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Adipogenic differentiation of human adipose derived mesenchymal stem cells in 3D architectured gelatin based hydrogels (ArcGel)

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Abstract

Polymeric matrixes mimicking multiple function of the ECM are expected to enable a material induced regeneration of tissues. Here, we investigated the adipogenic differentiation of human adipose derived mesenchymal stem cells (hADSCs) in a 3D architectured gelatin based hydrogel (ArcGel) prepared from gelatin and L-lysine diisocyanate ethyl ester (LDI) in an one-step process, in which the formation of an open porous morphology and the chemical network formation were integrated. The ArcGel was designed to support adipose tissue regeneration with its 3D porous structure, high cell biocompatibility, and mechanical properties compatible with human subcutaneous adipose tissue. The ArcGel could support initial cell adhesion and

survival of hADSCs. Under static culture condition, the cells could migrate into the inner part of the scaffold with a depth of 840 ± 120 μm after 4 days, and distributed in the whole scaffold (2 mm in thickness) within 14 days. The cells proliferated in the scaffold and the fold increase of cell number after 7 days of culture was 2.55 ± 0.08. The apoptotic rate of hADSCs in the scaffold was similar to that of cells maintained on tissue culture plates. When cultured in adipogenic induction medium, the hADSCs in the scaffold differentiated into adipocytes with a high efficiency (93 ± 1%). Conclusively, this gelatin based 3D scaffold presented high cell compatibility for hADSC cultivation and differentiation, which could serve as a potential implant material in clinical applications for adipose tissue reparation and regeneration.

Key words: Mesenchymal stem cells, gelatin based scaffold, adipose tissue regeneration, adipogenic differentiation

1. Introduction

A variety of factors including age, disease, trauma, tumor removal, congenital malformation, and burns could lead the loss of subcutaneous adipose tissue, which would affect the patients physiologically and psychologically [1-3]. However, restoring the structure and function of defective adipose tissue still presents a clinical challenge in reconstructive surgery. Currently, two approaches of treatment are commonly adopted to repair a defect of soft tissue. The first one is fat grafting or lipofilling [3], in which the autologous adipocytes are injected to the position of soft tissue defects. This procedure does not involve potentially toxic materials and is easily available, with a generally satisfying result in short term [1]. However, the long term outcomes of this approach are unpredictable due to the inner necrosis of the transplanted tissue and the high resorption rate [3,4]. The second approach is the transplantation of filler materials such as silicone, hyaluronic acid, or calcium hydroxyapatite based materials. Despite the capacity of supporting soft tissue repair and restoration, the clinical use of such filler materials are limited because of further documented complications, such as foreign-body reactions, which potentially affects function [5], uncontrollable degradation over time [6], as well as the risk of immunogenicity [7]. To overcome those disadvantages, strategies for adipose tissue reconstruction are being investigated, focusing on the most important aspects for the success of adipose tissue reconstruction - the stem cells with adipogenic differentiation potential and the scaffold materials mimicking function of the extracellular matrix (ECM) of adipose tissue.

Adipose derived stem cells (ADSCs) are multipotent mesenchymal stem cells isolated from adipose tissue. ADSCs show the ability to differentiate towards adipogenic, osteogenic,

chondrogenic, myogenic and cardiomyogenic lineage [8,9]. Previous research found that ADSCs differentiated into adipocytes and to replenishing lost volume of soft tissue through proliferation and maturation into adipocytes, which makes them ideally suitable for adipose regeneration [10]. In addition to self-renewal and differentiation, ADSCs could secrete several growth factors including vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), basic fibroblast growth factor (FGF-2), and insulin-like growth factor 1 (IGF-1)[11,12], some of which could promote angiogenesis and adipose tissue regeneration[13].

