RGD-Functionalized Polyethylene Glycol Hydrogels Support Proliferation and In Vitro Chondrogenesis of Human Periosteum-Derived Cells

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Abstract

The combination of progenitor cells with appropriate scaffolds and *in vitro* culture regimes is a promising area of research in bone and cartilage tissue engineering. Mesenchymal stem cells (MSCs), when encapsulated within hydrogels composed of the necessary cues and/or preconditioned using suitable culture conditions, have been shown to differentiate into bone or cartilage. Here, we utilized human periosteum-derived cells (hPDCs), a progenitor cell population with MSC characteristics, paired with protease-degradable, functionalized polyethylene glycol (PEG) hydrogels to create tissue-engineered constructs. The objective of this study was to investigate the effects of scaffold composition, exploring the addition of the cell-binding motif Arginine-Glycine-Aspartic Acid (RGD), in combination with various *in vitro* culture conditions on the proliferation, chondrogenic gene expression, and matrix production of encapsulated hPDCs. In growth medium, the hPDCs in the RGD-functionalized hydrogels maintained high levels of viability and demonstrated an enhanced proliferation when compared to hPDCs in non-functionalized hydrogels. Additionally, the RGD-containing hydrogels promoted higher glycosaminoglycan (GAG) synthesis and chondrogenic gene expression of the encapsulated hPDCs, as opposed to the non-functionalized constructs, when cultured in two different chondrogenic media. These results demonstrate the potential of hPDCs in combination with enzymatically degradable PEG hydrogels functionalized with adhesion ligands for cartilage regenerative applications.

Keywords

Matrix metalloproteinase; bone morphogenetic protein; transforming growth factor beta; cell encapsulation; mesenchymal stem cells

Introduction

Treatments for the repair of damaged articular cartilage have shortcomings due to donor site morbidity, poor mechanical and biological integration, the need for multiple surgeries, and high cost, which have led research groups to try to find alternative methods to treat joint surface defects.[1] The concept of tissue engineering, where cells are isolated and cultured, combined with an appropriate scaffold, stimulated with biochemical cues, and introduced into the host's body, has been gaining interest in the field of regenerative medicine.[2] Using this approach, mesenchymal stem cells (MSCs) in combination with 3D biomaterials have been investigated.

MSCs are mesodermal derived progenitor cells that have multi-lineage differentiation capabilities and thus can form tissues such as bone, cartilage, and fat.[3-5] Although there are several different sources for these progenitor cells, a cell population that is attractive is derived from the periosteum. The importance of the periosteum as a whole tissue graft has long been investigated and shown potential for orthopedic applications.[6,7] The cells within the inner cambium of the periosteum have demonstrated strong MSC-like characteristics with multi-lineage potential.[8,9] When compared to MSCs from other sources such as bone marrow (BM-MSCs), synovium (SDCs), and adipose tissue (ADCs), periosteum-derived cells (PDCs) possessed a higher proliferative capacity and contained the highest number of progenitor cells when seeded at low densities.[10-12] PDCs have also displayed osteochondrogenic potential *in vivo*, when seeded on calcium phosphate scaffolds.[13] Furthermore, human PDCs (hPDCs) have shown chondrogenic potential when cultured *in vitro* in micromass conditions,[14] thus making them an attractive cell source to test in 3D hydrogel systems. Similar to other cell sources, harvesting of PDCs also has shortcomings. PDCs are involved in bone homeostasis by generating osteoblasts that enable appositional bone growth and, in combination with osteoclasts, help the modeling

and remodeling of the cortical bone.[15] Harvesting from this region could alter tissue homeostasis, but as aforementioned, PDCs possess a high proliferative capacity[10] and hence a relatively small amount of tissue would be required. Furthermore, harvest of small tibial periosteal flaps has been FDA approved, and they have been used for autologous chondrocyte implantation to repair damaged cartilage for several years.[16] Although hPDCs have garnered attention due to their relative ease of isolation, expansion potential, and multi-potency at the single cell level *in vitro* [8,9], there are few studies investigating hPDCs cultured in a 3D system, let alone encapsulated in polyethylene glycol (PEG) hydrogels. Thus, the evaluation of the behavior of hPDCs in PEG hydrogels represents one novel aspect of the present work.

A 3D environment, via aggregation or pellet structures, has been proven to be crucial for the differentiation of MSCs down the chondrogenic lineage.[17-19] However, pellet structures tend to guide the MSC population towards hypertrophy, an undesired outcome that has been shown to be circumvented through the use of a 3D hydrogel system.[20] In addition to providing a direct means of creating a sufficiently sized repair construct, hydrogels mimic the native environment of the cells and can be modified to the cells' specific needs, reducing the divide between 2D cell culture and *in vivo* conditions.[21] For instance, it has been demonstrated that chondrocytes, the native cell type in articular cartilage, de-differentiate under 2D conditions and prefer a 3D system to maintain their phenotype.[22] Hence, we hypothesized that encapsulation within a tailored 3D biomaterial will aid in the *in vitro* chondrogenic differentiation of hPDCs.

Synthetic hydrogels, more specifically PEG hydrogels, have an ease of modification, inherent anti-immunogenic properties, and an ability to imbibe large amounts of water, which together make them an attractive choice for cartilage tissue engineering.[23,24] On the other hand, hydrogels made from naturally derived materials have the advantage of inherent bioactivity due to the

presence of cell adhesive and protease sensitive domains. Although PEG hydrogels intrinsically exhibit minimal biological activity, they can be altered by the addition of peptides and growth factors to enhance their biological relevance, thus leading to a system with mechanical, chemical, and biological properties that are easy to control. For example, MSCs, which are anchorage-dependent, have shown low viability in unmodified synthetic PEG hydrogels.[25] To address this limitation, several groups have incorporated adhesive ligands found in the extracellular matrix (ECM), such as Arginine-Glycine-Aspartic Acid (RGD), into PEG hydrogels, thus enhancing the encapsulated cells' response.[25-28] Another vital aspect of a successful 3D hydrogel construct is its ability to be degraded and remodeled. PEG hydrogels incorporating matrix metalloproteinase (MMP)-sensitive peptides in the hydrogel backbone allowed for enhanced proteolytic degradation and cellular invasion,[29] with the degradation rate dependent on the specific peptide sequence used.[30,31] Following these results, several researchers have incorporated MMP-sensitive substrates into PEG-based scaffolds for cartilage tissue engineering.[32,33] The controlled degradation led to enhanced, uniform ECM production, facilitating a more directed differentiation of the encapsulated cells.[32,33]

