PERFUSION CELL CULTURE REVEALS A PARACRINE OR AUTOCRINE SIGNALLING PATHWAY INVOLVED IN ADIPOSE-DERIVED STEM CELL DIFFERENTIATION INTO ADIPOCYTES

Mette Hemmingsen[1], Peder Skafte-Pedersen[1], David Sabourin[1], Rasmus Find Andersen[1], Anita L. Sørensen[2], Philippe Collas[2], and Martin Dufva[1]*

[1] *Department of Micro- and Nanotechnology, Technical University of Denmark, DENMARK and*
[2]*Institute of Basic Medical Sciences, Stem Cell Epigenetics Laboratory, University of Oslo, NORWAY*

ABSTRACT

Microfluidic cell culture has advantages compared to conventional static cell culture regarding spatial and temporal control of near-cell environment. Differentiation of adipose-derived stem cells (ASC) into adipocytes was studied under microfluidic perfusion culture conditions. Constant removal of cell released components from a culture had large negative impact on differentiation. Adding supernatant from cells, undergoing differentiation into adipocytes in static culture, to the perfusion medium restored efficient differentiation. The results suggest that a paracrine or autocrine signalling pathway is involved in differentiation of ASC into adipocytes.

KEYWORDS: Perfusion cell culture, Adipose-derived stem cells, Differentiation, Paracrine, Autocrine

INTRODUCTION

Microfluidic cell culture is an emerging field in stem cell research [1] owing to advantages compared to conventional static cell culture. By having constant perfusion of medium over the cell culture, the near-cell environment can be precisely controlled with respect to for example chemical stimuli via the cell culture medium. Constant perfusion will remove secreted factors from the cells and thereby disturb cell to cell signalling, which normally would be considered as a drawback as compared with static cell cultures.

ASC are an abundant source of adult stem cells with multipotent differentiation properties [2], no ethical or immunoreactive considerations, and potential for tissue regeneration and engineering. Differentiation of ASC into adipocytes is regulated by a network of transcription factors induced by insulin, glucocorticoids, agents elevating cAMP, and serum factors [3]. However, little is known about the role of paracrine or autocrine signalling in adipogenesis, and conventional static culture systems have been unsuitable for such investigations. In this study, we use a previously described microfluidic cell culture system [4] to disrupt paracrine and autocrine signalling pathways during stem cell differentiation. The possibility of disrupting signalling pathways is necessary for identification of cell released factors.

EXPERIMENTAL

The microfluidic system (Fig. 1a) [4] consists of three miniaturized eight-channel peristaltic pumps, liquid reservoirs and an exchangeable microfluidic chip with up to 24 culture chambers (Fig. 1b). Glass vials, lids, and PTFE tubing (BOLA 1810-01, Bohlender GmbH) were sterilized by autoclaving before assembling the system. Tubes connecting the liquid reservoirs to the pumps and the cell culture chip were filled separately with Milli-Q water to remove bubbles, before clicking on the cell culture chip to the system base plate. Inlet and outlet reservoirs were coupled with PTFE tubing (BOLA 1810-10) and supplied with sterile filtered air added 5% CO_2. To avoid formation of bubbles, a pressure of 0.3 bar was put on the flow system during the whole system preparation and cell culture period, only interrupted when for instance changing liquid reservoirs.

