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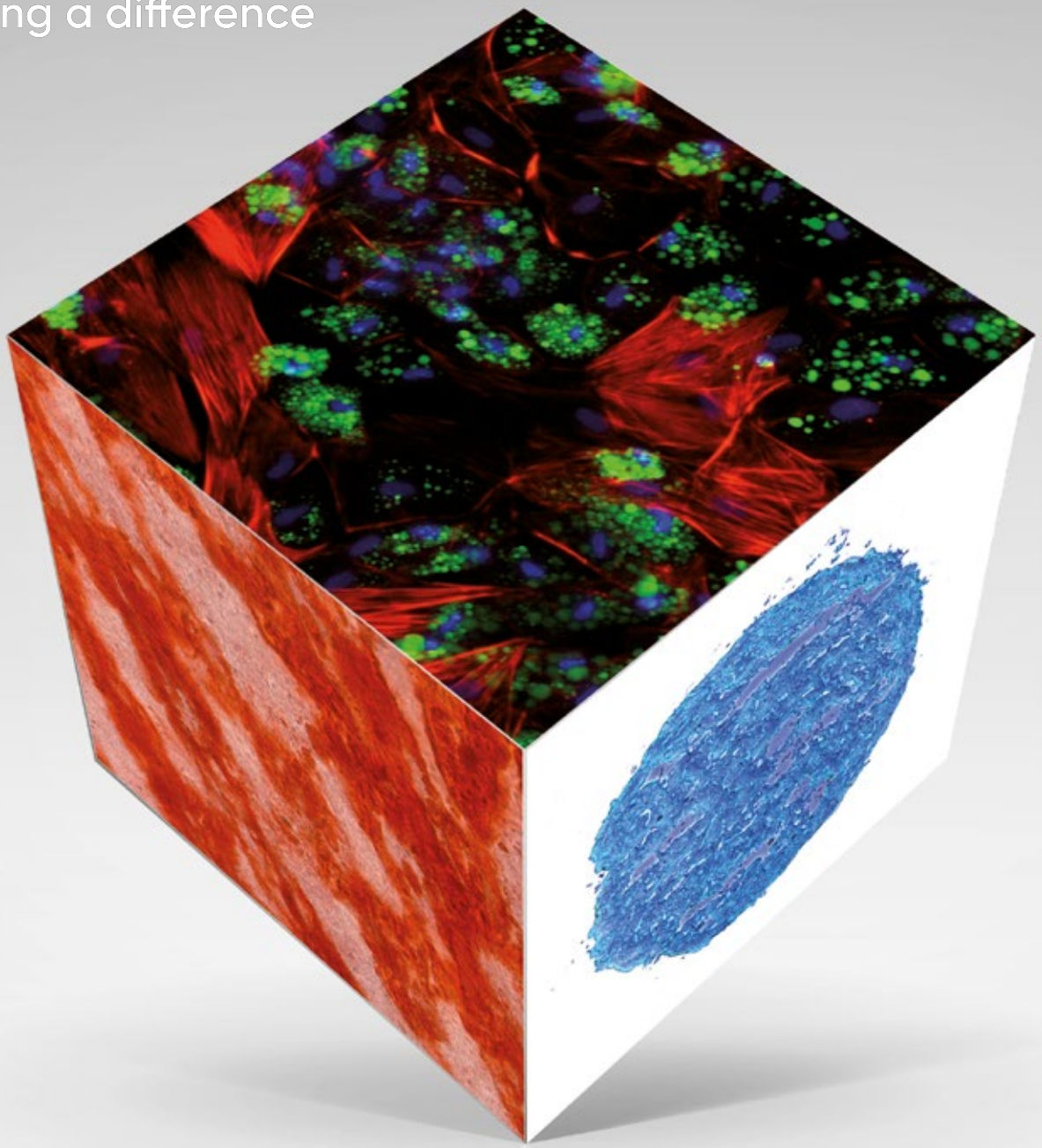

APPLICATION NOTE

Cultivation and Differentiation of Human Adipose Derived Mesenchymal Stem Cells with CELLSTAR® and CELLCOAT® Cell Culture Products

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1/ INTRODUCTION

With their ability to differentiate into many different cell types, mesenchymal stem cells (MSCs) hold great promise for the treatment of diseases and the replacement of injured or lost tissues and organs in humans. In this role MSCs compete with embryonic stem cells (ESCs) and the induced pluripotent stem cells (iPCs). Nevertheless, the postulated existence of adult MSC niches; hence the availability of autologous MSC sources, qualifies these cells as outstanding candidates for future stem cell therapy (for review see NIRMALANANDHAN AND SITTAMPALAM, 2009).

