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Assessment of cartilage regeneration on 3D collagen-polycaprolactone scaffolds: Evaluation of growth media in static and in perfusion bioreactor dynamic culture

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ABSTRACT

Efforts on bioengineering are directed towards the construction of biocompatible scaffolds and the determination of the most favorable microenvironment, which will better support cell proliferation and differentiation. Perfusion bioreactors are attracting growing attention as an effective, modern tool in tissue engineering. A natural biomaterial extensively used in regenerative medicine with outstanding biocompatibility, biodegradability and non-toxic characteristics, is collagen, a structural protein with undisputed beneficial characteristics. This is a study designed according to the above considerations. 3D printed polycaprolactone (PCL) scaffolds with rectangular pores were coated with collagen either as a coating on the scaffold's trabeculae, or as a gel-cell solution penetrating scaffolds' pores. We employed histological, molecular and imaging techniques to analyze colonization, proliferation and chondrogenic differentiation of Adipose Derived Mesenchymal Stem Cells (ADMSCs). Two different differentiation culture media were employed to test chondrogenic differentiation on gelated and non gelated PCL scaffolds in static and in perfusion bioreactors dynamic culture conditions. In dynamic culture, non gelated scaffolds combined with our in house TGF-β_2 based medium, augmented chondrogenic differentiation performance, which overall was significantly less favorable compared to StemPro™ propriety medium. The beneficial mechanical stimulus of dynamic culture, appears to outgrow the disadvantage of the "weaker" TGF-β_2 medium used for chondrogenic differentiation. Even though cells in static culture grew well on the scaffold, there was limited penetration inside the construct, so the purpose of the 3D culture was not fully served. In contrast dynamic culture achieved better penetration and uniform distribution of the cells within the scaffold.

1. Introduction

Cartilage, a connective tissue which is found in many structures throughout the body i.e. in the synovial joints, spine, ribs, external ears, nose and airways, is formed by chondroblasts that differentiate into chondrocytes and secrete a cartilaginous extracellular matrix (ECM). ECM is a heterogeneous fibrillar network of glycosaminoglycans (GAGs) and proteins that provides mechanical support and resistance during cartilage deformation [1].Compared to other tissues, injured cartilage has limited intrinsic healing capacity and demonstrates an inability to produce sufficient amount of extracellular matrix, due to its avascular, aneural and alymphatic nature. Cartilage injuries, therefore, result to changes in ECM physicochemical properties, caused by alterations in its structural components. These changes are associated with increased catabolic activity and inflammation of the joints [2].Consequently many surgical techniques and cell-based therapies

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have been introduced over the last decades [3], aiming to enhance cartilage repair and regeneration. Till today, full reconstruction of large cartilage defects has not yet been accomplished and, most importantly, the mechanical properties of the native cartilage needed to withstand the loadings of daily human activities cannot be reproduced [4–6].

Nowadays, part of the efforts in regenerative medicine is directed towards the construction of biocompatible scaffolds and the determination of their micro environment, which will lead to the ideal scaffold, mimicking the *in vivo* cellular environment [7–9]. Stem cells, selected from various sources, are encouraged to colonize, proliferate and finally differentiate to chondrocytes [10,11]. 3D scaffolds are made from materials either natural in origin or synthetic or even combination from both types, aiming to support cell growth and differentiation [12–17]. Furthermore, the use of bioreactors alters conditions in cell cultures, to better resemble the *in vivo* environment [18,19].

Synthetic semi-crystalline polycaprolactone (PCL) is attracting researchers' attention, as a biomedical material because of its biodegradability, biocompatibility, mechanical strength and elasticity. PCL scaffolds have been shown to support both proliferation and chondrogenic differentiation of stem cells. However, due to PCL's hydrophobic nature there is an increasing interest in the development of PCL composite/blended collagen scaffolds, aiming to enhance hydrophilicity [20–22]. Collagen is a structural protein with undisputed beneficial characteristics that is found in excess throughout the human body. It is extensively used as a natural biomaterial in tissue engineering, presenting outstanding biocompatibility, biodegradability and non-toxic characteristics. Collagen has been reported to enhance MSCs proliferation [23] and it can be used as a sponge, hydrogel, film or membrane scaffold [24,25].

In this study we used 3D printed PCL scaffolds with rectangular pores, coated with collagen either as a coating on the scaffold's trabeculae, or as a gel-cell solution penetrating the pores of the scaffolds. We analyzed adhesion, proliferation and chondrogenic differentiation of Adipose Derived Mesenchymal Stem Cells (ADMSCs) over time. Two different chondrogenic media were tested under static and dynamic culture conditions using perfusion bioreactors. We tested scaffold and bioreactor synergy, which is attracting growing attention as an effective tool in tissue engineering.

2. Materials and methods

2.1. Isolation, expansion and characterization of adipose derived mesenchymal stem cells (ADMSCs)

Human adipose tissue (~70 ml) was harvested, from a lipoaspiration procedure, under Papageorgiou Hospital Review Board approved protocols, 263-7/12/2016, and patient informed consent. Cells were isolated and expanded in MSC medium, consisting of a-MEM, 15% FBS, 2 mM Glutamine, 0.1 mM L-ascorbic acid phosphate, 100U/ml penicillin, 100 mg/ml streptomycin. They were characterized as Mesenchymal Stem Cells by a Guava® easyCyte 8 H T flow cytometer (Merck-Millipore, Darmstadt, Germany). Cells expressed CD90 and CD73 (used as positive markers), whereas CD45 was used as a negative marker. Unstained cells were used as a control to set the gates and analysis region as described in our previous work [26,27].

2.2. Chondrogenic differentiation

Routinely on the 7th day of expansion, MSC medium was replaced with chondrogenic media to induce differentiation. Two different media were applied for 26 days: in the first one we employed an in-house TGF-β_2 based culture medium (a-MEM, with 15% FBS, 0.1 mM L-ascorbic acid phosphate, 2 mM Glutamine, ITS, 100 nM dexamethasone and 10 ng/ml transforming growth factor-β_2, 100U/ml penicillin and 100 mg/ml streptomycin). For the second condition, we used the StemPro™ Chondrogenesis Differentiation Kit (Thermo Fisher Scientific). Media changes were performed every 2–3 days as needed.

