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# Grooved poly(lactide-co-trimethylene carbonate) substrates in tenogenic media maintain human tendon derived cell phenotype in culture – A preliminary report☆

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ABSTRACT

Tissue engineering strategies for tendon repair and regeneration rely heavily on the use of tendon derived cells. However, these cells frequently undergo phenotypic drift *in vitro*, which compromises their therapeutic potential. In order to maintain the phenotype of tendon derived cells *in vitro*, microenvironmental cues (biophysical, biochemical and/or biological in origin) have been used to better imitate the complex tendon microenvironment. Herein, the influence of planar and grooved (groove width of ~1.0 μm, groove depth of ~1.4 μm and distance between groves of ~1.7 μm) poly(glycolide-co-ε-caprolactone) substrates with elastic modulus of 7 kPa and poly (lactide-co-trimethylene carbonate) substrates with elastic modulus of 12 kPa on human tendon derived cell response was assessed, using planar tissue culture plastic substrates of 3 GPa elastic modulus as control, in both basal and tenogenic media. At day 17, the grooved 12 kPa poly(lactide-co-trimethylene carbonate) substrate induced the highest deposition and alignment of collagen type I in tenogenic media. At day 17, the grooved 12 kPa poly(lactide-co-trimethylene carbonate) substrate and the tissue culture plastic induced the highest deposition and the tissue culture plastic and the planar 7 kPa poly(glycolide-co-ε-caprolactone) induced the lowest alignment of tenascin C in tenogenic media. Also at day 17 in tenogenic media, the grooved 12 kPa poly(lactide-co-trimethylene carbonate) substrate induced the upregulation of most tenogenic genes (COL1A1, COL3A1, MKX, TNMD). Our data further support the notion of multifactorial tissue engineering for effective control over cell fate *in vitro* setting.

## Introduction

In clinical setting, tendon tissues are inherently difficult to regenerate or repair due to their low vascularity and cellular activity [1]. Considering that tissue grafts and biomaterials have limited capacity to restore native tendon function [2], it is expected that tissue-engineering approaches will provide a functional therapy in the years to come [3,4]. To this end, it is essential to be able to maintain tendon derived cell (TDC) phenotype *ex vivo*, the cell population of choice for tendon engineering therapies [5], but a cell population that readily loses its

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function in culture [6].

To mimic the *in vivo* microenvironment of the tendon niche *in vitro*, physical, chemical and biological cues are under intense investigation [7,8], with physical signals leading the race, due to their relative simplicity in implementation, scalability and clinical translation. In particular, micro- and nano- grooved topographies have been shown to induce physiological elongated TDC morphology, to stimulate tendon-specific extracellular matrix (ECM) synthesis and anisotropic deposition and to upregulate tendon-specific genes [9–12]. Despite the undeniably beneficial effects of topographical cues on TDC phenotype maintenance, the rigidity of the substrates (another key modulator of cell, including TDC [13], fate [14,15]) has been largely underappreciated. As a result, when TDCs were grown on rigid substrates, even with physiologically relevant topography, trans-differentiation was inevitable, as was evidenced by upregulation of chondrogenic and osteogenic genes [16].

In recent years, the combined effect of surface topography and substrate stiffness on cell function has been the subject of multiple investigations (including TDCs [17]), the findings of which have been recently summarised [18,19]. Notwithstanding the significant advancements, it is worth noting that most research endeavours in this space are carried out using non-degradable polymers (e.g. polydimethylsiloxane, PDMS, [20–23]), which are of little value in the development of implantable devices or very rigid substrates, such tissue culture plastic (TCP) polystyrene [24,25] with ~ 3 GPa elastic modulus. Considering that mechanical properties mismatch between targeted tissue and implanted biomaterial is a significant contributor of implant failure [26–29], it is imperative to assess the combined effect of substrate rigidity and surface topography on cellular function using biodegradable materials of clinical relevance.