Finally, the success of MSC-laden constructs can be improved via appropriate priming *in vitro*. Several researchers have demonstrated an enhanced chondrogenic differentiation of MSCs when stimulated with growth factors such as bone morphogenetic protein-2 or -6 (BMP-2, BMP-6) or transforming growth factor-beta 1 or 3 (TGF-β1, TGF-β3).[17,19,34-37] The chondrogenic priming of MSC-laden constructs seems to provide a suitable environment for *in vitro* chondrogenic differentiation.[26,28,34,38] hPDCs also display a favorable outcome when stimulated with these growth factors, either in a micromass culture system or seeded on a 3D scaffold.[14,39] However, these cells have not been tested within hydrogels, and we believe encapsulation of hPDCs within

a hydrogel is an alternative *in vitro* culture approach worth investigating. Therefore, herein, hPDCs were encapsulated as a single cell suspension in protease-degradable, functionalized PEG hydrogels to create tissue-engineered constructs. The objective of this study was to investigate the effects of scaffold composition, specifically exploring the addition of the cell-binding motif RGD, in combination with *in vitro* priming with medium containing either BMP-2 or TGF-β1 on proliferation, chondrogenic differentiation, and matrix production of encapsulated hPDCs.

Materials and Methods

PDC Culture: Cells were isolated from donors of similar age (two male and two female donors, aged 13.7 ± 2.5 years), as previously described, and pooled together based on identical *in vitro* growth kinetics and *in vivo* bone-forming capacity.[14] The pooled hPDCs were plated at 5000 cells/cm^2 and sub-cultured after expansion. At passage 8, the pooled cells were harvested for encapsulation within the hydrogels. All procedures were approved by the Ethical Committee for Human Medical Research (KU Leuven), and the donors' informed consents were obtained.

Hydrogel Preparation and Cell Encapsulation: 6.5 % (w/v) PEG hydrogels were made via a Michael Type addition reaction as previously described (Fig. 1).[30] Briefly, 20 kDa 4-arm PEG-Vinyl Sulfone (VS) (JenKem) was dissolved in 0.3 M HEPES buffer, pH 8. Next, the adhesion peptide containing a free thiol (Ac-G**C**GYGRGDSPG-NH_2; prepared by solid phase peptide synthesis and purified via HPLC-MS) was reacted with the PEG-VS for a final concentration of 150 μM (Fig. 1A). For hydrogels without RGD, an equal volume of buffer was added. Next, a cell suspension was mixed with the di-thiol, MMP-sensitive crosslinker, Ac-G**C**REGPQGIWGQER**C**G-NH_2 (Biomatik or PeptideSynthetics). Finally, the PEG-VS, with or without RGD functionalization, was reacted with the crosslinker and cell suspension. The final

cell concentration within the hydrogels for the proliferation and viability analysis was $1x10^6$ cells/mL, and for the chondrogenic differentiation assays, it was $10x10^6$ cells/mL. Hydrogel disks were formed by sandwiching drops of the hydrogel precursors between hydrophobic SigmaCote (Sigma) treated slides and incubating them for 30 min at 37 °C (Fig. 1B).

Culture Conditions for Proliferation or Differentiation: Cell-laden hydrogels were placed in 24-well plates (Fig. 1C) with growth medium (GM) [high-glucose Dulbecco's modified Eagle's medium (DMEM Glutamax; Invitrogen) supplemented with 10 % fetal bovine serum (FBS; BioWhittaker), 1 % sodium pyruvate (1 mM; Invitrogen), and 1 % antibiotic–antimycotic solution (AB; Invitrogen)] throughout the proliferation experiment or with one of two different chondrogenic media: 4C [DMEM/F12 (Invitrogen) with 5 % FBS, 1 % AB, 10 ng/ml TGF-β1 (PeproTech), 1X Insulin Transferin Selenium + premix (ITS+; BD Biosciences), 100 nM dexamethasone, and 100 µg/ml ascorbic acid][40] or a chemically defined, serum-free medium with 100 ng/ml BMP-2 (CDM-BMP2).[41] All media were replenished every 2-3 days.

Cell Viability Assay: A Live/Dead analysis was performed using an Invitrogen kit after 1 week and 4 weeks of *in vitro* culture in GM. The hPDCs within the different PEG hydrogel constructs hydrogels were stained with calcein AM and ethidium homodimer-1 (ETH-1). The samples were imaged using an Olympus FluoView confocal microscope with 10X objective at step size of 10 µm and a total thickness of 500 µm. Imaris software was used to quantify the images in the 3D view, and all images are represented as the maximum intensity of the z-stack.

Cell Morphology (DAPI/Phalloidin Staining): The hydrogel-cell constructs were stained in a solution of Alexa Fluor 488-labeled Phalloidin (0.8 U/ml; Molecular Probes) and 4,6-diamidino-2-phenylindole (DAPI; 2.5 µg/ml; Thermo Fisher) to look at actin filaments and nuclei,

respectively. The samples were imaged with an Olympus FluoView confocal microscope with 10X objective with a step size of 10 µm and a total depth of 500 µm. ImageJ software was used to analyze and quantify cell spreading and circularity of the encapsulated cells.

Picogreen/QuanIT DNA Quantification: The DNA quantity of 3 hydrogels per condition and per time point was calculated using the Picogreen DNA QuanIT kit (Invitrogen). The hydrogels were first degraded in a Proteinase-K (0.5 mg/ml; Sigma Aldrich) digestion buffer, mixed with the reagents provided in the kit, and subsequently read using the Qubit Fluorometer.

Dimethylmethylene blue (DMMB) GAG Assay: At each time point, the same digested samples used in the DNA assay were used for the DMMB assay. 1,9–dimethylmethylene blue chloride (Sigma) was dissolved in ethanol and then added to a 0.04 M NaCl/glycine solution, pH 3, for a final concentration of 46 µM DMMB. The samples were loaded into a 96 well plate, along with the DMMB dye solution, and the absorbance was read at 570 nm. The GAG concentration was calculated from a standard curve generated using a serial dilution of chondroitin sulfate (Sigma).

