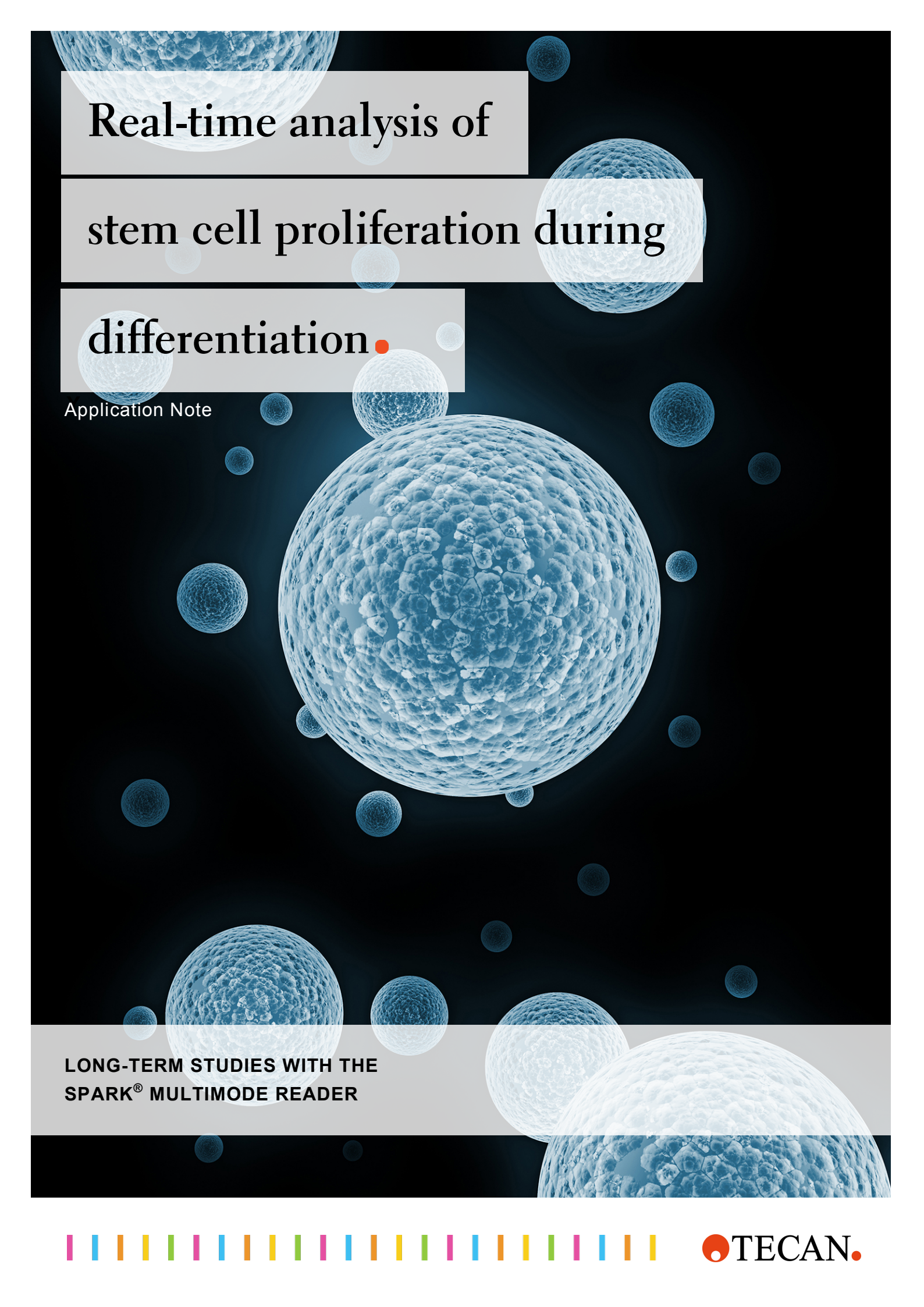




Real-time analysis of stem cell proliferation during differentiation.

Application Note

LONG-TERM STUDIES WITH THE
SPARK® MULTIMODE READER



INTRODUCTION

Mesenchymal stem cells (MSC) are multipotent adult stem cells originating from perivascular progenitor cells [1]. They have a high capacity for self-renewal and can be differentiated *in vitro* towards adipocytes, chondrocytes, neurons and osteoblasts [2].

As terminal differentiation of cells is associated with a loss in self renewal, this process is believed to counteract cell proliferation [3]. Understanding the molecular mechanisms cells use to switch between proliferation and differentiation is of central importance for human stem cell research.