Since the discovery of MSCs as colony forming fibroblasts by Friedenstein (FRIEDENSTEIN ET AL., 1970) and the first detailed description of their differentiation potential (PITTENGER ET AL., 1999) a lot of insight into their niche biology as well as signaling pathways of self-renewal versus differentiation has been gained. Today, MSCs remain a topic of intense research with the minimum molecular criteria defining an MSC being still investigated and debated. Thus it may not surprise that non-molecular criteria, namely the ability to adhere to plastic surfaces, and the capability to differentiate into bone, cartilage and fat cells are still accepted for the definition of the MSC phenotype. Beside that, more and more molecular markers are established, whose absence (CD11b, glycophorin-A, CD56) or presence (Stro-1) is characteristic for MSCs (for review see KOLF ET AL., 2007).

Plastic-adherent, multipotent cells can be isolated from many adult tissue types such as bone marrow, muscle, bone, tendon, and fat tissue; and even from tissues of non-mesodermal origin (DA SILVA MEIRELLES, 2006). Among these sources fat tissue is particularly easy to access and readily available, which makes adipose tissue derived stem cells (ADSCs) very attractive for research and regenerative medicine (box 1). ADSCs are highly proliferating *in vitro* and share their multilineage differen-

Human adipose derived mesenchymal stem cells (ADSCs) may be isolated from sources as easily available as human fat tissue collected during liposuction procedures. ADSCs have been shown to differentiate into mesenchymal cell types, including adipocytes, osteoblasts, and chondrocytes (FRASER ET AL., 2006A) as well as non-mesenchymal cell types, such as neuronal and glial progenitors, liver cells and endothelial cells (FRASER ET AL., 2006B). Recently, several suppliers of primary cells have included cryopreserved ADSCs into their portfolio, such as Invitrogen™ Life Technologies with its StemPro® human ADSCs. Taking advantage of such commerical cell sources leaves the isolation and pre-characterisation of the cells in the supplier's responsibility for maximum convenience of the scientist experimenting with these cells. Invitrogen's StemPro® human ADSCs have well characterised cell surface protein profiles and are guaranteed to be at least bi-potent. The two different isolations of StemPro® human ADSCs used in this study could both be differentiated along the chondrogenic, osteogenic, and adiopogenic lineage.

tiation potential with MSCs of other origin (STREM ET AL., 2005).
Here, ADSCs were cultivated under condition maintaining their stemness or inducing their differentiation along the chondrogenic, osteogenic, or adipogenic lineage. Such *in vitro* differentiation of MSCs may not only be conducted by well-defined growth factor cocktails present in the cultivation medium, but also by other parameters such as the type of cell culture substrate (OH ET AL., 2008).

The aim of this study was to provide protocols for the cultivation and directed differentiation of ADSCs on CELLSTAR® (tissue culture treated) and CELLCOAT® (Collagen Type I) cell culture substrates with the cell type, the cultivation media and substrates being well-matched. TC treated CELLSTAR® plates were found excellently suited for the cultivation of ADSCs under conditions maintaining their pluripotency over at least 5 passages, whereas chondrogenic, osteogenic and adipogenic differentiation could be induced using growth factor enriched cultivation media from Invitrogen. CELLCOAT® Collagen Type I coated plates enhance the establishment of osteogenic characteristics such as calcium deposition in the extracellular matrix and expression of alkaline phosphatase.