In addition to ADSCs, the scaffold is of great importance for soft tissue reconstruction. By now, a variety of natural and synthetic materials have been used to produce scaffolds with the aim to support tissue repair and regeneration. When used in combination with stem cells, such a scaffold material should at least fulfill several requirements. First, the high cell compatibility is necessary to allow for adhesion, proliferation and migration of stem cells, and later for support adipocyte survive. Second, the material should have the mechanical properties comparable to native adipose tissue, to facilitate surgical handing and support adipogenic differentiation of transplanted stem cells. Third, a 3D porous structure might facilitate the accommodation of cells. At last, the scaffold should be biodegradable, which is critical to provide space for cell proliferation, guide tissue development, and enable the replacement of the materials by autologous or transplanted cells [14,15]. Different materials have been processed into scaffolds for soft tissue engineering. Polylactic acid (PLA), polyglycolic acid (PGA) and the copolymer poly[lactide-*co*-(glycolic acid)] PLGA [16-19] are the most commonly explored synthetic materials. Collagen, silk fibroin, ECM secreted by hADSCs, decellularized human placenta, fibrin, gelatin, hyaluronic acid, and matrigel belong to the category of natural materials applied in this context [20]. Compared to synthetic polymers, the materials of natural origins, especially collagen and gelatin, offer clinically appropriate properties to mimic soft tissues. Collagen is abundantly present in native adipose ECM. It has been proved that both collagen and gelatin could support adipogenesis of stem cells [21-24].

In this context, the aim of this study was to evaluate the potential of a 3D architectured gelatin based hydrogel (ArcGel), consisting of gelatin and lysine connected by urea junction units, as scaffold for adipose tissue regeneration. Given the low cytotoxicity of gelatin from natural origin, the ArcGel scaffold was expected to support hADSC adhesion and survival with high cell viability. The low immunogenicity of gelatin [25] might decrease the risk of strong body reaction following implantation. The ArcGel showed a porous morphology, with the pore sizes of 272±114 μm in the dry state determined by SEM, which might allow for accommodating

hADSCs and providing space for cell proliferation. The ArcGel might be suitable for adipogenic differentiation of hADSCs regarding its macroscopic mechanical properties, as it showed an elastic modulus of 3.1 ± 0.2 kPa determined by oscillatory rheological measurement, which is compatible to human subcutaneous adipose tissue. The water uptake of the ArcGels was 983 ± 37 wt.%, and the ArcGel could degrade *in vitro* within a few weeks [26]. Therefore, the ArcGel might have potential advantages upon degradation *in vivo*, including providing more space for cell proliferation and adipose tissue development, and facilitating the replacement of the scaffold material by native tissue. To this end, the cell biocompatibility of the ArcGel for hADSC culture as well as its potential for supporting adipogenic differentiation of hADSCs were investigated *in vitro*.

2. Materials and methods

2.1 ArcGel scaffold

The gelatin based 3D architectured hydrogel (ArcGel) was prepared using a one-step approach as reported previously [26], in which the formation of an open porous morphology and the chemical functionalization were integrated. In brief, LDI (8-fold molar excess of NCO groups compared to NH_2 groups of the gelatin) was added to a 10% aqueous solution of gelatin (Type A, 200 bloom, with low endotoxin content, GELITA AG, Eberbach, Germany) and PEO-PPO-PEO triblock copolymer (Pluronic® F-108, Sigma-Aldrich Chemie, Steinheim, Germany) under mechanical stirring in a flat flange cylindrical jacketed vessel with bottom outlet valve (HWS Labortechnik, Mainz, Germany) at 45 °C. After 6 minutes, the obtained ArcGel was collected and freeze-dried. To perform cell culture study, the dried ArcGel was cut into discs with 10 mm in diameter and 2 mm in thickness. Before cell seeding, the air inside the ArcGel samples was removed by infiltrating the samples with cell culture medium followed by a vacuum degassing process.

2.2 Human adipose derived mesenchymal stem cells

The hADSCs were isolated from human adipose tissue as described previously [27]. The adipose tissue was obtained by abdominal liposuction from a female donor after informed consent (No.: EA2/127/07; Ethics Committee of the Charité - Universitätsmedizin Berlin, approval from 17.10.2008). The hADSCs were cultured in the human adipose-derived stem cell medium (Lonza, Walkersville, MD, USA) at 37 °C in a humidified atmosphere containing 5% CO_2, and the medium was changed regularly to maintain cell growth.

