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The regulation of expanded human nasal chondrocyte re-differentiation capacity by substrate composition and gas plasma surface modification

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Abstract

Optimizing re-differentiation of clinically relevant cell sources on biomaterial substrates in serum containing (S+) and serum-free (SF) media is a key consideration in scaffold-based articular cartilage repair strategies. We investigated whether the adhesion and post-expansion re-differentiation of human chondrocytes could be regulated by controlled changes in substrate surface chemistry and composition in S+ and SF media following gas plasma (GP) treatment. Expanded human nasal chondrocytes were plated on gas plasma treated (GP+) or untreated (GP−) poly(ethylene glycol)-terephthalate–poly(butylene terephthalate) (PEGT/PBT) block co-polymer films with two compositions (low or high PEG content). Total cellularity, cell morphology and immunofluorescent staining of vitronectin (VN) and fibronectin (FN) integrin receptors were evaluated, while post-expansion chondrogenic phenotype was assessed by collagen types I and II mRNA expression.

We observed a direct relationship between cellularity, cell morphology and re-differentiation potential. Substrates supporting high cell adhesion and a spread morphology (i.e. GP+ and low PEG content films), resulted in a significantly greater number of cells expressing $\alpha_5\beta_1$ FN to $\alpha_V\beta_3$ VN integrin receptors, concomitant with reduced collagen type II/I mRNA gene expression. Substrates supporting low cell adhesion and a spherical morphology (GP− and high PEG content films) promoted chondrocyte re-differentiation indicated by high collagen type II/I gene expression and a low percentage of $\alpha_5\beta_1$ FN integrin expressing cells.

This study demonstrates that cell–substrate interactions via $\alpha_5\beta_1$ FN integrin mediated receptors negatively impacts expanded human nasal chondrocyte re-differentiation capacity. GP treatment promotes cell adhesion in S+ media but reverses the ability of low PEG content PEGT/PBT substrates to maintain chondrocyte phenotype. We suggest alternative cell immobilization techniques to GP are necessary for clinical application in articular cartilage repair.

Keywords: Chondrocyte; Gene expression; Surface modification; PCR; Fibronectin; Vitronectin; Cartilage tissue engineering

1. Introduction

The ability of polymeric substrates to influence the attachment, proliferation and gene expression in anchorage dependent cells has long been established [1–5]. More recently, with the development of tissue engineering strategies, aimed at combining reparative cells and

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porous scaffolds for repairing or regenerating damaged tissues, such as articular cartilage defects, the role of cell–biomaterial interactions in regulating specific cellular functions has received considerable attention [6–10].

Scaffold-based therapies for repairing articular cartilage defects require attachment and retention of large numbers of chondrogenic cells within a three-dimensional (3D) environment to regenerate damaged extracellular matrix (ECM) [6]. In order to obtain suitable numbers of cells to seed within porous scaffolds of clinically relevant size (0.5–12.5 cm^2) [11], extensive in vitro cell expansion is required. It is well established that during expansion in monolayer, chondrocytes lose their phenotype, or de-differentiate [1,12], resulting most noticeably in a change in collagen synthesis from type II to type I, as well as a change in cell morphology from a spherical to a flattened, fibroblast-like appearance [13–15]. These series of events in which chondrocytes interact with biomaterial substrates is typically initiated by surface adsorption of serum-derived proteins followed by integrin-mediated cell adhesion. Subsequent morphological and cytoskeletal changes to the cell, as well as interactions with ions, proteins and other molecules from the culture medium, influence differentiation capacity which is ultimately reflected in the amount and quality of ECM proteins synthesized by the cell. For example, serum proteins such as vitronectin (VN) have been shown to be involved in chondrocyte adhesion to polymer substrates [16], whereas, the presence of fibronectin (FN) has been shown to induce chondrocyte de-differentiation [17–19]. However, in light of current clinical trends to move away from culture media containing animal-derived serum components to more defined media, thereby reducing the risk of disease transfer [20], substrates and surface treatment mechanisms which may influence cell adhesion and (re)differentiation in a serum-free (SF) culture environment are of particular interest. Therefore, in developing successful cell-based technologies, the optimization of biomaterial substrates and/or associated surface modification techniques which regulate, or are ''instructive'' for, adhesion and re-differentiation of de-differentiated (i.e. expanded) human chondrocytes is of significant clinical importance and has not been extensively studied.

Our group has been investigating a number of model systems to study chondrocyte–material interactions using two-dimensional (2D) poly(ethylene glycol)-terephthalate—poly(butylene terephthalate) (PEGT/PBT) block co-polymer substrates [21,22] and porous 3D scaffolds for articular cartilage repair [6,23,24]. The advantage of these co-polymer systems is that during synthesis, the molecular weight (MW) of poly(ethylene glycol) (PEG) and the weight percent (wt%) ratio of hydrophilic PEGT and hydrophobic PBT blocks can be tailored, making it possible to modulate substrate properties such as wettability [25], swelling, biodegradation rate [26], mechanical properties [24] and protein adsorption [21]. Optimizing cell attachment to hydrophilic PEGT/PBT substrates, however, is problematic in tissue engineering applications [27] where high seeding efficiencies of expanded cells are required to elicit repair tissue. Protein adsorption studies performed on 2D hydrophilic PEGT/PBT films presenting a low PEG MW exhibited a low FN to VN adsorption ratio and resulted in poor cell attachment, but an increased ability to maintain primary human articular chondrocyte phenotype [21]. In contrast, hydrophobic PEGT/PBT substrates with high PEG MW have shown that preferential surface adsorption of FN compared to VN was positively correlated with chondrocyte attachment and promoted de-differentiation. Studies investigating the influence of 2D PEGT/PBT substrate composition on the adhesion and re-differentiation of expanded human chondrocytes, a more clinically relevant cell source, and in S+ and SF media have not been reported.

