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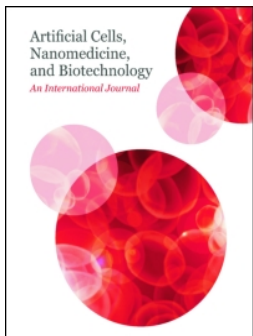
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Adipose tissue-derived ECM hydrogels and their use as 3D culture scaffold

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ABSTRACT

Adipose tissue has the therapeutic capacity in the form of a fat graft, for example, for treatment of irradiation-induced scars and difficult to heal dermal wounds. For large-scale clinical application, an off-the-shelf product is warranted. In recent years, ECM-derived hydrogels are postulated to harbour therapeutic capacity and might even replicate the beneficial effects of adipose tissue. In normal homeostasis, the natural ECM acts as a deposit of growth factors, that releases them over time. In the healing of lesions, this might promote cell accumulation and proliferation which in turn stimulates angiogenesis and repair. The decellularization of tissue and the generation of hydrogels may leave cytotoxic traces. Therefore, our research assessed the cytotoxic effect of human adipose tissue-derived ECM hydrogels on connective tissue cells i.e. fibroblasts. The results showed no cytotoxicity, meaning the hydrogels caused no cell death. Cell migration and survival were observed when cultured in ECM hydrogels and followed for 7 days. Cell survival in the hydrogel was confirmed with CFDA staining and also cells showed the ability to penetrate and migrate throughout the gel. We conclude that ECM hydrogels are promising to use as innovative therapy for wound healing.

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Extracellular matrix (ECM); hydrogel; adipose tissue; adipose tissue-derived stromal cells (ASC)

Introduction

Adipose tissue has a beneficial role in wound healing [1,2]. Yet the underlying pathways are not completely understood. This renders adipose tissue a novel, intriguing and exciting territory in science, especially in the field of wound healing.

Lipografting is used in reconstructive- and plastic surgery to restore loss of volume after breast surgery or to treat burn wounds and augment wound healing [3,4]. Short term results of lipografting are good, however, on the long-term; graft survival is variable, ranging from 20–90% loss of volume after injection [1]. In this way, the clinical efficacy of lipografting is only temporary. To improve tissue grafting, scientists implemented a technique where they combined adipose tissue-derived stromal cells (ASC) with fat tissue [5].

The adipose tissue comprises parenchyma (adipocytes) and stroma. Stroma comprises vasculature (endothelial and smooth muscle cells as well as pericytes, adipose tissue-derived stromal/stem cells (ASCs) and fibroblasts embedded and held together by the extracellular matrix (ECM). The stroma is also known as the stromal vascular fraction (SVF). In general, most studies focus on ASCs as optimal candidates for the repair of tissue damage or to facilitate angiogenesis, for example in orthopaedics and cardiology [6–9]. The secretome of ASCs stimulates angiogenesis which augments local tissue perfusion. This paracrine action of ASCs comprises secretion of pro-regenerative factors such as VEGF, FGF, and

HGF. In addition, ASCs contribute to repair by differentiation into parenchymal or stromal cells, such as smooth muscle cells, pericytes, fibroblasts, adipocytes or osteoblasts, depending on the damaged tissue [8]. We have shown that ASC-derived pericytes both promote and stabilize vascularization *in vitro* and *in vivo* in a NOTCH2-dependent fashion [10,11]. In these studies, we also cultured ASCs in 3D matrices of commercial extracellular matrix i.e. Matrigel[®].

In the last 5 years, there is a new, promising player arising in this field: the stromal vascular fraction [12–14]. Mechanical isolation of SVF is faster and easier than the isolation and culture of ASCs [15]. SVF is shown to improve wound healing rates faster compared to the injection of ASCs alone because it contains multiple cell types including vasculature, fibroblasts and ECM, which are important in the wound healing process. Moreover, ECM serves as a slow growth factor reservoir to which cells are able to attach to and can be supported by in growth and proliferation.

In the past years, ECM has been studied increasingly and different regenerative therapies, with the use of ECM, are developing in various medical fields [16]. ECM comprises the non-cellular component of the tissue, occupies the space between cells and functions as a scaffold to give structural support to cells. ECM is composed of extracellular macromolecules secreted by professional connective tissue cells such as fibroblasts. The two main classes of macromolecules in

ECM are (1) strongly negatively charged polysaccharides such as glycosaminoglycans (GAGs) and proteoglycans (PGs) that embed the (2) proteinaceous fraction of ECM such as collagens. Collagen is the most abundant protein; and comprises about 30% of the tissue mass (percentage varies within different tissue types) and there are 28 different types of collagens. PGs can either stimulate or inhibit angiogenesis in a context-dependent manner. By virtue of their charge, PGs and GAGs are very hydrophilic molecules that regulate tissue volume by water retention which makes ECM, in fact, a natural hydrogel [17]. Moreover, ECM serves as a slow release reservoir of growth factors that regulate the behaviour of proximal cells. This ranges from suppression of apoptosis by joint action ECM molecules and growth factors to proliferation, differentiation and migration. Growth factor binding to the GAGs in the ECM depends on the presence of the so-called aminoterminal heparin-binding domain but also plain electrostatic or Van der Waals interactions may keep soluble factors bound to ECM.