This application note describes the real-time analysis of human stem cell differentiation using cell-based assays developed by PromoCell for the sensitive and reproducible assessment and quantification of cell viability, proliferation and apoptosis in a fast and convenient manner. It shows that the initiation of *in vitro* differentiation in human MSC does not immediately result in a stop of cell division, but can under certain conditions transiently induce proliferation. The experiments were established on Tecan's Spark multimode microplate reader, an instrument platform that offers tailor-made solutions to suit virtually any life science research application. It has a variety of functions and features for cell-based analyses, including an automated cell imaging module and environmental control features such as CO₂, temperature and humidity control right inside the reader.

MATERIAL & METHODS

Stem cell culture and differentiation

Human mesenchymal stem cells from adipose tissue (hMSC-AT, PromoCell C-12977) were propagated in MSC Growth Medium DXF (PromoCell, C-28019) on cell culture vessels pre-coated for 20 min at 37°C with 10 µg/ml of human fibronectin (PromoCell, C-43060) in HEPES Buffered Saline Solution (PromoCell, C-40020). For differentiation, MSC were resuspended at $2 - 5 \times 10^4$ cells/mL in DXF medium, and 100 µl per well were plated on black 96-well plates with µClear bottom (Greiner Bio-One, 655 087). For counting, vessel coating, seeding and growth of MSC the Spark 10M multimode plate reader was used, equipped with its proprietary cell chip adapter, injector system and humidity cassette. After adhesion of MSC, DXF medium was replaced with 200 µl/well of the following ready-to-use differentiation media:

- adipogenic (PromoCell C-28016)
- osteogenic (PromoCell C-28013)
- chondrogenic (PromoCell C-28012)
- neurogenic (PromoCell C-28015)

Differentiation of MSC was monitored for up to 60 hours. Cells were seeded into the microplate and left to adhere over night. The plate was then placed into the Humidity Cassette, with the fluid reservoirs of the plate filled with deionized water. To avoid edge effects due to evaporation, the outer wells of the plate were filled only with culture medium without cells. The Spark 10M was set to 37°C and 5% CO₂. Cell differentiation was monitored by automated measurement of absorbance, cell confluence, luminescence or fluorescence at defined intervals.

Reagents and measurements

The Colorimetric Cell Viability Kit I (WST-8, PromoCell, PK-CA705-CK04) was used to determine the number of viable cells during cell proliferation. This assay detects the bioreduction of the tetrazolium salt WST-8 into a yellow-colored formazan dye by living cells and can be used for long-term, real-time measurements because WST-8 does not have any adverse effects on cell growth and viability. For analysis of cell proliferation, the Cell Proliferation Kit III (488-HTS, PromoCell, PK-CA724-488HTS) was used to measure incorporation of the thymidine-analog EdU during DNA synthesis. Caspase-3 activity was quantified using the Caspase-3 Fluorometric & Colorimetric Assay Kit (PromoCell, PK-CA707-30008-1). All kits were used according to the manufacturer's instructions.

RESULTS

The balance between stem cell self-renewal and differentiation is carefully regulated and terminal differentiation of stem cells is believed to stop proliferation [3]. However, little is known about how fast stem cells react to differentiation stimuli *in vitro*. Thus, the goal of this study was to monitor and characterize changes in cell proliferation within the early stages of *in vitro* MSC differentiation.



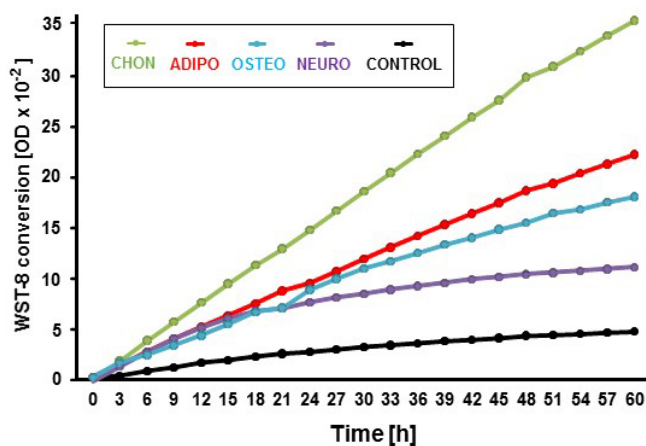


Figure 1: Real-time analysis of viable cell numbers upon differentiation of stem cells by measurement of WST-8 conversion.

In order to perform a real-time analysis, MSC were cultivated inside the Spark 10M multimode plate reader, and subsequently subjected to chondrogenic, adipogenic, osteogenic or neurogenic differentiation stimuli. In addition, cell viability was monitored by measuring the conversion of the non-toxic tetrazolium salt WST-8 to the yellow-colored formazan by living cells (Figure 1). Within 60 hours after induction, differentiating MSC showed increasing rates of WST-8 conversion relative to control MSC which had been kept in non-differentiating DXF medium. This result suggests either an increase in living cell numbers or in cell viability during the initial phase of differentiation.