2.3 Cell distribution and migration in ArcGel

For seeding hADSCs on ArcGel, the disc-shaped scaffold was first put into the 48-well tissue culture plate (TCP). Then, 5×10^4 cells suspended in 500 µL growth medium (Lonza, Walkersville, MD, USA) were added into each well. After culturing the cells for 4 days and 14 days, the samples were washed with PBS and fixed using 4 wt% paraformaldehyde (ThermoFisher Scientific, Waltham, USA) then permeabilized with 0.1wt% Triton X-100 (Sigma–Aldrich, St. Louis, MO, USA). After blocking with 3 wt% BSA solution, ActinRed™ 555 (Life Technologies, Darmstadt, Germany) and SYTOX Green Nucleic Acid Stain (Life Technologies, Darmstadt, Germany) were used to stain F-actin and nuclei, respectively. The stained samples were scanned on a confocal laser scanning microscope (LSM 780, Carl Zeiss, Jena, Germany) using the mode of z-stack multilayer scanning, and the images were processed using ZEN 2012 software (Carl Zeiss, Jena, Germany). A longitudinal sectional view of the samples was achieved by cutting the samples before scanning, in order to investigate the cell migration into the scaffold.

2.4 Cell proliferation

The proliferation rate of hADSCs in the ArcGel scaffold was measured using a cell count kit (CCK-8) (Dojindo Molecular Technologies Inc., Munich, Germany) at indicated time points. In brief, for each well the old medium was replaced with 500 µl of fresh medium, followed by adding 50 µl of CCK-8 solution. After 2 hours of incubation at 37 ºC, 100 µl of medium/CCK-8 mixture was transferred from each insert into a transparent 96-well tissue culture plate, and the absorbance was measured at a wavelength of 450 nm and a reference wavelength of 650 nm using a microplate reader (Infinite 200 PRO, Tecan Group Ltd., Männedorf, Switzerland). A standard curve, which was generated by measuring the absorbance of a series of samples with known cell numbers, was used to calculate the number of hADSCs in the ArcGel.

2.5 Cellular apoptosis

Cellular apoptosis test was conducted to study the apoptotic rate of cells cultured in ArcGel. Following cell seeding in the scaffold, the caspase-3/7 activation, which is a key marker of cell apoptosis, was measured at day 1, 4 and 7 using a Caspase-Glo @ Kit (Promege, Madison, USA). In brief, the culture medium was replaced with new medium (360 µL/sample), followed by adding Caspase-Glo@ 3/7 reagent (300 µL/sample) and shaking orbitally at 300 rpm for 30 seconds. After incubation for 90 min at room temperature, 200 µL liquid of each sample was transferred into 96-well TCP and the luminescence intensity was measured using a microplate reader (Infinite 200 PRO®, Tecan Group Ltd., Männedorf, Switzerland). The result was given

as relative light units (RLU) normalized with cell number, which was measured by CCK-8.

2.6 Adipogenic differentiation

The cells were seeded on the scaffold and cultured in adipose-derived stem cell growth medium for 14 days. Then the medium was replaced with adipogenic induction medium (Life Technologies, Darmstadt, Germany) for adipogenic differentiation. After 14 days of adipogenic induction, the samples were fixed with 4 wt% paraformaldehyde, followed by Oil Red O (Sigma–Aldrich, St. Louis, MO, USA) staining to detect the lipid droplets in the adipogenically differentiated cells [28]. Further, FABP-4 immunostaining was performed to quantify the adipogenically differentiated cells in the scaffold. In brief, after 21 days of adipogenic induction, the samples were fixed with 4 wt% paraformaldehyde and permeabilized with 0.1 wt% Triton X-100 solution. Then, the cells were blocked with 3 wt% BSA and stained using rabbit anti-human FABP-4 monoclonal antibody (Merck Millipore, Darmstadt, Germany) and Alexa Fluor® 488 labeled IgG antibodies (Life Technologies, Darmstadt, Germany). Cell nuclei were stained by SYTOX Green Nucleic Acid Stain (Life Technologies, Darmstadt, Germany). The stained samples were scanned with a confocal laser scanning microscope (LSM 780, Carl Zeiss, Jena, Germany) using the mode of z-stack multilayer scanning, and the images were processed using ZEN 2012 software (Carl Zeiss, Jena, Germany). Then, the cells were counted to calculate the percentage of differentiated cells (FABP-4 expressing cells/total cells × 100%).