A manner to implement the extracellular matrix as a treatment or bioengineering tool is to produce ECM hydrogels. Various human or porcine tissues qualify to produce organ-specific ECM hydrogels including heart [18,19], lung [20], pancreas [21], skin [22], and adipose tissue [23]. We surmise that these ECM hydrogels may act to retain growth factors with the benefit that these are released with distinct kinetics over time. The released growth factors are able to induce angiogenesis by stimulation of endothelial cells to form new blood vessels which, in turn, stimulates tissue regeneration resulting in wound healing [24]. Wound healing is especially impaired in diabetic patients because of decreased peripheral blood flow leading to diabetic ulcers formation. Diabetes affects ASCs and SVF, therefore, the clinical use of an ECM-derived hydrogel from healthy donors is more suitable for patients suffering from diabetes mellitus.

ECM hydrogels may also be applicable for 3D culturing, 3D bioprinting and in vivo tissue regeneration [25]. These can be in a donor-independent fashion and over a species barrier while SVF which can be used only as an allogeneic transplant otherwise immunological rejection would occur. More preliminary studies are needed before ECM hydrogel can enter clinical trials. The decellularization of adipose tissue precedes the generation of ECM hydrogels, the latter is done by mild digestion under low pH with pepsin. In particular, decellularization involves multiple steps with harsh chemicals such as detergents that are incompatible with the survival of cells. Therefore, the aim of this research was to assess the influence of human adipose tissue-derived ECM hydrogels on viability, proliferation and migration of therapeutic cells i.e. ASCs and fibroblasts.

Materials and methods

Fractionation of adipose tissue procedure

The fractionation of adipose tissue (FAT) procedure was performed as previously described [15]. Briefly, adipose tissue was harvested during normal liposuction procedures and transferred cooled from the operation room. Informed

consent was obtained according to the local ethical committee of the University Medical Center of Groningen. For the FAT procedure, liposyrates were warmed to room temperature (RT) and divided into 50 ml tubes. Then, liposyrate was centrifuged at $956 \times g$ for 3 min. at RT to separate adipose tissue into three layers: oil, adipose tissue and infiltration fluid. The oily fractions, as well as the infiltration fluid, were discarded. Next, the centrifuged adipose tissue was placed in a 10 ml syringe and connected to the fractionator (a luer to luer connector with three holes of 1.4 mm inside). An empty 10 ml syringe was connected on the other side of the fractionator. Adipose tissue was pushed 30 times forwards and back and the adipocytes were mechanically disrupted. Finally, adipose tissue was centrifuged again at $956 \times g$ at RT for 3 min. The second round of centrifugation yielded four fractions: oily fraction, SVF and infiltration fluid containing a small pellet. SVF was collected and washed with phosphate-buffered saline (PBS) and stored at -20°C until further use (Figure 1).

Decellularization of stromal vascular fraction

SVF was frozen with 50% ethanol/water in a -80°C fridge for 2 h and thawed for 30–60 min. for four cycles [26]. After thawing, the 50% ethanol/water mixture was replaced every time. Next, the SVF was incubated with 0.05% trypsin/0.05 mM ethylenediaminetetraacetic acid (EDTA) (1:1 v/v) under constant stirring (Bambino machine) at 37°C for 90 min. Then, samples were washed with phosphate buffered saline (PBS) and sonicated (70W) with 0.5% sodium dodecyl sulphate (SDS) at 46°C for 20 min. Samples were centrifuged and washed with PBS to completely remove SDS. Finally, samples were lyophilized and subsequently immersed in xylene and placed on a rolling bench for 17 min. Afterwards, samples were washed with PBS and subsequently washed with 100% ethanol until the solution became clear. Samples were incubated with DNase solution (1:1 v/v) (LS002007, Worthington, final concentration of $30 \mu\text{g/ml}$ DNase in 1.3 mM MgSO_4 and 2 mM CaCl_2) overnight at 37°C . Next day, samples were washed with PBS and again lyophilized. Finally, samples were ground to a fine powder with an UltraTurrax device (PM Tamson Instruments) and stored at -80°C until further use (Figure 1).

Gelation of decellularized adipose derived extracellular matrix

ECM (20 mg) was mixed with porcine pepsin powder (2 mg, 3,200 I.U. Sigma-Aldrich) in 1 ml 0.01 M hydrochloric acid (HCl). ECM was digested under constant stirring on a magnetic stirring device at 500 rpm at RT for 6 h. Afterwards, pH was raised to 7.4 to neutralize the pepsin with $100 \mu\text{l}$ of 0.1 M sodium hydroxide (NaOH) to reach a final concentration of 0.01 M NaOH. The pre-gel solution was buffered with $110 \mu\text{l}$ of 10X PBS to reach a final concentration of 1X PBS. Then, the pre-gel solution was mixed well and incubated for one hour at 37°C to allow for gelation (Figure 1).