2.3. Scaffold fabrication

An additive manufacturing method was used by Fused Deposition Modeling (FDM) process with a benchtop 3D-Printing machine PRUSA i3 (USA) to create PCL scaffolds. Cylindrical scaffolds, 10 mm in diameter and 3 mm in height, were composed from 10 layers of material, 300 μm thickness each, interconnected with a rectilinear layer pattern and infill density of 65%, thus resulting to rectangular pores of 200 μm on the final constructs. Slice3r software was used for creating this geometry pattern. The printing temperature was set at 145 °C and the extrusion speed at 60 mm/s. PCL, with an average molecular weight of 50 kDa and a density of 1145 g/cm^3, was purchased from 3D4 MAKERS (The Netherlands). After fabrication PCL scaffolds were immersed in a 4 M NaOH bath, for approximately 20 h at room temperature (RT), to increase their surface hydrophilicity and clean their fibers [28–30]. Control PCL scaffolds were incubated in PBS for 20 h at RT. A total of 45 scaffolds were fabricated for this study.

2.4. Seeding ADMSCs on scaffolds

Scaffolds after the NaOH bath were designated as NaOH-PCL and represent the first group of our study. Remaining scaffolds were further treated by collagen type I coating designated as Coat-PCL group, or by the gelation method designated as Gel-PCL group.

2.4.1. NaOH-PCL scaffolds seeded for static culture

NaOH-PCL scaffolds were seeded with 2×10^5 cells/scaffold. Equal number of cells was applied on both scaffolds' surfaces by flipping it over. Cell seeded scaffolds were then incubated for 2 h at 37 °C, 5% CO_2 to allow for cell adhesion before the expansion medium was added (1.5 ml/scaffold). NaOH-PCL scaffolds were transferred to a new 24-well plate after 24 h and fresh expansion medium was added.

2.4.2. Coat-PCL scaffolds seeded for static culture

NaOH treated scaffolds were immersed in collagen type I solution (50 μg/ml in 0.02 N acetic acid) for 1 h at 37 °C, 5% CO_2. The excess solution was aspirated and the scaffolds were air dried in the flow hood for 10 min. Subsequently, Coat-PCL scaffolds were then seeded with 2×10^5cells/scaffold, applied on each surface; incubated for 2 h at 37 °C, 5% CO_2 to allow for cell adhesion before the expansion medium was added (1.5 ml/scaffold). After 24 h, seeded Coat-PCL scaffolds were transferred to a new 24-well plate and fresh expansion medium was added.

2.4.3. Gel-PCL scaffolds seeded for static culture

The Gel-PCL method briefly: For the preparation of 1 ml gelation solution we mixed 650 μl dH_2O with 100 μl 10XPBS. To this, we added 250 μl collagen and the pH of the resulting solution was adjusted to 7.4 with few drops of 1 N NaOH (6 μl). The final solution was kept on ice to avoid early gelation according to manufacturer's instructions (BD inc Europe). ADMSCs were mixed with the resulting solution and were added to the scaffold designated as Gel-PCL group. The cells were calculated so that each scaffold received 2×10^5 cells. The gel-cell solution was then applied onto the scaffold inside a 24-well plate taking care to cover it. After a 2 h incubation at 37 °C expansion medium was added. After 24 h the Gel-PCL scaffolds were transferred to a new 24-well plate and fresh expansion medium was added.

2.4.4. Cell seeding for the dynamic culture

For dynamic culture NaOH-PCL scaffolds were seeded with 2×10^5 cells/scaffold. The cell suspension was applied on the scaffold, delivering equal number of cells on both scaffolds' surfaces by flipping it over. Cell seeded scaffolds were then incubated for 2 h at 37 °C, 5% CO_2 to allow for cell adhesion. Seeded NaOH-PCL scaffolds were then placed

in the perfusion bioreactors (BOSE, TA Instruments). Expansion medium (80 ml) was added to every bioreactor. A peristaltic pump (MASTERFLEX L/S, Cole-Parmer, Thermo Fisher Scientific, USA) was used to keep the fluid flow. In this experiment, a constant continuous flow rate of 1 ml min^{-1} was induced for each scaffold.

2.5. Cell viability, proliferation and differentiation

We used the Viability/Cytotoxicity Assay kit for Animal Live & Dead Cells (Biotium, CA, USA), according to the manufacturer's instructions. ADMSCs/scaffold constructs were double stained with calcein AM and ethidium homodimer, staining living and dead cells, respectively. After adding both dyes, the constructs remained at RT in the dark for 30 min, they were washed with PBS and were visualized by a confocal upright fluorescence microscope (Nikon D-Eclipse 80i C1). The constructs were captured as z-stack images using the EZ - C1 3.20 software.

2.6. Histological evaluation and scanning electron microscopy

For histology, specimens were fixed in 10% v/v neutral buffered formalin overnight at RT. After fixation, constructs were dehydrated in graded ethanol series, immersed in a toluole bath for 5 min and then embedded in paraffin at 55 °C for 60 min. After blocking, paraffin cubes were cut transversely into 10 μm thick sections using a Leica RM2235 rotary microtome with low profile disposable blades (DB80LX). Sections were mounted on SuperFrost microscope slides (MenzelGläser, SuperFrost® Plus, Menzel GmbH & Co KG, Germany). Selected sections, from equal distances throughout the whole scaffold, were heated at 60 °C for 30 min, deparaffinized, hydrated and then stained with Hematoxylin and Eosin. Sections were dehydrated and covered.

Sections for Alcian Blue staining were acquired as described above. Selected sections were heated at 60 °C for 30 min, deparaffinized, hydrated and then stained in an alcian blue solution (1% w/v pH = 2.5, adjusted with 3% glacial acetic acid) for 30 min. Sections were dehydrated and covered.