In addition, the expression level of FABP-4 protein was determined using ELISA. The cells were washed with PBS and lysed using cell lysis buffer (ThermoFisher Scientific, Waltham, USA) after 14 or 21 days of induction in ArcGel. The amount of FABP-4 was measured using a FABP-4 ELISA kit (Abcam, Cambridge, UK) according to the manufacture's instruction. The experiment was performed in triplicates. The hADSCs cultured on TCP and/or in growth medium were included as controls.

2.7 Statistics

ArcGel samples from three batches and hADSCs from one donor were used in this study. The number of replications was larger than three as indicated in the figure legend of Figs. 2 and 4. The results were expressed as mean ± standard deviation (SD). Statistical analysis was performed using independent-samples T-test and a significance level (Sig.) <0.05 was considered statistically significant.

3. Results

3.1 Cell distribution and migration into the ArcGel

After 4 and 14 days of cultivation in growth medium, the cells were stained to investigate the cell distribution and migration into the ArcGel. The cells could attach and spread on the scaffold. The density of cells on the material surface increased with the culture time, as shown in top-view images of day 4 and day 14 (Fig. 1 A), indicating the cell growth on the scaffold. Further, the cells could migrate into the scaffold after cell attachment, due to the high porosity of the ArcGel. After 4 days of cultivation, the cells migrated into the scaffold for 840±120 μm; and after 14 days, the cells distributed in the whole scaffold with a thickness of 2 mm (Fig.1B).

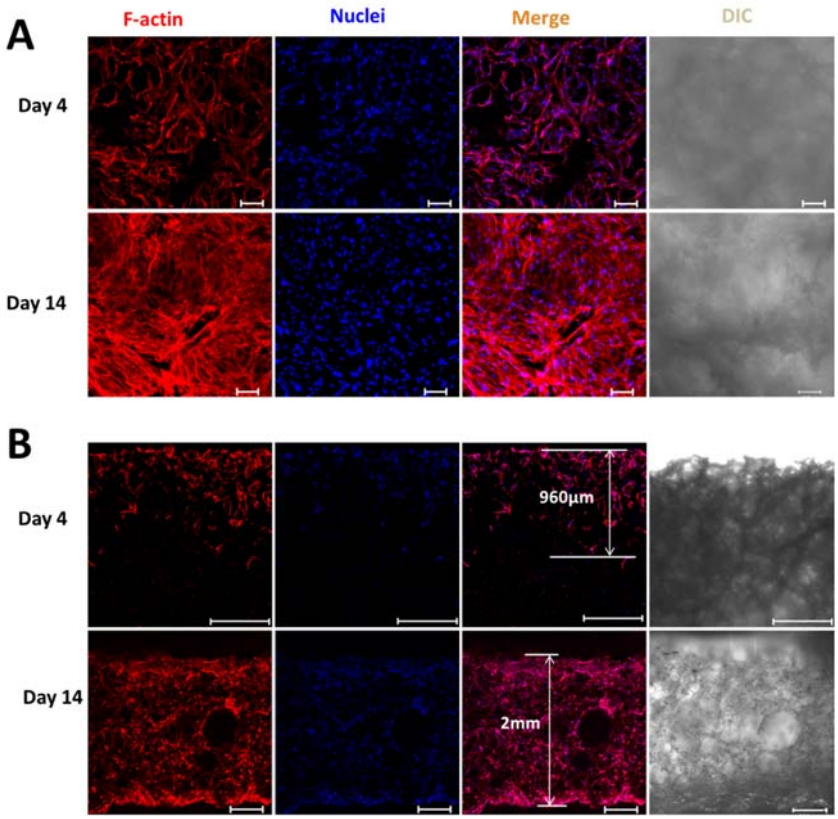

Fig. 1. The distribution and migration of hADSCs into the ArcGel. hADSCs were cultured in the ArcGel for 4 days and 14 days followed by fluorescence staining to visualize the actin cytoskeleton (red) and nuclei (blue). (A) Top-view images showed hADSCs could attach and grow on the ArcGel surface (bar = 100 μm). (B) Longitudinal section-view images indicated the hADSCs migration into the ArcGel (bar = 500 μm, DIC: differential interference contrast microscopy).