Surface modification via glow discharge gas plasma (GP) is used commercially to treat tissue-culture polystyrene (TCPS) surfaces, and has been shown to enhance cell attachment, growth and differentiation [28,29]. These features are reportedly caused by etching and incorporation of hydroxyl and carboxyl groups in the most superficial layers (i.e. at a nano-scale level) during GP treatment through a cascade of chemical reactions [25,30]. GP surface modification has been shown to enhance bone progenitor-cell attachment and function on 2D polymer films and 3D polymer scaffolds [31,32], potentially making it a simple and highly desirable technique for modification of polymeric scaffolds, particularly for bone tissue engineering, without changing the underlying bulk biomaterial properties (e.g. strength, biodegradation). In preliminary studies using primary bovine chondrocytes, we observed increased attachment and chondrogenesis on GP treated 3D PEGT/PBT scaffolds compared with untreated scaffolds [27], indicating possible advantages for cartilage tissue engineering applications. However, the effect of GP treatment on the attachment and phenotypic expression of expanded human chondrocytes has not been investigated. Studies have shown that argon GP treatment of PEGT/PBT block co-polymers resulted in the preferential removal of PEG blocks, as well as significantly increasing substrate hydrophilicity due to the introduction of polar functional groups at the surface [25]. Given that more hydrophilic PEGT/PBT surfaces tend to enhance maintenance of chondrocyte phenotype [21,33], GP treatment of varying compositions of PEGT/PBT substrates may also regulate phenotypic expression of expanded chondrocytes exposed to these surfaces. Furthermore, we hypothesized that physico-chemical modification of the upper-most

surface of PEGT/PBT substrates via GP treatment may influence cell adhesion and re-differentiation via mechanisms independent of adsorption of serum-derived proteins. To test this hypothesis, we evaluated adhesion and chondrocyte phenotype in serum-containing S+ and SF media, both shown to stimulate re-differentiation of expanded human chondrocytes [34].

For clinical tissue engineering applications, it is unclear what role GP treatment has in modifying substrate properties and whether surface hydrophilicity, protein adsorption, or the bulk properties of the substrate are responsible for eliciting different adhesion and phenotypic responses in human chondrocytes. Therefore, in this study we chose specifically to evaluate the re-differentiation capacity of expanded (i.e. de-differentiated) human nasal chondrocytes by culturing (i) on varying compositions of PEGT/PBT films, (ii) with (GP+) or without (GP−) GP treatment, and (iii) in S+ or SF media. Cell adhesion and proliferation were investigated by means of DNA quantification and cellular expression of FN and VN integrin receptors was analyzed by immunofluorescence. Post-expansion re-differentiation capacity was assessed by quantification of collagen types I and II gene expression using real-time polymerase chain reaction (PCR) with TCPS and pellet culture techniques used as negative and positive controls for re-differentiation potential, respectively.

2. Materials and methods

2.1. Material

PEGT/PBT co-polymers were obtained from IsoTis S.A. (Bilthoven, The Netherlands) with a composition denoted as *a*/*b*/*c*, where *a* represents the PEG MW (g/mol), and *b* and *c* represent the wt% of the PEGT and PBT blocks, respectively (Fig. 1). Cell–substrate interactions were evaluated on PEGT/PBT co-polymer films produced from 1000/70/30 and 300/55/45 compositions.

PEGT/PBT co-polymer resin was dissolved in 10% chloroform (Sigma Aldrich) and films, approximately 50 μm in thickness, were solution-cast using a technique described previously [33]. Following overnight drying steps in air and vacuum oven (50 °C), circular PEGT/PBT films were punched out to fit Ø10.5 mm 12-well plates. Due to the swelling behaviour of hydrophilic 1000/70/30 films (approximately 20%), Ø8.5 mm discs were punched out whereas for hydrophobic 300/55/45 films with limited swelling behaviour (approximately 2%), Ø10 mm discs were punched out.

2.2. Gas plasma (GP) treatment

Films for GP treatment were placed in a cylindrical, radio-frequency glow-discharge glass bell chamber (Harrik Scientific Corp, NY). A vacuum was applied to the chamber (0.01 mbar) and subsequently flushed four times with argon (purity ⩾99.999%, Hoekloos B.V., The Netherlands). The films were then treated under an argon plasma (0.1–0.2 mbar) for 30 min. GP treated and untreated 1000/70/30 and 300/55/45 films were vacuum sealed in foil purged with nitrogen and sterilized by γ-irradiation (minimum dose 25 kGy) at Isotron B.V. (Ede, The Netherlands). Samples were stored in foil at −20 °C prior to use.

2.3. Tissue culture

Human hyaline cartilage from the nasal septum was obtained from Medisch Centrum Alkmaar (The Netherlands) after informed consent. Nasal chondrocytes were isolated and pooled from two patients (aged 26 and 36) via collagenase digestion (collagenase type-II, Worthington), plated on TCPS (NUNC) at a density of 3500 cells/cm^2 and culture-expanded until passage two. In order to obtain a suitable number of cells, an expansion medium was used containing DMEM (Invitrogen) supplemented with 10% fetal bovine serum (FBS, Sigma Aldrich), 0.29 mg/ml L-glutamine (Sigma Aldrich), 1 mM sodium pyruvate (Invitrogen), 0.1 mM non-essential amino acids (NEAA, Sigma Aldrich), 100 units/ml penicillin (Invitrogen), 100 μg/ml streptomycin (Invitrogen), 10 mM HEPES buffer (Invitrogen), 1 ng/ml TGFβ_1 (R&D Systems), 5 ng/ml bFGF-2 (R&D Systems), and 10 ng/ml PDGF-bb (R&D Systems). This specific combination of growth factors has previously been shown to enhance human chondrocyte proliferation and re-differentiation capacity [35]. Expanded human nasal chondrocytes were selected for this study since they represent a clinically relevant cell source, and have been shown to de- and re-differentiate in a similar manner to articular chondrocytes [36], but also proliferate faster and exhibit a greater chondrogenic capacity after expansion [34].

GP+ and GP− 1000/70/30 and 300/55/45 films were placed in sterile 12-well plates (NUNC) and allowed to equilibrate overnight at 37 °C in appropriate S+ or SF media (described

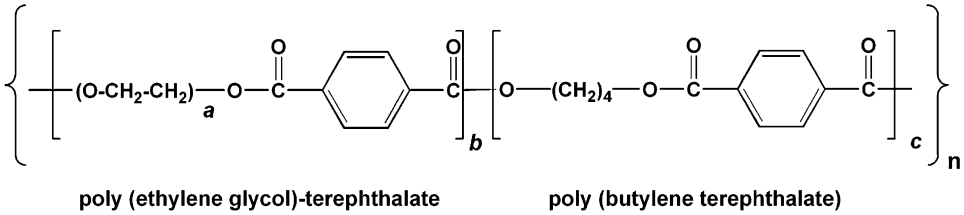

Fig. 1. Chemical structure of PEGT/PBT block co-polymers. The composition is denoted as *a*/*b*/*c*, where a represents the PEG MW (g/mol), and *b*/*c* represent the wt% ratio of the PEGT to PBT blocks, respectively.