2/ MATERIALS

Description	Supplier	Item No.
Alcian blue 8GX	Sigma-Aldrich	A3157
Alizarin red S	Sigma-Aldrich	A5533
Alkaline buffer solution	Sigma-Aldrich	A9226-100ML
Bradford reagent	Sigma-Aldrich	B6916
CELLCOAT® 96 well plate, Collagen Type I, µClear®	Greiner Bio-One	655 956
CELLSTAR® 96 well plate, TC treated	Greiner Bio-One	655 180
CELLSTAR® TC flask, TC treated T175	Greiner Bio-One	660 175
CELLSTAR® TC flask, TC treated T25	Greiner Bio-One	690 175
CELLSTAR® TC flask, TC treated T75	Greiner Bio-One	658 175
Complete Lysis M	Roche Diagnostics	4719956001
Cryo.s cryogenic tubes, 2 ml, internal thread	Greiner Bio-One	126 263
DAPI	Sigma-Aldrich	D8417
Fetal calf serum	Invitrogen Life Technologies	10270-106
Formalin	Sigma-Aldrich	HT5014
Gentamicin	Biochrom	A2717
Goat serum	Sigma-Aldrich	G9023
Hydrochloric acid	Sigma-Aldrich	84420
Histoprime® AP kit	Linaris	ÊSA5100
Isopropanol	Carl Roth	9866.3
LipidTOX™ Green neutral lipid staining	Invitrogen Life Technologies	H34475

Description	Supplier	Item No.
L-lanyl-L-glutamine	Biochrom	K0302
MesenPRO RS™ basal medium	Invitrogen Life Technologies	12747-010
MesenPRO RS™ growth supplement	Invitrogen Life Technologies	12748-018
NaOH	Carl Roth	6771.3
Oil Red O	Sigma-Aldrich	O0625
PBS	Biochrom	L1825
Phalloidin-TRITC	Sigma-Aldrich	P1951
4-Nitrophenol	Sigma-Aldrich	N7660
4-Nitrophenyl phosphate disodium salt hexahydrate	Sigma-Aldrich	P5994
StemPro® adipocyte basal medium	Invitrogen Life Technologies	A10410-01
StemPro® adipogenesis supplement	Invitrogen Life Technologies	A10065-01
StemPro® chondrogenesis supplement	IInvitrogen Life Technologies	A10064-01
StemPro® Human Adipose-Derived Stem Cells	Invitrogen Life Technologies	R7788-110
StemPro® osteocyte/chondrocyte basal medium	Invitrogen Life Technologies	A10069-01
StemPro® osteogenesis supplement	Sakakura Finetek	A10066-01
Tissue-Tek® O.C.T.™ compound	Invitrogen Life Technologies	4565
Triton® X-100	Sigma-Aldrich	T8787
TrypLE™ Express	Invitrogen Life Technologies	12604-013

3/ METHODS

3.1/ EXPANSION AND SUBCULTIVATION

ADSCs were expanded and cultivated on TC treated CELLSTAR® cell culture flasks in growth medium (MesenPRO RS™ basal medium with 2 % MesenPRO RS™ growth supplement, 2 % FCS and 2 mM L-alanyl-L-glutamine) to reach about 80 % confluency. For passaging, cells were washed with pre-warmed PBS (37 °C) and detached with 0.05 ml TrypLE™ Express recombinant protease per cm^2 cultivation surface (7 min, 37 °C). TrypLE™ Express was then inhibited by adding growth medium. Cells were collected by centrifugation, transferred into fresh growth medium and subcultivated with a splitting ratio of 1:3. All cell cultures were carried out in cell culture incubators at standard conditions (37 °C, 90 % humidity, 5 % CO_2). ADSCs were passaged up to 5 times without loss of their trilineage differentiation potential.

3.2/ CRYOCONSERVATION

For cryoconservation aliquots of 2 ml cell suspension containing 7 x 10^5 cells in 20 % DMSO/growth medium were frozen with a 1 °C/min temperature gradient. Long term storage was carried out in 2 ml Cryo.s cryogenic tubes over liquid nitrogen. For recovery of frozen stocks, Cryo.s were quickly thawed, their content transferred to 10 ml prewarmed growth medium, cells collected by centrifugation and transferred into fresh growth medium.

3.3/ CHONDROGENIC DIFFERENTIATION

For chondrogenic induction, a droplet of 5 µl chondrogenic medium (StemPro® osteocyte/chondrocyte basal medium with 10 % StemPro® chondrogenesis supplement and 10 mg/ml gentamicin) containing 80000 ADSCs was added to the center of each well of a tissue culture treated 96 well CELLSTAR® plate. After 2 hours of incubation at 37 °C cells had formed micromasses and 100 µl chondrogenic medium was added per well. Long-term cultivation was carried out at standard cell culture conditions with medium exchanges every 3rd to 4th day.