3.2 Cell proliferation and apoptosis

Cell proliferation in the ArcGel was studied by quantifying the cell number at different time

points (Fig.2A). The scaffold showed a high capacity for allowing hADSC proliferation. After 7 days of cultivation in grow medium, the fold increase of cell number was 2.55 ± 0.08. After 10 days, the fold increase of cell number reached to 5.75 ± 0.42. In order to study the influence of ArcGel scaffold on cell apoptosis, the apoptotic level was evaluated at day 1, 4 and 7 (Fig.2B). A decreasing tendency of cell apoptosis level was observed in all groups, i.e. ArcGel, TCP+ArcGel and TCP. There was no significant difference of apoptotic level in the tested three groups.

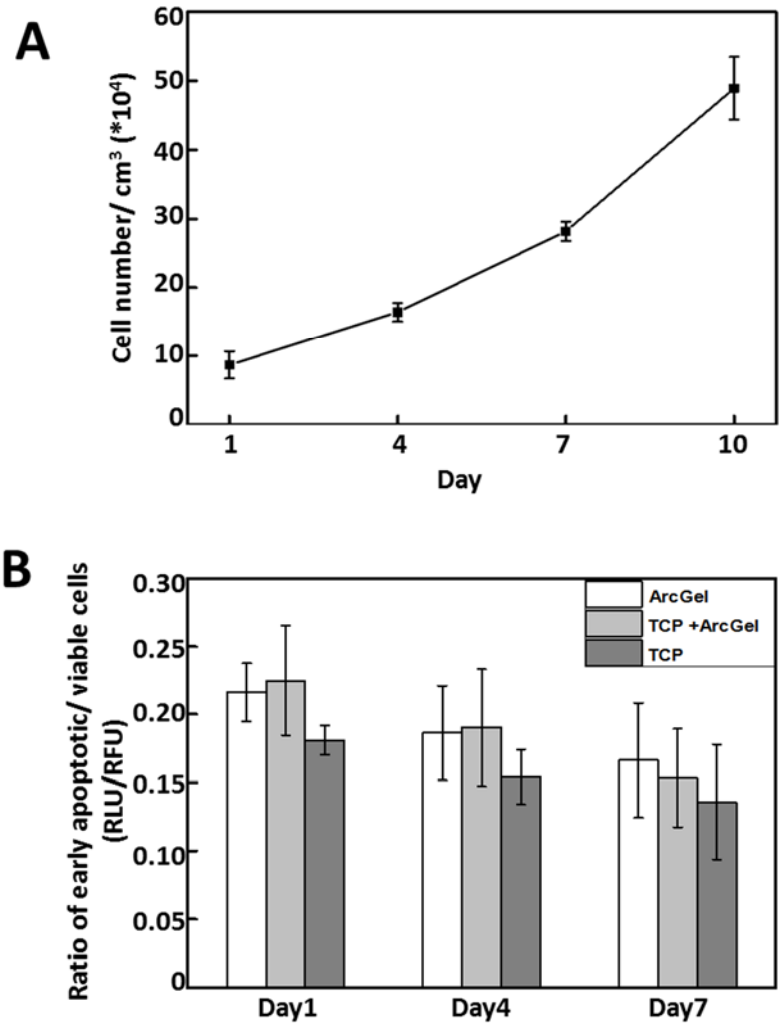

Fig. 2 Cell proliferation and apoptosis in ArcGel. (A) Proliferation of hADSCs on ArcGel. (B) The apoptosis of hADSCs on different substrates. (ArcGel: cells seeded on ArcGel; TCP+ ArcGel: cells seeded on TCP, with ArcGels added to the culture medium; TCP: cells seeded on TCP) (n = 3).