below). Expanded chondrocytes were trypsinized (0.15% trypsin–EDTA, Invitrogen), seeded at a density of 10,000 cells/cm^2 on GP treated (GP+) or untreated (GP−) PEGT/PBT films and cultured in S+ or SF media, both shown to stimulate re-differentiation of human nasal chondrocytes [34]. Specifically, the S+ medium contained DMEM-Glutamax (Invitrogen) supplemented with 10% FBS, 0.1 mM NEAA, 100 units/ml penicillin, 100 μg/ml streptomycin, 10 mM HEPES buffer, 0.1 mM ascorbic acid 2-phosphate (Invitrogen), and 10 μg/ml insulin (Sigma Aldrich). The SF medium contained DMEM-Glutamax supplemented with ITS+1 (1×, Sigma Aldrich), 0.1 mM NEAA, 100 units/ml penicillin, 100 μg/ml streptomycin, 10 mM HEPES buffer, 0.1 mM ascorbic acid 2-phosphate, 1.25 mg/ml human serum albumin (HSA, Sigma Aldrich), 10^{-7} M dexamethasone (Sigma Aldrich), 10 ng/ml TGFβ_1 (R&D Systems). Pellet cultures (0.5×10^6 cells/pellet) and TCPS (10,000 cells/cm^2) served as positive and negative controls for cell re-differentiation, respectively, with the abovementioned S+ and SF media used in both cases.

2.4. Evaluation

2.4.1. DNA content

To determine the number of cells adhering to the various polymer substrata, films were harvested at days 3 and 10 ($n=3$) and frozen at −80 °C. Films were placed in a solution containing proteinase-K (1 mg/ml), pepstatin-A (10 μg/ml) and iodoacetamide (185 μg/ml) (Sigma Aldrich) overnight at 56 °C to digest the cells and ECM. Quantification of total DNA was performed with a Cyquant dye kit (Molecular Probes) as per manufacturer's instructions using a fluorescent plate reader (Perkin Elmer). Total cell number was determined by calculating the DNA content per cell from a cell standard using known concentrations of expanded (P2) human nasal chondrocytes taken prior to seeding of PEGT/PBT substrates. DNA/cell data were consistent with previously published values indicating human articular chondrocytes contain 11.6±2.7 pg DNA per cell [37].

2.4.2. Histology

Pellets were harvested at day 10 ($n=3$) and fixed overnight in 0.14 M cacodylate buffer (pH = 7.2–7.4) containing 0.25% glutaraldehyde (Merck). Samples were then dehydrated in sequential ethanol series, plastic embedded in glycol-methacrylate (Merck) and cut using a microtome to yield 5 μm thick sections. Sections were stained with haematoxylin (Sigma Aldrich) and fast green (Merck) to visualize cells/cell nuclei and safranin-O (Sigma Aldrich) to visualize extra-cellular glycosaminoglycan (GAG).

2.4.3. Scanning electron microscopy (SEM)

To examine cell morphology, samples harvested at day 3 and day 10 ($n=3$) were fixed and dehydrated as described above, and critical point dried from liquid carbon dioxide using a Balzers CPD 030 Critical Point Dryer. Films were then sputter-coated (Cressingdon) with a thin gold layer and studied using a Philips XL30 Environmental Scanning Electron Microscope.

2.4.4. Collagen type II/I gene expression

Samples harvested at day 10 were analyzed by quantitative real-time PCR for collagen types I and II messenger RNA (mRNA) expression ($n=3$). Films were carefully rinsed in PBS and frozen in Trizol reagent (Life Technologies) at −80 °C. RNA were extracted from films using 250 μl Trizol (Life Technologies) according to the manufacturer's instructions. cDNA were generated from total RNA using reverse-transcriptase Stratascript (Stratagene). Real-time PCR reactions were performed and monitored using a ABI prism 7700 Sequence Detection System and the Sequence Detector V program (Perkin Elmer Applied Biosystems). The PCR master mix was based on AmpliTaq Gold DNA polymerase (Perkin Elmer Applied Biosystems). In the same reaction, cDNA samples were analyzed both for collagen type I or collagen type II and for a housekeeping gene (18S ribosomal RNA), using previously described sequences of primers and probes [35]. Each cDNA sample was assessed at least in duplicate, with collagen types I and II mRNA expression levels normalized to the corresponding 18S rRNA levels. Since collagen type II is a typical marker of differentiated chondrocytes in hyaline cartilage, as opposed to collagen type I, expressed in de-differentiated chondrocytes, we used the ratio of mRNA levels of collagen type II to I as a chondrocyte differentiation index.

2.4.5. Integrin receptor detection

FN and VN integrin receptors were detected by immunofluorescence. At days 3 and 10, films ($n=3$) were fixed for 15 min with 10% formalin (Sigma), rinsed with phosphate buffered saline (PBS, Invitrogen), and cells permeabilized with 0.2% Triton X-100 for 20 min. Films were rinsed in PBS and blocked for 15 min with 0.1 M glycine, and for a further 15 min using SF protein block (Dako). Films were again rinsed in PBS and incubated separately with monoclonal antibodies directed against the $\alpha_5\beta_1$ FN receptor (clone P1D6, dilution 1:500, Covance); or against the $\alpha_V\beta_3$ VN receptor (anti-CD61, dilution 1:100, Pharmingen). Films were rinsed in PBS and further incubated for 30 min with a goat anti-mouse secondary antibody conjugated with Alexa 488 (dilution 1:100, Molecular Probes). The films were rinsed three times in PBS and mounted with Vectashield (Vector Labs, CA) containing DAPI to visualize cell nuclei. Slides were examined under a fluorescence microscope (Nikon Eclipse E600) using a F–T filter (FITC/Texas Red, Nikon) for integrin receptors detection (labelled green) and a quad-band filter (XF 113-2, Omega Opticals) for DAPI stained cell nuclei (labelled blue). Representative images for FN and VN receptors using both filters were captured at the same exposure using a digital camera (Sony Corporation, Japan) and merged using Paintshop Pro (Jasc Software) yielding a single image locating cell nuclei (blue) combined with either FN or VN integrin receptor staining (green). To quantify the number of adherent cells expressing FN and VN receptors, all immunofluorescence images were analyzed and the percentage of FN or VN expressing cells out of the total number of cells in each image set were counted.

