DNase I, 70µl RDD buffer), centrifuged, and the new flow-through discarded. The next requirement was the addition of 500µl of Buffer RPE, centrifuged for 2mins at 8000 *g* to wash the column to dry the RNeasy silica-gel membrane, and the flow-through discarded. To elute, the RNeasy column was transferred to a new 1.5ml collection tube, 30-50µl of RNase-free water were pipetted directly onto the RNAeasy silica-gel membrane, and the tube was centrifuged for 1min at 8000rpm. Finally, the flow-through was taken, and applied to the mini column again. After centrifuging for 1min at 8000rpm, the flow-through was stored.

The RNA quantification and the purity analysis were made with a NanoDrop Lite Spectrophotometer (Thermo Scientific, UK). On average we obtained 20ng/µl of not completely pure RNA, which was below the limit for running a qPCR. Therefore were used the RNA extraction with iScript method as described below.

RNA extraction with iScript method

30µl of iScript were added in each empty channel of the chamber, and after 1 minute, the RNA iScript lysate was collected.

q-PCR

cDNA was made from the RNA collected after the FSS experiment, using the 96 well Thermal cycler (Applied Biosystems, USA) for 20min at 55°C and 10min at 72°C; 9µl of RNA sample and 20µl of Reverse Transcription Premix (Primer design, UK). Two different methods were used for q-PCR analysis.

1) TaqMan Method: 5µl of Master mix, 0.5µl of Primer mix (Forward, Reverse, TaqMan Probe, 2:2:1), 0.5µl of Nuclease-free H_20, and 4µl of cDNA were used to a total volume of 10µl.
2) SYBR Green Method: 5µl of Master mix, 1µl of Primer mix (Forward,

Reverse, Water, 1:1:8), and 4µl of cDNA were employed to a total volume of 10µl.

Analysis was conducted by the ΔΔCt method and normalizing against 18S and B2M genes.

Gene	Forward 5'→3'	Reverse 5'→3'
PAR2	GGCACCATCCAAGGAACCAATA	GGAAGGAAGACAGTGGTGAGTT
COX2	GCTGTTCCCACCCATGTCAA	AAATTCCGGTGTTGAGCAGT
IL6	ACCCCCAATAAATATAGGACTGGA	GAAGGCGCTTGTGGAGAAGG
TGFB1	CGACTCGCCAGAGTGGTTAT	CGGTAGTGAACCCGTTGATGT
MMP13	AAATTATGGAGGAGATGCCCATT	TCCTTGGAGTGGTCAAGACCTAA
18S	CGAATGGCTCATTAAATCAGTTATGG	TATTAGCTCTAGAATTACCACAGTTATCC
B2M	TTCTGGCCTGGAGGCTATC	TCAGGAAATTTGACTTTCCATTC

Table 1. Primers sequences

ELISA

The coating solution was prepared by diluting the coating antibody to 1µg/mL with Coating Buffer (8.0g NaCl, 1.13g Na_2HPO_4, 0.2g KH_2PO_4, 0.2g KCl, 0.1% ProClin™; q.s. to 1.0L with distilled H_2O. pH to 7.4), and used to coat a EIA/RIA flat bottom 96 well plate with 100µl/well. The covered plate was incubated overnight (12-18hours) at 4° C. The wells were aspirated and washed 1 time with 300µl of Wash Buffer (0.2g KH_2PO_4, 1.9g $K_2HPO_4.3H_2O$, 0.4g EDTA, 0.5mL Tween 20; q.s. to 1.0L with distilled H_2O. pH to 7.4.) per well. Following the wash, the plate was inverted and taped on absorbent paper to remove excess liquid. Then, the plate was then blocked with 200µL per well of Assay Buffer (8.0 g NaCl, 1.13 g Na2HPO4, 0.2 g KH2PO4, 0.2 g KCl, 5.0 g bovine serum albumin (fraction V), 1 mL Tween 20; q.s. to 1.0 L with distilled H2O, pH to 7.4) for 1 hour at room temperature. 100µL of standards in triplicate, and samples were pipetted into designated wells. Immediately, 50µL of the working detection antibody were added into each well (Anti-Human IL-6 Biotin (0.025mg/0.125mL) diluted to 0.16µg/mL with Assay Buffer), and incubated for 2 hours at room temperature with continual shaking (700rpm). Then, the wells were aspirated and washed 5 times using the method previously explained. After washing, 100µL of the working streptavidin-HRP solution (diluted to 1/2500 in Assay Buffer) were added per well and incubated for 30 minutes at room temperature with continual shaking (700rpm). After aspirating and washing again 5 times using the previous method, 100µL of the TMB (Tetramethylbenzidina) substrate were added to each well, and the plate was incubated for 30 minutes at room temperature with continual shaking (700rpm). Then 100µL of Stop Solution (1.8N H_2SO_4) was added to each well, and the absorbance was measured at 450nm (reference absorbance: 650nm) within 30 minutes of adding Stop Solution.

Statistical analysis

Data were tested for normality (Sigmastat 2.03; SPSS) and, depending on the result, analysed with parametric or non paremetric tests. One-way analysis of variance (ANOVA) was utilized for multiple comparisons whilst two-way ANOVA was used to compare different groups and treatments. All Pairwise Multiple Comparison Procedures (Student-Newman-Keuls Method). Data was expressed in graphs as mean±SEM.

RESULTS

PAR2 detection in mice model

It was previously demonstrated that PAR2 expression is higher in OA cartilage [15, 34], and therefore, we wanted to verify whether PAR2 levels differ from healthy to osteoarthritic chondrocytes in an *in vivo* model of OA. For that reason sham operated and DMM (destabilization of the medial meniscus) C57BL/6J mice were used. After the immunostanning treatment, stanning chondrocytes were quantified giving a statistically significant increase of PAR2 protein levels in DMM mice compared with sham operated mice, as shown in Figure 6.