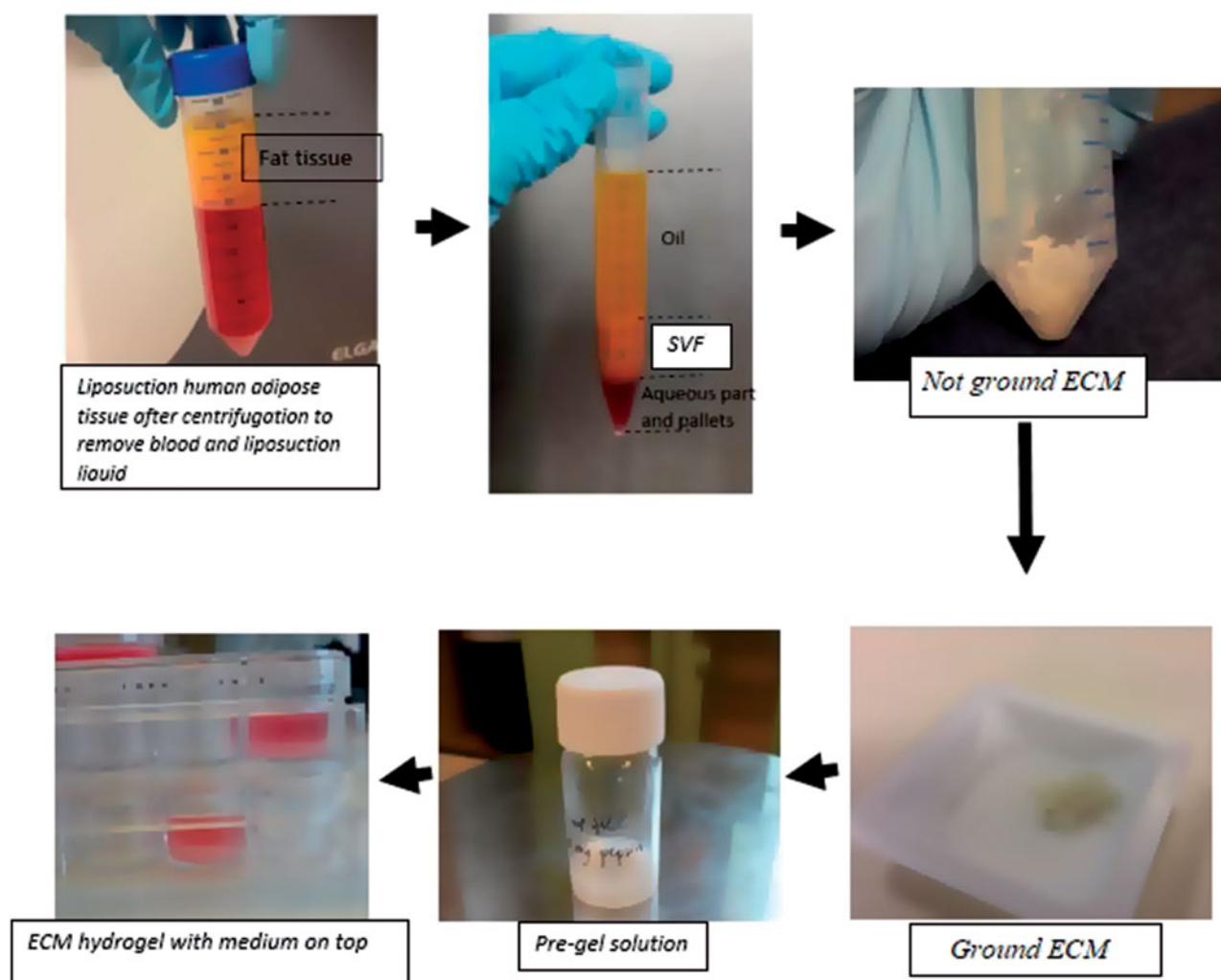


Figure 1. Overview of preparation method of an ECM hydrogel from human adipose tissue. SVF: stromal vascular fraction; ECM: extracellular matrix.

Immortalization and lentiviral tagging of ASCs

Cultured human ASCs (pool of five donors, 1 million at passage 4) were transfected with 1 μ g pMC1neo-polyA (Stratagene) which is a plasmid encoding the large T antigen of SV40 with neomycin as selectable marker [27,28]. At 48 h post-transfection, transfected cells were selected by adding 250 μ g/ml geneticin (G418) to the medium. After \sim 3 weeks, colonies that remained were picked, subcultured and propagated in medium with 250 μ g/ml G418 for a second round of selection. Stable cell lines were propagated and stored in liquid nitrogen. For the current study, clone iADSC13 was used and characterized. Tagging with CMV promoter-driven reporter genes respectively EGFP (green fluorescence) or dTomato (red fluorescence) was with third generation VSV-pseudotyped replication-deficient lentiviruses. Up to three rounds of transduction were done to increase the fraction of reporter-expressing ASCs. These were named iADSC13EGFP and iADSC13dTomato respectively. Additionally, transfected cells were FACS-sorted, propagated and cryopreserved in liquid nitrogen. Between 80 and 95% of the sorted ASCs showed reporter expression that was detectable with a fluorescence inversion microscope, which sufficed for the experiments.