2.5. Statistics

Data were represented as a mean±standard deviation ($n=3$). To test levels of significance in cell content, collagen

II/I mRNA ratio and FN/VN expressing cells, one-way analysis of variance (ANOVA) was performed using SigmaStat statistical software (SPSS Inc.). If tests for normality and variance passed, then one-way ANOVA was performed, whereas if variance tests failed, then one-way ANOVA-on-ranks was performed with Student–Newman–Keuls post hoc tests for significance ($P<0.05$) used in both cases.

3. Results

3.1. Cell content

DNA analysis showed that after 10 days culture in S+ media, the number of cells adhering to GP+ 1000/70/30 and 300/55/45 films was significantly greater compared to GP− films. There was no significant difference in cell number between TCPS, GP+ 1000/70/30 and GP+ 300/55/45 substrates after 3 days (data not shown). However, after 10 days, TCPS had significantly higher numbers of adherent cells than all other substrates, while GP+ 1000/70/30 and GP+ 300/55/45 films maintained a similar cell content (Fig. 2A). Among all conditions tested, the lowest number of cells was found on GP− 1000/70/30 films, which exhibited a significantly lower cell content than GP− 300/55/45 films. These results demonstrate greater cell content on low PEG MW compositions (300/55/45) compared to high PEG MW compositions (1000/70.30); however, following GP treatment, significantly greater cell content was observed irrespective of PEGT/PBT composition.

Due to the absence of potent cell attachment proteins in SF media, cell numbers were significantly reduced on TCPS, GP− and GP+ substrates compared with S+ media (Fig. 2A). GP treatment of PEGT/PBT films did not significantly enhance cell content per film over TCPS in the absence of serum. When comparing cell number between films in SF media, no significant difference was observed between GP+ and GP− 300/55/45 films, or between GP+ and GP− 1000/70/30. However, cell content was significantly greater on 300/55/45 films than 1000/70/30 films, suggesting that substrate composition and not GP treatment was responsible for increased cell number in the absence of serum.

When the data were expressed in terms of cell proliferation (Fig. 2B), the number of cell doublings between days 3 and 10 in S+ media was greatest on GP− 300/55/45 (1.8) films, followed by GP+ 300/55/45 (1.5), GP+ 1000/70/30 (1.3) and GP− 1000/70/30 (0.8), compared with 1.6 cell doublings on TCPS. Cell proliferation remained limited in SF media, with greatest cell doublings observed on GP− 300/55/45 (0.5) and GP+ 300/55/45 (0.4), compared with 0.7 cell doublings on TCPS (Fig. 2B). No cell proliferation was observed on treated and untreated 1000/70/30 substrates between day 3 and day using SF media, and was likely due to limited cell adhesion (Fig. 2A). Therefore, out of all the substrates, TCPS and GP− 300/55/45 films provided greater cell proliferation capacity in both S+ and SF media, whereas GP− 1000/70/30 provided the least cell proliferative capacity. Comparing treated and untreated substrates, GP seemed to only influence cell proliferation in S+ medium on 1000/70/30 films.

3.2. Cell morphology

Cell morphology was assessed for each of the substrates and media conditions at days 3 and 10. Since cell morphology was generally similar in S+ or SF media at each of the time points, therefore, only samples from day 10 are illustrated (Fig. 3). With respect to the number of adherent cells in S+ medium, SEM analysis confirmed our DNA results, with surfaces of GP+ 1000/70/30 (Fig. 3A) and GP+ 300/55/45 films (Fig. 3B) and TCPS (Fig. 3E) confluent with chondrocytes, exhibiting a spread morphology and numerous pseudopodia. Fewer cells were present on GP− 300/55/45 films, again exhibiting a spread morphology (Fig. 3D). Very

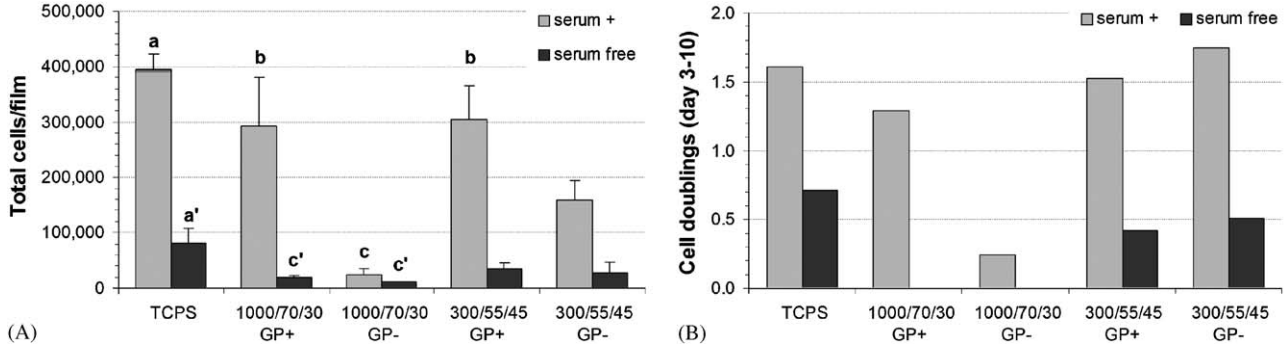

Fig. 2. (A) Total cell content per film at day 10 cultured on gas plasma treated (GP+) and untreated (GP−) substrates in serum containing (S+) or serum-free (SF) media compared with TCPS. (B) Cell doublings on each of the substrata between days 3 and 10. Significant differences in S+ (a, b, c) and SF media (a′, b′, c′) denoted by: a, a′⩾ all substrates; b, b′⩾ GP− films; c, c′⩽300/55/45 films.