Herein, the influence of planar and grooved (groove width of 1.0 μm, groove depth of 1.5 μm and distance between groves of 1.6 μm) poly (glycolide-co-ε-caprolactone), PGCL 10/90, substrates with elastic modulus of 7 kPa and planar and grooved (groove width of 1.0 μm, groove depth of 1.4 μm and distance between groves of 1.8 μm) poly (lactide-co-(trimethylene carbonate), PLTMC 80/20, substrates with elastic modulus of 12 kPa on human TDC response, in both basal and tenogenic media, was assessed and correlated to cells cultured on planar TCP with ~ 3 GPa elastic modulus.

## Materials and methods

### *Materials*

The polymeric substrates (206.0 ± 37.0 μm thick PGCL 10/90 and 210.5 ± 38.6 μm thick PLTMC 80/20) were produced via compression moulding and the topography was induced via imprinting lithography, as has been described in detail previously [30–32]. Atomic force microscopy and scanning electron microscopy micrographs of the planar and grooved substrates are provided in **Supplementary Figure S1**. All tissue culture plastics were purchased from Sarstedt (Ireland). All chemicals, cell culture media and reagents were purchased from Sigma Aldrich (Ireland), unless otherwise stated.

### *Cell culture*

Human tendons were obtained from patients without tendinopathy undergoing tendon surgeries at Galway University Hospital, Galway, Ireland and at Bon Secours Hospital, Galway, Ireland. Appropriate licences, ethical approvals and informed consent forms were in place (Licence Reference: CA1046). Human TDCs were extracted using the migration method, following established protocols [33–35]. Briefly, tendon segments were supplemented with 5 ml of culture media containing Dulbecco's modified Eagle medium, 10% foetal bovine serum and 1% penicillin-streptomycin (PS) and placed at 37 °C in a humidified atmosphere of 5% $CO_2$. Culture medium was changed every three days. After a few days, the first colonies of TDCs were noticed around the tendon sections. When the migrated TDCs reached 80–90% confluency, they were treated with trypsin / ethylenediaminetetraacetic acid solution and sub-cultured in T-175 tissue culture flasks until passage 2. From passage 3 to 4, the cells were cultured in basal media, composed of MesenCult™ ACF Plus Medium (StemCell Technologies, UK), 1% L-glutamine and 1% PS. At passage 5, the cells were trypsinised, seeded on the scaffolds and allowed to attach and spread for 3 days in basal media. At day 4, the media were changed to either basal media or tenogenic media [basal media supplemented with tenogenic supplement (StemCell Technologies, UK)]; thereafter the media were changed every 3 days. Protein synthesis and gene expression were assessed at day 6 and day 17. A schematic representation of the experimental design is provided as **Supplementary Figure S2**.

### *Protein synthesis and deposition analyses*

At day 6 and day 17, the cells were briefly washed with phosphate buffered saline (PBS) and fixed with 4% paraformaldehyde for 20 min at room temperature. Cells were washed again with PBS and non-specific site interactions were blocked with 3% bovine serum albumin in PBS for 30 min. The cells were incubated overnight at 4 °C with the primary antibodies for collagen type I and tenascin C (**Supplementary Table S1**), after which, they were washed 3 times with PBS, followed by 30 min incubation at room temperature with appropriate secondary antibodies (**Supplementary Table S1**). Nuclei were counterstained with Hoechst Fluorescent Stain. To determine matrix composition at each time point, fluorescence area per image was quantified using ImageJ software (NIH, USA). Three replicates per conditions were imaged and three fields of view were taken from each well (total of 9 images analysed per experimental group).

