Non-Invasive Method for Monitoring Real-Time Oxygen Concentrations during Hematopoietic Stem/Progenitor Cell (HSPC) Culture

Summary

The SDR SensorDish® Reader has allowed for the quantitation of *in vitro* oxygen concentrations during the culture of hematopoietic stem/progenitor cells (HSPC) isolated from human umbilical cord blood. The influence of simulated physiological oxygen concentrations on HSPC proliferation and cell cycle status, as well as expression of markers of stem cell phenotype or differentiated cell types, was examined concurrently with the monitoring of real-time oxygen concentrations in the cell culture media. This study has demonstrated the usefulness of this system for examinations of cell phenotype and function in conditions that effectively mimic the *in vivo* oxygen environment.

Oxygen Concentrations Rise during Embryonic Development

Oxygen concentrations change dramatically during human embryonic development. These alterations in oxygen concentration are most striking in the developing placenta as it grows and invades maternal uterine tissue and ultimately taps into oxygenated maternal blood (reviewed in[1]. Trophoblast cells are one of the earliest cell types to form in the human placenta and, thus, must develop in a variety of oxygen concentrations. Similarly, hematopoietic and vascular progenitor cells can first be found within the early placenta in a low oxygen environment[2]. As development proceeds, oxygen tensions rise and both trophoblast and progenitor cells adapt to the rising oxygen concentrations[3,4]. Interactions between trophoblast and vascular cells are extremely complex and have not been adequately described. Alterations in oxygen concentration are known to have direct effects on the differentiation of trophoblast and vascular cells. Furthermore, pathological placental development has significant effects on in utero oxygen tensions. Abnormal placental oxygen concentrations likewise affect growth and development of the fetus and could eventually lead to an increased risk of fetal and maternal morbidity and mortality. For this reason, it is extremely important that investigations into the effects of oxygen concentration on placental growth and vascular development continue. An *in vitro* culture system that effectively mimics these alterations in physiological oxygen concentrations during *in vitro* cell culture would prove irreplaceable in future studies.

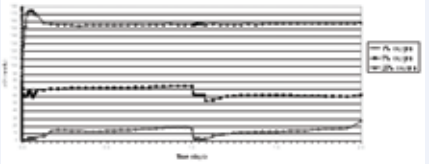
HSPC Culture in Physiological Oxygen Concentrations

For the analysis of cellular processes during placental vascular development, an *in vitro* model was developed using CD133+ cells isolated from human umbilical cord blood, called hematopoietic stem/progenitor cells (HSPC). Two different oxygen concentrations were used for this *in vitro* model to simulate physiological oxygen tensions at specific time-points during placental development. Traditional cell culture conditions in ambient oxygen were likewise examined and compared. Crucial to this investigation was a non-invasive method for monitoring real-time oxygen concentrations in the HSPC culture media during experimentation. To that end, the SDR SensorDish Reader from PreSens Precision Sensing GmbH was effectively employed to measure oxygen concentrations while conserving the sterile *in vitro* environment. Low oxygen environments in this cell culture study were achieved using a hypoxia chamber (Billups-Rothenburg, USA) inserted into a traditional CO_2 incubator (Figure 1). Prior to cell culture, the chamber was infused with gas mixtures containing either 1% or 8% O_2 according to the manufacturer's protocol. HSPC cultures in ambient oxygen concentrations (20%) were also compared. This setup allowed for specific physiological oxygen concentrations to be monitored and

Figure 1: The SDR SensorDish® Reader from PreSens Precision Sensing GmbH was to monitor *in vitro* oxygen concentrations during HSPC culture. HSPC were cultured in OxoDish® 24-well plates in a hermetically sealed, humidified cell culture chamber containing low oxygen gas mixtures (1% or 8% O2, 5% CO2) or atmospheric oxygen (20% O2) (A). OxoDishes® were placed on the SDR SensorDish® Reader and the chambers incubated at 37°C for the specified time. Connection of the SDR to computer (B) allowed for non-invasive, real-time measurements of oxygen concentrations in HSPC culture media during the experimental period.

maintained *in vitro* (Figure 2). HSPC culture dynamics in physiological oxygen concentrations could also be compared to traditional HSPC culture in the hyperoxic environment of ambient oxygen. The effects of oxygen concentration of HSPC function and differentiation were likewise compared in this study.