RNA Extraction and Quantitative PCR (qPCR) for Chondrogenic Markers: The total RNA from the hydrogels was isolated using a trizol/ethanol/chloroform RNA extraction method and quantified using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific). Subsequently, 500 ng of RNA was converted into complementary DNA (cDNA) with Oligo(dT) 18 primer using the RevertAid Hminus First Strand cDNA Kit (Fermentas). SYBR Green (Applied Biosystems) primers (Table 1) and a Rotor-Gene sequence detector were used for qPCR for chondrogenic gene markers such as *SOX-9*, aggrecan *(ACAN*), type II collagen (*COL2A1*), and type X collagen (*COL10A1*). At each time point, each independent triplicate sample was

measured in duplicate. Expression was calculated using the $2^{-\Delta\Delta CT}$ method, normalized to the house keeping gene, β-actin (*β-ACT*), and further normalized to time point 0.

Statistical Analysis: Quantitative results are expressed as mean ± standard deviation. Cell spreading and circularity statistical analysis was conducted using a basic student T-test. For the Live/Dead and DNA quantification assays, a two-way ANOVA was performed. For the GAG production and chondrogenic gene expression assays, the statistical analysis was performed using a basic student T-test to assess the effect of time and further using a two-way ANOVA at each time point to see the interaction between the medium formulations and hydrogel compositions. Results were considered significant with *p*-values below 0.05.

Results

hPDC Viability in PEG Hydrogels Cultured in Growth Medium: After 1 week of *in vitro* culture, there was a slight difference in the viability of the hPDCs between the hydrogel formulations with and without RGD (Fig. 2A). The hydrogels functionalized with RGD maintained an average cell viability of around 85 %. On the other hand, the hydrogel constructs without RGD displayed a lower cell viability of about 65 %. After 4 weeks of *in vitro* culture, the encapsulated hPDCs favored the RGD-functionalized constructs as the cells within these hydrogels maintained a viability of 85 %. In contrast, the viability of the cells cultured in the hydrogels without RGD demonstrated a drop to about 40 %, in addition to being significantly lower than that for cells cultured in the constructs with RGD ($p<0.001$). Furthermore, as seen in confocal images at 4 weeks, the dead cells in both construct formulations seemed to be spread throughout the hydrogel and were not indicative of a necrotic core (Fig. 2B). Overall, the hPDCs maintained a

high level of viability when encapsulated as a single cell suspension within the PEG hydrogels functionalized with RGD, as opposed to when seeded within hydrogels without RGD.

Morphology of hPDCs in PEG Hydrogels in GM: After 4 weeks of *in vitro* culture promoting cell growth, there was a qualitative difference in cell morphology and spreading of the hPDCs encapsulated within the two hydrogel formulations (Fig. 3). The cells within the hydrogels containing RGD displayed a more spread morphology than the rounded nature of the hPDCs within the hydrogels without RGD. Quantitatively, hPDCs seeded within hydrogels containing RGD spread significantly more than cells seeded in hydrogels without RGD at 4 weeks (cell area: 400 μm^2 vs. 215 μm^2, respectively, $p<0.05$) (Fig. 3E). A similar trend, although not significant, was observed when looking at the circularity, where a score of 1 indicates a perfectly round cell. The cells within the RGD hydrogels demonstrated a lower score (0.6) than the cells seeded in the hydrogels without RGD (0.8) (Fig. 3F).

Effect of Hydrogel Composition on Proliferation of Encapsulated hPDCs: After 1 week of culture in GM, the DNA content between the two different hydrogel compositions showed no significant difference (Fig. 4A). However, at 4 weeks, there was a significant difference in DNA content between construct compositions, where the hydrogel with the RGD motif displayed an approximately a 10-fold higher DNA content when compared to the hydrogel without RGD ($p<0.001$). Furthermore, when comparing the DNA content in both hydrogels at 4 weeks to their respective formulations at the 1 week time point, the hydrogel constructs without RGD displayed a 2-fold drop in DNA content ($p<0.001$), which is consistent with the drop in cell viability measured in these hydrogels, whereas the hydrogels containing RGD demonstrated an approximately 5-fold increase in DNA content ($p<0.001$). Thus, not only time but also the

hydrogel composition had a significant impact on the proliferation of the encapsulated cells when cultured in GM, and the hPDCs proliferated better in the RGD-containing hydrogels.

When looking at the DNA content at 0 weeks for the hydrogels cultured in the two different chondrogenic media, 4C and CDM-BMP2, there was no significant difference among the hydrogel groups and the different culture conditions (Fig. 4B). After 4 weeks, the hydrogels cultured in the serum-free CDM-BMP2 medium maintained their DNA content levels for both of the hydrogel compositions. On the other hand, the hydrogels cultured in 4C medium displayed a significant drop in DNA content over time ($p<0.01$), in addition to having significantly lower DNA content than the hydrogels cultured in the CDM-BMP2 medium ($p<0.001$).

New Matrix Production under Chondrogenic Differentiation vs. Proliferative Conditions: When the cell-hydrogel constructs were cultured in either the 4C or CDM-BMP2 chondrogenic medium, both hydrogels with and without RGD saw a significant increase in GAG production by the encapsulated hPDCs over the culture period (Fig. 5; $p<0.001$). In addition, at 4 weeks, the hydrogels with RGD significantly outperformed their counterparts in 4C medium, producing close to 2.5X more GAG/DNA ($p<0.001$). Moreover, when looking at the hydrogel formulations cultured in the CDM-BMP2 medium, the cells within the RGD-containing hydrogels synthesized 1.5X more GAG/DNA than the cells cultured in the hydrogels without RGD at 4 weeks. Further, both the hydrogels without RGD and with RGD in the CDM-BMP2 medium displayed a significantly lower GAG/DNA content ($p<0.01$ and $p<0.001$, respectively) when comparing with stimulation with 4C medium. In contrast, the cells encapsulated in the hydrogels and cultured in GM did not produce significantly more GAG in either hydrogel formulation over time. Thus, culturing the constructs in chondrogenic medium enhanced matrix synthesis and accumulation.