3.3 Adipogenic differentiation of hADSCs in ArcGel

The ADSCs could differentiate into adipocytes in the ArcGel in adipogenic induction medium.

Similar to the cells on TCP, formation of lipid droplets was observed in hADSCs in ArcGel after 14 days of adipogenic induction, as shown in oil red O staining images (Fig. 3). The quantification of FABP-4, the marker of adipogenic differentiation, indicated a high differentiation rate of hADSCs in ArcGel. Around 93 ± 1% hADSCs were FABP-4 positive, based on the fluorescence images of stained cells (representative images in Fig. 4A). The images of longitudinal section-view showed that even the cells inside the ArcGel underwent differentiation (Fig. 4B). This result was confirmed by FABP-4 quantification using ELISA. After 21 days of cultivation in adipogenic induction medium, hADSCs in ArcGel presented similar FABP-4 expression level as those cultured on TCP, and was higher than that after 14 days of induction (Fig. 4C). The cells did not undergo differentiation in normal growth medium in either ArcGel or TCP, indicating that there was no spontaneous differentiation.

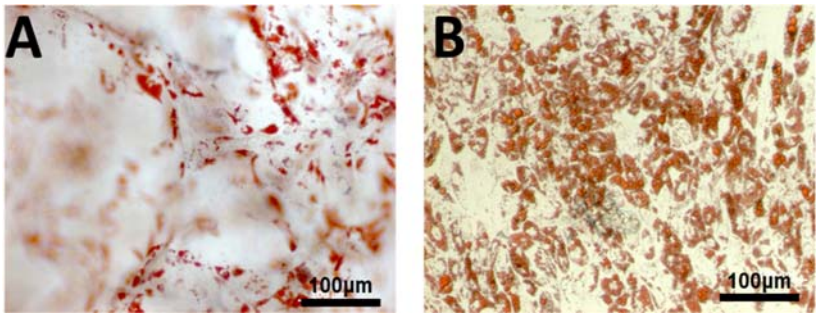

Fig.3 Oil red O staining of adipogenically differentiated hADSCs in ArcGel (A) and on TCP (B) after 14 days of cultivation in adipogenic induction medium.