Characterization and differentiation potential of immortalized ASC

Flow cytometry

Immortalized ASC, lentivirally tagged with either EGFP or dTomato, were analyzed using flow cytometry (FACS) for CD surface marker expression. Cells were stained with the following anti-human monoclonal antibodies: CD31- phycoerythrin/cyanine7 (Pe/Cy7; eBioscience, Vienna, Austria), CD45-fluorescein isothiocyanate (FITC; IQ Products) and CD90-allophycocyanin (APC; BD Bioscience, San Jose, CA). CD29-APC (eBioscience), CD44-FITC (BD Bioscience) and CD105-Pe/Cy7 (eBioscience). For controls, we used the following monoclonal antibodies: Mouse IgG1 kappa-Pe/Cy7, Mouse IgG1 kappa-APC (both eBioscience), IgG2b FITC (BD Bioscience) and IgG1 FITC (IQ products). Cells were mixed with the antibodies and incubated for 30 min.

Adipogenic, osteogenic and smooth muscle cell differentiation assay

Basal medium DMEM (BioWhittaker Walkersville, MD) containing 10% foetal bovine serum (FBS), 1% penicillin/streptomycin and 1% glutamine was used. Immortalized ASC,

tagged with EGFP or dTomato, were cultured to confluency and medium was changed to promote differentiation. For adipogenic differentiation this was basal DMEM plus 0.1 μM dexamethasone, 1 nM insulin, 0.5 mM isobutylmethylxanthine. For osteogenic differentiation this was basal DMEM plus 0.1 μM dexamethasone, 10 mM β -glycerophosphate and 0.05 mM ascorbic acid. Finally, smooth muscle cell differentiation was in basal DMEM plus with 10 ng/ml TGF- β 1. After 14 days culture in the differentiation medium, cells were fixed with 2% PFA and stained for Oil Red O (Sigma-Aldrich, St. Louis, MO) for adipogenic differentiation, Alizarin Red (Sigma Aldrich) for osteogenic differentiation and Phalloidin-FITC (Invitrogen, Thermo Fisher Scientific, Renfrewshire, Scotland) in DAPI for 30 min for smooth muscle cell differentiation.

Colony formation assay

Tagged ASCs were seeded at, respectively, hundred and thousand cells per well of a six-well culture plate in duplicate and were cultured for 14 days. Cells were washed with PBS and fixed with 2%PFA in PBS for 15 min. Cells were washed well with PBS and stained with Crystal Violet (Sigma-Aldrich). Plates were scanned and the ability to form colonies was assessed by determining the area of the colonies.

Histological characterization of acellular matrix by haematoxylin and eosin

Cryo-sections of 12 μm were made from snap-frozen adipose tissue, SVF and ECM derived from the same donor ($n = 17$). Samples were stained with haematoxylin solution for 5 min. After staining, samples were washed with tap water for 5 min. Afterwards, samples were stained with eosin solution for 10 min. and subsequently washed with tap water for 5 min. Finally, samples were mounted with Aquatex and visualized under light microscope (Leica Microsystems, DM IL).

MTT conversion assay for cell viability

Hydrogels ($n = 3$) in triplicate were prepared in a 24 well plate with a volume of 0.5 ml. One millilitre of culture medium (basal DMEM) was placed on top of each hydrogel and collected every 24 h for 4 days. Conditioned medium from each hydrogel for each time point was used in triplicate. A serial twofold dilution series of the hydrogel-derived medium was used.

Human dermal fibroblasts (PK84) were cultured in 96 well plates in culture medium (as previously described) until confluency. Culture medium was replaced with the dilution series of hydrogel-derived medium for 48 h. As a positive control for cytotoxicity, a twofold serial dilution series of puromycin (Gibco, 10 mg/ml) in the medium was used, starting at 10 μl per well. Normal culture medium served as a negative control

After 48 h, 5 mg/ml MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) in PBS was added to each well. Plates were incubated at 37 °C for 3 h. Afterwards, the

medium was removed and the purple formazan crystal dissolved in 200 μl of dimethyl sulfoxide (DMSO) by careful mixing. Optical density was measured at 650 nm and 585 nm. The difference in optical density between 650 nm and 585 nm was plotted against the log dilution to generate cytotoxicity graphs. Results were analyzed with GraphPad Prism 7 (CA, USA) using a nonlinear regression for a dose-response inhibition. The half maximal inhibitory concentration (IC_{50}) was used as an indicator of cytotoxicity. The IC_{50} value indicates how much of the substance is needed to induce 50% of cell death.