3.4/ OSTEOGENIC AND ADIPOGENIC DIFFERENTIATION

For osteogenic and adipogenic differentiation 3500 ADSCs in 100 µl growth medium were seeded per well of a tissue culture treated 96 well CELLSTAR® plate. Having reached 80 % confluency (after 3-4 days in culture) medium was exchanged to osteogenic medium (StemPro® osteocyte/chondrocyte basal medium with 10 % StemPro® osteogenesis supplement and 10 mg/ml gentamicin) or adipogenic medium (StemPro® adipocyte basal medium with 10 % StemPro® adipogenesis supplement & 10 mg/ml gentamicin). Long-term cultivation was carried out at standard cell culture conditions with medium exchanges every 3rd to 4th day.

3.5/ ALCIAN BLUE STAINING

Micromass cultures (spheroids) were harvested, fixed for 7 min in 4 % icecold formalin, washed with PBS, embedded in Tissue-Tek® compound and shock frozen at -80 °C. Frozen spheroids were cryosectioned to obtain 16 µm sections. Sections were mounted onto glass slides, dried, washed three times with PBS and stained with Alcian blue solution (1 % in 0.1 N HCl) for 35 min at room temperature. After washing in PBS, nuclei were counterstained with 0.01 mg DAPI/ml PBS. Alcian blue stains mucopolysaccharides and glycosaminoglycans which are typically expressed in cartilage tissue.

3.6/ ALIZARIN RED S STAINING

For detection of calcium deposition in the extracellular matrix, Alizarin red S staining was performed. For that, cell cultures were fixed for 30 min with 4 % formalin at room temperature. After two washing steps with deionised water cell cultures were stained with Alizarin red S solution (1.5 % in citrate buffer, pH 4.2) for 30 min at room temperature followed by another four washes with deionised water.

3.7/ ALKALINE PHOSPHATASE ENZYME HISTOCHEMISTRY

Alkaline phosphatase staining was carried out using the Histoprime® AP substrate kit. Cell cultures were fixed for 2 min with 4 % icecold formalin. After two washes with PBS, cell cultures were stained for 45 min at room temperature with freshly prepared Histoprime® substrate solution followed by another washing step with PBS.

3.8/ ASSESSMENT OF ALKALINE PHOSPHATASE ACTIVITY IN CELL LYSATES

Cells were lysed using 70 µl freshly prepared Lysis-M buffer per well of a 96 well plate. Lysis was carried out for 5 min at room temperature. 30 µl of each cell lysate was transferred into the wells of a fresh 96 well plate containing 30 µl 4-nitrophenyl phosphate solution (10 mM) and 30 µl alkaline buffer solution per

well. Plates were incubated for 15 min at 37 °C. AP reaction was stopped by adding 3 N NaOH and the absorbance at 410 nm was measured. A dilution series of 4-nitrophenol (15 - 115 U/l) was used as a standard.

3.9/ ASSESSMENT OF TOTAL PROTEIN CONTENT

The total protein content (cell mass and matrix deposition) in lysates of cell cultures was assessed using the Bradford method (BRADFORD 1976).

3.10/ PHALLOIDIN/LIPIDTOX STAINING

Cell cultures were fixed for 5 min with 4 % formalin at room temperature. After two washes with PBS cells were permeabilised incubating for 5 min with 0.1 % Triton® X-100. After another two washing steps with PBS, cells were incubated with 5 % goat serum in PBS.

Subsequently, staining solution containing goat serum (5 %), Phalloidin-TRITC (5 µg /ml), LipidTOX™ Green (1x) and DAPI (10 µg/ml) in PBS was added and cells were incubated for 1 h at room temperature in the dark. After one washing step with PBS cells were analysed and microphotographed using a fluorescence microscope.

3.11/ OIL RED O STAINING

For Oil Red O staining, cell cultures were fixed for 5 min with 4 % formalin at room temperature. After two washes with PBS cells were incubated with 60 % isopropanol for 5 min and stained with Oil Red O solution (2 mg/ ml in deionised water) for 5 min at room temperature.

Stained cells were then washed with tap water until the water appeared clear without traces of unbound dye. Cells were microphotographed using a phase contrast microscope.