Morphology of seeded ADMSCs on uncoated and collagen-coated scaffolds was observed visualizing sample surfaces and cross sections by means of Scanning Electron Microscopy (SEM). Scaffolds were fixed with 3% v/v glutaraldehyde, rinsed, post-fixed with osmium tetroxide, rinsed again and then were dehydrated in increasing concentrations (30%–100% v/v) of ethanol in water. The samples were dried, sputter-coated with carbon and observed under a SEM (JEOL JSM-6390 LV) at an accelerating voltage of 20 kV.

2.7. Assesment of Chondrogenic differentiation of ADMSCs seeded on PCL scaffolds

Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) was used to assess up- or down-regulation of chondrogenic markers (the transcription factor SOX-9 and Aggrecan-ACAN) during long-term culture with both chondrogenic media (TGF-β_2 and StemPro™) and under two different culture conditions, static and dynamic. More specifically, total RNA was extracted from the constructs after 26 days in chondrogenic media using Nucleo-ZOL (Macherey Nagel, Düren, Germany), according to the manufacturer's instructions. The RNA purity and concentration were measured using a Nano Drop spectrophotometer (Epock, Biotek, Biotek instruments, Inc, Vermont, U.S.A). The obtained RNA samples were then reverse transcribed as previously described. The results were adjusted by amplification efficiency (LinRegPCR) and normalized against one housekeeping gene (succinate dehydrogenase complex, subunit A, flavoprotein-SDH-A) F: GCATGCCAGGGAAGACTACA R:GCCAACGTCCACATAGGACA found to remain stable during differentiation processes of cells.

2.8. Statistics

All data were analyzed with analysis of variance (ANOVA) for repeated measurements. Multiple comparisons between groups were performed with Tukey's post-hoc test, using Prism 6.0 Software (GraphPad, CA, USA). Differences between means were considered statistically significant when $^{**}p < 0.01$,$^{***}p < 0.001$, $^{****}p < 0.0001$ and $\alpha = 0.05$. Data were presented as mean ± SD for $n = 3$ biological replicates.

3. Results

3.1. Scaffold porosity

Scaffolds' porosity was measured by the gravimetric method [31–36]. The material density was calculated using the bulk and the true density of the scaffold (n = 3), according to the Eqs. (1) and (2) below,

$$\rho_{scaffold} = \frac{mass}{volume} \quad (1)$$

$$Total\ porosity,\ P_t = 1 - \frac{\rho_{scaffold}}{\rho_{material}} \quad (2)$$

where: $\rho_{scaffold}$ = apparent density of the scaffold, and $\rho_{material}$ = density of the material. The volume was calculated by measuring the diameter and the height of the samples with micro-calipers and a micrometer, (Table 1). Results are presented as mean ± SD of three independent measurements.

3.2. Evaluation of cell viability and proliferation

The purpose of the first phase of the experiment was to distinguish the optimal methods to acquire the best functional hydrophilic PCL scaffolds for cartilage tissue regeneration. We tested three different methods to increase PCL scaffolds' surface hydrophilicity, treatment with 4 M NaOH, coating with Collagen type I (rat tail, BD) and direct loading a cell-gel onto the NaOH treated scaffolds (gelation method). Cell viability and proliferation was compared to ADMSCs loaded onto control-untreated PCL scaffolds.

Untreated-control scaffolds exhibit sparse and isolated minimal cell growth, seemingly originating from a single cell attachment/colony, (Fig. 1B). Cell attachment on the 2nd day and proliferation on the 7th, seem to be best supported on Gel-PCL scaffolds, that appear with the highest concentration of living cells. NaOH-PCL and Coat-PCL scaffolds, both exhibit comparable cell attachment and proliferation (Fig. 1C,D,F,G), although it is markedly diminished compared to the one attained on the Gel-PCL scaffolds (Fig. 1E,H).

Table 1
Scaffold fabrication and geometrical characteristics, pore sizes and scaffold porosity.

Scaffold design characteristics					
Scaffold design pattern	Fiber diameter μm	Layer height μm	Infill density (%)	Pore Shape/size geometry	Scaffold porosity (%)
Rectilinear	400	300	65	Rectangular/200μm	82.2 ± 0.22