Chondrogenic Gene Expression of Encapsulated hPDCs in PEG Hydrogels: Chondrogenic gene expression was analyzed using quantitative PCR (Fig. 6). First, the effect of the 4C medium was explored, and after 1 week as well as 4 weeks of culture, the constructs showed an increase for all the chondrogenic markers: *SOX-9*, *COL2A1*, *ACAN*, and *COL10A1*. The *SOX-9* expression (Fig. 6A), an early marker for chondrogenesis, displayed close to a 2.5-fold increase at 1 week of culture in 4C medium for both hydrogel formulations. After 4 weeks, the *SOX-9* expression of the encapsulated hPDCs saw a significant rise in both hydrogel formulations ($p<0.01$). Similar to the *SOX-9* expression, culturing the cell-laden hydrogels with this medium for 4 weeks did not produce a significant difference in *COL2A1* gene expression between the hydrogel compositions. At 1 week, there is a 2-fold increase in *COL2A1* expression for both hydrogel formulations in 4C medium (Fig. 6B). Furthermore, after 4 weeks of culture, there was a significant increase compared to 1 week ($p<0.01$), resulting in an almost 15-fold increase for both formulations. For *ACAN* (Fig. 6C), after an initial increase at 1 week observed in both hydrogel formulations cultured in 4C medium, there was no significant difference in fold expression levels from 1 to 4 weeks as well as between the two hydrogel types at 4 weeks. Lastly, when looking at *COL10A1* (Fig. 6D), a marker for hypertrophy, already at 1 week, the cells cultured in the hydrogels without RGD displayed close to a 300-fold increase and the cells cultured in the hydrogels with RGD demonstrated close to a 120-fold increase. After 4 weeks of *in vitro* culture in the 4C medium, the *COL10A1* expression levels did not change; however, the difference between the hydrogel compositions became significant ($p<0.001$).

On the other hand, when the constructs were cultured in CDM-BMP2 medium, they displayed a marked difference in gene expression when compared to the constructs cultured in 4C medium. Although both formulations showed a significant increase over time ($p<0.01$), in the RGD-

containing hydrogels, *SOX-9* expression (Fig. 6A) showed a significantly higher fold increase when compared to the similar hydrogel formulation cultured in 4C medium ($p<0.001$). Additionally, at 4 weeks, the hPDCs encapsulated in the RGD-containing hydrogels and cultured in CDM-BMP2 medium demonstrated a significant upregulation of *SOX-9* when compared to the cells within the constructs without RGD ($p<0.001$). When analyzing the *COL2A1* gene expression (Fig. 6B) after 4 weeks in CDM-BMP2 medium, the gene expression level of the encapsulated cells increased significantly in both hydrogel formulations ($p<0.001$). The hydrogels without RGD displayed a 50-fold increase, and the RGD-containing hydrogels demonstrated close to a 200-fold increase. At the 4 week time point, the cells encapsulated in the RGD-containing constructs had a significantly higher expression level than the cells within the hydrogels without RGD ($p<0.01$). Moreover, the cell-hydrogel constructs cultured in the CDM-BMP2 medium significantly outperformed the same constructs in the 4C medium ($p<0.001$). Next, when looking at the *ACAN* expression levels (Fig. 6C) in CDM-BMP2 medium, the RGD-functionalized hydrogels already witnessed a 10-fold increase at 1 week and continued to maintain it at 4 weeks. These same constructs had significantly higher *ACAN* expression levels at 1 and 4 weeks compared not only to the cell-laden hydrogels without RGD ($p<0.05$ and $p<0.01$, respectively) but also to the same hydrogel formulations cultured in the 4C medium ($p<0.01$). In regard to the *COL10A1* expression levels in the CDM-BMP2 medium, at 1 week, the constructs displayed a 5-fold increase, which was significantly lower than the constructs in the 4C medium ($p<0.01$). However, after 4 weeks in CDM-BMP2 medium, the constructs without RGD and with RGD displayed a significant increase ($p<0.01$) to 100-fold and-300 fold, respectively.

Discussion

A promising tissue engineering scaffold should maintain the viability of the cells and allow for their expansion *in vitro*. Herein, PEG scaffolds with an MMP-sensitive crosslinker combined with an integrin-binding sequence were shown to not only help viability and proliferation but also support chondrogenic differentiation of the hPDCs that were encapsulated within, depending on the *in vitro* culture conditions. Hydrogels incorporating RGD preserved a higher viability and enhanced cellular proliferation of encapsulated hPDCs when cultured in GM, when compared to hydrogels without RGD. These same hydrogels also promoted more spreading of the cells. For similar purposes, several groups have investigated PEG-based scaffolds in combination with different cell sources to enhance cartilage formation *in vitro.*[28,29,32,42] While the novelty of the present study is the use of hPDCs in 3D PEG hydrogels, it is apt to compare these cells and their behavior to that of other MSCs, specifically BM-MSCs. MSCs have been shown to have a better viability, adhesion, and proliferation in degradable PEG hydrogels with the incorporation of an adhesion peptide.[25,27,28,43] One reason could be that MSCs in general and also hPDCs are anchorage dependent cells. It has been shown that in the absence of integrin-binding ligands, anchorage dependent cells can undergo anoikis, an apoptotic progression that results from the lack of cell and matrix interactions.[44,45] MSCs interact with RGD via the $\alpha_v\beta_3$ integrin, and cellular attachment leads to tyrosine phosphorylation and subsequent activation of focal adhesion kinase (FAK). FAK plays a vital role in transmitting the adhesion dependent cell survival signal within the cell. The high viability of ~85 % observed for the hPDCs within the RGD-functionalized hydrogels can also potentially be attributed to the mechanism of presentation of RGD, as was previously shown with MSCs.[27] In that study, RGD that was tethered via a single

link to the PEG network (as done here) was shown to promote 80 % MSC survival as compared to only 60 % survival seen when the peptide was connected via two links to PEG.[27]

Further, the hydrogels helped to maintain the viability and support the proliferation of hPDCs when the cells were encapsulated as a single cell suspension. Previous studies have used clusters or aggregates, which have been shown to play a role in MSC survival,[46,47] whereas we have shown that the hydrogel constructs provide the necessary adhesion points and space via degradation for the hPDCs to spread and proliferate over time. The proliferation rate is such that the RGD-containing hydrogels, when starting with a low cell density and cultured in regular GM for 4 weeks, were able to reach the initial DNA content of the cell-laden constructs used for the differentiation experiments. This indicates that this 3D hydrogel construct has the potential to be used as a single culture system, allowing for both expansion and differentiation. In addition, these 3D hydrogels could be used to encapsulate PDC micro-aggregates or spheroids, similar to approaches that have shown promising outcomes with human BM-MSC spheroids that demonstrated a significantly higher viability, DNA content, and a lower caspase activity in RGD-modified alginate hydrogels.[37]