Figure 2: Actual oxygen tensions measured in the HSPC culture media during a two-day experimental period in atmospheres containing low (1% or 8% O_2) or atmospheric oxygen (20% O_2). A representative graph demonstrating *in vitro* oxygen tensions measured with the SDR in combination with OxoDishes® during two-day HSPC culture in various oxygen environments. An average oxygen tension of 10.2 (± 5.5 mmHg) was encountered when HSPC were cultured in 1% O_2. In 8% O_2, the average oxygen tension in the media was 65.0 (± 7.5 mmHg). When HSPC were cultured in ambient oxygen (20% O_2) the average oxygen tension in HSPC culture media was 157.9 (± 9.2 mmHg). To obtain a relatively stable low oxygen environment, the atmosphere was replaced one time per hour for three hours at 24-hour intervals.

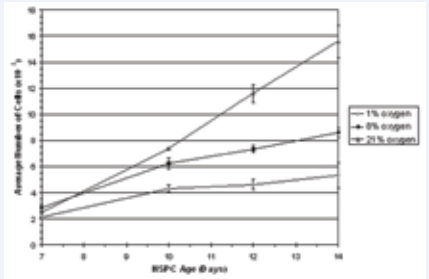


Analysis of the Effects of Physiological Oxygen Concentrations on HSPC Cultures

CD133+ HSPC cells isolated from umbilical cord blood were cultivated for up to one week in special 24-well OxoDishes®. Every 2-3 days, cells and culture media were removed, briefly centrifuged (1500 rpm; 5 min) and cells were carefully resuspended in media that had preincubated for 1 h in the appropriate oxygen concentrations (1%, 8% or 20%). Real-time oxygen concentrations were monitored online with the SensorDish Reader during the entire incubation period. Concurrently, HSPC cells were cultivated in either 24- or 6-well plates, depending on assay, under identical conditions. When media was exchanged, an aliquot was removed and cell number determined using a Casy® Counter. Flow cytometric measurement of DNA content allowed for the examination of HSPC cell cycle status following culture in physiological (1% or 8%) or ambient (20%) oxygen concentrations. Similarly, apoptosis levels were quantified using the FITC-Annexin V Apoptosis Kit (BD) in combination with flow cytometry. At the protein level, expression patterns of extracellular markers of stem/progenitor or differentiated cells were analysed using multi-parametric flow cytometry. The following characteristics were observed from HSPC cultured in the described oxygen concentrations.

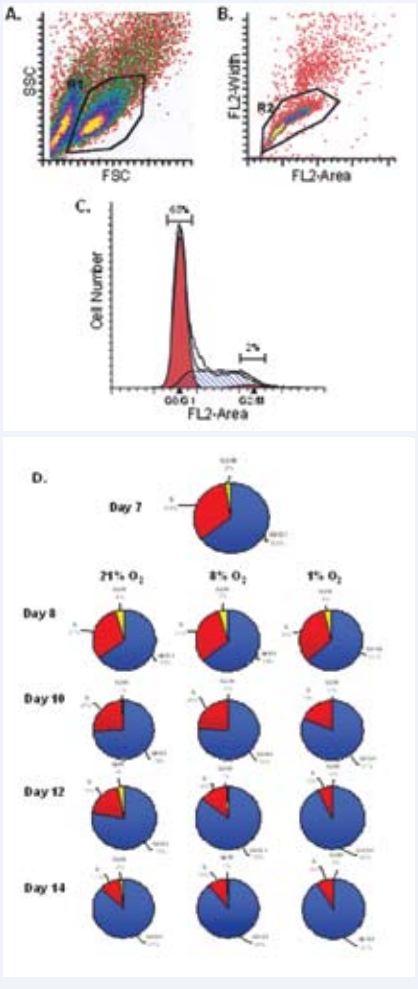
In hyperoxic environments, HSPC displayed an increased rate of proliferation. HSPC growth curves decreased in an oxygen concentration-dependent manner, with the lowest cell proliferation seen in environments containing 1% O_2 (Figure 3).