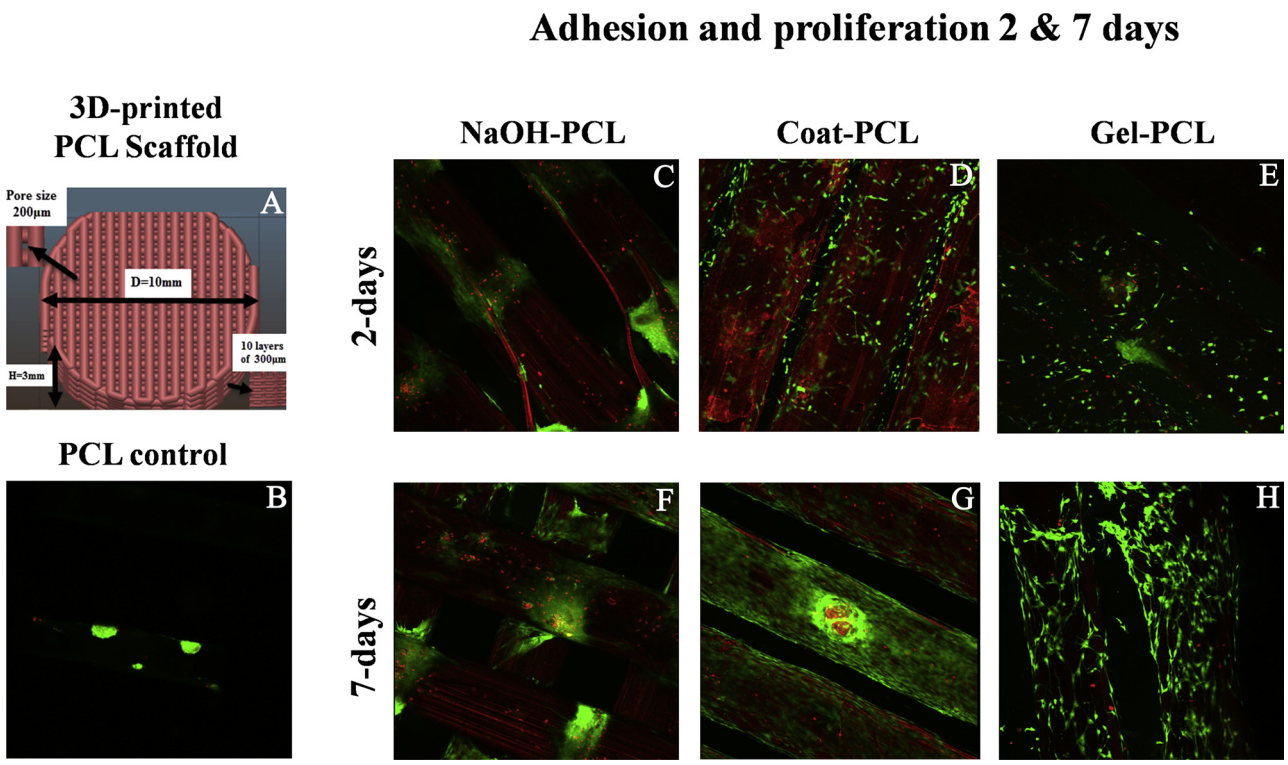

Fig. 1. Confocal images of the adipose derived stem cells seeded in untreated and treated PCL scaffolds. Live/dead fluorescent staining with calcein AM/ethidium homodimer showed high levels of cell viability of ADMSCs. Scaffold geometry from 3D Printing, **(A).** Cell adhesion on the control untreated scaffold, **(B)** and on all treated scaffolds, **(C,D,E)** after 2 days in MSC growth medium. Cell proliferation on all treated scaffolds after 7 days in MSC growth medium, **(F,G,H)**. Confocal images captured at 10X magnification.

3.2.1. Assessment of different chondrogenic media in both static and dynamic culture

Based on the results obtained from cell viability assessment on the three different constructs we decided to continue with NaOH-PCL and Gel-PCL scaffolds. We used these scaffolds to assess the efficacy of two different media towards chondrogenic differentiation of ADMSCs. The media used were an in house TGF-β_2 based culture medium and StemPro™.

After 26 days of static culture, cell viability remained high, indicating that both StemPro™ and TGF-β_2 based culture media are adequate to support cell growth. StemPro™ medium seems to be more supportive for cell growth but the difference is marginal and not always notable (Fig. 2.A.III). We also observed that in Gel-PCL scaffolds with StemPro™, cells appeared within a membranous sheet attached on the surface of the construct (Fig. 2.A.IV). Cell penetration in the NaOH-PCL scaffolds was higher, but nevertheless, also limited.

NaOH-PCL scaffolds were cultured in perfusion bioreactors under a constant fluid flow rate, in TGF-β_2 based culture medium. We assessed proliferation on the 7th day and differentiation the 26th day of culture. Proliferation and cell viability on the 7th day was markedly enhanced compared to the other scaffolds in static culture. Both, live and dead cells were more abundant on the 26th day of differentiation (Fig. 2.B.II), compared to the scaffolds in static culture. In a vertical cross section of the scaffold, the penetration of cells was the highest observed (Fig. 2.B.III).

3.3. Histochemical staining & cartilage identification (HE, Alcian blue)

We studied the sections of treated scaffolds (NaOH-PCL, Gel-PCL and bioreactor scaffolds) on the 7th day of culture in MSC growth medium. The cells usually appear in small groups, predominantly to the top and bottom surfaces of the scaffolds. The cell clusters are relatively scarce, with small cells having little cytoplasm present. Their nuclei are round and small. The cells are closely situated to each other forming tight, three dimensional groups (Fig. 3.A.I-III). Even though extracellular matrix is not abundant, as demonstrated by Alcian blue stain, it is often clearly observed near the groups of cells (Fig. 3.B.I-III).

The cells observed in scaffolds after 26 days of culture appeared more mature, in sheets of tissue with more extracellular matrix present. The cells were larger and round, oval or spindle shaped with a recognizable cytoplasm (Fig. 3.A.IV-VII).

The scaffolds cultured within the bioreactor appear with the most cell clusters and extracellular matrix, whereas the scaffolds cultured in static culture appear with fewer cells, as shown in Fig. 3B.VIII. The cells observed within their clusters in both gelation scaffolds (TGF-β_2 and StemPro™) are scarcer compared to the NaOH scaffolds, with the TGF-β_2 scaffold appearing with more extracellular matrix than the StemPro™. The newly formed tissue in both gelation scaffolds often appears with a characteristic margin made up from parallel, spindle-shaped cells, very close to each other. This gives the impression of a perichondrium (Fig. 3.A.IV,V). The scaffolds without gelation (TGF-β_2 and StemPro™) appear with a higher cell density and less extracellular matrix compared to the gelation scaffolds (Fig. 3.A.VI,VII,VIII). The cells cultured with TGF-β_2 appear mostly oval, with looser connections, whereas the cells cultured with StemPro™ are usually closely situated, spindle shaped cells (Fig. 3.A.V,VII).

3.4. Macroscopic characteristics and cell morphologies inside the PCL scaffolds-SEM analysis

SEM images in Fig. 4 show cell attachment and growth on NaOH-PCL and Gel-PCL scaffolds. Images were taken after 26 days of culture in both chondrogenic media (TGF-β_2 and StemPro™) and both on static and dynamic culture conditions. Visualization was succeeded on all scaffolds and our data show that ADMSCs demonstrated good attachment and generally an alignment with the scaffold's fibers (Fig. 4.A.II,V,