For a 3D biomaterial to be successful in tissue engineering applications, the cells also have to be able to undergo differentiation and lead to tissue formation. For chondrogenesis, the cellular morphology transition from a spread shape to a rounded shape has been indicated to be a crucial process.[48,49] Thus, it is not surprising that the incorporation of an adhesion sequence into 3D hydrogels impedes chondrogenesis of BM-MSCs.[38,50] However, the results in the present study seem to contradict this notion. Here, hPDCs encapsulated in a PEG hydrogel with 150 μM RGD displayed higher GAG/DNA synthesis and higher chondrogenic gene expression when cultured *in vitro* in different chondrogenic media, when compared to PEG hydrogels without RGD. The

positive results with RGD here could be due to the fact that the mentioned studies were conducted in alginate[50] or agarose hydrogels and used high concentrations of the adhesion peptide, whose prolonged exposure to the cells can be detrimental to differentiation.[38] The low RGD concentration and the enzymatically degradable nature of the PEG hydrogels utilized within this study are plausible reasons for the results, as it has been also shown that the disappearance of the adhesion peptide over time helps enhance the chondrogenic capability of MSCs in PEG hydrogels.[38] Here, the local degradation of the 3D hydrogels by proteases expressed by the encapsulated cells, which is dependent on the specific peptide sequence in the crosslinker,[30,31] should lead to the release of RGD, thus reducing the prolonged exposure to the administered binding motifs. Furthermore, the results demonstrated herein align themselves to other studies where the incorporation of these ligands has supported *in vitro* chondrogenesis.[28,51,52] Additionally, the degradation of the hydrogels would also lead to swelling of the constructs and allow for greater diffusion of the stimulatory factors present within the media as well as secreted GAGs, as previously observed with chondrocytes in PEG hydrogels;[33] however, this increase in permeability would be countered by the ability of the newly synthesized ECM to sequester growth factors.

When looking at the different chondrogenic culture conditions, the general trend was that RGD-containing hydrogels were associated with an enhanced effect on the initiation of chondrogenesis of hPDCs; however, the preferred medium formulation for *in vitro* cartilage formation was not clearly distinguishable. For this study, each of the media was chosen for specific reasons. CDM-BMP2 was developed from selected components that, when combined, were able to re-express the cartilage phenotype and maintain chondrocytes under serum free conditions.[41] Additionally, this medium supplemented with BMP-2 displayed promising initial results for the chondrogenic

differentiation of hPDCs within our labs.[39] The 4C medium, composed of TGF-β1, ascorbic acid, dexamethasone, and ITS+, was optimized for ATDC-5 cells, a chondrogenic cell line derived from murine teratocarcinoma. ATDC-5 cells have demonstrated excellent *in vitro* chondrogenic capabilities,[40] thus making the 4C medium an attractive choice to investigate with hPDCs. The GAG/DNA synthesis of the cells in the RGD-containing hydrogels was higher when cultured in the 4C medium. The components TGF-β1 and dexamethasone have been shown to increase GAG production of MSCs in pellet culture[17] as well as when MSCs are encapsulated in 3D hydrogels [53,54]. On the other hand, when looking at gene expression, especially for *COL2A1*, the cells cultured in the RGD-functionalized hydrogels in CDM-BMP2 medium significantly outperformed the constructs cultured in the 4C medium. This can be possibly due to the presence of parathyroid hormone (PTH) and BMP-2 in the CDM-BMP2 medium. PTH, at low to moderate concentrations, has been implicated with an increase in expression of *SOX-9* and *COL2A1* by MSCs.[55,56] The interplay between culture conditions and the structural environment is an important factor when it comes to the differentiation of hPDCs *in vitro*. For instance, our group has also been able to show that when hPDCs were cultured in 2D flasks, in the CDM-BMP2 medium, chondrogenic markers were upregulated at an early time point.[57] On the other hand, when hPDCs were cultured in a micromass system, not only was BMP-2 vital, but also the addition of BMP-6, growth differentiation factor-5 (GDF-5), TGF-β1, and fibroblast growth factor-2 (FGF-2) was required for achieving optimal GAG production and chondrogenic differentiation of hPDCs *in vitro*.[14] Importantly, we have shown that these hPDC-hydrogel constructs cultured in CDM-BMP2 medium, which is serum-free, could be relevant towards clinical translation.

In summary, the RGD-modified, enzymatically degradable PEG hydrogel system presents a new biomaterial option for the 3D *in vitro* chondrogenic differentiation of hPDCs. The results gathered in this study demonstrate that RGD functionalization does promote cell viability and proliferation as well as a more spread morphology of the hPDCs. Remarkably, the conditions do not hinder the process of initiating *in vitro* chondrogenesis of hPDCs. Thus, this RGD-containing 3D PEG hydrogel system combined with hPDCs might be used for a cell-based treatment for cartilage or bone regeneration and is an avenue to be further explored.