Figure 3: Growth curve demonstrating the effects of physiological oxygen on HSPC cell number. HSPC (Day 7) were cultured in low oxygen environments (1% or 8% O_2) or ambient oxygen (20% O_2). HSPC cell number was quantified on Days 10, 12, and 14 with a Casy ® Counter. HSPC number expanded in ambient oxygen (open triangles). On the other hand, HSPC growth dose-dependently decreased in low oxygen with the greatest decrease in proliferation seen in 1% O_2 (grey diamonds). HSPC culture in 8% O_2 (black squares) significantly decreased cell number when compared to hyperoxic conditions.



Flow cytometric analysis of HSPC DNA content allowed for the analysis of cell cycle status. Results from this investigation clearly demonstrated that the number of cells either resting (e.g. G0) or in G1 increased significantly when oxygen concentration in the environment decreased (Figure 4). Flow cytometry was also used to measure apoptosis levels in HSPC cultured in decreasing oxygen concentrations. Oxygen concentration appeared to have no significant effect on HSPC apoptosis in this experiment (data not shown). Taken together, these results suggest that when HSPC are cultured in conditions that mimic the *in vivo* environment, cell proliferation decreases, most likely due to an increase in the number of resting cells (G0) and without any significant effects on cell death.

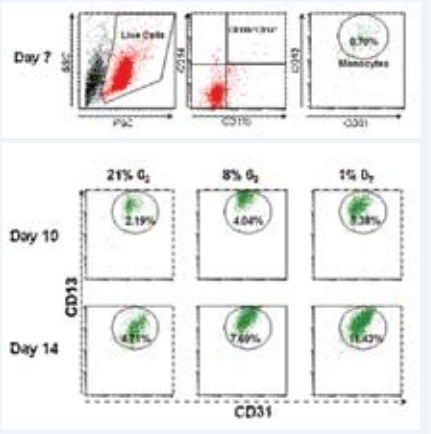
Figure 4: Flow cytometric cell cycle analysis of expanded HSPC (Day 7) and HSPC cultured in physiological oxygen concentrations. HSPC were expanded in HSPC-GM for 7 days, fixed and stained with propidium iodide (PI). Stained cells were then subjected to flow cytometry. Live cells (R1) were gated based on FSC and SSC (A). Single cells (R2) were gated and cell aggregates resulting from fixation were excluded (B). Cell ploidy was determined based on the stochastic integration of PI into HSPC DNA (C). Results from this experiment demonstrate that following expansion 65% of HSPC were 1n (G0/G1) while 2% of HSPC were 2n (G2/M). The remainder of the cells were in the S phase of the cell cycle (33%). HSPC were grown in 1%, 8% or 20% O_2 and cell cycle status determined on days 7, 8, 10, 12 and 14 (D). Low oxygen dose-dependently increased the number of resting G0/G1 HSPC over the test period. Subsequent reductions in cycling G2/M and S cells were measured.



