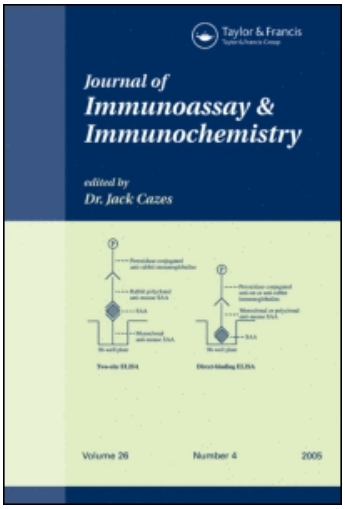

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Optimized Flow Cytometric Analysis of Endothelial Progenitor Cells in Peripheral Blood

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Abstract: Although flow cytometry is a rapid and convenient way to measure the number of circulating endothelial progenitor cells (EPC), there is no standard technique for preparation and measurement. The aim of this study was to present an optimized preparation method for EPC measurement which should serve as a standard to facilitate the comparison of the results in stem cell investigations by different research groups.

We have looked for the preparation method which delivered the best immunostaining with the directly conjugated antibodies against VEGF R2, CD133, CD34, and CD45. In order to test the sensitivity of the method, we determined the number of EPC in the peripheral blood of volunteers by flow cytometry and by cell culture assay. Furthermore, we have evaluated the influence of different durations of conservation on the EPC cell count.

The pre-treatment of blood samples with 0.2% formaldehyde for 30 minutes delivers the best immunostaining, and blood samples can be stored overnight at 4°C without loss of counting rate for EPC. We found an excellent correlation ($r = 0.98$) between the flow cytometric measurement and the cell count of the cell culture method.

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The presented protocol for the flow cytometric measurement of EPC in the peripheral blood can be used as a diagnostic or prognostic tool; we propose this protocol as the standard for EPC quantification.

Keywords: Endothelial progenitor cells (EPC), Flow cytometry, Optimisation, Standard protocol

INTRODUCTION

Endothelial progenitor cells (EPC) are bone marrow derived stem cells which exhibit characteristic endothelial surface markers and properties.[1] It has been shown that endothelial progenitor cells are capable of contributing to re-endothelialization and neo-vascularization in tissue injury and that injected EPCs home to sites of ischemia and augment neo-vascularization.[2]

EPCs are defined as cells positive for both haematopoietic stem cell markers, such as CD34, and an endothelial marker protein such as VEGF R2.[3] Circulating EPCs express, with different intensities, a variety of markers which are typical for the endothelial lineage. These markers include platelet endothelial cell adhesion molecule-1 (CD31), CD146, VE-cadherin, von Willebrand factor, endothelial NO synthase and, on stimulation, E-selectin.[4–6] In general, early EPCs in the bone marrow, or immediately after their migration into the systemic circulation, are positive for CD133/CD34/VEGF R2, whereas circulating EPCs are positive for CD34/VEGF R2/CD31/VE-cadherin.[7] They obviously lose CD133 and begin to express von Willebrand factor.[7]

Quantification of EPCs in the peripheral blood is necessary to understand the pathogenesis of a variety of vascular diseases and to elucidate how risk factors contribute to vascular changes. Flow cytometry is a rapid and convenient way to assess the number of circulating EPCs.

As it would be a great logistical advantage if the specimens can be stored after staining for hours, so that the flow cytometric measurement can be done in an extended window of time, we have looked for a suitable preparation method.

In several studies, the number of the EPCs in the peripheral blood has been determined using flow cytometry, although there is no consensus as to the best method.[8–13] In this study, we have examined the effects of preparation procedures for flow cytometry on the binding of CD133, VEGF R2, CD34, and CD45 antibodies and we have compared the results of the EPC number from flow cytometric measurement, using the preparation technique proved to exhibit the best antibody binding, with the results from EPC culture assay. Further, we have studied the stability of the antibody staining after storage over night at 4°C.

The aim was to present an optimized preparation method for EPC measurement in peripheral blood so that this technique could be a standard for EPC-measurement, which facilitates the comparision of the results in stem cell investigations from different research groups.