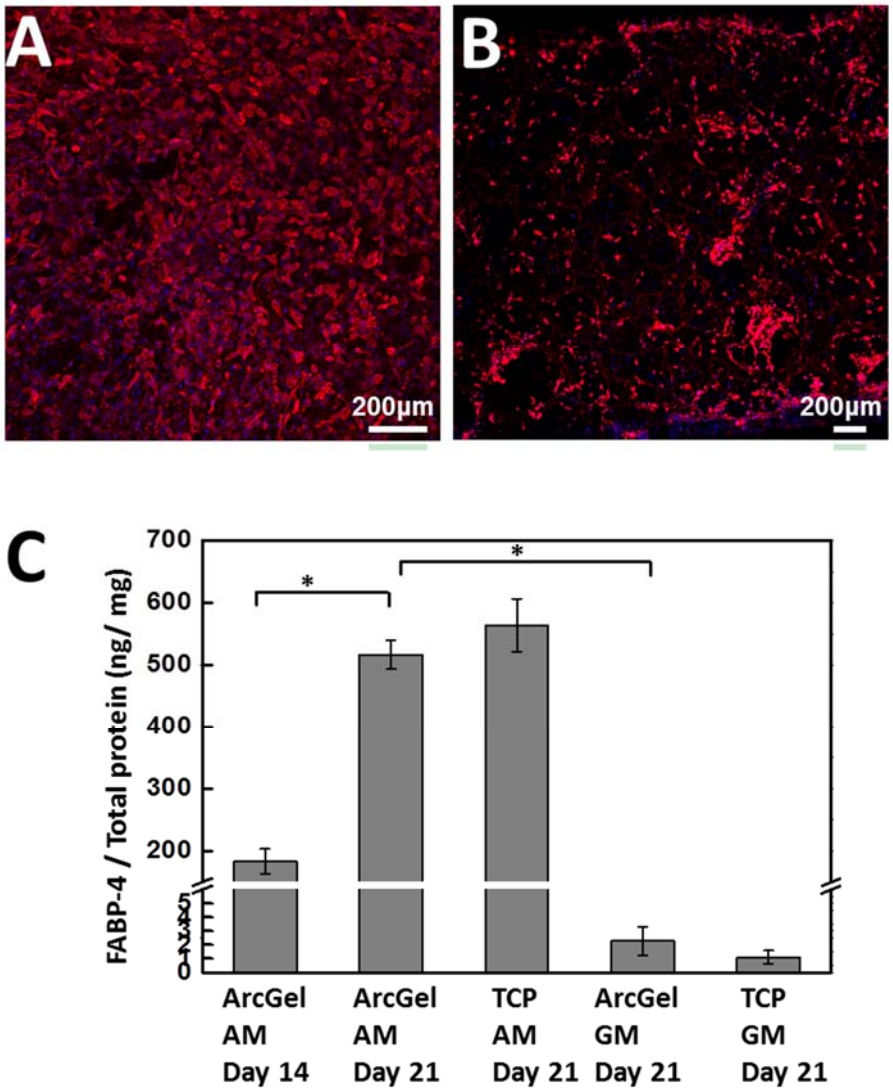

Fig.4 FABP-4 expression of adipogenically differentiated hADSCs. Top-view (A) and longitudinal section-view (B) images of FABP-4 expressing cells in ArcGels. Cells were cultured in adipogenic induction medium for 21 days followed by immunostaining (red: FABP-4; blue: nuclei) (C) The quantification of FABP-4 expression of cells cultured in ArcGel, or on TCP (AM: adipogenic induction medium; GM: growth medium; n = 3; *Sig< 0.05).

4. Discussion

In this study, we show that ArcGel could serve as a promising scaffold for adipose tissue regeneration, attributing to its high cell compatibility for hADSC cultivation and adipogenic differentiation. The scaffold was produced using gelatin, which contains Arg-Gly-Asp (RGD) sequences that promote cell adhesion and proliferation [29,30]. Gelatin is soluble in aqueous solutions at ≥ 35-40 °C, but can be stabilized as earlier shown through crosslinking by

glutaraldehyde [31], or genipin [32], or by promoting the reaction of gelatin functional groups using agents such as 1-ethyl-3-(3'-dimethylaminopropyl) carbodiimide hydrochloride (EDC) [33]. However, some of these crosslinking agents are highly toxic, which might lead to cell death caused by crosslinker residuals. Here, L-lysine diisocyanate ethyl ester (LDI) was employed as starting material that reacts with amino groups in gelatin and with water under decarboxylation, giving rise to the formation of direct crosslinks and of oligourea crosslinks and grafts, all contributing to the stabilization of the system [34]. IR spectra of the final, washed ArcGels did not display bands indicative for unreacted isocyanate groups. The hADSCs could attach on the ArcGel and exhibit the typical MSC morphology. The cells could proliferate on the scaffold without alteration of their adipogenic differentiation potential. These results indicated high cell compatibility of ArcGel for hADSC culture and differentiation. Apoptosis is the programmed cell death, by which the cell undergoes intentional suicide [35]. Apoptosis can be initiated through different pathways. In the intrinsic pathway, the cells die because they sense cell stress, while in the extrinsic pathway the cells undergo apoptosis upon receiving signals from other cells [36]. Here, we investigated the apoptosis of hADSCs seeded directly in the ArcGel and in the ArcGel leached medium (culturing cells in TCP with the presence of ArcGel in medium). The apoptotic rate of hADSCs in the ArcGel and in the ArcGel leached medium were similar to that of cells cultured on tissue culture plate, suggesting that no strong cellular apoptosis was induced by either the ArcGel or the small molecules resulting from ArcGel degradation.