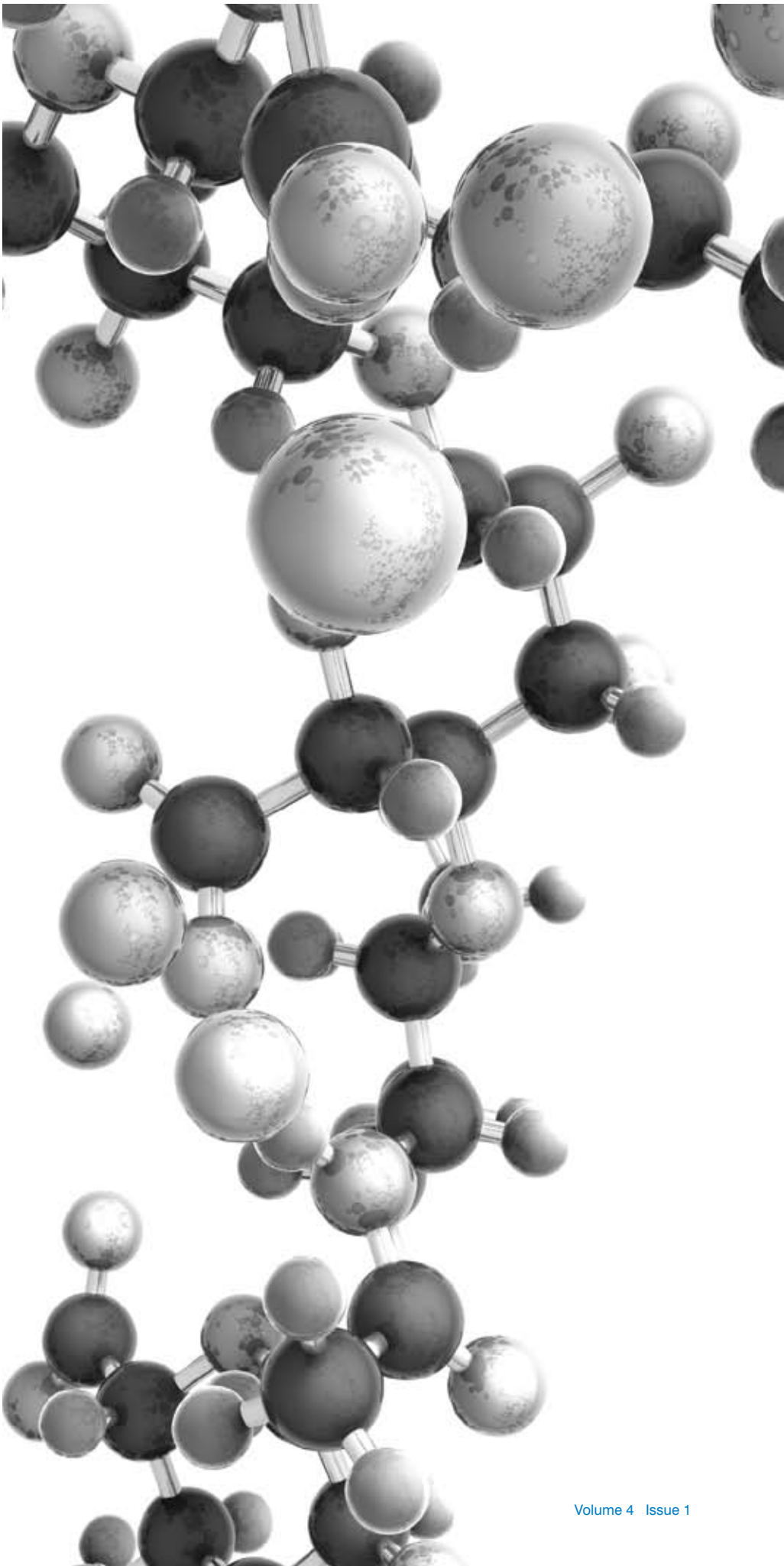
Multiparametric flow cytometry was employed to examine HSPC lineage marker analysis. Expression of the stem/progenitor cell markers (e.g. CD34, CD133, and CD117) were not significantly changed during

culture in physiological oxygen concentrations (data not shown). Conversely, based on the expression of four extracellular markers ($CD11b^+CD13^+CD14^+CD31^+$), the percentage of monocytes in the culture was greatly increased with decreasing oxygen concentration (Figure 5).