EXPERIMENTAL

Samples

EDTA-blood samples taken from volunteers were obtained as part of a routine diagnostic or screening procedure after informed consent had been obtained. The blood from 20 healthy volunteers (11 women and 9 men; mean age 40.4 years, SD 18.9) were included in the study. The study protocol was approved by the local Ethics Committee of the University of Münster.

Sample Preparation

For analysing EPCs samples of umbilical cord blood, of bone marrow, and of whole blood were either stained without fixation, stained after incubation for 30 minutes with 0.2% buffered formaldehyde,[14] or were fixed with 2% formaldehyde for 30 minutes after staining and lysing of red blood cells as described previously.[9]

After washing with PBS, $4 \times 50\,\mu L$ aliquots were incubated for 30 minutes in the dark with the phycoerythrin (PE)-conjugated monoclonal antibodies against human VEGF R2 (R&D Systems, Wiesbaden, Germany), human CD133 (Miltenyi Biotec, Bergisch-Gladbach, Germany) or human CD 45 (R&D Systems, Wiesbaden, Germany), and with the fluorescein isothiocyanate (FITC)-conjugated monoclonal antibodies against human CD34 (Serotec, Düsseldorf, Germany). Isotype-matched antibodies served as controls (Serotec, Düsseldorf, Germany). After staining, cells were washed with PBS, lysed with IO-Test 3 lysing solution according to the manufacturers instructions (Beckmann Coulter, Marseille, France), and resuspended in PBS.

To investigate whether storage of prepaired blood samples has an effect on detection of EPCs, they were stained in two sets, identically; one set was analysed immediately after preparation and the other set was stored at 4°C and analysed 17 h later.

Volumetric Flow Cytometry

The double-labelled samples were diluted 1:18 by phosphate-buffered saline (PBS, PAA Laboratories, Pasching, Austria) to 1 mL. The tubes were analyzed cytometrically, within 2 h of preparation, on a PAS III flow cytometer (Partec GmbH, Münster, Germany) equipped with a 20-mW 488-nm argon ion laser. Threshold was set at the lower end of the forward scatter (RN1) (Fig. 1a). Gates were set at forward scatter (FSC) and sideward scatter (SSC), including mononuclear cells and excluding PMNLs (R1) (Fig. 1a). Cells inside this gate were further analysed with regard to their fluorescence properties. A gate (R2) was set around the region

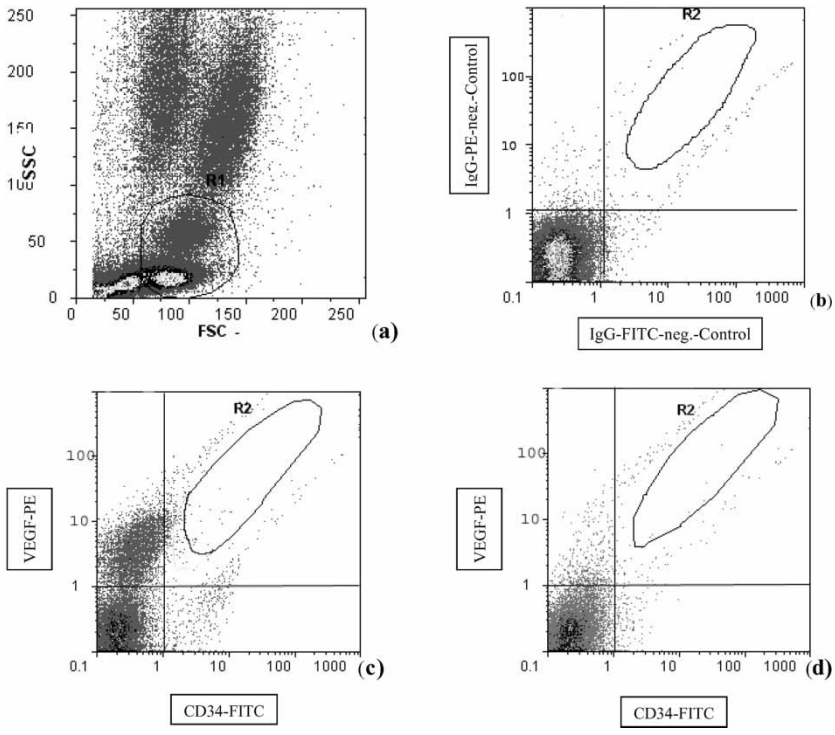

Figure 1. (a) Density plot of the flow cytometric analysis of umbilical cord blood with forward (SSC) and side light scatter (FSC). The gate was set on the mononuclear/lymphocyte fraction. (b) Density plot of IgG negative Control. c,d) Density plot of PE-conjugated anti-VEGF R2-antibodies versus FITC-conjugated anti-CD34-antibodies. Samples were treated with 0.2% formaldehyde before staining and lyses (c) and with 2% formaldehyde after staining and lyses (d). The percentage of positively stained cells with VEGF R2 was dramatically reduced after treatment with 2% formaldehyde (d).