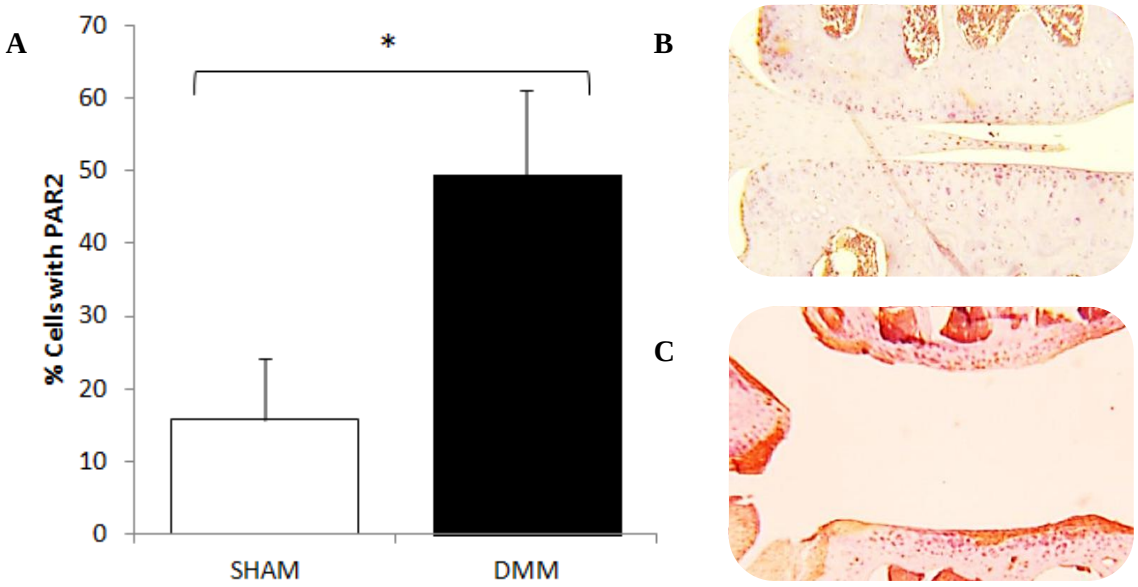

Figure 6. PAR2 levels in DMM and sham operated mice. *This figure shows the percentage of chondrocytes (A) whose PAR2 levels were detected by the immunostaning on sham (B) or DMM (c) operated mice, where PAR2 stain shows choncrocytes in brown colour. *P<0.05.*

Mechanical stimulation of SW1353 cells

PAR2 showed to be increased in the cartilage of the *in vivo* mouse model of OA (Figure 6), which induced us to test whether mechanical stimulation upregulates PAR2 presence and expression *in vitro*. We therefore conducted a series of experiments where different levels and types of FSS were generated onto SW1353 cells in culture. In comparison to static cultures, cells under FSS showed to have a statiscically significant increased level of PAR2 within the cells, as quantified in immunofluorescence (Figure 7).

As results confirmed that FSS can also generate an increase of PAR2 levels *in vitro*, it was questioned whether PAR2 levels in the cells were dependent on the types and intensities of FSS. Thus, the different mechanical stimulations were analysed separatedly by the same immunoflourescence quantification method and compared with the rest. As shown in Figure 7D, two main groups of FSS can be distinguished. Static culture, Steady 5dyns/cm^2, and Oscillating 5dyns/cm^2, present similar PAR2 low levels. By contrast, Steady 20dyns/cm^2, Oscillating 20dyns/cm^2, Pulsatile 5 and 20dyns/cm^2 have similar PAR2 levels, higher than those

observed within the first group. Despite this noteworthy trend, only Pulsatile 5dyns/cm^2 FSS has statistically significant differences with the amounts of PAR2 in the Static culture, suggesting that the flow under these conditions was generating a more consistent effect on PAR2.