Live/dead staining of ASCs on hydrogels

Hydrogels ($n = 3$) in triplicate were prepared in a 24 well plate with a volume of 0.5 ml Hydrogels were incubated with 1 ml of basal medium (as previously described in 2.5) for 8 days and replaced every day. A suspension of 300 μl of culture medium containing 200,000 immortalized ASCs was added on top of each hydrogel. Immortalization was performed with the large T antigen of SV40 and lentivirally tagged (Harmsen lab, described in 2.4) with enhanced GFP (green, iASC13EGFP) or with dTomato (red, iADSC13dTomato). Cells were evaluated for 7 days. Culture medium was changed every day and micrographs were taken every day. After 7 days, a live/dead staining was performed with CFDA-SE (1:2000), PI (1:500) and DAPI (1:5000) for iASC13GFP. For iADSC13dTomato only CFDA-SE and DAPI were used, PI staining was not used as it has the same wavelength as already pre-stained cells, therefore they are both the same colour and it cannot be differentiated. As a control, 1300 μl of culture medium containing 200,000 iASC13EGFP in a 24 well plate was used. Samples were visualized with an immunofluorescence microscope (EVOS[®] FL Cell Imaging System). After evaluation, samples were fixed with 2% paraformaldehyde in PBS for 30 min. Finally, samples were embedded in paraffin for immunohistology staining.

Immunohistology staining of ASCs inside hydrogels

From the above-mentioned paraffin embedded hydrogels (2.6), 4 μm sections on adhesive slides were made with Leica Reichert-Jung 2055 microtome. Samples were deparaffinized and incubated overnight with 0.1 M Tris/HCl solution (pH 9.0) at 80 °C. Next day, samples were stained with haematoxylin for 4 min. and mounted with Aquatex. Results were evaluated with a light microscope (Leica Microsystems, DM IL).

Results

Characterization of immortalized ASC

Immortalized ASC retain ASC characteristics

A mean of 98.9, 99.0, 95.9 and 23% of the immortalized ASCs showed expression of CD29, CD44, CD90 and CD105. Endothelial marker CD31 and common leukocyte marker CD45 was not detected. These results show that the

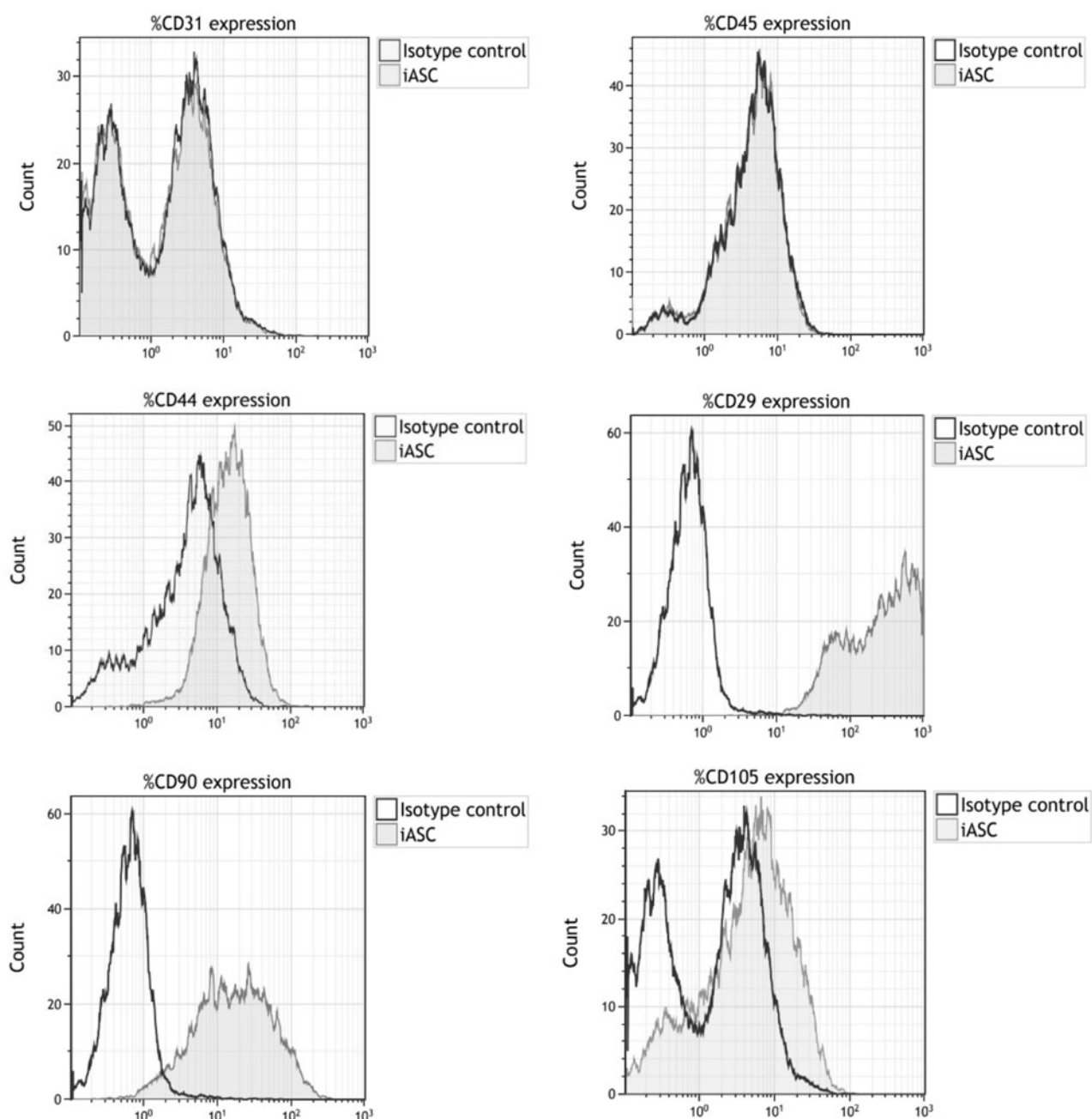


Figure 2. Representative data of CD-surface marker expression in immortalized ASCs.

lentivirally tagged immortalized ASC have a similar surface marker expression as ASC (Figure 2) [15].