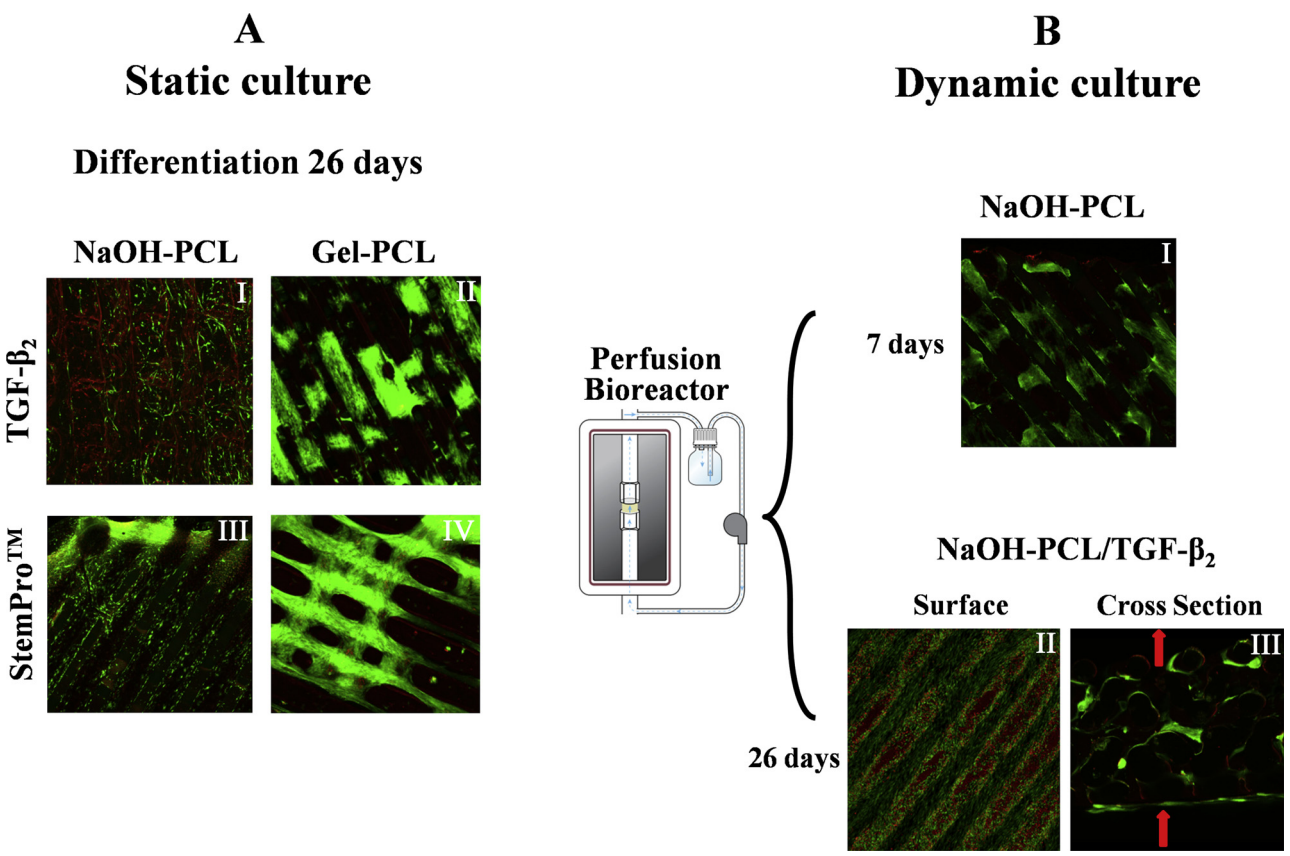

Fig. 2. Confocal images of the adipose derived stem cells seeded in treated PCL scaffolds both on static and dynamic culture. Live/dead fluorescent staining with calcein AM/ethidium homodimer showed high levels of cell viability of ADMSCs in static culture, **(A.I-IV)**. Cell proliferation on the 7th day of dynamic culture within perfusion bioreactors, **(B.I)** and cell viability after 26 days of culture in TGF-β_2, **(B.II)**. Cross sections of scaffolds showed greater cell infiltration in perfusion dynamic culture, **(B.III).**

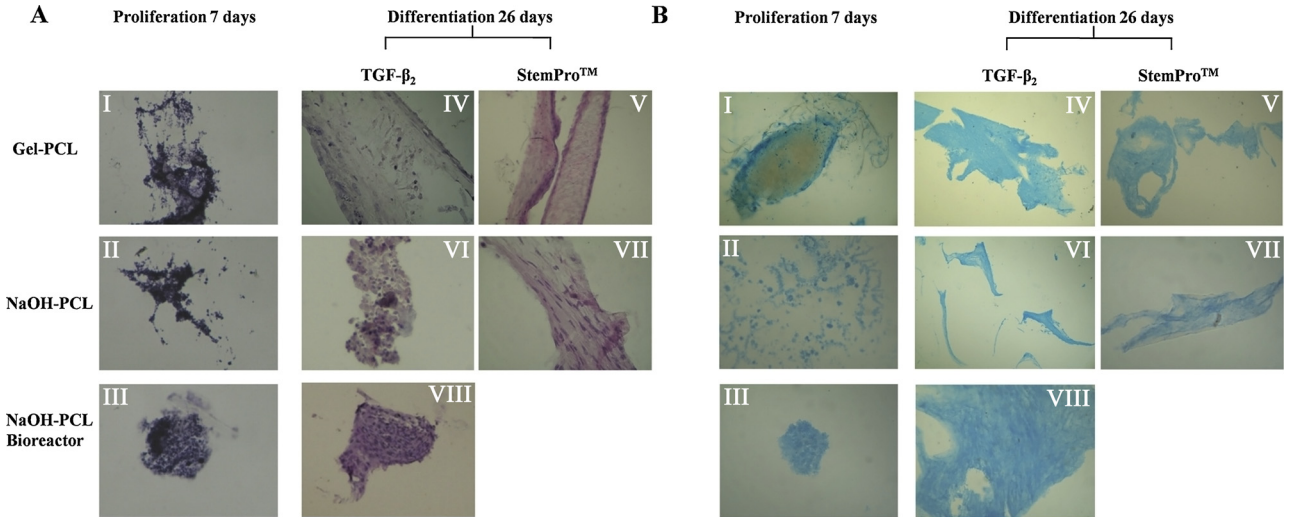

Fig. 3. Hematoxylin-Eosin (H&E) and Alcian blue staining of the adipose derived stem cells (ADMSCs) seeded in polycaprolactone (PCL) scaffolds treated with collagen (Gel-PCL) or NaOH (NaOH-PCL) and cultured in MSC medium for 7 days or in two different chondrogenic medium (TGF-β_2/StemPro) for 26 days, under static or dynamic culture conditions. Histological findings from H&E staining confirmed that the ADMSCs were more mature after 26 days of culture in both chondrogenic media than those cultured in MSC medium **(A)** Proteoglycan synthesis was confirmed by alcian blue staining after 7 days of culture in MSC medium indicating that GAG content in ADMSCs was greater in scaffolds cultured in chondrogenic medium than in MSC medium **(B)**. H&E, magnification X40; Alcian blue, magnification X40.