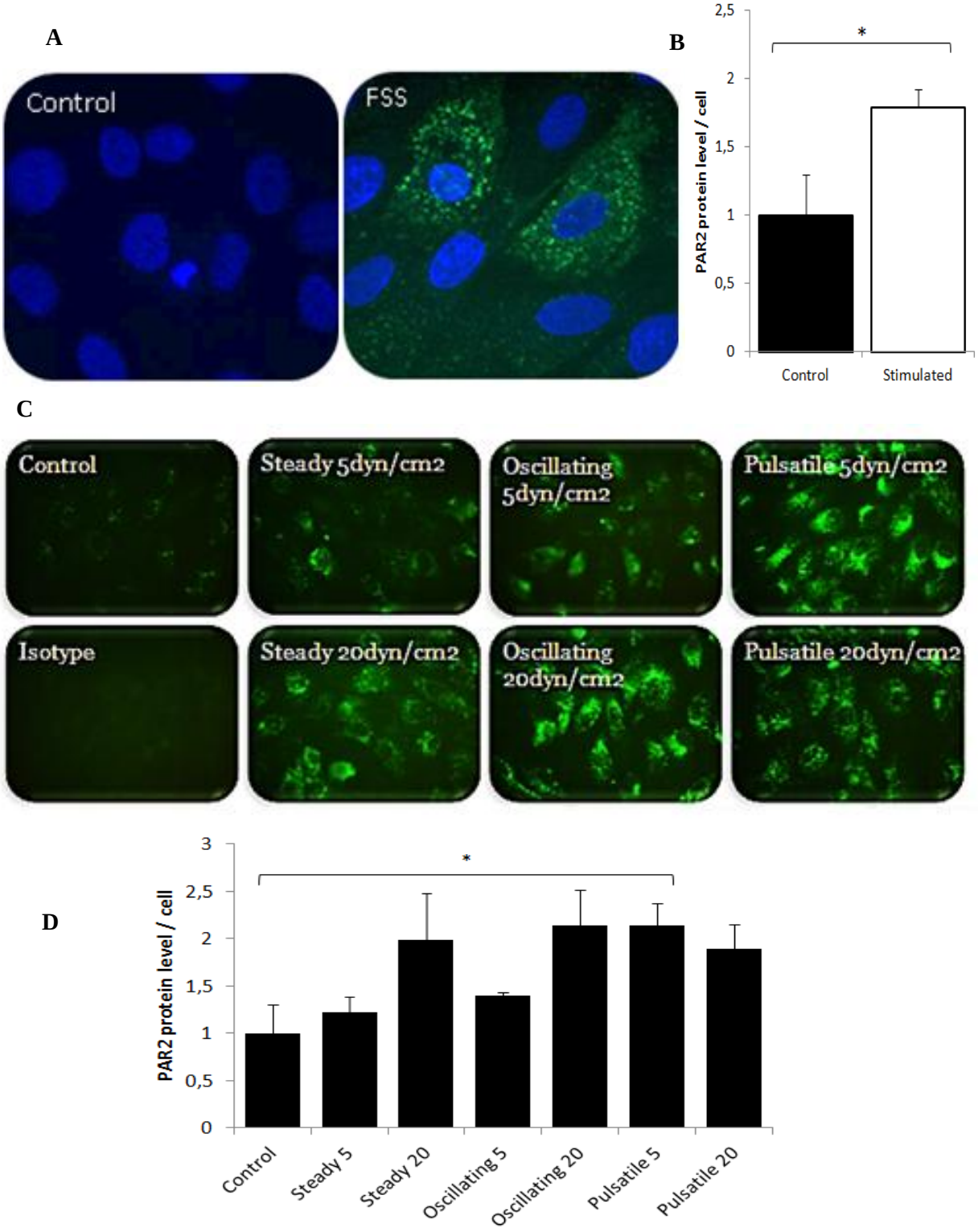

Figure 7. PAR2 protein levels induced by FSS. *Figures A) and C) are representative images of the SW1353 cells taken under the fluorescence microscope. Figures A) and B) only compare FSS against Control, whereas Figure C) and D) distinguish between the different types and intensities of flows used in the experiment. *P<0.05.*

SW1353 3D imaging and PAR2 localization

Where PAR2 was located was unknown so far. Theoretically, PAR2 detected levels could be thought to be increased due to its movement to the membrane. However, we wanted to confirm where PAR2 was mostly located after 1 hour of FSS, whether on the membrane, internalized, or both. Using an Inverted Spinning Disk Confocal Microscope was observed that PAR2 actually was not on the membrane but within vesicles in the cytoplasm, as it can be seen in Figure 8 and in the full video (https://youtu.be/AZn1B-YT6TY). The cells were fixed and not permeabilised, which should have made internalization of the antibody difficult, yet this was observed in all the samples, except in the control, where little or no PAR2 staining could be seen. Surprisingly, no PAR2 at all was detected on the membrane.