4/ RESULTS AND DISCUSSION

Here, the suitability of CELLSTAR® and CELLCOAT® products for the cultivation and differentiation of human ADSCs was assessed with a focus on:

1. **the possibility to propagate ADSCs in adherent culture without loss of their tri-lineage differentiation potential,**
2. **the onset of early markers for osteogenic or adipogenic differentiation and their characterisation by cytochemical stainings,**
3. **the accelerated establishment of osteogenic characteristics, such as alkaline phosphatase expression on Collagen Type I coated CELLCOAT® plates.**

4.1/ CELL EXPANSION AND PRESERVATION OF TRI-LINEAGE DIFFERENTIATION POTENTIAL

Prior to any differentiation experiment ADSCs must be expanded. Here, TC treated CELLSTAR® cell culture flasks (T25, T75 and T175) and growth promoting, differentiation inhibiting MesenPRO RS™ medium were used for cell expansion. Cells were grown up to 80 % confluency and passaged up to five times[1] before setting up differentiation experiments. As shown in figure 1, ADSCs did well maintain their tri-lineage differentiation capability after pre-cultivation and expansion on TC treated CELLSTAR® substrates and the actual differentiation experiments being performed in TC treated CELLSTAR®

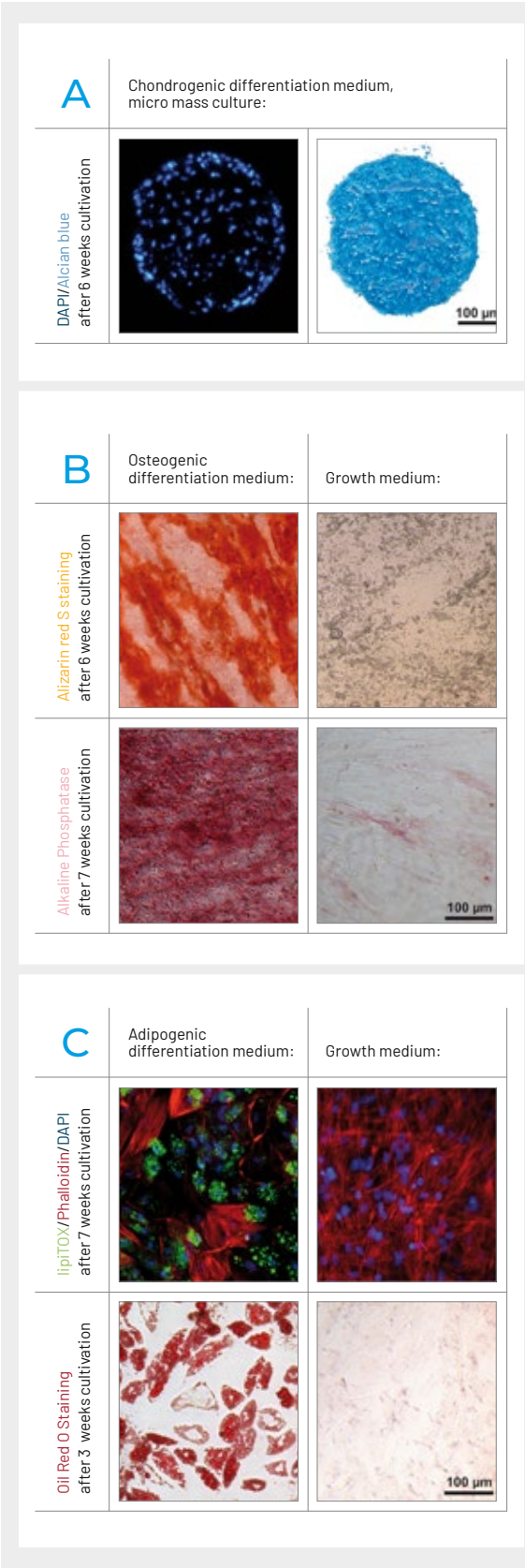

Figure 1: ADSCs culture under conditions promoting stemness or differentiation.

A: Micromass culture in chondrogenic medium allowes for the chondrogenic differentiation of ADSCs with cell nuclei distributed over the entire diameter of the spheroid and slightly enriched in its periphery (DAPI staining to the left). Cartilage specific acidic mucopolysaccharides and glycosaminoglycans spread over the entire spheroid (Alcian blue staining to the right).