B.VII). Extracellular matrix (ECM) was produced on and between the pores of the scaffolds and the cells formed a tissue-like network (Fig. 4.A.II, B.VII). Cells were found mostly on the top surfaces of all scaffolds with the highest density appearing in the Gel-PCL/StemPro™ scaffold (Fig. 4.A.II). Cells on the NaOH scaffolds cultured in the bioreactor, exhibited a higher penetration underneath the top layers, as shown in Fig. 4 B.VIII. The differentiated cells on day 26 were usually large (~10 μm) and round-shaped (Fig. 4.A.I, B.VIII).

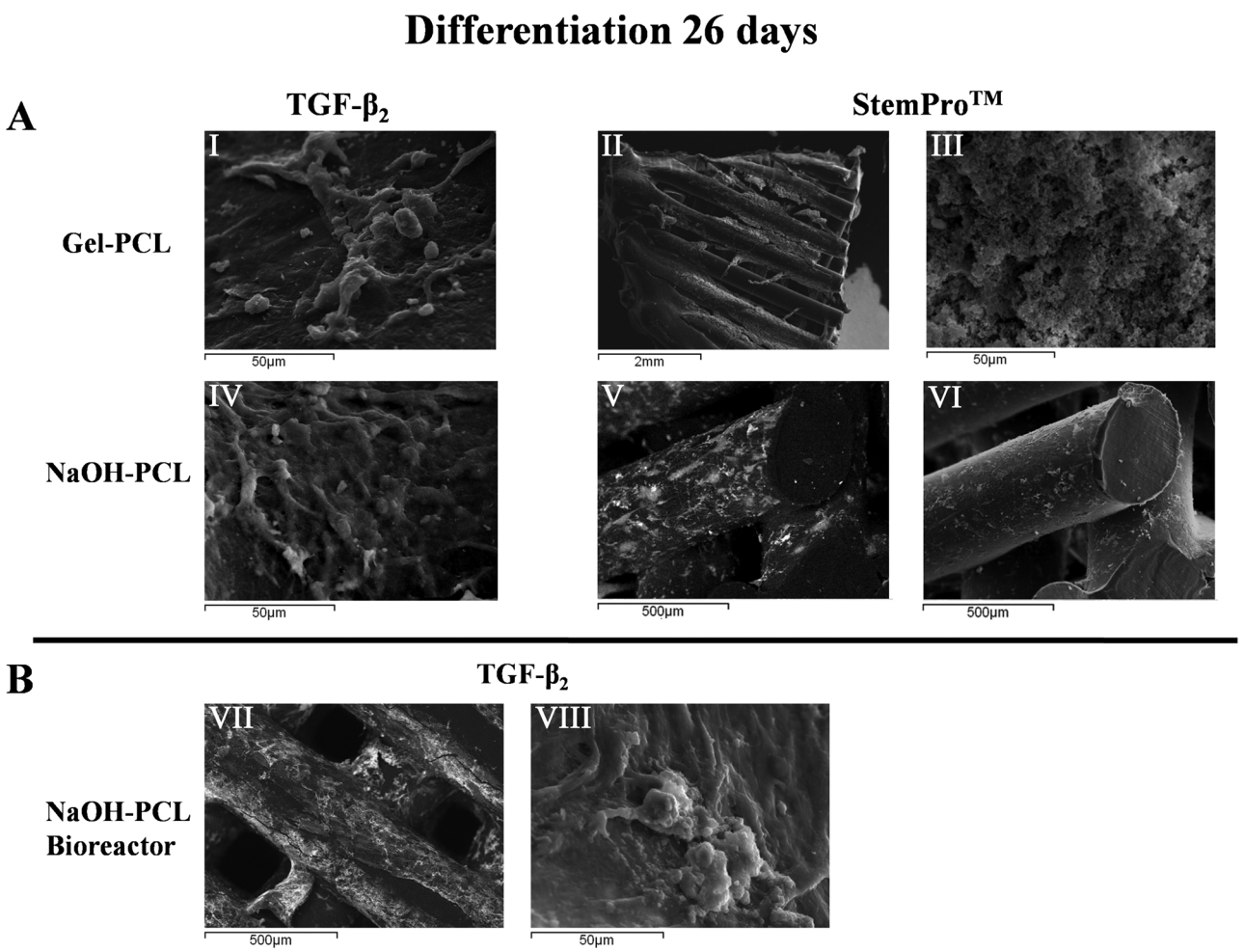

Fig. 4. Scanning electron microscopy (SEM) of the adipose derived stem cells (ADMSCs) seeded in **(A)** coated polycaprolactone scaffolds (Gel-PCL) for 26 days in TGF-β2 or StemPro chondrogenic medium, under static culture conditions (I-III), **(B)** uncoated polycaprolactone scaffolds (NaOH-PCL) for 26 days in TGF-β2 or StemPro chondrogenic medium, under static culture conditions (IV-VI), and **(C)** uncoated polycaprolactone scaffolds (NaOH-PCL) for 26 days in TGF-β2 chondrogenic medium, under dynamic culture condition (VII-IX).

3.5. Chondrogenic marker expression in the ADMSC/PCL scaffolds

To clarify whether the presence of collagen protein on the surface of the PCL scaffolds affects MSCs differentiation potential, ADMSCs were induced to differentiate towards chondrogenic lineage in the presence of either StemPro™ or TGF-$\beta_2$based chondrogenic media. After 26 days of *in vitro* static or dynamic culture, ADMSCs that underwent chondrogenic differentiation were examined for chondrogenic marker expression, ACAN (a major structural component of cartilage that represents the major proteoglycan in the articular cartilage) and SOX-9 (a master transcription factor that plays a crucial role in chondrogenesis).