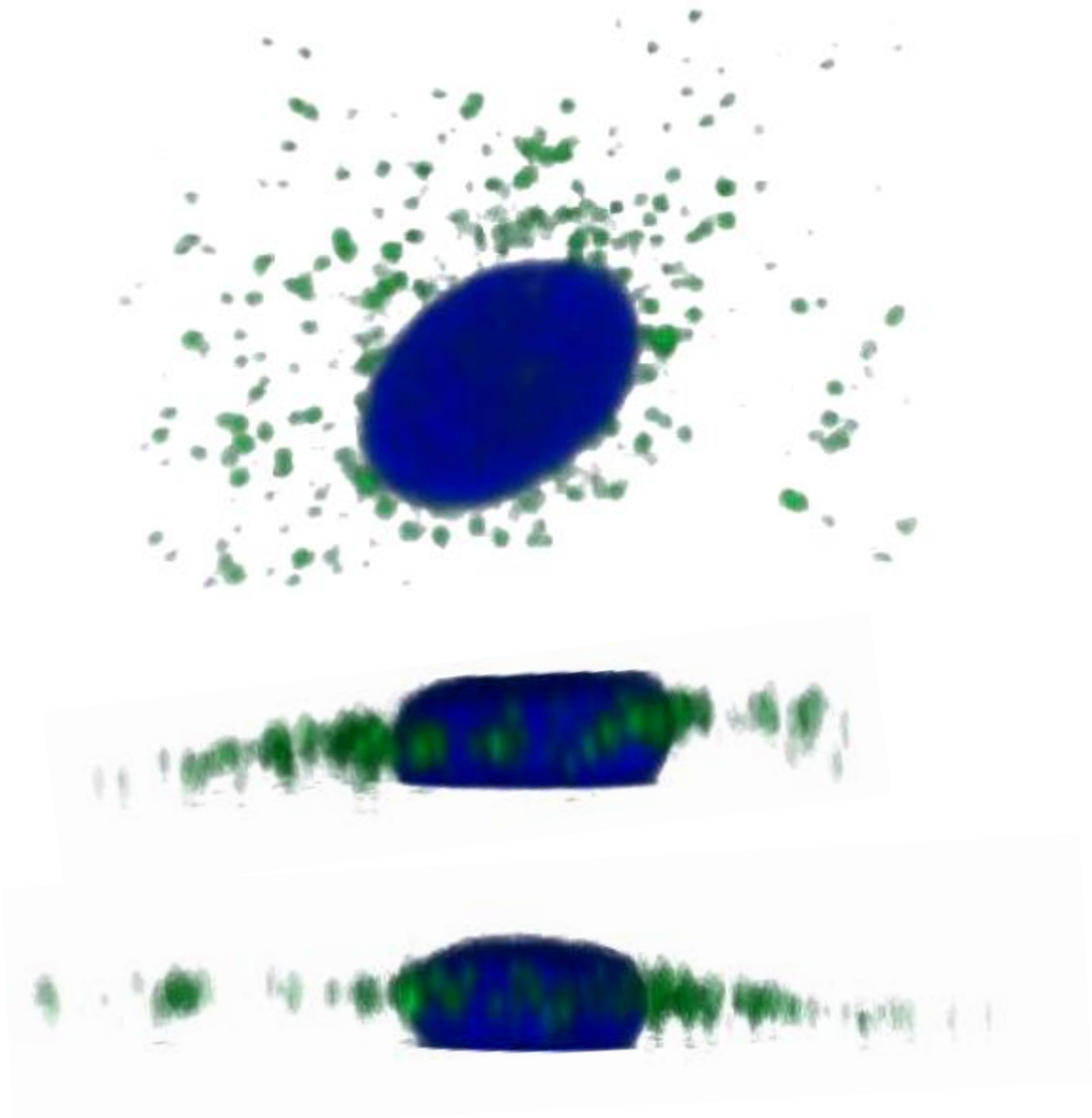

Figure 8. PAR2 localization. *These images of SW1353 were taken after 1hour of FSS and immunofluorescence treatment by an Evolve Delta 512 EMCCD Camera adapted to the Inverted Spinning Disk Confocal Microscope. Nuclei can be seen in blue color, and PAR2 in green color.*

Gene expression after 1hour of Fluid Flow

Since PAR2 presence in the cytoplasm, was increased depending on the FSS, *PAR2* gene expression, as well as some other OA related gene expression (*COX2, IL-6, TFGβ1*, and *MMP13*), were quantified. After one hour of mechanical stimulation no statistically significant differences in gene expression was detected in SW1353 cell line.

Nevertheless, according to the data displayed in Figure 9 some remarkable differences between gene expression can be appreciated depending on FSS, highlighting the trend observed in *COX2* gene expression, which is stimulated under Oscillating and Pulsatile flows, especially under 20dyns/cm^2.

PAR2 gene expression remained unchanged regardless of the treatment, suggesting that the increased PAR2 protein level within the cell is not due to an increase in the synthesis of PAR2. *MMP13, IL-6, and TGFB1*, apparently do not follow any particular trend, and their non statistically significant differences may be influenced by the variability within the experiments.

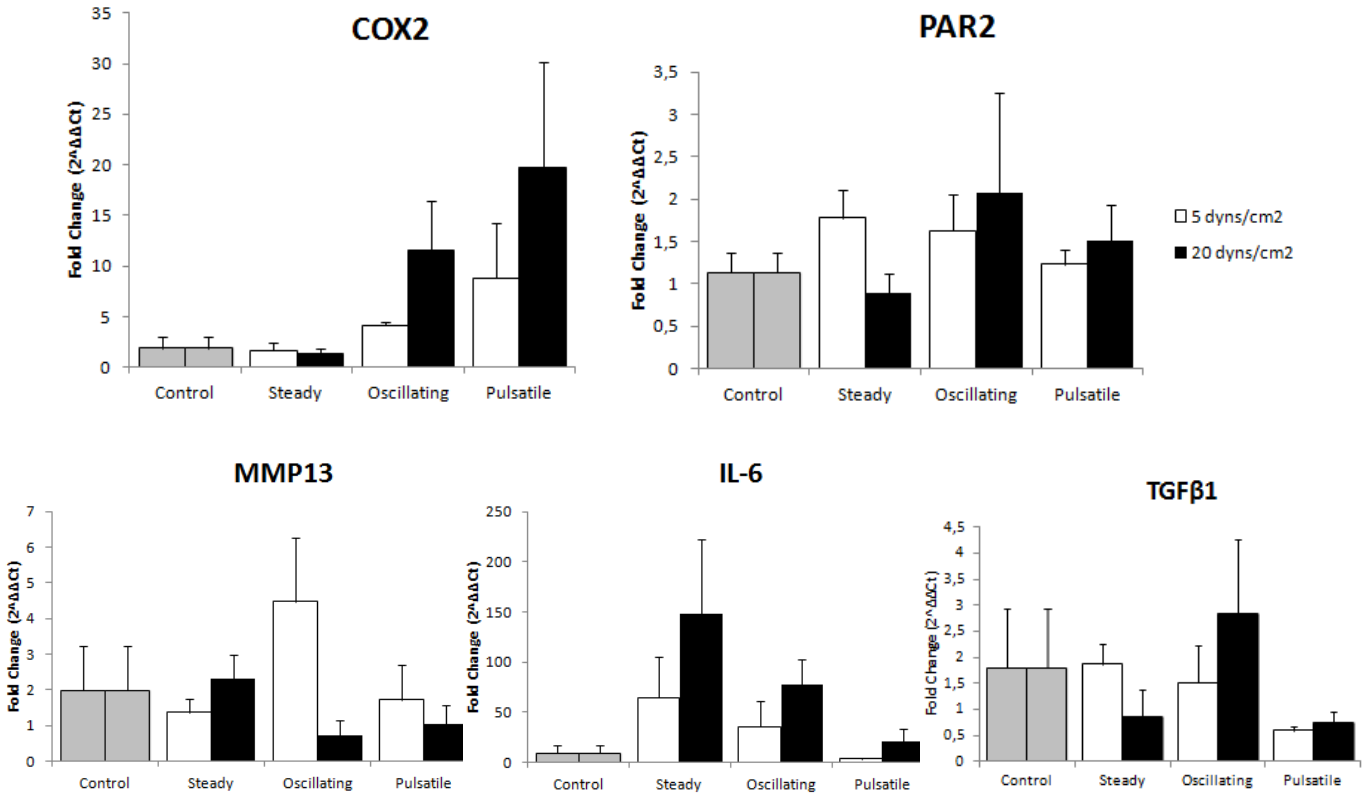

Figure 9. PAR2, COX2, IL-6, TGFB1 and MMP13 expression induced by FSS. *Statistically significant was considered when *P<0.05.*