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Figures and Tables

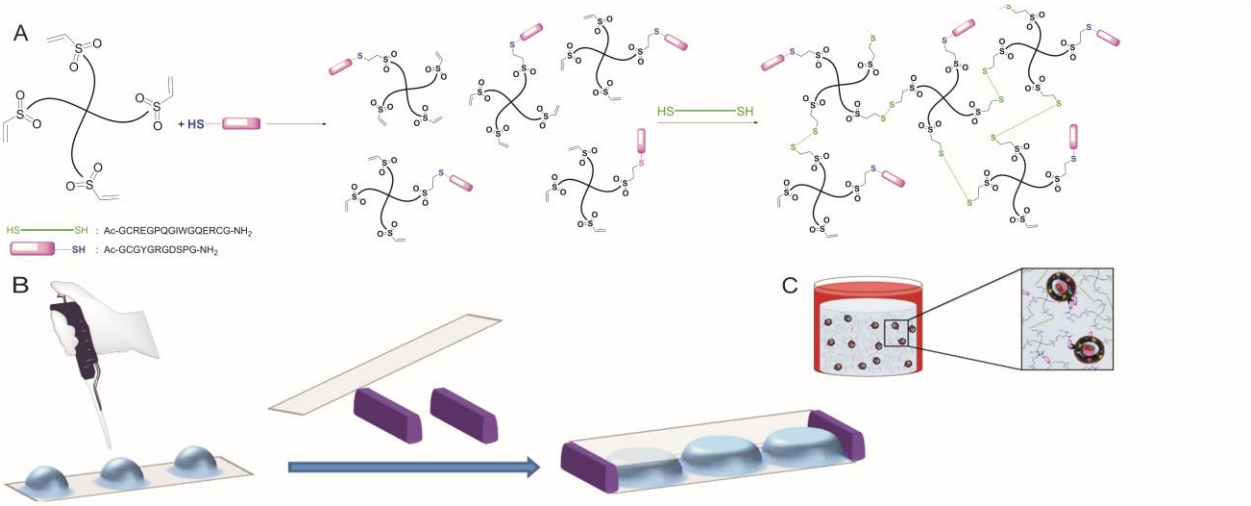

Fig. 1. Synthesis of protease-degradable PEG hydrogels for hPDC encapsulation. (A) Michael type addition reaction of 4-arm PEG-VS functionalized with integrin binding sequence (RGD) and crosslinked via a di-thiol crosslinker containing a proteolytically degradable substrate. (B) Hydrogels were formed by sandwiching drops of cell-containing hydrogel solution between hydrophobic, SigmaCote treated slides with spacers to create disks and incubated for 30 min at 37 °C. (C) Cell-laden hydrogels were then placed in well plates with respective medium, which was replenished every 2-3 days.

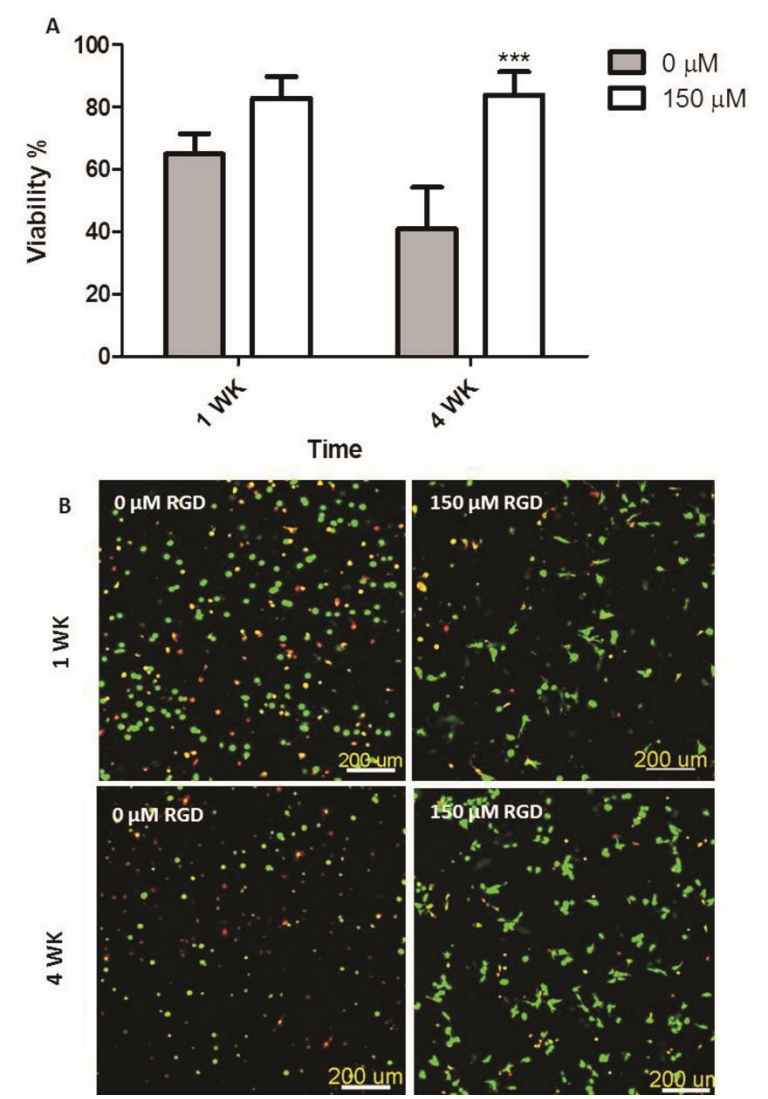

Fig. 2. hPDC viability within RGD-functionalized and non-functionalized, protease-degradable PEG hydrogels cultured in GM. (A) Quantification of the viability of hPDCs encapsulated in hydrogels with 0 µM RGD vs. 150 µM RGD. Results are presented as mean ± standard deviation (n=3; ***p<0.001). (B) Live/Dead staining via calcein AM (green, live cells) and ethidium homodimer-1 (red, dead cells). Confocal images depicted as maximum intensity of 500 µm z-stack using 10X objective (scale bar = 200 µm).