containing the double positively stained cells for the combinations: CD34-FITC/ VEGF-R2-PE, CD34-FITC/CD133-PE, and CD34-FITC/CD45-PE (Fig. 1c, d). Isotype-matched antibodies served as controls (Fig. 1b).

The PAS III flow cytometer is able to analyse an exact sample volume and, therefore, enables all EPC quantification directly.

Purification, Cultivation, and Characterization of EPCs

Mononuclear cells were isolated by density gradient centrifugation with Histopaque-1077 (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) from 1 mL of EDTA-anticoagulated peripheral blood, and mononuclear cells were plated on 4-well culture dishes coated with human fibronectin (Sigma-Aldrich

Chemie GmbH, Steinheim, Germany) in Earl's Medium 199 (PAA Laboratories, Pasching, Austria) supplemented with 20% FCS and 10 ng/mL hrVEGF (Chemicon, Temecula, California, USA). All cell cultures were maintained at 37°C with 5% CO_2 in a humidified atmosphere. After 4 days in culture, nonadherent cells were removed by washing with PBS; adherent cells were analysed cytochemically. Cells were fixed with 4% buffered paraformaldehyde for 20 minutes, stained with 4′,6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich Chemie GmbH, Steinheim, Germany) and unconjugated monoclonal antibodies against von Willebrand Faktor (vWF; Dako, Glostrup, Denmark), and counted per well by fluorescence-microscopy. Two or three independent investigators evaluated the number of EPCs.

Statistical Analysis

Data were presented as mean $\pm$ standard deviation (SD). The t-test was used to calculate differences between the parameters obtained for leukocytes analysed live (without fixation), for those analysed after fixation procedures, and between the values obtained for cell culture. Linear regression analyses were performed to investigate the correlations between the cell count obtained in the cell culture and obtained by the flow cytometric measurement.

RESULTS

Effect of Fixation on Detection of EPCs by Flow Cytometry

As shown in Fig. 1c, d, there is a dramatic loss in detection of positively stained cells for anti-CD34-FITC, as well as anti-VEGF R2-PE after treatment with 2% formaldehyde, in comparison to the preparation with 0.2% formaldehyde. These findings were also observed for the binding of CD133 antibodies in peripheral blood, umbilical cord blood, and bone marrow samples from healthy volunteers after different procedures (see (Tables 1a–c). When the samples were treated with 2% formaldehyde, after staining and lysis, the percentages of cells which appeared to be stained positively with CD34, VEGF R2, or CD133 were significantly reduced ($p < 0.05$) when compared both with samples which had been treated with 0.2% formaldehyde before staining and lyses, and those that were analyzed untreated (no fixation). Particularly, for PE-conjugated VEGF R2, these decreases were more marked and highly significant ($p < 0.01$) (Tables 1a–c).

There were, however, no significant differences between the percentage of cells which were positively stained with the PE-CD45-antibody when samples were prepared using 0.2% or 2% formaldehyde (Tables 1a–c).

No significant differences were observed in the percentage of cells that were positively stained with the PE-CD133, PE-VEGF R2, and FITC-CD34 antibodies

Table 1a. Effect of preparative procedures (untreated, 0.2% and 2% formaldehyde) on the binding of CD34, VEGF R2, CD133, and CD45 antibodies to leucocytes in peripheral blood

	Positively stained cells (%)		
Antibody	Untreated	0.2% Formaldehyde	2% Formaldehyde
CD34	3.3 (±0.7)	2.9 (±1)	**1.3 (±0.6)**[a]
VEGF R2	5 (±0.9)	5.3 (±2.9)	**1.9 (±0.6)**[a]
CD133	3.2 (±0.8)	3.2 (±2.1)	**1.4 (±0.5)**[a]
CD45	77.8 (±8.2)	79.7 (±11)	79.5 (±6.7)

Results are the means ± SD (n = 20).
[a]Untreated versus 2% formaldehyde: $p < 0.05$.

when the samples were treated with 0.2% formaldehyde before staining, in comparison with the untreated samples (Tables 1a–c). The counted EPCs by flow cytometry did not significantly differ from the untreated samples and the samples that were pre-treated with 0.2% formaldehyde.