### *Gene expression analysis*

Total RNA was isolated using the RNeasy Plus Micro Kit (Qiagen, Germany) according to the manufacturer's protocol. Briefly, samples were disrupted in Buffer RLT and homogenised. Ethanol was then added to the lysate and the samples were transferred to the RNeasy Micro spin column. All bind, wash and elution steps were performed by centrifugation in a microcentrifuge. Total RNA was retained in the membrane (bind step), contaminants were efficiently washed away (wash step) and high-quality RNA was eluted in RNase-free water (elution step). RNA concentration and purity were determined using a NanoDrop 1000 (ThermoFisher Scientific, Ireland). Samples with RNA purity values of 260/280 ratio ~1.8 and 260/230 ratio ~1.9 were used for qPCR experiments. RNA integrity was assessed with an Agilent 2100 Bioanalyser (Agilent Technologies, Ireland). Samples with RNA integrity (RIN) values of $> 8$ were used for qPCR experiments. Samples with RIN $< 8$ were excluded from the study. 1 μg total RNA was reverse transcribed using the iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories, Ireland). 5 ng cDNA were subsequently analysed by qPCR on a StepOnePlus™ Real-Time PCR System (ThermoFisher Scientific, Ireland), using TaqMan primer probe assays (IDT, Belgium) and TaqMan Gene Expression Mastermix (ThermoFisher Scientific, Ireland). The amplification conditions were 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. qBasePlus v. 2.4 (Biogazelle NV, Belgium) was used to perform geNorm analysis to determine the optimal number of reference genes. CQ values were analysed and normalised relative quantities were calculated by normalising the data to the expression of three validated endogenous control genes (HPRT1, TBP, EIF2B1; assay IDs are provided in **Supplementary Table S2**) with qBasePlus v. 2.4 (Biogazelle NV, Belgium) [36]. Nanog Homeobox (NANOG), SRY-box 2 (SOX2), early growth response 1 (EGR1), early growth response 2 (EGR2), scleraxis (SCX), collagen type I (COL1A1), collagen type III (COL3A1), mohawk homeobox (MKX), tenascin C (TNC) and tenomodulin (TNMD) were analysed by qPCR in basal and

tenogenic media at day 6 and day 17 (assay IDs and function of each molecule assessed are provided in **Supplementary Table S2**). Osteogenic [osteopontin (SPP1) and bone sialoprotein (BSP)], chondrogenic [SRY-Box Transcription Factor 9 (SOX9) and type II collagen (COL2A1)] and adipogenic [fatty acid-binding protein 4 (FABP4) and CCAAT/enhancer-binding protein alpha (CEBPA)] genes were analysed by qPCR in basal and tenogenic media at day 17 (assay IDs are provided in **Supplementary Table S2**).

### *Statistical analysis*

Data are expressed as mean ± standard deviation. Statistical analysis was performed using GraphPad v6.01 (GraphPad Software Inc., USA). One- or two- way ANOVA was used for multiple comparisons and a Tukey *post hoc* test was used for pairwise comparisons after confirming that the samples followed a normal distribution (Kolmogorov-Smirnov test) and had equal variances (Bartlett's and Levene's test for homogeneity of variances). When either or both of these assumptions were violated, nonparametric tests were used for multiple (Kruskal-Wallis test) and pairwise (Mann-Whitney test) comparisons. Statistical significance was accepted at $p < 0.05$.

## Results

### *Protein synthesis analysis*

In basal and tenogenic media, qualitative and quantitative immunocytochemistry analyses revealed that the PLTMC 80/20 G induced the highest ($p < 0.05$)% area of deposited collagen type I at day 17 (the longest time point assessed) and the highest ($p < 0.05$)% of collagen type I with 90° alignment at both day 6 and day 17 (Fig. 1).

Qualitative and quantitative immunocytochemistry analyses revealed that the PLTMC 80/20 P induced the highest ($p < 0.05$)% area of deposited tenascin C at day 17 in basal media; the PLTMC 80/20 G