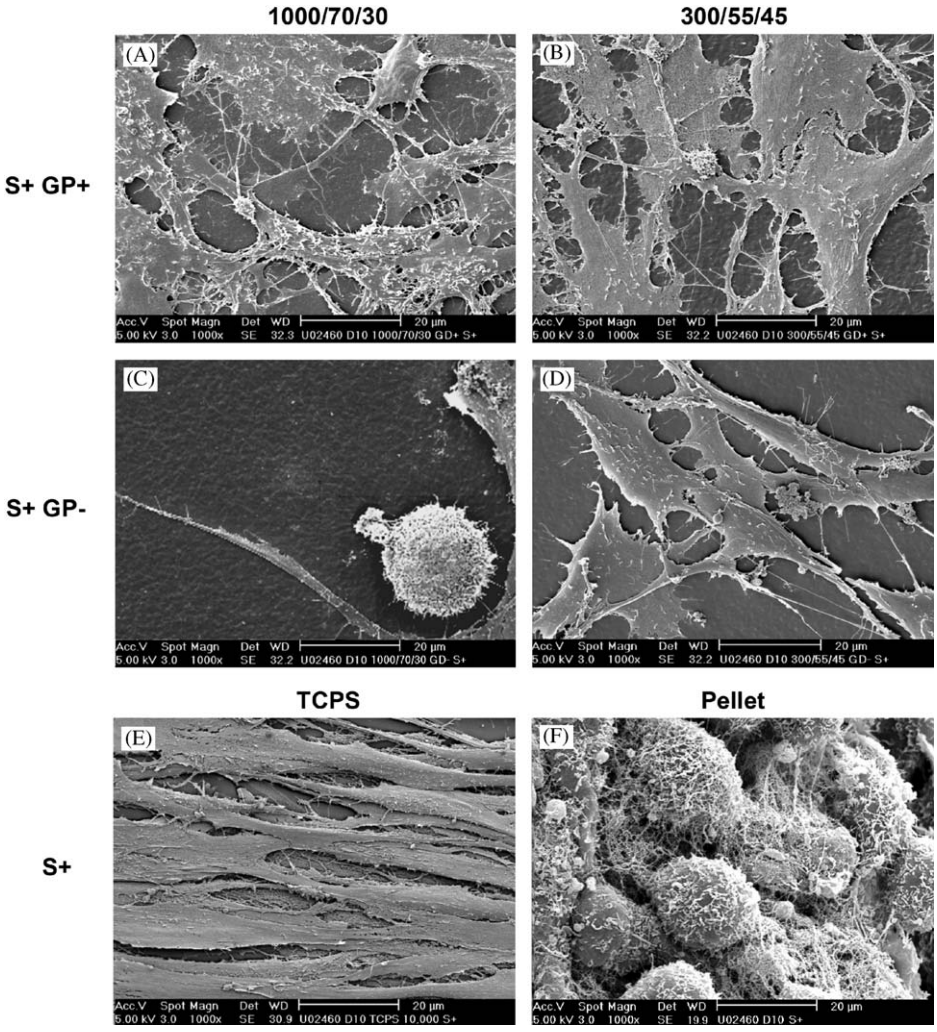

Fig. 3. (A–D) SEM micrographs (× 1000) showing cell morphology of expanded human chondrocytes after 10 days culture in S + medium on 1000/70/30 (A, C) and 300/55/45 substrates (B, D) either with (A, B) or without (C, D) GP treatment. (E) TCPS and (F) pellet controls in S + media.

few cells were adhered to GP− 1000/70/30 films and tended to exhibit a spherical appearance (Fig. 3C) similar in morphology to pellet cultured cells (Fig. 3F). These chondrocytes resisted adhesion and spreading, instead forming aggregates of 10–20 cells resembling a micron-scale pellet above the substrate. These observations are more clearly illustrated and discussed later in Fig. 5 following immunofluorescent staining.

3.3. Cell re-differentiation capacity

Pellets cultured in S + and SF media were positive for cell re-differentiation after 10 days, as indicated by spherical cells embedded in lacunae and intense safranin-O staining (inset Fig. 4) of sulphated GAG resembling that of articular cartilage. Furthermore, the re-differentiation index, taken as a ratio of collagen types II/I mRNA, was significantly higher in S + and SF pellets compared with cells cultured on TCPS for 10 days (Fig. 4). These results confirmed that pellet cultures represented a positive control for cell re-differentiation compared to TCPS, and were consistent with previous studies describing human nasal re-differentiation using similar culture conditions described in this study [34].

Although fewest cells adhered to GP− 1000/70/30 films in S + medium compared with all other conditions (Fig. 2A), these substrates promoted chondrocyte re-differentiation in 2D most similar to that shown in pellet cultures, resulting in collagen II/I mRNA ratios 2- and 3-orders of magnitude greater than GP− 300/55/45 and both GP + compositions respectively (Fig. 4). However, when treated with GP, both 1000/70/30 and 300/55/45 films exhibited only marginally higher collagen II/I mRNA ratios than TCPS in S + media, suggesting that GP treated PEGT/PBT films tend to promote a more de-differentiated, fibroblast-like phenotype and lower collagen II/I mRNA ratio (Fig. 4). While all substrates showed a higher re-differentiation index than TCPS in SF media, no significant differences in collagen II/I mRNA ratios between groups were observed (except

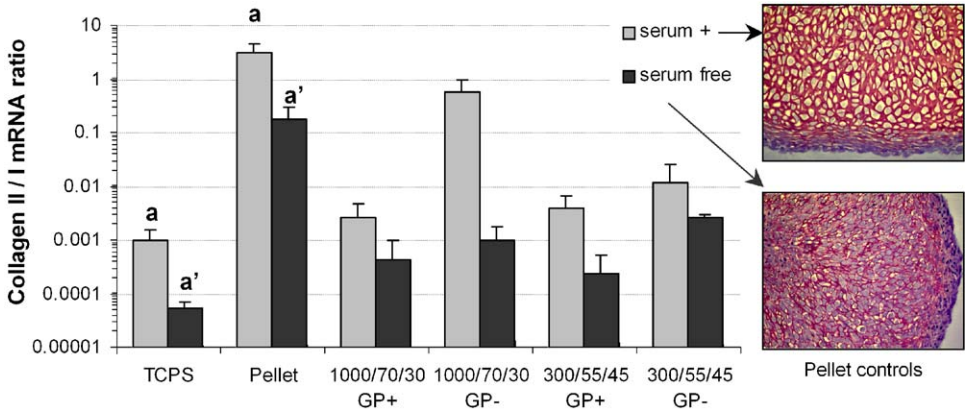

Fig. 4. Re-differentiation potential (collagen type II/I mRNA ratio) of expanded (P2) human nasal chondrocytes on gas plasma treated (GP+) and untreated (GP−) substrates after 10 days culture in S+ or SF media. Negative control for re-differentiation = TCPS; positive control for re-differentiation = pellet. Inset pictures show positive safranin-O staining in pellet cultures in both S+ and SF media (× 100). Significant difference between substrates denoted by: S+ media = a; SF media = a′.

between TCPS and pellet cultures), and these ratios were all noticeably lower than in S+ medium. Interestingly, more cells were present on TCPS substrates in SF media at day 10 compared with PEGT/PBT films (Fig. 2) and yet the films exhibited greater re-differentiation capacity. Although re-differentiation (Fig. 4 inset) and high collagen II/I mRNA ratio were observed in pellets cultured in SF medium, the chondrogenic index for cells cultured on GP− 1000/70/30 films was not significantly enhanced as compared to other substrates. While we demonstrated that expanded (i.e. de-differentiated) human nasal chondrocytes are capable of responding to changes in substrate composition and chemistry and re-express a chondrogenic phenotype, our results confirm the general paradigm that substrates which promote adhesion and proliferation of chondrocytes (e.g. GP+ and GP− 300/55/45 films), limit cell re-differentiation capacity.