Relationship of EPC Quantification Between FACS Analyses and Cell Culture Assay

Peripheral blood mononuclear cells were cultured and differentiated as described above. At day 4, EPCs appeared with a typical spindle-shaped morphology.[6] EPCs were further characterized by staining with anti-von Willebrand Faktor (vWF). At day four, 85% of the attached cells stained positively for this marker.

Results for the direct comparison of FACS-analyses and cell culture assay (EPC-assay) are presented in Figure 2a. The number of EPCs measured by

Table 1b. Effect of preparative procedures (untreated, 0.2% and 2% formaldehyde) on the binding of CD34, VEGF R2, CD133, and CD45 antibodies to leucocytes in umbilical cord blood

	Percentage of positively stained cells		
Antibody	Untreated	0.2% Formaldehyde	2% Formaldehyde
CD34	1.7 (±0.2)	1.9 (±0.6)	**1 (±0.2)**[a]
VEGF R2	2.8 (±1.2)	4 (±1.4)	**1.4 (±1.3)**[a]
CD133	2.4 (±0.2)	2.7 (±0.1)	**1.3 (±0.1)**[a]
CD45	72 (±5.7)	88.5 (±2.1)	67 (±1)

Results are the means ± SD (n = 10).
[a]Untreated versus 2% formaldehyde: $p < 0.05$.

Table 1c. Effect of preparative procedures (untreated, 0.2% and 2% formaldehyde) on the binding of CD34, VEGF R2, CD133, and CD45 antibodies to leucocytes in bone marrow

	Positively stained cells (%)		
Antibody	Untreated	0.2% Formaldehyde	2% Formaldehyde
CD34	3.7 (±0.3)	4.6 (±0.1)	**2 (±0.1)**[a]
VEGF R2	3.5 (±0.7)	5.5 (±0.1)	**1.2 (±0.1)**[a]
CD133	2.4 (±0.2)	2.8 (±0.1)	**0.9 (±0.1)**[a]
CD45	87.5 (±0.7)	82.5 (±3.5)	84 (±1.4)

Results are the means ± SD (n = 4).
[a]Untreated versus 2% formaldehyde: $p < 0.05$.

flow cytometry corresponds to the number of EPCs detected by cell culture assay. The correlation between the flow cytometric measurements and cell count of the cell culture method was excellent ($r = 0.98$, $p < 0.01$) (Fig. 2b).

Effect of Storage on Detection of EPCs by Flow Cytometry

As the pre-treatment with 0.2% formaldehyde leads to an EPC-count not different from the measurement of the untreated samples, as shown above, we investigated the effect of storage during 17 h on the detection of EPCs of samples pre-treated with 0.2% formaldehyde.

After a conservation period of 17 h at a temperature of 4°C in the dark, the pre-treated samples did not show any significant differences in the percentage of cells which were measured as stained positively, compared with the measurements of the identical samples immediately after staining (Table 2). The experiments were done with the PE or FITC-conjugated antibodies against CD34, VEGF R2, CD133, and CD45. The number of counted EPCs by flow cytometry did not significantly differ from the untreated and the pre-treated samples, as shown by regression analyses ($r = 0.96$, $p < 0.05$) (Fig. 3).