B: Cultivation of ADSCs in osteogenic medium induces osteogenesis as indicated by calcium deposition in the ECM (Alizarin red S staining, top left) and enhanced alkaline phosphatase (AP) activity (bottom left). Cultivation in growth medium yields no (top right) or little (bottom right) expression of the bone specific markers calcium and AP, respectively.

C: Adipogenesis correlates with breakdown of cytoskeletal structures (Phalloidin negative regions in top left panel) and the intracellular accumulation of fat vacuoles (LipidTOX™ staining top left, Oil Red O staining bottom left). Adipogenic characteristics are absent from ADSCs under growth promoting, differentiation inhibiting conditions (top and bottom right).

multiwell plates. The depicted results were obtained with passage five cells and are representative for both batches of StemPro® human ADSCs used in this study.

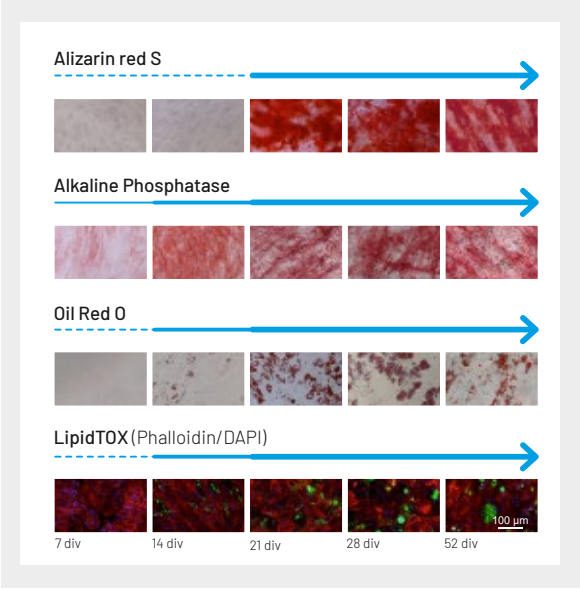

Figure 2: Onset of expression of cellular markers indicating osteogenic or adipogenic differentiation. Calcium deposition in the extracellular matrix could be detected by Alizarin red S staining as early as 21 days after ADSCs have been taken in culture with osteogenesis inducing medium. In contrast, alkaline phosphatase was already faintly expressed after 7 div (days in vitro) and prominent after 14 div. Intracellular lipid deposition indicative for differentiation along the adipogenic path was first detectable after 14 div in adipogenesis inducing medium by either Oil Red O or LipidTOX™ staining.

4.2/ TIME COURSE OF ADIPOGENIC AND OSTEOGENIC DIFFERENTIATION

In the course of this study, ADSCs were cultivated for prolonged periods on TC treated CELLSTAR® multiwell plates in cell culture media promoting their differentiation along the osteogenic or chondrogenic lineage. Easy-to-handle cytological staining protocols were found well suited for the assessment of early signs of cellular differentiation, such as calcium deposition and lipid accumulation in osteoblasts and fat cells, respectively.
In particular, alkaline phosphatase activity could be faintly detected as early as seven days after ADSCs have been taken in culture with osteogenic cell culture medium (seven days in vitro[2]). While alkaline phosphatase activity became strong after 14 div, calcium deposition was not detectable before 21 div **(figure 2)**.

In contrast, intracellular lipid accumulation as a first sign of adipogenic differentiation could be detected as early as 14 div in adipogenic medium. Both, LipidTOX and Oil Red O staining were found well suited for the detection of fat vacuoles in differentiating cells **(figure 2)**.

4.3/ ACCELERATED OSTEOGENESIS ON COLLAGEN TYPE I

Currently, many efforts in research are made in order to better understand the influence of cell-matrix interactions on cellular differentiation and to mimic structural features of the extracellular matrix for guiding stem cell differentiation (OH ET AL., 2008; CURRAN ET AL., 2006). Here, the effect of Collagen Type I on the development of osteogenic characteristics, such as enhanced alkaline phosphatase activity and protein deposition, has been examined. Whereas alkaline phosphatase activity remained on a low level when ADSCs were

[1] The provided maximum number of passages includes all passages prior to cryo preservation of the cells as well as passages after cell recovery from cryogenic storage.