Immortalized ASC retain cell differentiation capacity to adipocytes, osteoblasts and smooth muscle cells

Immortalized ASC successfully differentiated to adipocytes, osteoblasts and smooth muscle cells. This is coherent with the differentiation ability of normal, human isolated ASC (Figure 3) [15].

Immortalized ASC retain colony formation capacity

The number of colonies in each well consisting of more than 50 cells varied between 3–7%. This is coherent with results

reported from colony experiments with normal, human ASC, where the number of colonies should be between 0.1–5% [15].

Extracellular matrix derived hydrogels are non-cytotoxic

The serial sampling at 24 h intervals of possible cytotoxic elutes from hydrogels showed no difference in MTT conversion compared to medium controls. Therefore, no IC_{50} could be calculated for all time points of each hydrogel indicating that the hydrogels did not release any cytotoxic compounds over time (Figure 4). Results showed that positive control (puromycin) induced a strong cytotoxic response ($LogIC_{50}$ 3.1–3.6 mM).

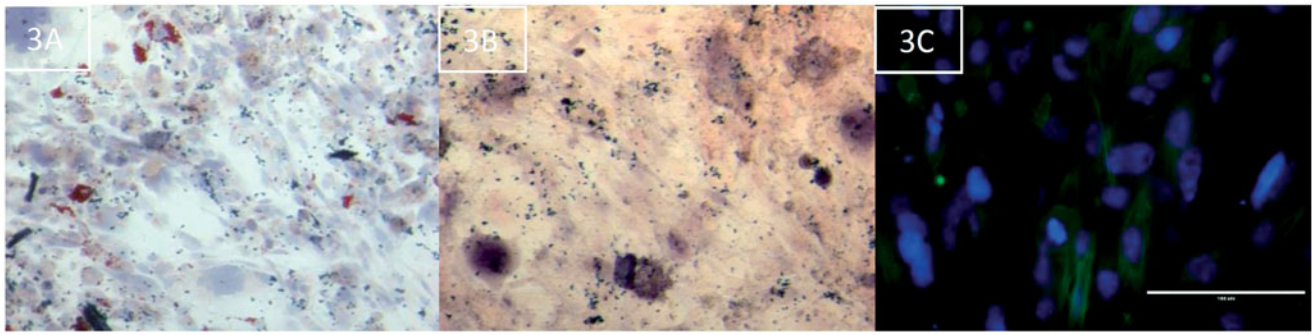


Figure 3. Immortalized ASC retain cell differentiation capacity to adipocytes, osteoblast, smooth muscle cells. (A) Immortalized ASC differentiated to adipocytes. (B) Differentiated to osteoblasts. (C) Differentiated to smooth muscle cells.

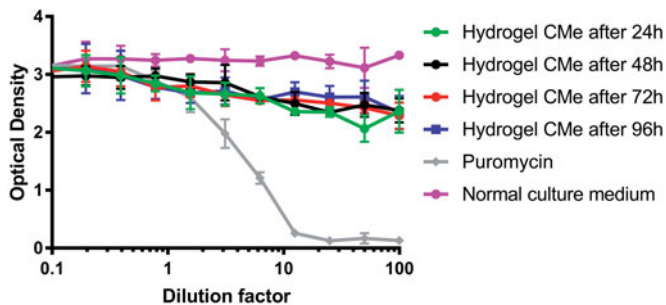


Figure 4. Optical density plotted against the serially diluted CMe from human adipose tissue-derived hydrogels after four different time points (log scale). PK84 fibroblasts were treated with hydrogel CMe or controls for 48 h. Puromycin was used as positive control. Normal culture medium was used as negative control. Results are presented as mean with standard error of the mean of triplicates of three independent donors. CMe: conditioned medium.

No dead cells present in extracellular matrix derived hydrogels

Live/dead staining showed no dead cells (PI-stained nuclei) in hydrogels nor control (Figure 5) ASCs appeared to adhere to the hydrogels and acquire the typical spindle-shape of mesenchymal cells; similar to the controls seeded on flat tissue culture plastic (Figure 5(B,D,F)). It appeared that ASCs were more stretched when cultured in hydrogels than on tissue culture plastic (Figure 5). Cell density did not appear to differ between ASC seeded on hydrogels as compared to tissue culture plastic controls, although quantification was not possible. The visualization even of fluorescently labelled ASCs (Figure 5(A,C,E)) on and in hydrogels is challenging due to the limited depth of field with the microscope.