Figure 5: Representative flow cytometry dot plots demonstrating the expression of extracellular markers for the monocyte lineage during HSPC culture in physiological oxygen concentrations. Expanded HSPC (Day 7) were incubated with fluorescently-labelled antibodies and subjected to multiparametric flow cytometric analysis. Live cells were gated based on size (FSC) and granularity (SSC). Cells coexpressing the monocyte markers CD11b and CD14 ($CD11b^+CD14^+$) were further gated. The majority of $CD11b^+CD14^+$ cells were contained within a population of $CD13^+CD31^+$ cells (monocytes). On Day 7, less than 1% of cells expressed all four markers (A). Cells expanded a further seven days (Day 14) were similarly analysed. The percentage of monocytes increased with decreasing oxygen concentration over this period (B).



Furthermore, the expression of important factors for vasculogenesis (e.g. chemokines) was affected by oxygen concentration. For example, when expression of the chemokine receptor, CXCR4, was examined in HSPC cultures grown in low oxygen it was noted that the number of CXCR4+ cells increased with decreasing oxygen concentration. In addition, the ligand for CXCR4 was shown to be expressed by placental stromal cells in low oxygen environments *in vivo* and *in vitro*[5]. Results from this study provide one of the first descriptions of HSPC culture conditions that effectively mimic the *in vivo* oxygen environment of the developing placenta. Furthermore, this study demonstrates evidence that oxygen concentration has a definite effect on



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stem/progenitor cell phenotype and function, and further substantiates the idea that oxygen plays a key role during placental development.

New Perspectives for Non-invasive Oxygen Monitoring with the SDR

Physiological oxygen concentrations during distinct time-points in human placental development could be effectively mimicked with surprising precision using two different gas mixtures, containing either 1% or 8% O_2 (5% CO_2). Measurements made in this study with the SDR SensorDish® Reader paired with OxoDishes® provide additional proof that traditional cell culture in atmospheric oxygen (20% O_2) creates a clearly hyperoxic environment with oxygen tensions well above normal pO_2 found in human tissues.

The use of low oxygen gas mixtures is not novel in placental research, and oxygen-reduced environments have previously been used to assess trophoblast development and functions[6-8]. One important difference, however, is that previous investigations did not include non-invasive, real-time oxygen monitoring. As clearly demonstrated in this investigation, oxygen concentrations in low oxygen cell culture systems are in a constant flux and are difficult to maintain at physiological levels. Accordingly, standardisation of experimental parameters (e.g. cell number, media depth and composition, gas replacement intervals, etc.) and extensive testing was required to ensure that actual physiological oxygen concentrations were reached and subsequently maintained. Using standardised protocols, variability between experiments was reduced. Furthermore, and in contrast to previously published results[6-8], the SDR oxygen monitoring system provided reliable data concerning *in vitro* oxygen levels during the entire experimental period.

The above-described study illustrates a successful attempt to analyse hematopoietic stem/progenitor cells functions in conditions that precisely mimicked physiological oxygen concentrations in their placental environment. Reproducible results from this study have demonstrated a clear connection between oxygen concentration and cell proliferation and cell cycle status. The significance of these results on stem/progenitor cell culture and function will be examined in further detail in the future. The SDR SensorDish® Reader has opened up the possibility of further *in vitro* studies in which defined, physiologically-relevant oxygen concentrations play an important role.

Future Directions for Research Involving the SDR SensorDish® Reader

In combination with *in vitro* experimentation, the SDR SensorDish® Reader has opened up additional application fields in defined oxygen environments. For example, tissue engineering for regenerative medicine or *in vitro* toxicology studies to test for cytotoxic agents could prove more useful when taken in the context of physiological oxygen concentrations. The SDR SensorDish® Reader could also be used to monitor oxygen concentrations while testing for useful pharmacological molecules. In addition, the pH sensor HydroDish® has made the SDR SensorDish® Reader an alternative for measuring pH values in the culture media under sterile conditions. With these applications, the SDR SensorDish® Reader has provided a new scale for instrumentation that allows stable cell culture conditions to be maintained. Furthermore, media changes or alterations in oxygen concentrations can be monitored and altered according to experiment. Using this system, research objectives will be reached in a timely manner, thereby reducing research costs.

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