Results in Fig. 5A show that under static culture conditions ADMSCs expanded in StemPro™ chondrogenic medium exhibited a significantly higher ACAN expression as compared to cells expanded in TGF-β_2 based medium. The expression of cells cultured in the same chondrogenic media (either StemPro™ or TGF-β_2) did not show any statistical difference among the scaffolds (gelated and non gelated). Dynamic cultured cells in TGF-$\beta_2$based medium exhibited a significantly higher ACAN expression ($p < 0,05$) compared to static cultured cells in the same media (both NaOH and Gel scaffolds). Compared to the cells in dynamic culture, only the cells in the gelated/StemPro™ scaffold performed better in terms of chondrogenic differentiation ($p < 0,05$).

The cells in the gelated/StemPro™ scaffolds exhibited a significantly higher SOX-9 expression compared to all other cells, which have shown a relatively low expression (Fig. 5B).

4. Discussion

4.1. Gelated and non gelated scaffolds in static culture

Scaffolds on cartilage tissue engineering applications serve as base constructs to host cells for proliferation and differentiation. They integrate with the cells and become part of the regenerated tissues, contribute to the mechanical properties of the engineered tissues and finally degrade *in vivo* in a time controllable manner.

PCL in this study was selected due to its physicochemical characteristics and because it can be controlled by 3D printing technology for customized scaffolds enabling and favoring tissue regeneration [37,38]. In this study we used cylindrical PCL scaffolds, 10 mm in diameter and 3 mm with rectangular pores of 200 μm proposed in the literature as optimal for ADMSCs-cartilage tissue engineering [39]. Despite its beneficial characteristics, PCL is a hydrophobic biomaterial whose surface lacks any chemical/biological structures to facilitate cell adhesion and therefore cartilage tissue regeneration. Herein, our first experimental goal was to distinguish the optimal methods to acquire the best functional hydrophilic PCL scaffolds for cartilage tissue regeneration. It has been reported that the hydrophobic characteristics of polycaprolactone can be altered by immersing PCL in 4 M NaOH [28–30], or by combining PCL with other materials, such as collagen [20,40]. We applied collagen on 4 M NaOH treated scaffolds by either of a process for surface coating (Coat-PCL scaffolds) or as a gel-cell

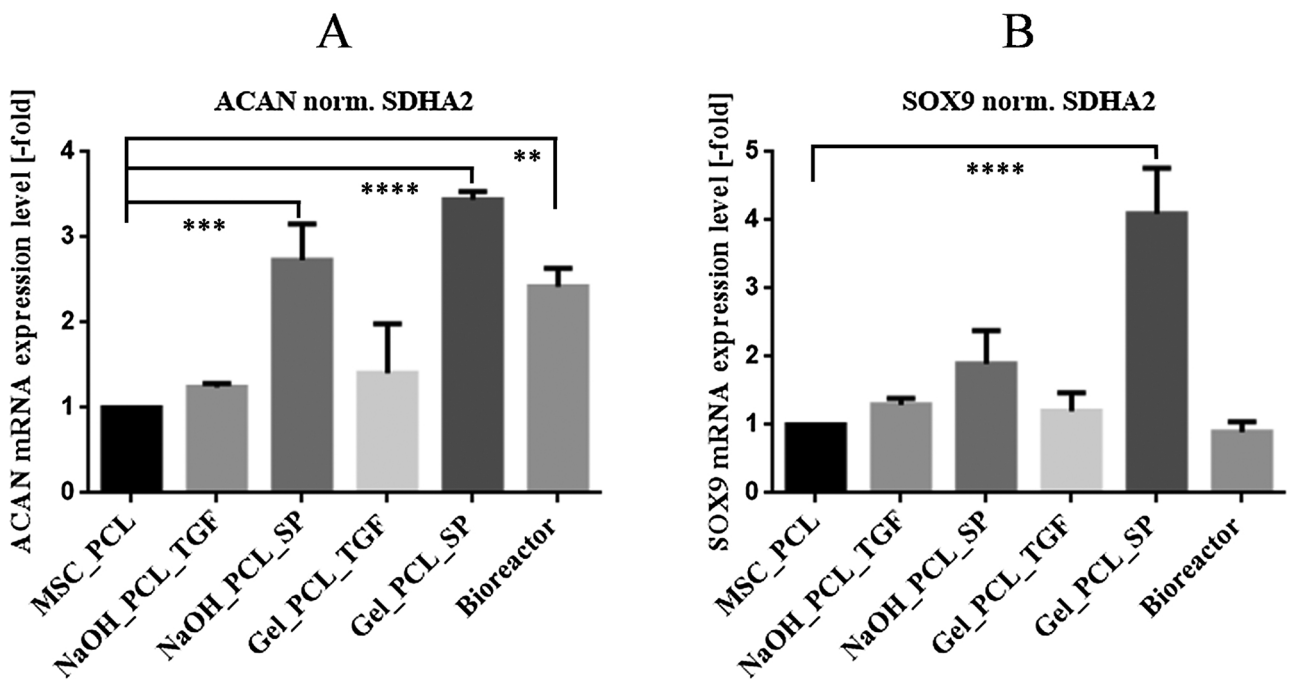

Fig. 5. Real-time PCR analysis of the expression of chondrogenic differentiation markers, *ACAN* **(A),** *SOX9* **(B)** in ADMSCs/scaffold constructs after 6 days exposure to MSC medium or after 26 days exposure to chondrogenic medium. *SDHA2*was used as housekeeping gene control. Values are means of n = 3 (± SD); **p < 0.01, ***p < 0.001, ****p < 0.0001.

solution (Gel-PCL scaffolds), thus obtaining a porous hydrophilic network or a gelated structure, respectively. Pertaining viability, the majority of cells remained viable in all treatments for both time points (2nd and 7th day of proliferation), exhibiting an insignificant number of dead cells. The results of the confocal microscope demonstrate that Gel-PCL scaffold is a better candidate for cell adhesion and proliferation than NaOH treated scaffolds or with the collagen coating treated scaffolds.