3.4. Integrin receptor detection

To study the effect of substrate composition and GP treatment on chondrocyte adhesion mechanisms, immunofluorescent staining for $\alpha_V\beta_3$ and $\alpha_5\beta_1$ integrin receptors for VN and FN, respectively was performed (Fig. 5) and used to quantify the percentage of cells expressing these receptors (Fig. 6). As discussed previously, spherical cell aggregates containing 10–20 cells formed on top of GP− 1000/70/30 films (Figs. $5B_1$ and B_2), whereas individual cells adhering to GP+ 1000/70/30 and 300/55/45 films (Fig. $5A_{1–4}$) exhibited a spread morphology. In S+ medium, greater cell numbers were observed on GP+ films (Fig. $5A_{1–4}$) than GP− films (Fig. $5B_{1–4}$), whereas in SF medium, greater cell numbers were observed on 300/55/45 films (Figs. $5C_3$–D_4) than 1000/70/30 films (Figs. $5C_1$–D_2), verifying DNA and SEM results.

In both S+ and SF media, the number of cells expressing FN and VN receptors (Fig. 6) was greater in GP treated films (Figs. $5A_{1–4}$ and $C_{1–4}$) than in untreated films (Figs. $5B_{1–4}$ and $D_{1–4}$), with the exception of spherical aggregates which formed on top of GP− 1000/70/30 films. As very few of these cells were physically adhering to the films, FN and VN integrin expression seen in these samples were exclusively not cell–substrate mediated, but more likely a result of cell–ECM interactions following matrix synthesis by cells within aggregates after 10 days culture (Figs. $5B_1$ and B_2). For this reason it was not possible to determine the percentage of cells expressing VN or FN integrin receptors for GP− 1000/70/30 substrates (Fig. 6). With the exception of these samples, the percentage of cells expressing the FN integrin receptor in S+ media on all substrates (Figs. $5A_2$ and A_4–B_4) was significantly greater in comparison to the percentage of VN expressing cells in corresponding substrates (Figs. $5A_1$, A_3–B_3 and 6). In SF media significant differences were only observed between percentages of FN and VN expressing cells on 300/55/45 substrates (Figs. $5C_3$–D_4 and 6). GP treatment (Figs. $5A_{1–4}$ and $C_{1–4}$) significantly increased the percentage of cells expressing both FN and VN receptors compared to corresponding GP− substrates irrespective of culture in S+ or SF media (Figs. $5B_{1–4}$ and $D_{1–4}$). While GP− 1000/70/30 films promoted the formation of spherical aggregates and no detectable cell–substrate VN/FN integrin receptor expression in S+ media (Figs. $5B_1$ and B_2), GP+ 1000/70/30 films promoted cell adhesion and spreading (Figs. $5A_1$ and A_2), with percentages of VN and FN expressing cells similar to GP+ 300/55/45 films (Fig. 6).

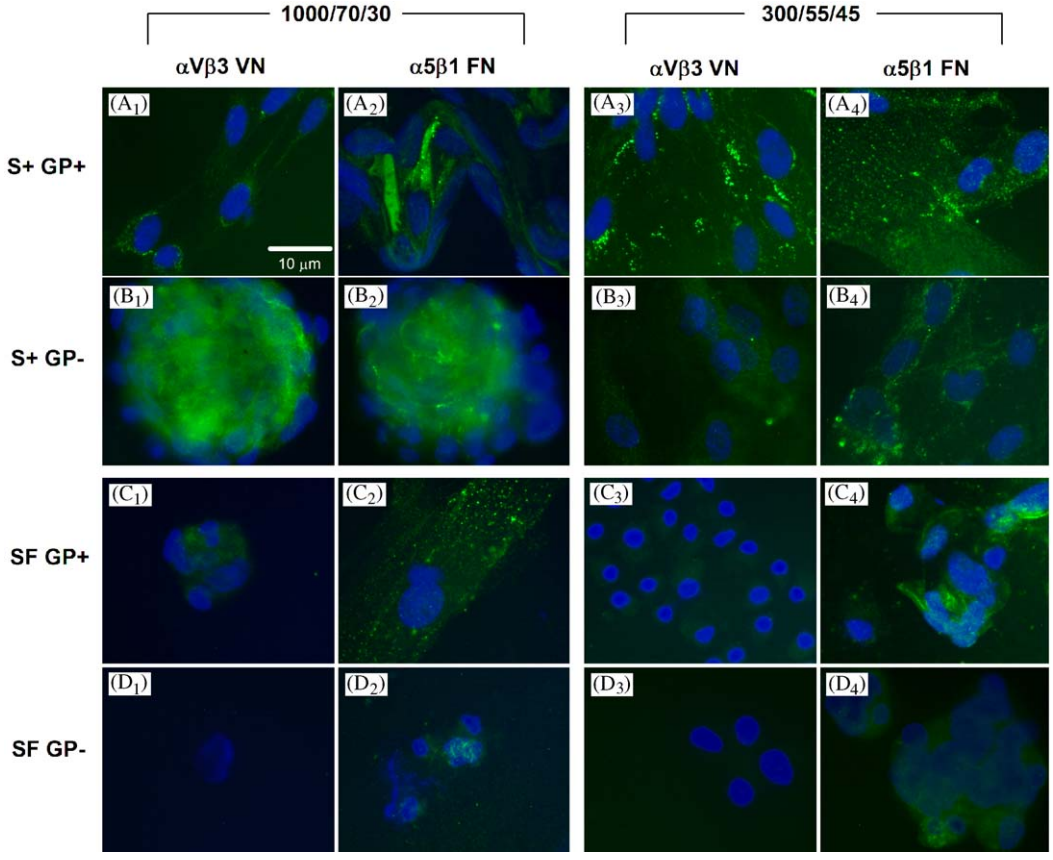

Fig. 5. Immunofluorescent staining for $\alpha_V\beta_3$ vitronectin (VN, A_1–D_1, A_3–D_3) and $\alpha_5\beta_1$ fibronectin (FN, A_2–D_2, A_4–D_4) integrin receptors in expanded human nasal chondrocytes at day 10. Cells were cultured on 1000/70/30 (A_1–D_2) and 300/55/45 films (A_3–D_4) either with (GP+, A_{1-4}, C_{1-4}) or without (GP−, B_{1-4}, D_{1-4}) gas plasma treatment and in S+ (A_1–B_4) or SF media (C_1–D_4). Cell nuclei stained blue with DAPI.