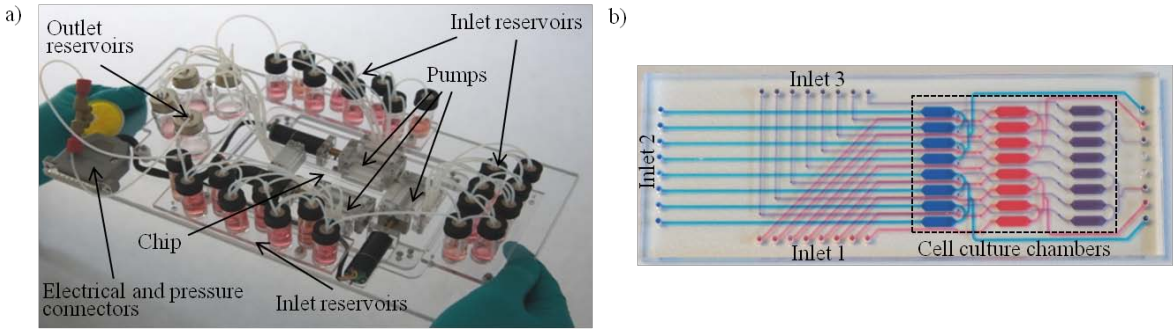

Figure 1: Microfluidic system for culture and functional studies of cells. a) The platform assembled with chip, 24 inlet and four outlet reservoirs. b) PMMA chip containing 24 parallel chambers with a height of 500 µm and a volume of 3.2 µL.

Prior to cell culture, the flow system was sterilized with 0.5 M NaOH for 20 min at a flow rate of 5.2 µL/min. NaOH was removed by washing with sterile water (W3500, Sigma) for 30 min at a flow rate of 5.2 µL/min. The surfaces of the cell culture chambers were coated with collagen (40 µg/ml in sterile water, C3867, Sigma) at 37ºC at a flow rate of 5.2 µL/min for 15 min followed by 195 nL/min for 45 min. Finally, the flow system was flushed with medium (DMEM/F-12+GlutaMax™ (31331, GIBCO) supplemented with 10% v/v newborn calf serum (N4762, Sigma), penicillin 100 U/mL, and streptomycin 100 µg/mL (P4333, Sigma)) for 30 min at a flow rate of 5.2 µL/min before cell loading. Uniform cell density over the entire culture chamber was obtained by loading the cells in culture medium supplemented 60% serum. To allow cell attachment, cells were perfused with medium supplemented 10% serum for 4 h at a flow rate of 33 nL/min at 37ºC. After this attachment phase, cells were perfused at a flow rate of either 33, 125, or 500 nL/min at 37ºC until completion of the experiment.

Differentiation was induced after 3 days of culture at a cell confluence of approximately 80-90%. The cells were perfused with either normal adipogenic differentiation medium (AM) (cell culture medium supplemented with IBMX (isobutylmethylxanthine) 0.5 mM (I5879, Sigma), dexamethasone 1 µM (D4902, Sigma), indomethacin 0.2 mM (17378, Sigma), and insulin 10 µg/mL (I9278, Sigma), or conditioned medium (CM) (a 1:1 mixture of medium from ASC undergoing adipogenic differentiation in static culture and AM with twice concentration of differentiation factors). Medium reservoirs were exchanged with fresh medium every third day.

Images of ASC differentiation were acquired at 10x phase contrast using a Zeiss Axio Observer.Z1 microscope connected to a Zeiss Axiocam MRm B/W camera. Quantification of lipid-filled droplets was carried out using ImageJ by marking areas of four or more pixels within a set range of gray-values and a circularization degree of 0.9-1.0, i.e. almost circular.

RESULTS AND DISCUSSION

Based on the assumption that constant flow conditions would disrupt a possible paracrine or autocrine signalling, we examined differentiation of ASC into adipocytes by perfusing ASC cultures with normal adipogenic medium at three different flow rates (33, 125, and 500 nL/min). Formation of lipid-filled droplets was used as an indicator of differentiation into adipocytes. Flow rates of 125 and 500 nL/min had a negative impact on differentiation compared to 33 nL/min (Fig. 2 upper panel and Fig 3). Flow rates of 33, 125, and 500 nL/min correspond to a resting time of medium in the cell culture chamber of 97, 26, and 6 min, respectively.