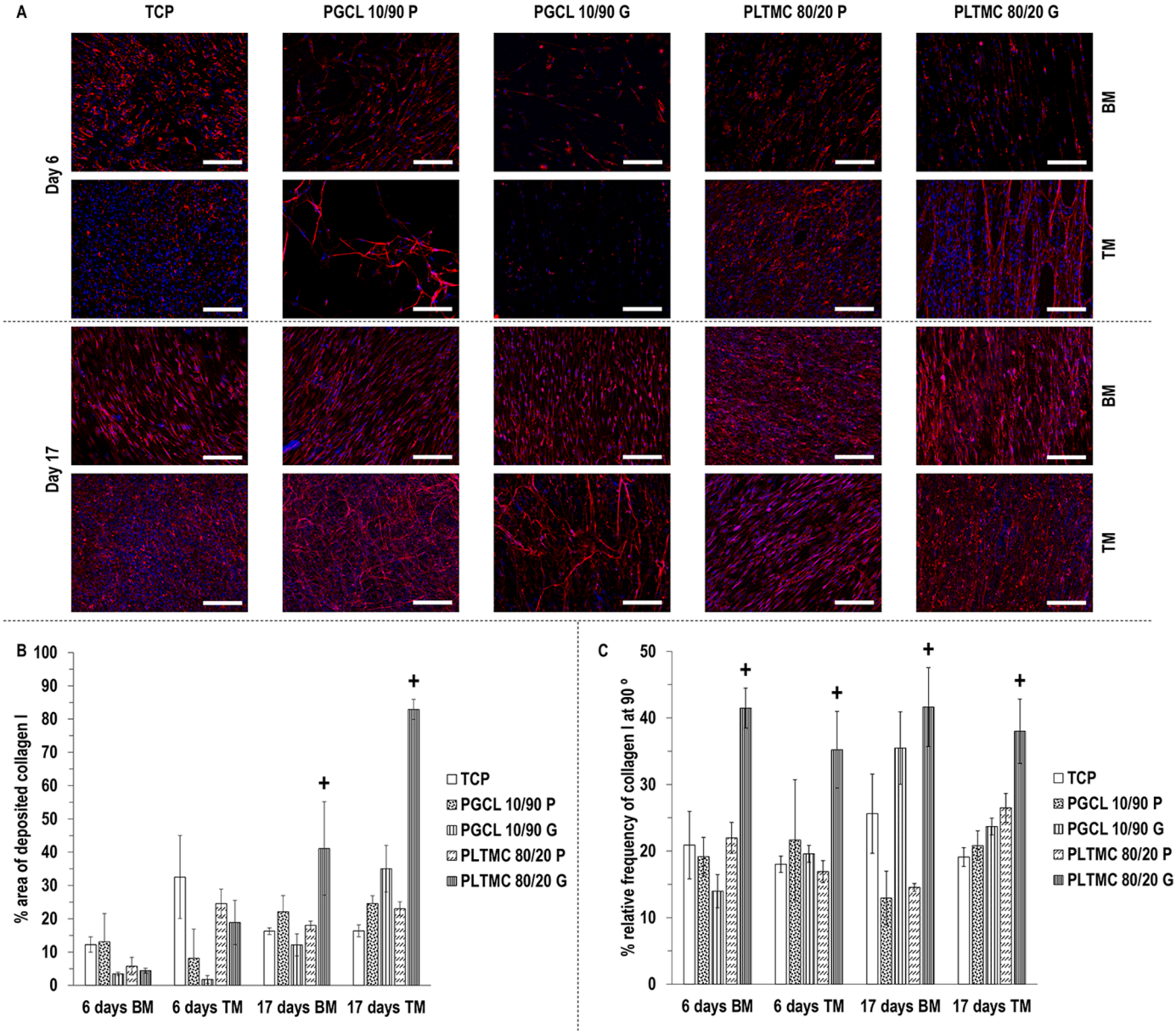


**Fig. 1.** Immunocytochemistry (A),% area of deposited collagen type I (B) and% relative frequency of collagen type I at 90° (C) of human TDCs at day 6 and day 17 in basal media (BM) and tenogenic media (TM) on 3 GPa TCP and planar (P) and grooved (G) 7 kPa PGCL 10/90 and 12 kPa PLTMC 80/20 substrates. Collagen type I: red. Nuclei: blue. Scale bars: 100 μm. + indicates highest ($p < 0.05$) value at a given time point and media.

induced the highest ($p < 0.05$)% area of deposited tenascin C at day 17 in tenogenic media; the PLTMC 80/20 G induced the highest ($p < 0.05$)% of tenascin C with 90° alignment at day 17 in basal media; the PGCL 10/90 P induced significantly ($p < 0.05$) lower than the other groups% of tenascin C with 90° alignment at day 17 in basal media (Fig. 2).