Our next goal was to evaluate how the given surface modification of PCL biomaterial influences chondrogenic differentiation of ADMSCs. On the 26th day of differentiation ACAN expression of the cells was documented. We compared the expression of cells in NaOH-PCL and Gel-PCL scaffolds cultured in StemPro™ medium. This way we compared only the effect of gelation of scaffolds on cell chondrogenesis. The difference was not significant ($p > 0,1$). We did the same with scaffolds in our TGF-β_2 based medium. Both NaOH-PCL and Gel-PCL scaffolds exhibit comparable expression of ACAN ($p > 0,5$). Taken together these data, we conclude that gelation treatment does not favor chondrogenic differentiation, as compared to treatment with only NaOH, which is in contrast with Ghorbani et.al study in which electrospun scaffolds of collagen-PCL exhibited increased levels of gene expression and GAG content compared to PCL scaffolds [41].

An important observation is that the cell growth in Gel-PCL scaffolds in StemPro™ medium, appear as a membranous sheet on the surface of the scaffold. This is likely because the living cells exhibited more connections amongst themselves and between the newly formed extracellular matrix (Fig. 3.A.IV). In the same scaffolds we observed limited penetration of cells within its interconnected pores. We postulate that this is attributed to the formation of the membrane like structure on the surface of the scaffold, preventing cell infiltration and colonization of the scaffold not allowing for 3D culture. This however is in contrast with the theory that collagenous materials in the porous constructions may prevent cells from falling through, resulting in a higher amount of cells retained inside [22,42].

Overall both Gel-PCL and NaOH-PCL scaffolds are favorable for cell viability and chondrogenic differentiation. Gel-PCL scaffolds may be best for cell adhesion and proliferation, but when combined with StemPro™, infiltration and colonization of the cells inside the scaffold may be limited due to the formation of a membrane on its surface.

4.2. Chondrogenic media and dynamic culture

Two different chondrogenic media were tested, TGF-β_2 based medium and StemPro™, both on Gel-PCL and NaOH-PCL scaffolds. After 26 days of static culture cell viability remained high. Both StemPro™ culture medium and TGF-β_2 based medium are beneficial for the cells. The chondrogenic potential of both media was examined by ACAN expression. All cells in StemPro™ scaffolds exhibited a significantly higher expression of ACAN compared to all cells in TGF-β_2 scaffolds (NaOH and gelated), in static culture ($p < 0,001$).

NaOH-PCL scaffolds were used in perfusion bioreactors and cells on the 26th day of culture exhibited the highest cell population and penetration observed with a more homogenous distribution on the surfaces and inside the scaffolds as well as presenting an abundant extracellular matrix, compared to the static cultures [43,44]. This however is in contrast to other studies where more cartilage deposition was observed in scaffolds under static conditions than in scaffolds under dynamic flow perfusion [45,46].

In the dynamic culture, we observed augmented cell growth and colonization of the scaffold. This cell “overgrowth” was also accompanied by increased number of dead cells (Fig. 3.B.II). We hypothesize that this observation can be attributed to the high shear stress stimuli created by the fluid flow which induce hypertrophy of cells as postulated by Kock et al. [45].

It has been reported that dynamic culture can also favor chondrogenic differentiation [47,48]. Cells growing in our perfusion bioreactors in TGF-β_2 based medium, exhibited significantly higher ACAN expression ($p < 0,05$) compared to cells growing in the same medium, in static cultures (NaOH and Gel scaffolds). Furthermore ACAN expression of cells in dynamic culture with NaOH/TGF-β_2 based medium was comparable to the one exhibited in NaOH/StemPro™, indicating that dynamic culture enhances the TGF-β_2 based medium to a better performance. This performance is also comparable to the one exhibited by the Gelated/StemPro™. The better performance of dynamic culture is

likely due to an efficient mechanical stimulus created by the fluid flow. This flow caters for nutrient transfer and controlled shear stress rates, activating the chondrocyte differentiation pathway [49–51]. The favorable dynamic culture conditions seem to alleviate the disadvantage of the "weaker" TGF-β2 based medium used in comparison to the better performing StemPro™ medium.

5. Conclusion

Dynamic culture favored chondrogenic differentiation even though it was performed in TGF-$\beta_2$based medium which overall was significantly less efficient compared to StemPro™. The beneficial mechanical stimulus of dynamic culture conditions seemed to outgrow the disadvantage of the "weaker" TGF-β_2 based medium used for differentiation. In addition, the dynamic culture achieved better penetration of cells within the scaffold. Although the cells on static cultures grew well on the surface of the scaffold, there was no adequate penetration inside the construct, so the purpose of the 3D cell-culture was not fully served.

With respect to personalized cartilaginous implant development, we are in need of future work focused on the identification of the most advantageous settings for optimal dynamic chondrogenic culture, aiming to narrow down the complexity of our methods towards minimal manipulation of our constructs.

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Ethics approval and consent to participate

The study has been approved by the Ethics Committee, of the Institutional Review Board of the School of Medicine, Faculty of Health Sciences, Aristotle University of Thessaloniki reference number 555/1-10-2015 and tissue donation was under Papageorgiou Hospital Review Board approved protocols 263-7/12/2016 signed informed consent according to the Helsinki Protocol.

Consent for publication

Not applicable.

Data availability statement

The datasets supporting the results of this article are included within the article

Conflict of interest

The authors declare that they have no competing interests.

Author contributions

AK conceived and coordinated the study, supervised experiments, analyzed and interpreted the data and drafted the manuscript. ED participated in the study design, provided adipose tissue samples and contributed to drafting of the manuscript. EA performed the cell culture and molecular analysis of the samples. KT designed the produced 3D printed scaffolds, performed the experiments participated in tissue culturing, analyzed and interpreted data and all contributed to drafting of the manuscript. MM performed the histochemical characterization, supervised the SEM analysis and drafted part of the manuscript. AB participated in methodology establishment, supervised the confocal analysis analyzed and interpreted data and critically reviewed the manuscript. All authors read and approved the final manuscript.

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