Gene expression after PAR2 artificial activation or inhibition

PAR2 can be artificially activated and inhibited. Therefore it was of interest to test whether OA related gene expression changes depending on activation or inhibition of PAR2 protein in SW1353 cells. After 1 hour of pulsatile 5dyns/cm^2 FSS, natural activation was mimicked for 2hours using a SLIGKV peptide and its inhibition by using SAM11 antibody. As in the previous experiment, *PAR2, COX2, IL-6, TFGβ1*, and *MMP13* genes expression was analyzed by mRNA extraction, reverse transcription, and qPCR.

When analyzing *PAR2* gene expression, one of the first things to be detected was its statistically significant increase of gene expression after no activating or inhibiting treatment, which was not observed in the previous fluid flow experiment, where no resting time was included in the assay. In contrast, under FSS, when PAR2 activating peptide was administrated *PAR2* gene expression was highly decreased compared to control (no treatment), and its expression was not affected compared to SLIG treated under static state, suggesting that activation of PAR2 after FSS hinders its expression. In cells treated with SAM11, *PAR2* gene expression shows that FSS induces a significant increase when compared to the static state (Figure 10A). However, external inhibition of PAR2 seems to hinder its expression, not only after Fluid Flow, but also under static condition. Nevertheless it is not statistically significant.

Whether this PAR2 external activation or inhibition could affect the expression of some related genes in SW1353 cell line was tested as well. The study resulted in a highly significant stimulation of *COX2* expression after activation of PAR2 under no fluid flow (static state). Moreover, despite not statistically significant, it seems to be a trend of *COX2* hindering its expression after 1 hour of FSS and two hours of resting time, no matter the treatment, compared to the static state (Figure 10B).

IL-6 shows significantly increased expression (~20 fold) after the external activation of PAR2 in static conditions. This effect was not observed under FSS, where activation of PAR2 did not yield increased levels of *IL-6* (Figure 10C).

Furthermore, in order to analyze whether the increase of *IL-6* gene expression produces the expected consequent increase of IL-6 protein production several ELISA assays were accomplished. Nevertheless, the assessment did not reveal any statistically significant change in IL-6 protein levels (Figures 10D and 10E).

We also analysed *TGFβ1*. Its expression seems to be increased under no FSS, but this tendency is not statistically significant if examinated individually by treatments (SLIG or SAM11). Nonetheless, *TGFβ1* gene expression of cells under static state (including control, and treated cells, SLIG and SAM11) presented a statistically significant raise compared to those that underwent FSS (Figures 10F and 10G).

Finally, *MMP13* showed no significant changes in FSS, either PAR2 activation or inhibition in SW1353 cell line. However, there is a trend whereby cells that underwent FSS appear to have a higher level of *MMP13* gene expression, but it is not statistically significant when all the data was pooled attending to the type of flow (Figures 10H and 10I).