*Gene expression analysis*

Gene expression analysis data are provided in Fig. 3. At day 6 in basal media, the PGCL 10/90 P and the PLTMC 80/20 G upregulated (fold change ≥ 2.0) two genes (NANOG, SOX2), the PGCL 10/90 G and the PLTMC 80/20 P upregulated (fold change ≥ 2.0) three genes (NANOG, SOX2, EGR1) and the TCP upregulated (fold change ≥ 2.0) three genes (SOX2, MKX, TNC). At day 6 in tenogenic media, the PGCL 10/90 P upregulated (fold change ≥ 2.0) seven genes (NANOG, SOX2, EGR1, EGR2, SCX, COL1A1, TNC), the PLTMC 80/20 P upregulated (fold change ≥ 2.0) six genes (NANOG, SOX2, EGR2, SCX, COL1A1, TNC), the PGCL 10/90 G upregulated (fold change ≥ 2.0) five genes (SOX2, EGR2, SCX, COL3A1, TNC), and the TCP and PLTMC 80/20 G upregulated (fold change ≥ 2.0) four genes (EGR2, SCX, MKX, TNC and EGR2, SCX, COL3A1, TNC, respectively). At day 17 in basal media, the PGCL 10/90 G upregulated (fold change ≥ 2.0) five genes (SCX, COL3A1, SOX9, FABP4, CEBPA), the PGCL 10/90 P upregulated (fold change ≥ 2.0) three genes (NANOG, SOX9, CEBPA), the PLTMC 80/20 P and the PLTMC 80/20 G upregulated (fold change ≥ 2.0) two genes (SOX9, CEBPA) and the TCP upregulated (fold change ≥ 2.0) one gene (CEBPA). At day 17 in tenogenic media, the PLTMC 80/20 G upregulated (fold change ≥ 2.0) five genes (COL1A1, COL3A1, MKX, TNMD, FABP4), the TCP (COL1A1, COL3A1, MKX, FABP4), PGCL 10/90 G (COL1A1, COL3A1, MKX, FABP4) and PLTMC 80/20 P (NANOG, SOX2, MKX, FABP4) upregulated (fold change ≥ 2.0) four genes and the PGCL 10/90 P upregulated (fold change ≥ 2.0) two genes (MKX, FABP4). At day 17, SPP1, BSP and COL2A1 were not detected independently of the media