[2] The term „days in vitro" is abbreviated with „div" in what follows and stands for the duration of cell culture in differentiation media until fixation and cytochemical characterisation of he cultivated cells.

cultivated in growth medium, the total protein content of these cultures increased slightly from 20 div onwards. This effect may mainly be ascribed to an increase in total cell number and some non-specific extracellular protein deposition. After 38 div the total protein content of the ADSC cultures was found slightly higher on TC treated CELLSTAR® plates as compared to Collagen Type I coated CELLCOAT® plates **(figure 3)**.

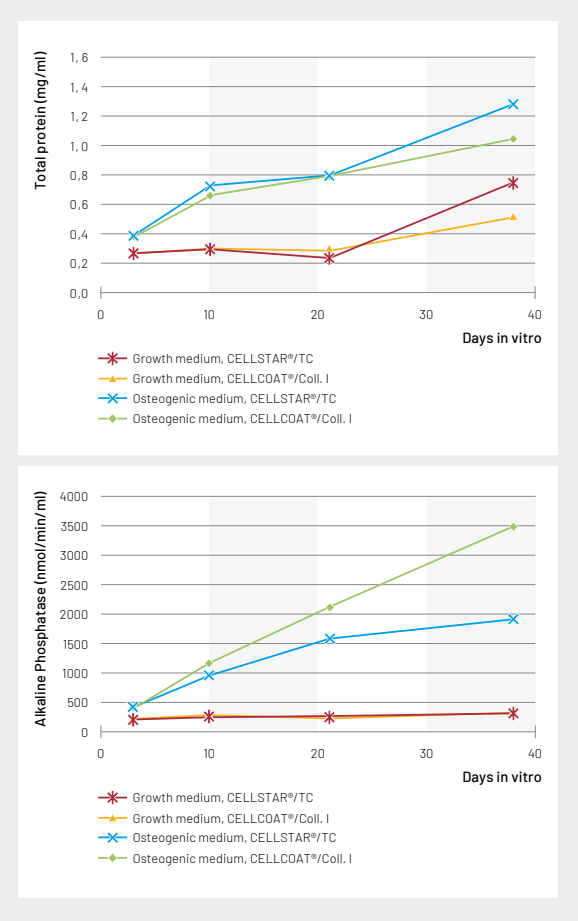

Figure 3: Time course of total protein content and alkaline phosphatase activity of ADSC cultures cultivated on different cell culture substrates.
ADSCs cultivated in osteogenesis inducing cell culture medium yield up to three times more total protein content as compared to the cultivation in growth promoting/differentiation inhibiting medium (upper panel). When cultivated in osteogenic medium on CELLCOAT® Collagen Type I coated cell culture plates, ADSCs develop up to 100 % more alkaline phosphatase activity as compared to the cultivation on TC treated CELLSTAR® plates. This effect does not occur with cell culture medium allowing for cell proliferation and inhibiting differentiation (lower panel).
Error bar = standard error of the mean.

When cultivated in osteogenesis inducing medium, ADSC cultures developed much stronger alkaline phosphatase activity and greater total protein contents as compared to the cultivation in growth medium. In the context of osteogenesis, this increased protein deposition may most likely be attributed to the synthesis of bone specific extracellular matrix.

Interestingly, alkline phosphatase activity was up to twice as high with ADSCs cultivated on Collagen Type I coated CELLCOAT® plates as compared to TC treated CELLSTAR® plates. This effect became obvious as early as 10 div and very prominent after 38 div **(figure 3)**.

It is indicative for Collagen Type I accelerating the development of bone specific features in differentiating ADSCs. Furthermore, the observed osteogenic effect of CELLCOAT® Collagen Type I may not be considered autarkic – it is rather synergistic with the presence of osteogenic growth factors in the cultivation medium.

5/ CONCLUSION

Commercially available ADSCs are a valuable stem cell resource and may easily be expanded on TC treated CELLSTAR® cell culture labware without loss of their tri-lineage differentiation potential. Furthermore, cell culture products of the CELLSTAR® and CELLCOAT® line in combination with StemPro® differentiation kits were found excellently suited for the in vitro differentiation of ADSCs.

6/ LITERATURE

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FRICKENHAUSEN, GERMANY

PHONE +49 7022 948-0
FAX +49 7022 948-514
E-MAIL info@de.gbo.com

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