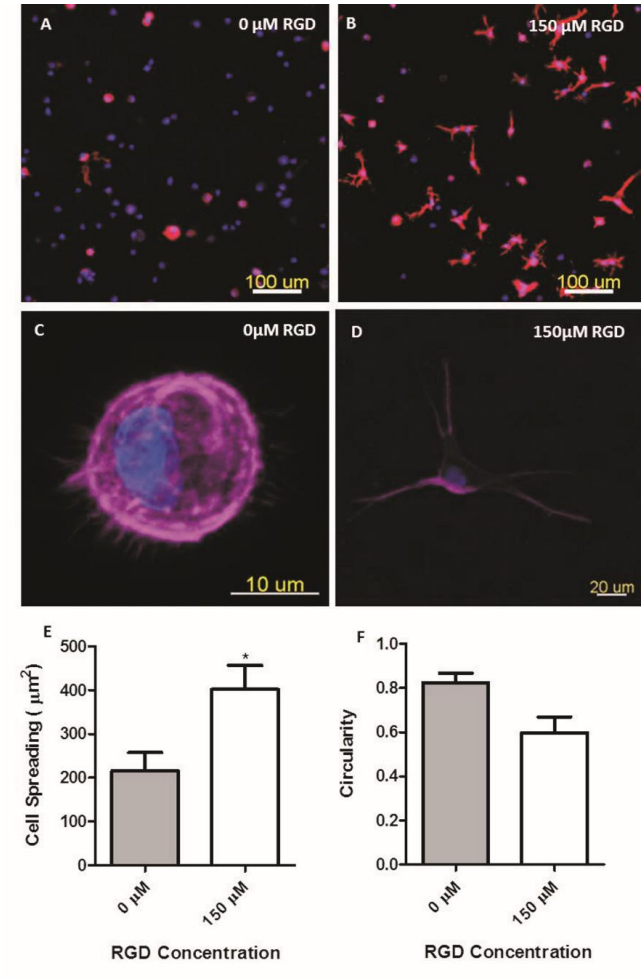

Fig. 3. Encapsulated hPDC spreading within protease-degradable PEG hydrogels cultured in GM. Confocal images are depicted as maximum intensity projections of the z-stacks. (A, C) DAPI/Phalloidin staining of hPDCs in hydrogels without RGD after 4 weeks of culture in GM imaged with 10X objective (A) and 40X Objective (C). (B, D) DAPI/Phalloidin staining of hPDCs in hydrogels with 150 µM RGD after 4 weeks of culture in GM imaged with 10X objective (B) and 40X objective (D). Blue color indicates the cell nuclei, and pink color indicates the actin filaments/cytoskeleton of the hPDCs. (E) Average area covered by each encapsulated cell was calculated using imageJ. (F) Average circularity of the encapsulated hPDCs was calculated using imageJ, and 1 indicates a perfectly round shape. Results in (E, F) are presented as mean ± standard deviation (n=3 images, *$p<0.05$).

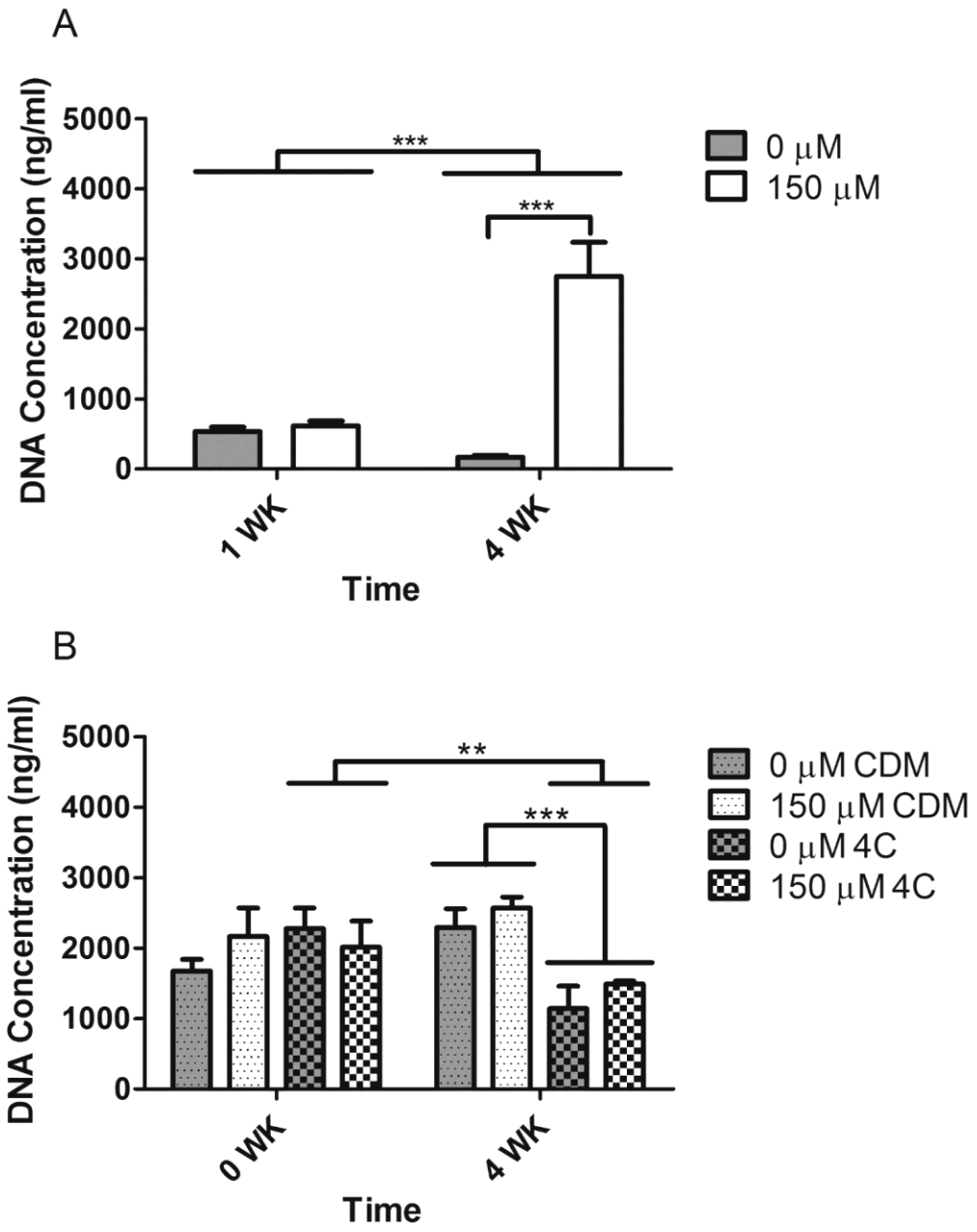

Fig. 4. DNA quantification of encapsulated hPDCs within 150 μM RGD-containing hydrogels and 0 μM RGD-containing hydrogels. (A) Hydrogels cultured in GM for 1 week and 4 weeks. (B) Hydrogels cultured in two different chondrogenic differentiation media (CDM and 4C) for 0 weeks and 4 weeks. Results are presented as mean ± standard deviation (n=3, ***$p<0.001$).