Histological confirmation of ASCs present inside the hydrogels

Haematoxylin-stained thin section of paraffin-embedded hydrogels confirmed the presence of ASCs inside the hydrogels (Figure 6A) and at the periphery below the gel surface (Figure 6(B)).

Discussion

The results of this research show that human adipose tissue-derived ECM hydrogel is non-cytotoxic and support adhesion, survival as well as migration and proliferation of immortalized

ASCs. These are promising results that warrant further implementation e.g. as a delivery vehicle of stem cells, in animal and later also clinical trials. Cells cultured in the 3D hydrogel environment showed different morphology with respect to extending and elongating structures compared to cell morphology in plain cell culture plastic 2D environment culture. We also showed that immortalized ASC show the same characteristics as normal ASC (CD markers, colony forming ability and differentiation ability).

ECM derived hydrogels can have various implementations. ECM hydrogels are currently being used as a 3D culturing model and as bioengineering printing bio-ink. As shown by this study, culturing of cells differs in a 2D environment as compared to a 3D environment, especially in morphology. A study by Sung et al. showed that fibroblast in 3D culture released more signalling molecules which resembles more precisely their behaviour in the human organism [29]. Therefore, a 3D ECM hydrogel culture system is a better representative of how processes in the human body function and, therefore, an ideal culture system to study the effect of treatments on different cellular processes. The ECM hydrogels will likely impact bioengineering, as bio-ink for printing organoids together with vascular and connective tissue cells.

Currently, ECM hydrogels from different origins have been tested in a variety of medical fields [18–22]. A study by Ghuman et al. induced a stroke in rats and subsequently injected an ECM hydrogel derived from adult porcine urinary bladder tissue into the necrotic stroke area [30]. This resulted in increased tissue regeneration in the brain after injection of an ECM hydrogel. The injected ECM hydrogel caused more cell infiltration as compared to the control group. Although different origins of the extracellular matrix are available, the use of human adipose tissue-derived ECM provides several advantages as compared to decellularized tissue from other origins. First, human adipose tissue is more easily accessible without adverse side-effects compared to other organs of the human body such as skin. Second, human adipose tissue is widely available since patients frequently donate liposuction adipose tissue. Third, the obtained adipose tissue-derived ECM can be used as both autologous as well as an allogeneic treatment modality, while autologous transplantation is not possible for most of the other decellularized organs of the