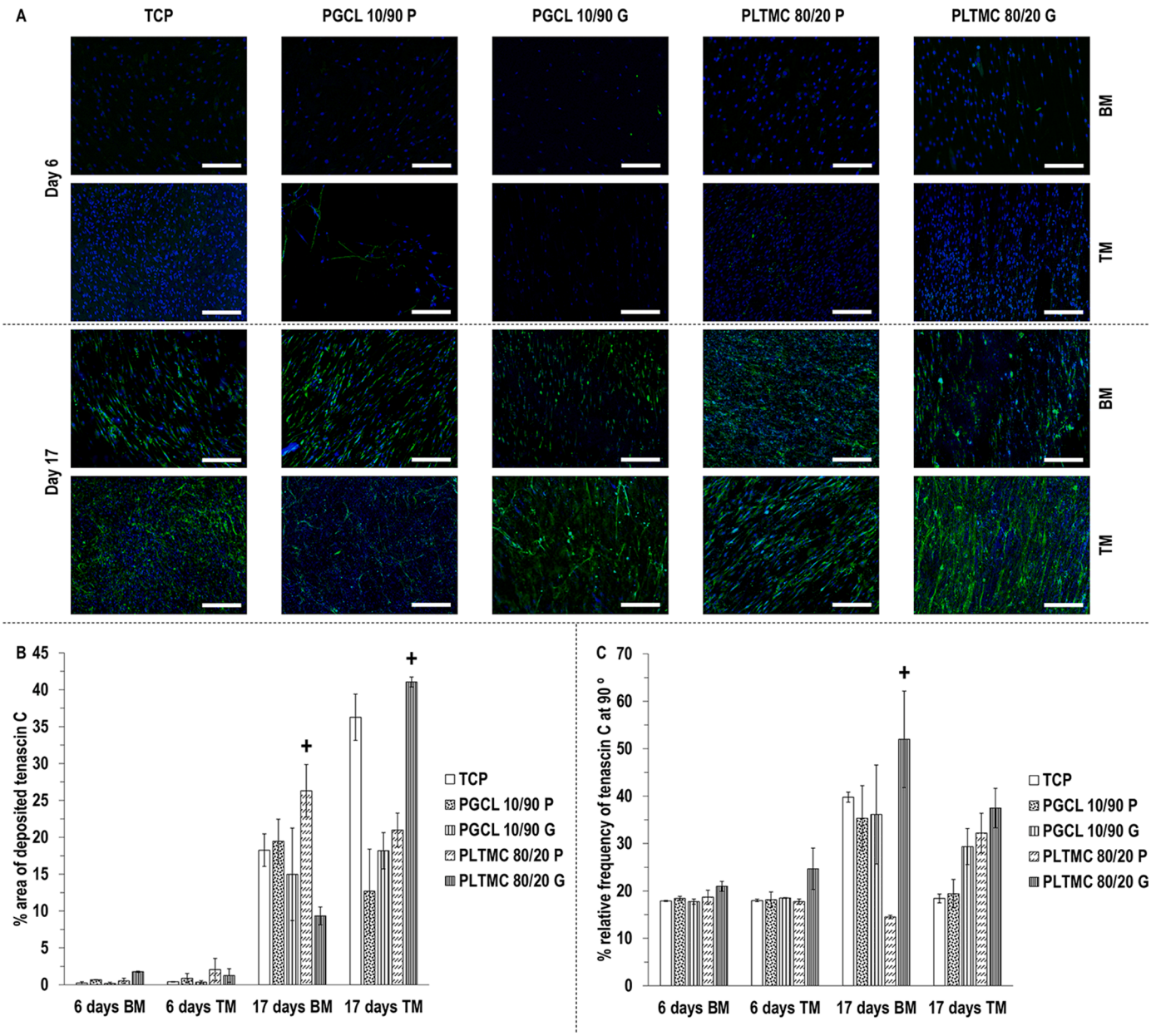


**Fig. 2.** Immunocytochemistry (A),% area of deposited tenascin C (B) and% relative frequency of tenascin C at 90° (C) of human TDCs at day 6 and day 17 in basal media (BM) and tenogenic media (TM) on 3 GPa TCP and planar (P) and grooved (G) 7 kPa PGCL 10/90 and 12 kPa PLTMC 80/20 substrates. Tenascin C: green. Nuclei: blue. Scale bars: 100 μm. + indicates highest ($p < 0.05$) value at a given time point and media.


**Fig. 3.** Gene analysis of human TDCs at day 6 and day 17 in basal media (BM) and tenogenic media (TM) on 3 GPa TCP and planar (P) and grooved (G) 7 kPa PGCL 10/90 and 12 kPa PLTMC 80/20 substrates. Red indicates upregulation (fold change $\geq$ 2.0), green indicates downregulation (fold change $<$ 2.0), white indicates not detected and blue indicates groups that did not produce sufficient quantities of RNA for analysis.

and the substrate used. At day 6, not sufficient quantities of RNA were obtained from samples allocated to osteogenic (SPP1, BSP), chondrogenic (SOX9, COL2A1) and adipogenic (FABP4, CEBPA) genes.

## Discussion

Although the combined effect of surface topography and substrate rigidity has been the subject of many investigations, most of the work to-date in this field has been carried out using non-degradable polymers. Thus, herein, the influence of planar and grooved PGCL 10/90 substrates with elastic modulus of 7 kPa and planar and grooved PLTMC 80/20 substrates with elastic modulus of 12 kPa on human TDC response, in both basal and tenogenic media, was assessed and correlated to cells cultured on planar TCP with ~ 3 GPa elastic modulus.