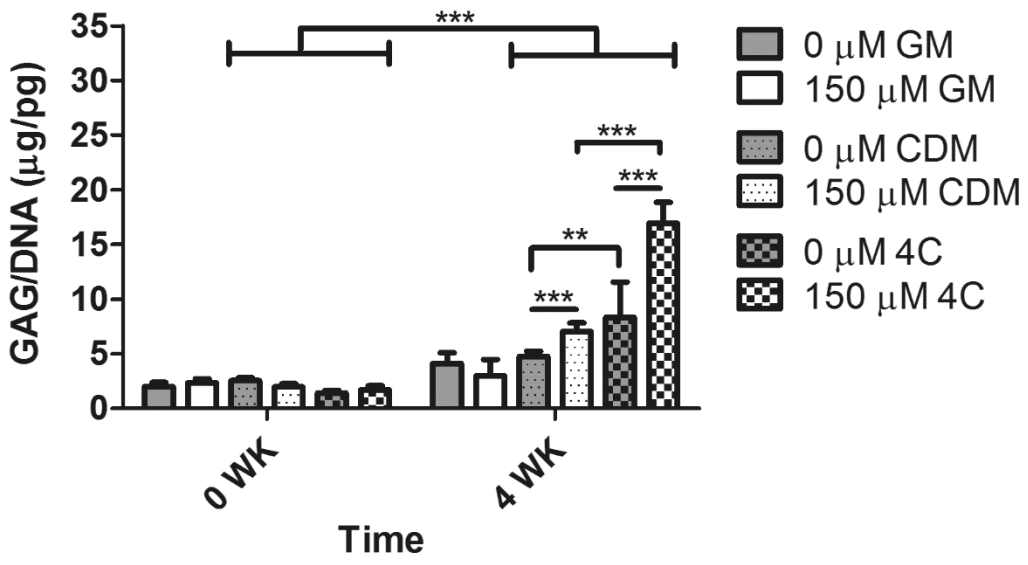

Fig. 5. GAG production of encapsulated hPDCs within 150 μM RGD-containing hydrogels and 0 μM RGD-containing hydrogels cultured in different media (GM, CDM, and 4C) normalized to DNA content. Results are presented as mean ± standard deviation (n=3, ***p<0.001, **p<0.01).

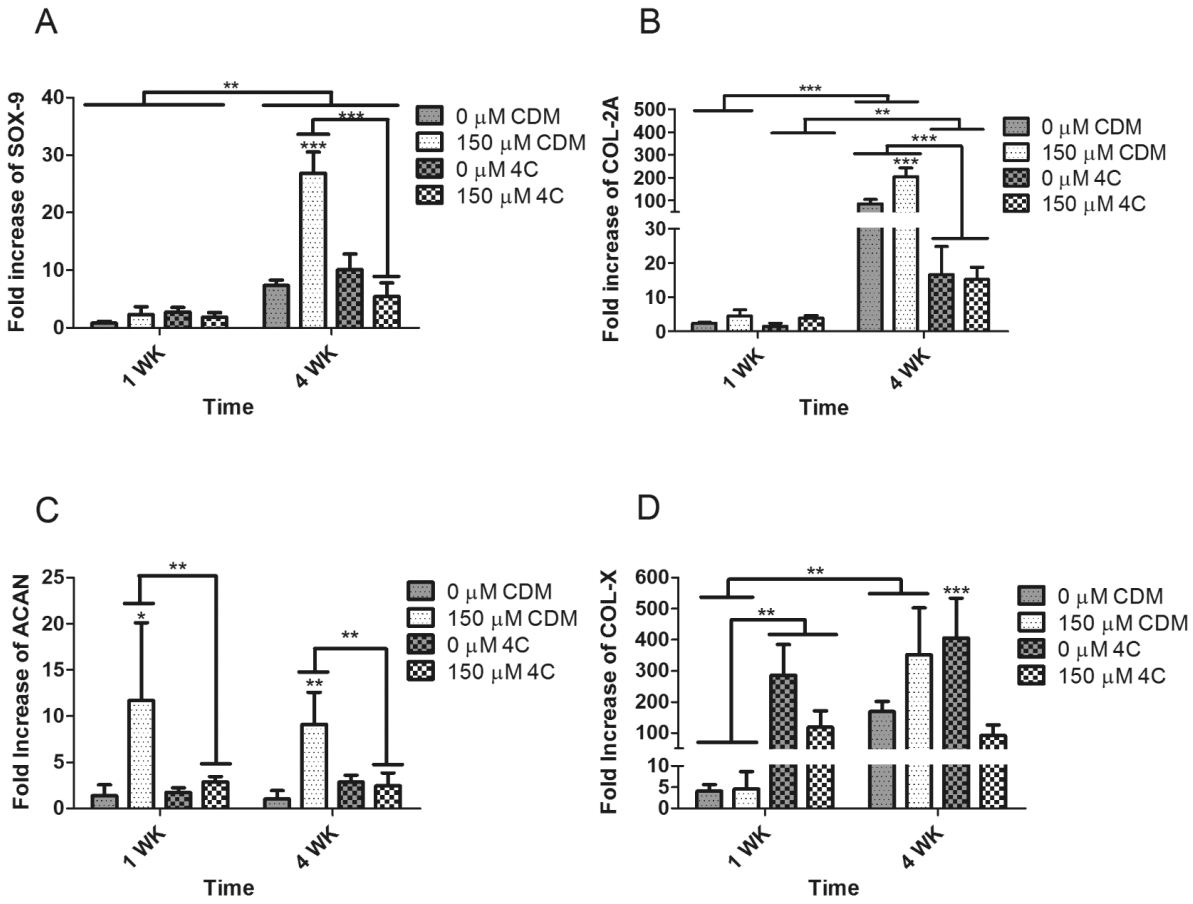

Fig. 6. Chondrogenic gene expression of hPDCs encapsulated in 150 μM RGD-containing hydrogels and 0 μM RGD-containing hydrogels cultured in two different chondrogenic media (CDM and 4C). Gene expression was normalized to time point zero and the housekeeping gene, β-Actin. (A) *SOX-9* (SOX-9); (B) *COL2A1* (COL-2A); (C) *ACAN* (ACAN); (D) *COL10A1* (COL-X). Results are presented as mean ± standard deviation (n=3, *p<0.05, **p<0.01, ***p<0.001).

Table 1. Primer sequences

Gene	Forward Sequence	Reverse Sequence
SOX-9	TGGAGACTTCTGAACGAGAGC	CGTTCTTCACCCACTTCCTC
COL-2A	GGCTTCCATTTCAGCTATGG	AGCTGCTTCGTCCAGATAGC
ACAN	GTCTCACTGCCCAACTAC	GGAACACGATGCCTTTCAC
COL-10A	ACGATACCAAATGCCCACAG	GTGGACCAGGAGTACCTTGC
β-Actin	CCCAGATCATGTTTGAGACCT	CCTGTAGATGGGCACAGT