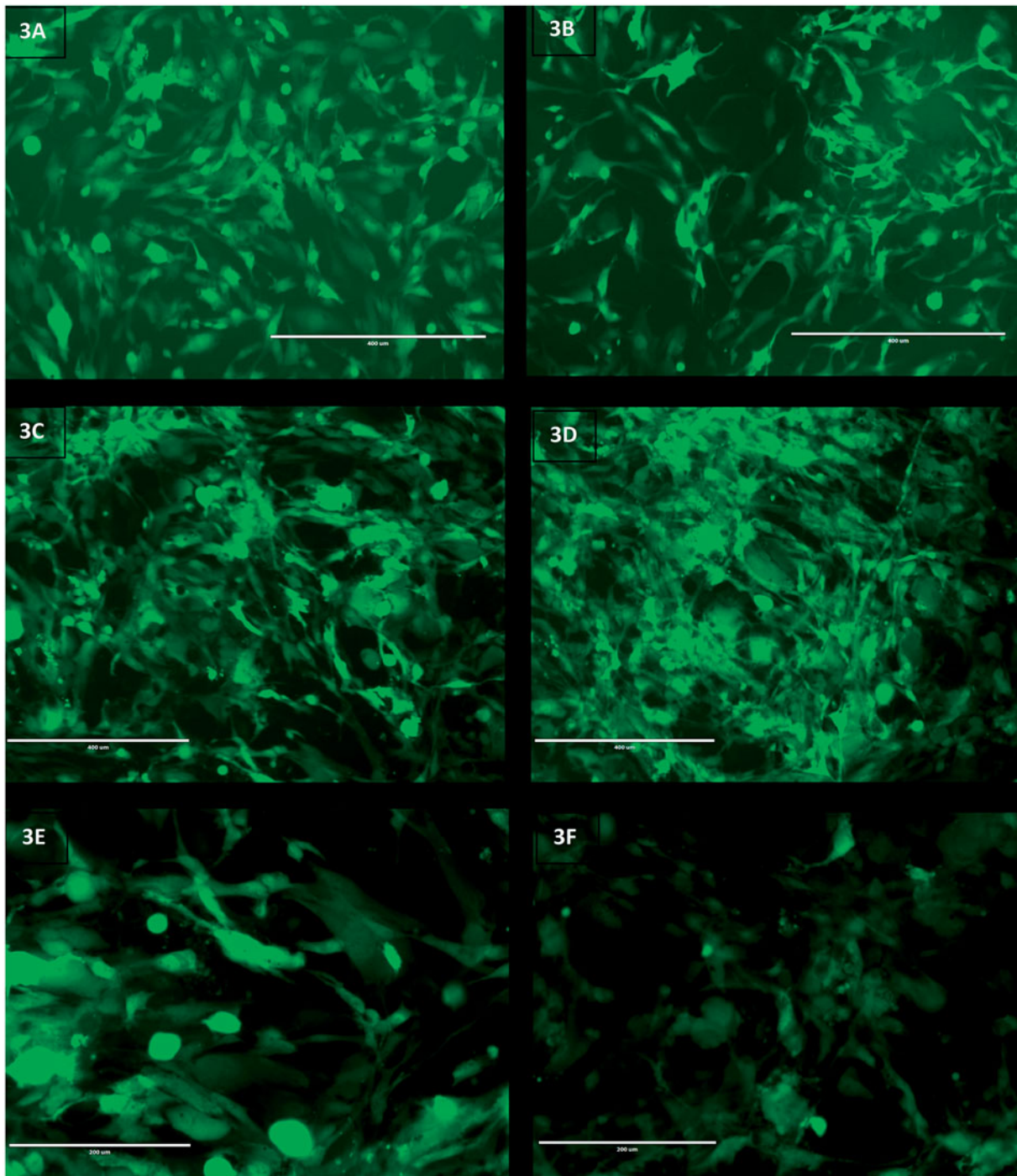


Figure 5. (A,C,E) Representative fluoromicrographs of iASC13EGFP cultured on top of human adipose tissue-derived ECM hydrogels after respectively 1, 6 and 6 days. (B,D,F) Representative fluoromicrographs of iASC13EGFP cultured on tissue culture plastic (control) for respectively 1, 6 and 6 days. Scale bar: A–D: 400 μm , E–F: 200 μm . iASC13EGFP: immortalized EGFP-tagged adipose derived stromal cells; ECM: extracellular matrix.

human body (e.g. heart, lung). Allogeneic transplantation is possible due to the lack of a cellular component causing an immune response after transplantation.

This study served to set up a platform technology and focused on the potential adverse influence of ECM hydrogels on two stromal cell types ASC and fibroblasts. Current research comprises of co-culture systems in ECM hydrogels of different organs and investigating processes relevant to tissue regeneration such as vascularisation. Thus, co-cultures of two or more different cell types are

under investigation. Besides, physical features of hydrogels such as matrix stiffness and viscoelasticity are under investigation now.

In conclusion, the results of this research showed that human adipose tissue-derived ECM hydrogels are not cytotoxic and support adhesion and (in)growth of human immortalized ASCs. These results are promising and warrant further research about the implementation of adipose tissue-derived hydrogels as e.g. a delivery vehicle of cell types or bioengineering printing bio-ink.

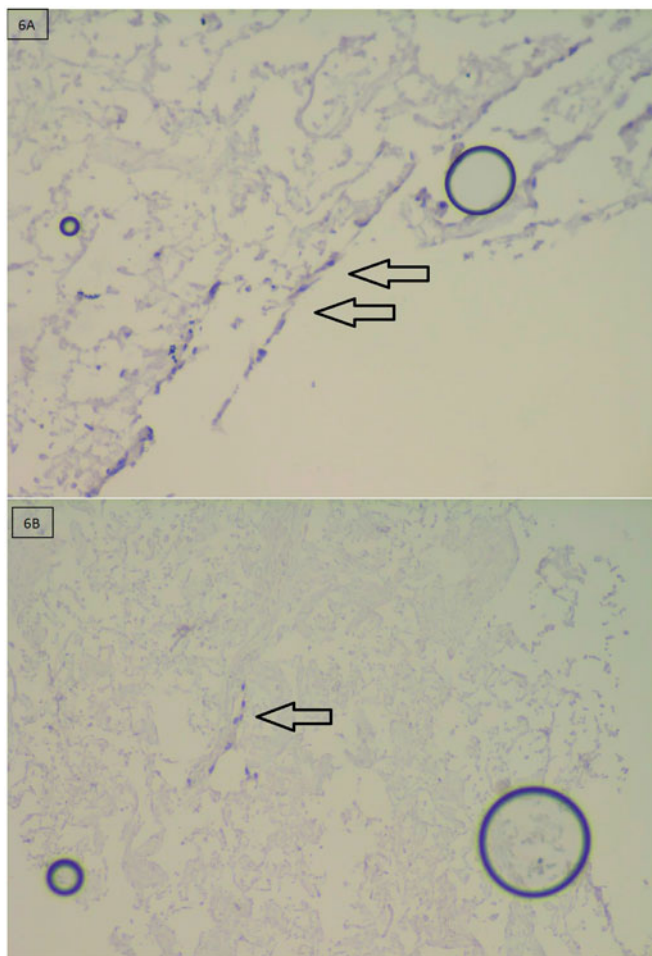


Figure 6. Hematoxylin-stained sections of iASC13EGFP cultured on top of human adipose tissue-derived ECM hydrogels after 6 days. Note that cells (visible by their dark blue nuclei) have migrated into the gel (arrows) at the periphery (A) and to the centre (B). iASC13EGFP: immortalized EGFP-tagged adipose derived stromal cells; ECM: extracellular matrix.

Disclosure statement

No potential conflict of interest was reported by the authors.

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