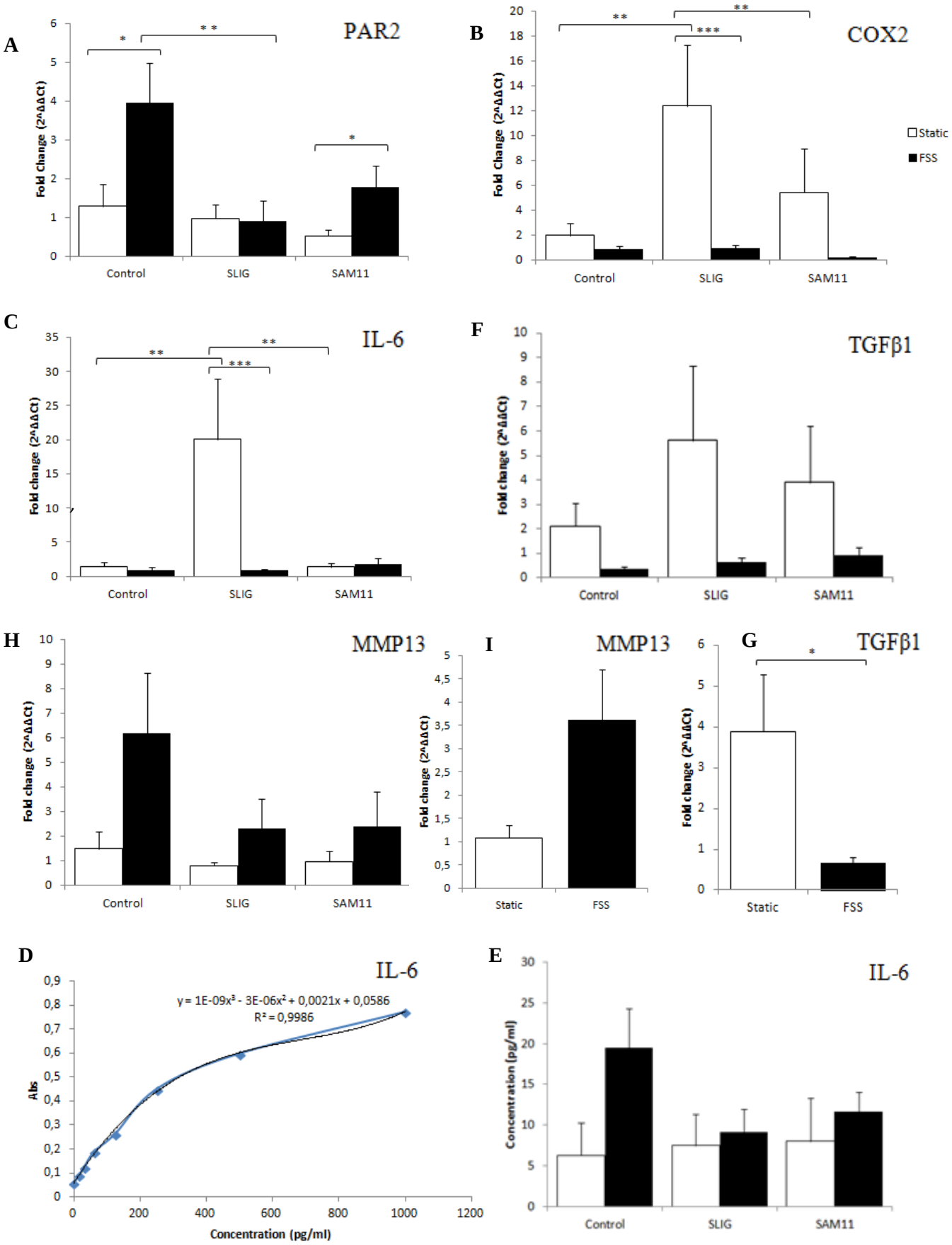

Figure 10. Gene and protein expression after PAR2 external activation or inhibition, and 2 hours of resting time. A) *PAR2 gene expression.* ***B)*** *COX2 gene expression.* ***C)*** *IL-6 gene expression.* ***D)*** *and* ***E)*** *IL-6 protein levels.* ***F)*** *and* ***G)*** *TGFβ1 gene expression.* ***H)*** *and* ***I)*** *MMP13 gene expression. *P<0.05, **P<0.01, and ***P<0.001. Two-way ANOVA statistical analysis. Static state in white, and FSS in black.*

DISCUSSION

Human osteoarthritis is not easily studied in humans. Thus, the induced DMM in mice was used as an *in vivo* model in order to test whether there was a relationship between PAR2 and OA, what it had been previously observed by W. R. Ferrell *et al.* [12]. The result was, as expected because of its theoretical importance in osteoarthritis development, an over expression of the PAR2 protein within the chondrocyte cells on the cartilage of femur and tibial condyles (Figure 6). The result was statistically significant, and highly convincing of the direct relationship between OA and presence of PAR2 protein in cells within the OA joint. *In vitro* results correlated the *in vivo* studies, but only shortly after FSS stimulation. PAR2 protein levels were increased when mechanical stimulation was applied (Figure 7). Furthermore, the type and the intensity of fluid flow showed different levels of PAR2 protein detected. The experiment revealed that not all types of flows and intensities have the same ability to mechano-stimulate the cells in the same manner. This connects with the previously mentioned chondroprotective effect of low levels of FSS [9].

Steady and Oscillating Flow at 5dyns/cm^2 showed very small differences with the Static control, which could be understood as a physiological pressure. Nevertheless, FSS generated at 20dyns/cm^2, including Steady, Oscillating and Pulsatile flows, revealed an elevated quantification of PAR2, which in this case could be referred to as a pathological pressure. Surprisingly, Pulsatile flow at 5dyns/cm^2 appeared to stimulate *PAR2* expression at the same level of the 20dyns/cm^2 fluid flows and it was the only one with statistically significant differences. When we examined the location of the PAR2 fluorescence in the cells we observed that it was concentrated in vesicles inside the cell, which suggests that PAR2 had internalized. This correlates with the fact that PAR2 proteins are stored in Golgi vesicles to have a rapid response to PAR2 activation [14]. Since we did not permeabilise the membrane, there are questions about how the antibody reached the cytoplasm. Nevertheless, K. Yamamoto *et al.* [21] reported that shear stress increase membrane fluidity in many cell types, which is also known to be directly related with membrane permeability [22], suggesting that FSS is the responsible of the cell membrane permeability to antibodies, what could alter PAR2 protein detected levels, opening a second hypothesis where the detected PAR2 protein might not be dependent on the synthesized PAR2, but on cell membrane permeability. There are also doubts about the efficiency of SAM11 as an anti-PAR2 antibody to use on fixed cells and tissue, where colleagues (personal communication) have encountered problems using this antibody to detect PAR2 on the cell membrane. Once the tertiary and quaternary structures of PAR2 are published it might be possible to investigate this matter further.

In order to know if the detected PAR2 protein of the previous experiment could have just been synthesized because of the stimulation or it was already present within the cells, *PAR2* gene expression was studied after one hour, and no changes in *PAR2* gene expression were seen. This finding suggest that the point of highest stimulation is the initial first surge of flow, so after 1 hour under flow the gene expression could be returning to normal because PAR2 protein levels could have reached the maximum, which was previously suggested to happen in 30 minutes by S. K. Böhm *et al.* [23]. On the other hand, *COX2* gene expression, a gene that has been reported to be stimulated by FSS not in a direct PAR2 dependent manner,

was observed to follow a trend where Oscillating 20dyns/cm^2 and Pulsatile flows appeared to have rising values of its expression, although none significant due to the high variability within the experiment. As indicated by P. Wang *et al*. [9], *COX2* (*PTGS2*) gene, encodes for an enzyme that sinthesises PGE2, which induce the expression of PICs and MMPs. Furthermore, H. Komatsu *et al.* [14] reported that PGE2 can inhibit PAR2 signal transduction by decreasing its surface expression. Thus, the data here presented suggest it could have occurred that PAR2 proteins were internalized into vesicles after its expression on the membrane responding to PGE2.