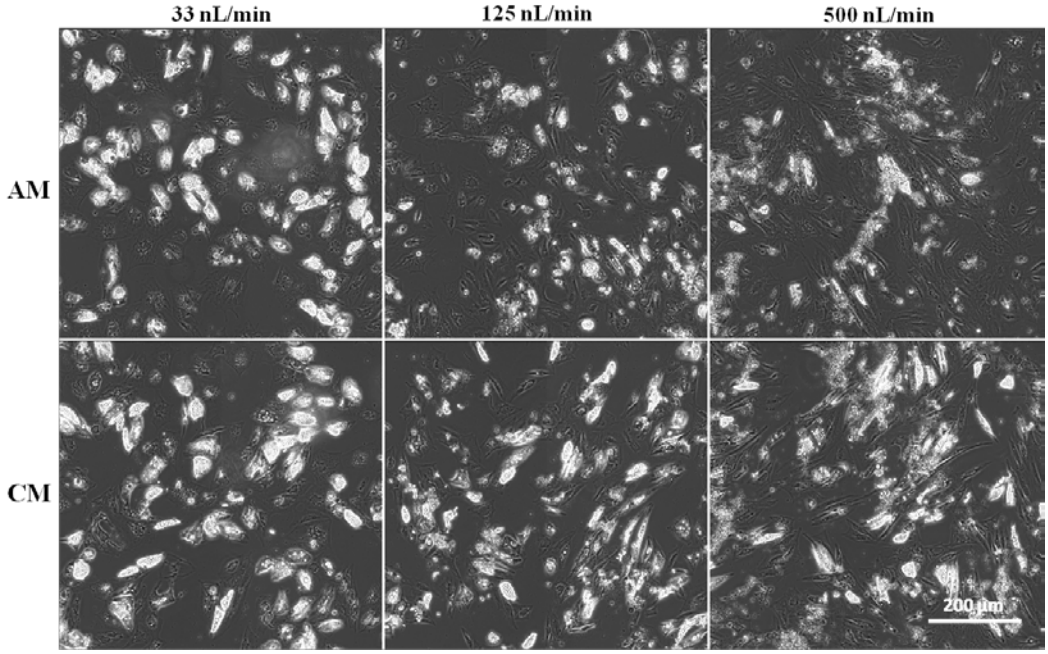

Figure 2: Conditioned medium restored differentiation of ASC into adipocytes. ASC was induced to differentiate by perfusing the cells at three different flow rates (33, 125, and 500 nL/min) with either normal adipogenic differentiation medium (AM) or conditioned medium (CM) (a 1:1 mixture of medium from ASC undergoing adipogenic differentiation in static culture and adipogenic differentiation medium with twice concentration of differentiation factors). 10x phase contrast images of differentiating ASC acquired after 16 days of differentiation.

To investigate whether the decreased formation of lipid-filled droplets was caused by removal of cell secreted signalling factors during differentiation, ASC were differentiated by perfusion with conditioned medium (AM added supernatant from

cells undergoing differentiation into adipocytes in static culture). Perfusion with CM increased the formation of lipid-filled droplets compared to ASC perfused with AM alone, as shown in Fig. 2 and Fig. 3. Importantly, the effect was dependent on flow rate. Almost no positive effect of perfusion with CM over AM was observed at 33 nL/min. By contrast, perfusion with CM resulted in up to two times more lipid-filled droplets than perfusion with AM for flow rates of 125 and 500 nL/min (Fig. 3b). This suggests an involvement of paracrine and/or autocrine signalling factors in differentiation of ASC into adipocytes. As control to perfusion with CM, cells were perfused with medium added twice the concentration of differentiation factors, but this resulted in cell death. Cells perfused with normal cell culture growth medium led to no differentiation at flow rates of 125 and 500 nL/min. However, some spontaneously differentiated cells were observed by perfusion with growth medium at 33 nL/min in the outlet end of the chamber (data not shown). The presence of differentiated cells only at 33 nL/min and in the outlet end of the chamber, where released factor concentration would be highest, further point to an involvement of paracrine and/or autocrine signalling factors in ASC differentiation into adipocytes.