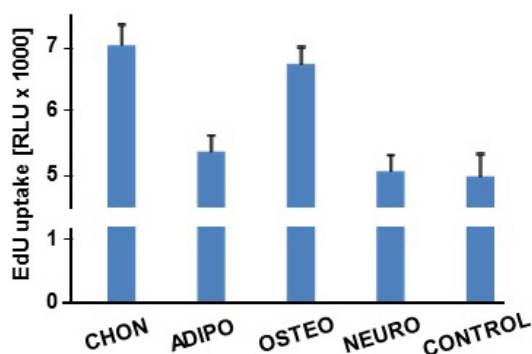


Figure 2: Stem cells differentiation can result in cell proliferation in the course of stem cell differentiation monitored by EdU uptake.

To be able to identify cell proliferation during the *in vitro* differentiation of MSC, the uptake of the thymidine-analog EdU during DNA synthesis was measured. As a result, differentiating MSC under chondrogenic and osteogenic conditions showed a significant increase in EdU uptake relative to control MSC (Figure 2). In contrast, MSC in adipogenic and neurogenic differentiation did not show alterations in the rate of DNA synthesis. Thus, the elevated

cell numbers during chondrogenic and osteogenic differentiation seem to be a result of cell proliferation.

The last set of experiments was targeted at investigating cell viability during *in vitro* differentiation, in order to exclude potentially dead cells from the results evaluation. Therefore, cellular caspase activity (as an indicator for apoptotic cell death) was tested shortly after transferring the cells to the various differentiation media. While most of the culture conditions did not result in any change, the chondrogenic differentiation led to a robust but transient caspase-3 activation which was however not followed by cell death induction (Figure 3). Thus, caspase-mediated cell death does not seem to influence viability of differentiated MSC.

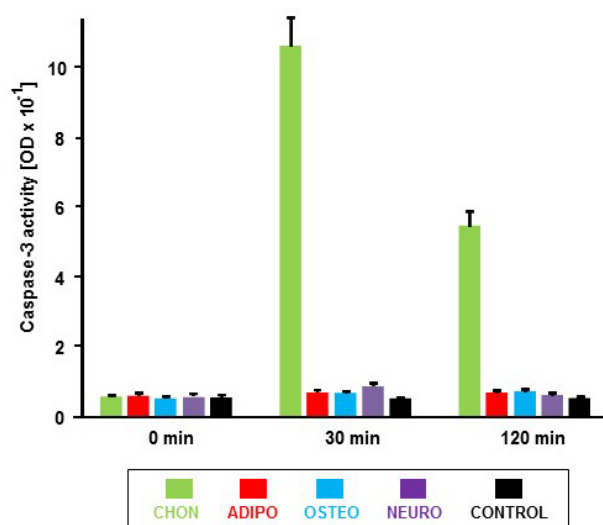


Figure 3: Caspase activation in the course of stem cell differentiation

DISCUSSION / CONCLUSION

It was found that within the first 60 hours after induction of *in vitro* differentiation towards adipocytes, osteocytes, chondrocytes and neurons human MSC showed a substantial elevation of cell numbers or cell viability compared to non-differentiated MSC. Elevated DNA synthesis during chondrogenic and osteogenic differentiation suggested the induction of cell proliferation. In contrast, cell proliferation during adipogenic and neurogenic differentiation was comparable to non-differentiated MSC. Here, an acceleration of cell metabolism – possibly induced by culture media with inducers for differentiation – was identified as cause for the elevated viability.

Furthermore, transient caspase activation was detected in chondrogenic differentiation of MSC, which, as previously reported, seems to be instrumental for differentiation but is

not associated with cell death [4]. Of note, differentiation of human MSC *in vitro* does not immediately slow down cell proliferation, but might in some cases transiently induce cell growth.

The application of the Spark 10M multimode reader for cell counting, plating and subsequent culture of stem cells during differentiation for up to several days enabled the automated multi-parameter, real-time analysis.

Differentiation of MSC *in vitro* with ready-to-use culture media worked reliable and robust. In addition, culture of stem cells in the presence of WST-8 was found to be a non-toxic and very sensitive measure for cell viability and cell numbers.

ACKNOWLEDGEMENTS

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ABBREVIATIONS

ATP	adenosine triphosphate
MSC	mesenchymal stem cells

About the authors

Associate professor Dr. Rüdiger Arnold teaches at the University of Heidelberg as a lecturer in several life science education programs. Dr. Arnold studied biology in Giessen and worked as a postdoctoral fellow at the Max - Plank - Institute of Physiology and Clinical Research in Bad Nauheim. Afterwards he joined the German Cancer Research Center and habilitated in immunology at the medical faculty of the Ruprecht Karls University in Heidelberg where he worked in the area of activation, differentiation, and apoptosis in leukocytes and lymphoma. Between 2011 and 2015 he headed the student research laboratory of the Heidelberg Life - Science Lab at the German Cancer Research Center. He also has many years of experience as a lecturer in training for professional qualifications tumor biology DIW-MTA.

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