Levels of *PAR2* gene expression after 2 hours of resting time appeared to be increased (Figure 10A), which was not observed within the non resting time assay. This 2 hours gap could have been used by the newly synthesized PGE2 or by any other mechanism to induce its internalization, generating a swelling in *PAR2* gene expression. Activation with external SLIG peptide, a fact that is reasonably faster than the biological production of PGE2, results in a reduced value of its gene expression compared to the non activated, suggesting that PAR2 levels of the membrane were replaced by the fully PAR2 deposits. Regarding at *COX2* gene expression after 2 hours of gap time (Figure 10B), it is noteworthy that its expression is not sustained in a highly activated level, and in fact, it is temporally induced in response to high fluid shear stress and then return to the basal level after a 2 hour of FSS exposure [9], whose expression could have been even repressed compared to static sate to balance the protein number within the cells.

IL-6, which appears to be one of the main mediators in the pathophysiology of OA but alone is not capable of stimulating cartilage degradation directly, is also upregulated in response to PGE2 by a mechanism that is not completely known in chondrocytes [9, 24]. We observed that after one hour of fluid flow its gene expression has no statistically significant changes, and it seems to follow no particular tendency, maybe an increased expression when mechanical loading is higher, 20dyns/cm^2 of flow (Figure 9), which could be explained since *COX2* is also upregulated by mechanical loading. When PAR2 is activated by SLIG peptide after no FSS, a marked increase of *IL-6* gene expression is detected (Figure 10C), suggesting that when PGE2 mediated PAR2 internalization is not produced, activation of basal PAR2 induces *IL-6* expression in a direct manner. Nevertheless, IL-6 protein levels were incomprehensibly not detected by ELISA assay (Figures 10D and 10E), because all the reported IL-6 results were under the detection threshold of the technique. The study was repeated several times. It was determined that the volume of total media used per well was excessive to allow detection of secreted IL-6 by the low number of cells in the well. Because of the experiment set up, we could not reduce the volume any further.

PGE2 regulates PICs mediated - MMP expression, depending on the concentration of PGE2 in joint cartilage, as well as by other mechanisms [9, 25, 26]. Low fluid shear stress could be chondroprotective and suppresses the expression of MMP13, a protein expressed under high FSS that produces collagen type II degradation and generates the breakdown of the extracellular matrix (ECM), contributing to the development of OA [9, 25]. Nevertheless, the results obtained in this experiment show no change in *MMP13* gene expression in response to FSS (Figure 9), neither under pathologic FSS, nor under the physiological one. Still, there is a trend to increased gene expression after FSS and 2 hours resting time (Figures 10H and 10I).

TGFβ1 is a homeostasis regulator for both subchondral bone and articular cartilage, and increasing evidence indicates altered TGFβ signaling is involved in the pathogenesis of OA development and may have dual role [9, 27]. It has been reported by G. Zhen *et al*. [27] to be upregulated in the early phase of OA, and other researchers disclosed that inhibition of its activity leads to development of OA [28], highlighting the hypothetical dual role. Our assay did not reveal any significant change in its expression after one hour of fluid flow (Figure 9), but it did after two hours resting time (Figures 10F and 10G), suggesting that *TGFβ1* is inhibited after mechanical stimulation.

Because of the intention of protein and gene expression analysis, it was used an *in vitro* model of SW1353 chondrocyte cells and fluid flow as the originator of the shear stress. Human chondrosarcoma cell line SW1353 was used as a model of primary human (adult articular) chondrocytes (PHCs), due to its appropriated culturing properties that make it be a very suitable *in vitro* model system and avoid the limitations of PHCs caused by the lack of a sufficient number, its variability between donors, and the temporal dedifferentiation and change in the gene expression pattern. Nevertheless, SW1353 cells were characterized as a cell line with only a limited potential to mimic PHCs, suggesting that their use as a substitute for chondrocytes is challenged and requires additional experimental evidence and validation. It has been suggested that they are not a good candidate *in vitro* system for studying chondrocyte anabolism, but they seem to be a valuable *in vitro* system for investigating catabolic gene regulation, despite the fact that mRNA expression levels of matrix-degrading enzymes, including MMP13 and other PICs, were also partly lower in SW1353 cells than those observed in adult articular chondrocytes. Furthermore, it was also reported by M. Gebauer *et al.* [29] that SW1353 cells can adopt an epithelial phenotype after long term culture. All this data together, added to the variability found in the gene expression analysis, suggest that resulting profiles of genes expression cannot be completely believed, and further experiments with fresh cell cultures are required, and probably better using primary chondrocytes. Nonetheless, it is important to remark that in OA there are strong functional interactions among the cartilage, synovium, and subchondral bone, and this complexity is difficult to achieve, and lacks in any *in vitro* model.

Additional experiments will be essential to gain deeper understanding in PAR2 regulation and its importance as a mechanotransductor, if it indeed is one, and its role in OA mediation. Time course experiments of gene expression, likewise immunofluorescence assays after permeabilisation and the usage of antibodies able to detect PAR2 on the membrane, as well as ELISAs could reveal important information about the precise moments of protein synthesis, confirming its presence thanks to ELISAs, and the location of PAR2 along the time course, avoiding the possible alterations originated by cell membrane permeability.

In conclusion, all this data together suggest that shear stress might induce PAR2 internalization, directly or via PEG2 as proposed by Komatsu *et al.* [14]. Thus, despite the required confirmations, this study would bring light into the largely unknown mechanism of PAR2 regulation and osteoarthritis development, revealing that although both PAR2 and COX2 seem to play very important roles in the mediation of OA progression, they might work together, as part of the same mechanism, or by two different but connected mechanisms that collectively potentiate OA disease.