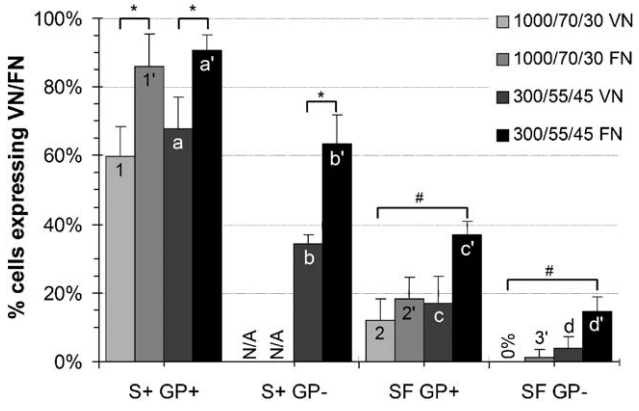

Fig. 6. Percentage of adherent human nasal chondrocytes expressing $\alpha_V\beta_3$ vitronectin (VN) or $\alpha_5\beta_1$ fibronectin (FN) integrin receptors determined from immunofluorescent images on gas plasma treated (GP+) and untreated (GP−) 1000/70/30 and 300/55/45 substrates. Significant difference denoted by: * = FN > VN; # = FN > all substrates; 1, 1′ ⩾ 2, 2′ ⩾ 3′; a, a′ ⩾ b, b′ ⩾ c, c′ ⩾ d, d′.

4. Discussion

The goal of this study was to evaluate whether controlled changes in substrate surface chemistry via GP surface modification and substrate composition can regulate the post-expansion re-differentiation of human chondrocytes in serum containing (S+) and serum free (SF) media.

We demonstrated that PEGT/PBT substrate composition had a significant effect on the cell content and re-differentiation capacity of expanded human nasal chondrocytes. While fewest cells adhered to GP− 1000/70/30 films in S+ medium compared with all other conditions (Fig. 2A), these substrates promoted chondrocyte re-differentiation in 2D most similar to those shown in pellet cultures, resulting in collagen II/I mRNA ratios 2- and 3-orders of magnitude greater than GP− 300/55/45 and both GP+ compositions, respectively (Fig. 4). GP treatment significantly enhanced cell adhesion on all substrate compositions in S+ media; however, the re-differentiation capacity of expanded human nasal chondrocytes was markedly reduced, resulting in collagen II/I mRNA ratio similar to TCPS controls.

While surface wettability, protein adsorption, or the bulk substrate properties have all been suggested to influence cell attachment and differentiation, the typical role of PEG likely plays a role in the series of cell and surface mediated events that influence expanded human

chondrocyte re-differentiation capacity on GP treated and untreated PEGT/PBT substrates. PEG incorporation at biomaterial surfaces is commonly employed to limit protein adsorption as a result of PEG hydrophilicity and high surface mobility [38,39]. In this study, PEG incorporation was achieved by synthesizing long (MW 1000) or short (MW 300) PEG chains within the 70:30 wt% or 55:45 wt% ratio of PEGT and PBT, respectively. In an argon GP, sputtering and ultraviolet etching play a predominant role in further modifying polymer surfaces [40]. For example, using high-resolution X-ray photoelectron spectroscopy [25], analysis of 1000/70/30 and 300/55/45 films after a 30 min argon GP treatment resulted in preferential etching of PEG blocks. The etching rate of the amorphous PEG phase was approximately four times higher than for the more crystalline PBT phase, thereby selectively removing mobile, hydrophilic PEG from PEGT/PBT co-polymer surfaces. This would suggest that wettability decreases with GP treatment, yet wettability has been shown to increase following argon GP treatment due to the introduction of polar functional groups by post-oxidation reactions of surface free radicals with oxygen in air [25]. Contact angles measured on water-equilibrated, untreated 1000/70/30 and 300/55/45 substrates were $39^\circ \pm 1^\circ$ and $48^\circ \pm 3^\circ$, respectively, whereas contact angle measurements for substrates GP treated under argon for 30 min were $34^\circ \pm 2^\circ$ and $24^\circ \pm 3^\circ$, respectively. In comparison, the contact angle of TCPS substrates has been shown to be $62^\circ \pm 2^\circ$ [41]. Substrate wettability has been suggested to influence chondrocyte adhesion and cell phenotype [42], and while in general hydrophilic GP+ 1000/70/30 and GP+ 300/55/45 substrates showed greatest cell content in this study, substrate wettability likely had little effect on chondrocyte re-differentiation. For example, we cannot assume hydrophilic substrates support re-differentiation since, according to contact angle data, the most hydrophilic substrates (GP+ 300/55/45 and 1000/70/30) actually showed least re-differentiation, whereas the most hydrophobic substrates (GP− 300/55/45 and TCPS) also had limited re-differentiation potential.

Independent of low or high PEG MW PEGT/PBT composition, GP treatment promoted significantly higher cell content as compared to untreated films, but only when serum was present in the culture medium. Whereas on untreated films, cell adhesion was significantly influenced by PEGT/PBT composition (Fig. 3), with significantly higher cell content observed on low PEG MW (e.g. GP− 300/55/45) as apposed to high PEG MW compositions (e.g. GP− 1000/70/30) in S+ medium and in SF medium (to a lesser extent). If preferential PEG etching occurs following GP treatment, thereby resulting in a PEG-deficient/PBT-rich surface (as described earlier), then additional PBT binding regions or changes in surface charge are not influencing physico-chemical mechanisms by which expanded chondrocytes adhere in the absence of serum, only the original co-polymer compositon. Although the mechanism is unclear, low PEG (i.e. GP− 300/55/45) surfaces in S+ and SF media, or PEG deficient (i.e. GP+ 1000/70/30, GP+ 300/55/45) surfaces in S+ media may be responsible for influencing cell–substrate interactions. It should be noted, however, that while this study of expanded human nasal chondrocytes represents the clinical environment, these cells represent a population of adherent cells selected from the TCPS expansion phase in S+ medium, and so are less likely adhere to substrates in SF media. Nevertheless, significant differences were still observed, and these cells clearly are capable of re-differentiating in SF conditions as shown in pellet cultures (Fig. 4 inset).