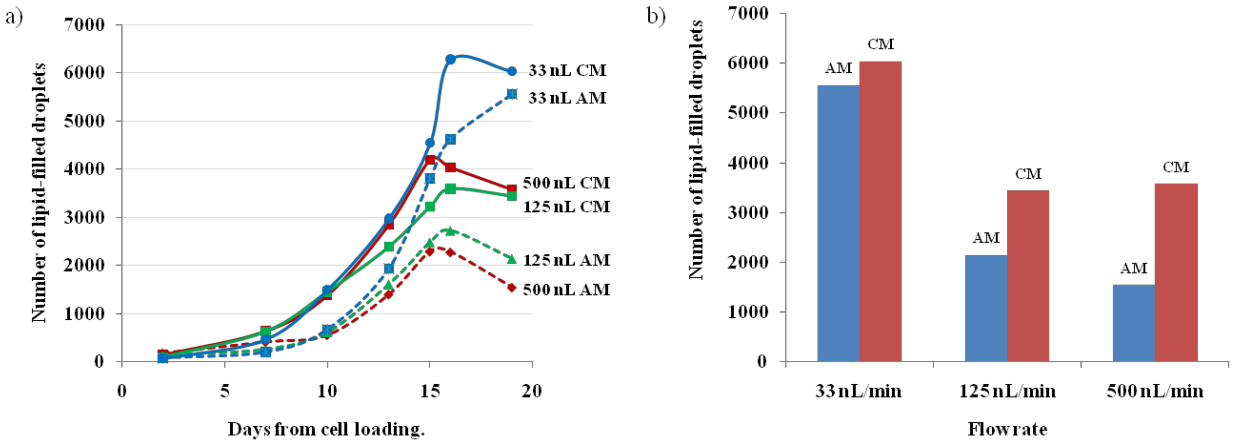

Figure 3: Effect of conditioned medium (CM) on differentiation of ASC into adipocytes was flow rate dependent. a) Number of lipid-filled droplets over time from cell loading. Induction of differentiation was started at day 3. Number of lipid-filled droplets were measured using ImageJ by marking areas of four or more pixels within a set range of gray-values and a circularization degree of 0.9-1.0, i.e. almost circular. b) Number of lipid-filled droplets at perfusion with CM compared to perfusion with AM at flow rates of 33, 125 and 500 nL/min. Data shown at day 19, i.e. after 16 days of differentiation.

CONCLUSION

We have shown that perfusion culture at flow rates of 125 and 500 nL/min have a negative impact on differentiation of ASC into adipocytes compared to perfusion at 33 nL/min. Furthermore, differentiation is restored when perfusing with adipogenic conditioned medium. This demonstrates that ASC differentiation into adipocytes is dependent on a paracrine and/or autocrine signalling pathway. Thus, we have proven that our microfluidic perfusion culture system is suitable for stem cell differentiation. It constitutes a flexible tool to fine-tune stem cell differentiation conditions, as well as investigate signalling pathways controlling differentiation**.**

ACKNOWLEDGEMENTS

This work was supported by Grant No. 2106-08-0018 "ProCell", under the Programme Commission on Strategic Growth Technologies, the Danish Agency for Science, Technology and Innovation.

REFERENCES

[1] Wu HW, Lin CC, Lee GB: *Stem cells in microfluidics.* Biomicrofluidics, 5(1):13401.

[2] Zuk PA, Zhu M, Ashjian P, De Ugarte DA, Huang JI, Mizuno H, Alfonso ZC, Fraser JK, Benhaim P, Hedrick MH: *Human adipose tissue is a source of multipotent stem cells*. Molecular biology of the cell 2002, 13(12):4279-4295.

[3] Farmer SR: *Transcriptional control of adipocyte formation.* Cell metabolism 2006, 4(4):263-273.

[4] Skafte-Pedersen P, Sabourin D, Hemmingsen M, Østergaard P, Blaga F, Dufva M: *A user-friendly, self-contained programmable microfluidic cell culture system for high quality microscopy*. MicroTAS: 2010.

CONTACT

*Associate Professor Martin Dufva, Department of Micro- and Nanotechnology, Technical University of Denmark, DTU Nanotech, Oersteds Plads, Building 345 East; tel: +45 4525 6324; martin.dufva@nanotech.dtu.dk