### *Protein synthesis and deposition analyses*

Starting protein analysis, the most notable finding was that at day 17, the grooved 12 kPa PLTMC 80/20 substrate induced the highest collagen type I deposition, especially in tenogenic media. Growth factors are frequently employed to enhance ECM synthesis [37]. It is interesting to note that only the grooved 12 kPa PLTMC 80/20 at both time points and in both media consistently induced aligned collagen type I deposition. Considering that both substrates had similar anisotropic topography, we believe that the observed difference is due to the elastic modulus difference (7 kPa PGCL 10/90 and 12 kPa PLTMC 80/20). Again, our data are in agreement with previous observations, where stiffer, as opposed to softer and similar in topography, substrates induced bidirectional cell and deposited ECM orientation (human tenocytes, PDMS, 1000 kPa, 130 kPa, 50 kPa, grooved topography groove depth of ~2000, ~1900 and ~1850 nm [17]). With respect to tenascin C, at day 17 in tenogenic media, the planar TCP and the grooved 12 kPa PLTMC 80/20 substrate induced the highest tenascin C deposition. We attribute this enhanced tenascin C deposition to the combined effect of topography and rigidity. A previous study reported increased tenascin C synthesis, when mouse adipose-derived stem cells were cultured on PDMS substrates with groove and ridge width of 10 μm and groove depth of 3 μm [38]. It is worth noting that in the case of tenascin C, when high amounts were deposited, independently of the substrate and media, the alignment was reduced. A similar observation has been reported previously for ECM deposition in the presence of macromolecular crowding; the authors speculated that the cells were not able to process fast enough the enhanced deposited ECM in an aligned fashion [39].

*Gene expression analysis*

With respect to gene analysis, firstly, in basal and tenogenic media, a significant reduction in tenogenic genes was observed form day 6 to day 17, in alignment with previous publications with regards to the susceptibility of TDCs to phenotypic drift [6,40,41]. Secondly, at day 17, in basal media, the expression of both chondrogenic and adipogenic genes was increased and in tenogenic media, the expression of adipogenic genes was increased. We attribute this to the TDC extraction protocol. Specifically, TDCs are customarily extracted using either the collagenase digestion [6] or the migration [42] protocol. It is evidenced that the migration protocol that was used herein does not necessarily provide a pure tenocyte population, but rather a mixed population of tenocytes and tendon stem cells, with the latter having the capacity to differentiate towards adipogenic and chondrogenic lineages [43,44]. Thirdly, it is interesting to note that in tenogenic media all substrates (including the TCP) at day 6 upregulated EGR2, TNC and SCX expression, which are considered early tenogenesis markers [45–49], whilst at day 17 upregulated MKX, which is considered a later tenogenic marker [50]. Lastly, we consider of significant importance that the grooved 12 kPa PLTMC 80/20 at day 17 in tenogenic media upregulated the expression of COL1A1, COL3A1 and MKX (as did the TCP and the grooved 7 kPa PGCL 10/90), but it was the only that upregulated TNMD, another late and prominent tenogenic marker [51–54].

## Conclusions

Contemporary tissue engineering utilises simultaneously biophysical cues and soluble factors to effectively control cell fate *in vitro*. In this context, herein we assessed the influence of planar 3 GPa tissue culture plastic and planar and grooved 7 kPa poly(glycolide-co-ε-caprolactone) and 12 kPa poly(lactide-co-trimethylene carbonate) substrates on human tendon derived cell response in basal and tenogenic media. At day 17 in tenogenic media, the grooved 12 kPa poly(lactide-co-trimethylene carbonate) substrate induced the highest deposition and alignment of collagen type I; induced the highest deposition (along with tissue culture plastic) of tenascin C; and upregulated the most tenogenic genes (COL1A1, COL3A1, MKX, TNMD). Our data advocate the use of a multifactorial approach to effectively control tendon derived cell function *in vitro*.

## Author contribution

SR and DIZ designed the study and wrote the manuscript. SR carried out experiments and analysed data. EMF was involved in the processing of the polymeric films. EP optimised tenogenic differentiation method. SHK optimised qPCR experiments. AO provided the silicon master moulds with grooved topography. All authors discussed the data and approved the final version of the manuscript.

## Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Sofia Ribeiro was an early career researcher recruited at Sofradim Production, Medtronic, France and registered for PhD at University of Galway, Ireland. Yves Bayon is an employee of Sofradim Production, Medtronic, France. All other authors declare no conflicts of interest.

## Data availability

Data will be made available on request.

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## Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.bea.2023.100098.

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