From SEM (Fig. 3) and FN/VN immunofluorescence data (Fig. 5, Fig. 6), there were clear relationships between cell morphology and re-differentiation capacity (Fig. 4). Substrates showing enhanced cell adhesion and higher percentages of $\alpha_V\beta_3$ VN and $\alpha_5\beta_1$ FN expressing cells (i.e. GP+ 1000/70/30, GP+ 300/55/45, GP− 300/55/45) resulted in low collagen II/I mRNA ratios more similar to TCPS (negative control), whereas substrates promoting a more spherical cell morphology (i.e. GP− 1000/70/30) in conjunction with no cell–substrate related FN/VN integrin expressing cells resulted in greater collagen II/I mRNA ratios, more similar to pellet cultures (positive control). Our results correlate well with studies using primary human articular chondrocytes which demonstrated expression of both $\alpha_V\beta_3$ VN and $\alpha_5\beta_1$ FN receptors in S+ medium on 300/55/45 films, but neither receptor was expressed on 1000/70/30 films [22]. Previous studies have shown that $\alpha_5\beta_1$ is a primary chondrocyte FN receptor. Knudsen and Loeser [43] showed that antibodies directed against α_5 or β_1 integrin subunits inhibited more than 80% of cell adhesion to FN, or more than 90% of chondrocyte adhesion to FN and types II and VI collagen, respectively. With respect to cell phenotype, the presence of FN has been shown to induce chondrocyte de-differentiation [17,19] and in particular, expression of the $\alpha_5\beta_1$ integrin receptor in conjunction with the ligand FN were shown to be up-regulated during chondrocyte de-differentiation, whereas the $\alpha_V\beta_3$ VN receptor or it's ligand were not up-regulated [18]. We observed a similar relationship with respect to the re-differentiation capacity of expanded human chondrocytes in this study. Cell–substrate mediated interactions supporting cell adhesion and a low collagen II/I mRNA ratio (i.e. GP+ 1000/70/30, GP+ 300/55/45, GP− 300/55/45) resulted in significantly higher percentages of cells expressing the $\alpha_5\beta_1$ FN integrin receptor, particularly in S+ medium following GP treatment (Fig. 6). In each case, the number of $\alpha_5\beta_1$ FN expressing cells was significantly greater than the number of $\alpha_V\beta_3$ VN expressing cells.

Again, one explanation for the observed differences in chondrocyte adhesion and re-differentiation on various PEGT/PBT substrates may be the role of PEG content, particularly in relation to adsorption of FN from serum. For example, Mahmood et al. [21] used SDS–PAGE and western blot analysis to investigate protein adsorption from serum on various compositions of porous PEGT/PBT granules in relation to adhesion and phenotypic expression of primary human articular chondrocytes on PEGT/PBT films. Preferential adsorption of FN over VN on low PEG MW substrates was correlated with in greater attachment and a de-differentiated phenotype, whereas a low FN to VN ratio on high PEG MW substrates was correlated with reduced cell adhesion and a more differentiated phenotype. Although expanded human nasal chondrocytes were used in this study, preferential FN adsorption and cell-mediated interaction via $\alpha_5\beta_1$ integrin receptors may describe why low PEG MW films (i.e. GP− 300/55/45) promoted adhesion and a low re-differentiation capacity, whereas high PEG MW films (i.e. GP− 1000/70/30) promoting a spherical cell morphology, and a high re-differentiation capacity, concomitant with a lack of cell–substrate mediated integrin receptor expression for $\alpha_5\beta_1$. While this study provides preliminary data on the mechanisms surrounding expanded human chondrocyte re-differentiation, further work is necessary to optimize the measurement of protein adsorption on various compositions of GP treated and untreated PEGT/PBT granules in order to verify these hypotheses.

Translation of re-differentiation data obtained from these studies on 2D films, particularly at the mRNA level, to 3D scaffolds at the protein level is a pertinent issue for tissue engineering applications. We demonstrated in this study that high collagen II/I mRNA ratio correlated with positive staining for GAG proteins. Furthermore, in a recent parallel study, we demonstrated that PEGT/PBT compositions that supported a chondrogenic phenotype in 2D in this study were also capable of promoting re-differentiation and cartilage ECM production in expanded human nasal chondrocytes in 3D PEGT/PBT scaffolds in vitro [23]. We would, therefore, expect that chondrocyte re-differentiation and collagen II/I gene expression data presented here would translate favourably to 3D PEGT/PBT scaffold architectures produced using GP treated and untreated 300/55/45 and 1000/70/30 compositions.

5. Conclusions

This study highlights the need to carefully assess cell–substrate interactions and the use of S+ or SF culture conditions to optimize the engineering of cartilage constructs, particularly with respect to surface modification to improve cellular adhesion whilst maintaining suitable re-differentiation capacity.

We demonstrated that PEGT/PBT composition had an effect on adhesion of expanded human nasal chondrocytes irrespective of whether S+ or SF media was used. We further demonstrated that PEGT/PBT composition affects re-differentiation potential of de-differentiated human chondrocytes, evidenced by greater collagen II/I mRNA in 1000/70/30 compositions which promoted a more spherical cell morphology. Surface modification via GP treatment provided a simple method to significantly improve expanded human chondrocyte adhesion on low PEG MW PEGT/PBT substrates in S+ media, but not in SF media However, cell–substrate interactions in S+ media resulting in significantly increased expression of $\alpha_5\beta_1$ FN integrin receptors following GP treatment was linked to a marked reduction in chondrocyte re-differentiation capacity. While GP treatment of polymer substrates may prove beneficial for bone tissue engineering applications [31,32], this study suggests that, for clinical cartilage tissue engineering strategies relying on post-expansion re-differentiation of expanded human chondrocytes, GP treatment may not be a suitable surface modification technique. As an alternative to these direct cell attachment mechanisms, and to support the potential clinical use of low PEG MW 1000/70/30 compositions, cell immobilization may be better achieved through the use of cell aggregation, hydrogel delivery or micro-porous coating techniques for example. In addition, future studies are necessary to further investigate the role of protein adsorption on GP surface modification of PEGT/PBT substrates and their subsequent influence on cell signalling and re-differentiation pathways on 2D substrates and 3D scaffolds.

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