CONCLUSIONS

- Both *in vivo* and *in vitro* results suggest a direct relation between PAR2 and osteoarthritis. OA DMM mice model showed an over expression of the PAR2 protein within the chondrocyte knee cells, and *in vitro* OA mimicked by Fluid Shear Stress showed an apparently increase of PAR2 protein levels in SW1353 human chondrocyte cell line.
- PAR2 protein levels in SW1353 cell line seem to be dependent on the type and the intensity of the fluid flow. Not all types of flows and intensities mechano-stimulate the cells in the same manner. Steady and Oscillating Flow at 5dyns/cm^2 showed very small differences with the Static control, which could be understood as a physiological pressure, whereas FSS generated at 20dyns/cm^2, including Steady, Oscillating and Pulsatile flows, revealed an elevated quantification of PAR2, which could be referred to as a pathological pressure. Pulsatile flow at 5dyns/cm^2 appeared to stimulate *PAR2* expression at the same level of the 20dyns/cm^2 fluid flows and it was the only one with statistically significant differences.
- Inverted Spinning Disk Confocal Microscope images of PAR2 within the cell, together with recent publications about membrane fluidity, suggest that FSS might turn membrane of SW1353 cells into permeable to antibodies. This would open a second hypothesis about the detected levels of PAR2 protein, which could not be dependent on the synthesized PAR2, but on cell membrane permeability.
- After 1 hour Fluid Flow stimulation PAR2 protein was found within vesicles inside the cells, which might suggest that PAR2 had internalized. This internalization could be in response to PGE2, attending to *PAR2* and *COX2* gene expression results too.
- *PAR2* gene expression seems to be stimulated after FSS and resting time, but repressed when it is externally activated. On the other hand, *COX2* looks to be temporally induced in response to high fluid shear stress and then return to the basal level after a 2 hour of FSS exposure, whose expression could have been even repressed compared to static sate to balance the protein number within the cells.
- When PAR2 is activated by SLIG peptide after no FSS, a marked increase of *IL-6* gene expression is detected, suggesting that when PGE2 mediated PAR2 internalization is not produced, activation of basal PAR2 could induce *IL-6* expression in a direct manner. Nonetheless, this result couldn't be corroborated by the ELISA assay.
- Neither *MMP13* nor *TGFβ1* revealed a change in gene expression after 1h of stimulation, but they seem to follow an opposite trend after two hours resting time and PAR2 treatment experiment, stimulating *MMP13* gene expression and inhibiting the *TGFβ1* one.

CONCLUSIONES

- Los resultados obtenidos tanto *in vivo* como *in vitro* sugieren una relación directa entre PAR2 y osteoartritis. Los ratones DMM (con desestabilización del menisco medial) usados como modelo de osteoartritis, mostraron una sobreexpresión de la proteína PAR2 en los condrocitos de la rodilla, y la línea celular de condrocitos humanos SW1353 estimulados por un esfuerzo de corte generado por fluido, también mostraron un incremento en los niveles de PAR2.

- Los niveles de la proteína PAR2 en la línea celular SW1353 dependen del tipo y de la intensidad de fluido que genera el esfuerzo de corte, de tal modo que no todos los tipos ni intensidades de fluidos generan una misma estimulación mecánica. El flujo constante y el flujo oscilante a 5dinas/cm^2 (0.5Pascales) muestran muy pequeñas diferencias con el control estático, que pueden ser considerados como presiones fisiológicas, mientras que el esfuerzo de corte a 20dyn/cm^2 (2.0Pa), tanto en flujo constante, como en oscilante y pulsátil, revelaron una gran cantidad de PAR2, lo que puede entenderse como presiones patológicas. El flujo pulsátil a 5dyn/cm^2 parece estimular a *PAR2* al mismo nivel que los flujos de 20dyn/cm^2, siendo el único flujo con diferencias estadísticas en la estimulación de *PAR2*.
- Las imágenes obtenidas con el microscopio confocal de disco giratorio, junto con las recientes publicaciones sobre la fluidez de la membrana, sugieren que el esfuerzo de corte producido por flujo de fluido podría inducir la permeabilización de la membrana de las células SW1353 frente a anticuerpos, lo que abriría una segunda hipótesis sobre los niveles detectados de la proteína PAR2, que podrían depender de la permeabilidad de la membrana, y no de la síntesis.
- Tras una hora de estimulación mediante el flujo del fluido, se observó que PAR2 se hallaba contenido en vesículas dentro de las células, lo que puede sugerir que se ha producido su internalización. Atendiendo a los resultados de la expresión génica de *PAR2* y *COX2*, la sugerida internalización podría ser en respuesta a PGE2.
- La expresión génica de *PAR2* parece estar estimulada tras el esfuerzo cortante por flujo y un periodo de reposo, y reprimida cuando la proteína es activada externamente. Por otro lado, *COX2* parece estar temporalmente inducido en respuesta a altos esfuerzos de corte, sin embargo, cuando este esfuerzo cesa, se observa que recupera los niveles basales después de dos horas de la exposición, llegando incluso a poder reprimirse su expresión para compensar el número de proteína en la célula.
- La activación de PAR2 por el péptido externo SLIG sin haberse producido ningún esfuerzo de corte previo, genera un marcado incremento de la expresión génica de *IL-6*, sugiriendo que cuando la internalización de PAR2 mediada por PGE2 no se está produciendo, la activación del PAR2 basal puede inducir la expresión de *IL-6* de manera directa. Sin embargo, esto no pudo ser corroborado mediante el ensayo de ELISA.
- Ni *MMP13* ni *TGFβ1* revelaron un cambio en su expresión génica tras la estimulación de una hora, a pesar de que parecen seguir tendencias opuestas en el ensayo con tratamiento de PAR2 y dos horas de reposo, en el que se estimula la expresión génica de *MMP13*, y se inhibe la de *TGFβ1.*

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