In addition to the chemical properties, physical properties of the scaffold material could affect stem cell behavior and fate. For example, the materials stiffness could specifically direct stem cell differentiation lineage [37,38], while the porosity, pore size and pore interconnection have been shown to directly affect cell migration, proliferation, differentiation, microvascular formation, oxygen and nutrients supply, as well as the exchange of metabolites [39-43]. Therefore, these parameters are important in the criteria of scaffold design. The elastic modulus of the ArcGel determined by rheology was 3.1 ± 0.2 kPa, which was relatively compatible with the human subcutaneous adipose tissue with an initial modulus of 1.6 ± 0.8 kPa measured by tensile testing [44]. Therefore, the ArcGel with such an elasticity at the macroscopic level might facilitate the differentiation of ADSCs towards adipogenic lineage. The parameter of local elastic modulus at the microscopic level is also critical for stem cell differentiation, as demonstrated in our previous work that the stiffer ArcGel with local elastic modulus of 1250 ± 140 kPa could promote *in vitro* osteogenic differentiation of MSCs and *in vivo* bone regeneration [26]. The ArcGel used in the present study had a local elastic modulus of 55 ± 30

kPa as measured by AFM indentation test [45], which was much lower than that of the osteogenesis-promoting ArcGel. Accordingly, a higher level of adipogenic differentiation of hADSCs was observed in the ArcGel of this study. However, it should be noted that the ArcGel used here is still stiffer than adipose tissue [46], suggesting the potential flexibility to regulate hADSC differentiation by tuning the ArcGel mechanical properties. The high porosity with appropriate pore size of ArcGel could provide enough space for hADSC accommodation, proliferation and differentiation. The pore-pore interconnection could allow for cell migration. Accordingly, the hADSCs seeded on scaffold surface migrated into the inner part of the scaffold and distributed in the whole scaffold after 2 weeks of culture. This result is of great importance for applying ArcGel in adipose tissue reconstruction. The ArcGel would not only facilitate the *in vitro* preparation of homogeneously stem cell-loaded 3D scaffold, but also allow for the migration of autologous cells into the scaffold after implantation, which might accelerate the process of soft tissue reconstruction. Notably, as revealed by our previous work [27], the pores merged during ArcGel degradation, resulting in the formation of new, larger and round pores but retaining the integrity of the 3D porous structure. These results suggested that at subsequent phase after implantation the ArcGel scaffold might be able to offer more space for cell proliferation and guide adipose tissue development.

hADSCs isolated from human aspirated fat tissue exhibit strong proliferation and adipogenic differentiation potential *in vitro*. The scaffold materials loaded with ADSCs must be able to support adipogenic differentiation of the cells to contribute to adipose tissue reparation. In this context, the adipogenic differentiation of hADSCs in ArcGels was assessed. When cells were cultured in growth medium, no adipogenic differentiation was observed, indicating that the ArcGel could maintain hADSC stemness and did not induce spontaneous differentiation. When adipogenic induction medium was applied, the hADSCs in the scaffold differentiated into adipocytes with a high efficiency (93 ± 1%). These results suggested the potential of the ArcGel to be used for adipose tissue reconstruction. According to the clinical requirement, different treatment approaches might be flexibly adopted: (i) direct implantation of the ArcGel, which might facilitate the adipogenic differentiation of autologous ADSCs, (ii) implantation of the ArcGel pre-loaded with ADSCs, which might undergo adipogenic differentiation in the body, (iii) implantation of the ArcGel pre-loaded with adipogenically induced ADSCs.

5. Conclusion

In this study, a 3D gelatin-based hydrogel (ArcGel) was evaluated for hADSC cultivation and adipogenic differentiation. The ArcGel showed high cell compatibility for hADSC adhesion,

spreading and proliferation. The cellular apoptosis level of hADSCs in ArcGel was similar to that on tissue culture plate. The high porosity of ArcGel with appropriate pore size could allow for hADSC accommodation and provide space for cell proliferation. Importantly, the pore-pore interconnection facilitated cell migration in the ArcGel. The hADSCs in the ArcGel could maintain their stemness in growth medium, and undergo adipogenic differentiation with a high efficiency in adipogenic induction medium. Conclusively, the ArcGel presented high cell compatibility for hADSC cultivation and differentiation, which could serve as a potential implant material in clinical applications for adipose tissue reparation and regeneration.

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