DISCUSSION

Since there is no standard technique for EPC-quantification in peripheral blood, the results of stem cell investigations will remain hardly comparable between research groups using different methods of preparation procedures and antibody combinations.[2] We have shown, that strong fixation with 2% formaldehyde, as practised by some groups,[8,9] leads to a significant

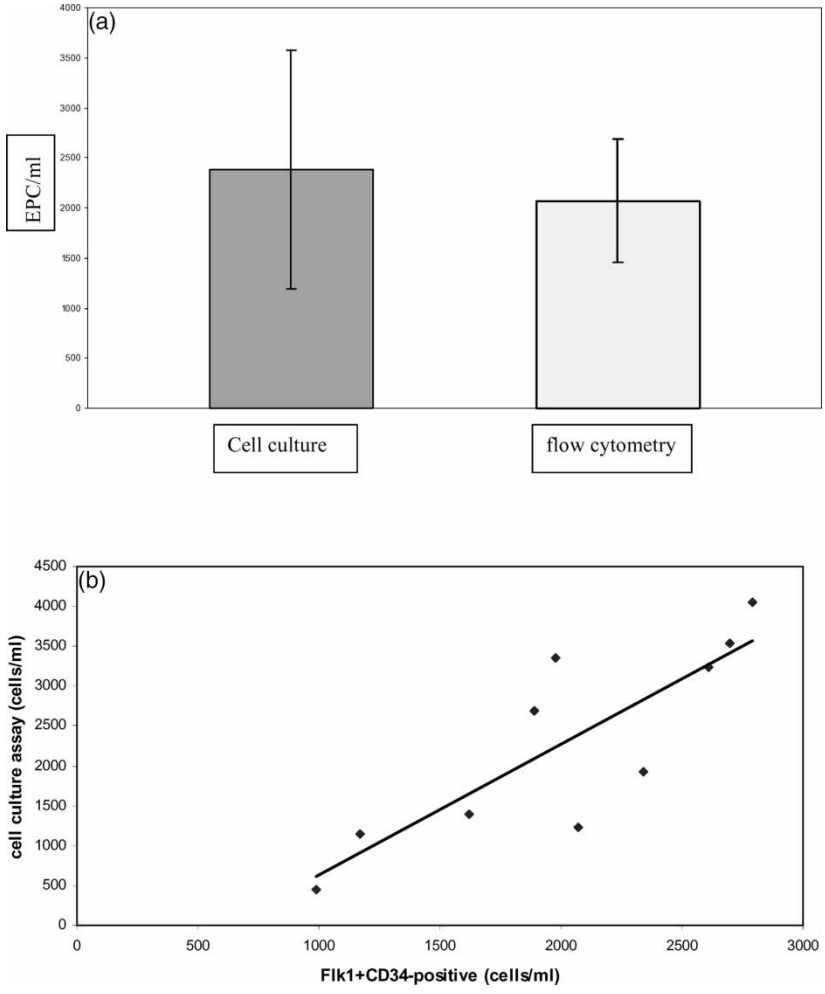

Figure 2. (a) Means of detected EPC/mL by cell culture assay and flow cytometric measurement (n = 11). (b) Comparison of the number of mononuclear cells from peripheral blood which were measured by FACS-analysis to be double positively stained with PE-VEGF R2 and FITC-CD34 with the number of adherent cells in an EPC-assay (cells/mL blood). The blood samples were taken of 11 volunteers. (Correlation coefficient: r = 0.85, n = 11).

reduction of the antibody binding. Therefore, the detection of EPCs was impaired after fixation with 2% formaldehyde.

Our presented method of preparation, with moderate fixation of the blood samples with 0.2% formaldehyde, and analyses of double positively stained mononuclear cells for PE-VEGF R2 and FITC-CD34, leads to highly reproducible results of numbers of EPCs per mL EDTA-blood and offers the

Table 2. Effect of conservation

Antibody	Positively stained cells (%)	
	Measured immediately	Measured 17 h later
CD34	1.9 (±0.6)	1.5 (±0.1)
VEGF R2	4 (±1.4)	4.2 (±0.5)
CD133	2.7 (±0.1)	3.7 (±1.1)
CD45	88.5 (±2.1)	91 (±2.8)

The effect of conservation over 17 h at 4°C of 0.2 % formaldehyde pre-treated samples after staining and lyses on the percentage of cells which appeared as stained positively with FITC-CD34, PE-VEGF R2, PE-CD133, and PE-CD45 antibodies to leucocytes. Results are the means ± SD (n = 20).

possibility to store the probes overnight before flow cytometric analyses. The results showed excellent correlation with the cell culture method (Fig. 3). The measured concentrations of EPCs in the peripheral blood matched the values reported in the literature.[7–10]

Discussion still goes on as to which marker proteins should be used to characterise the EPC fraction in peripheral blood. EPCs are defined as cells positive for both haematopoietic stem cell markers, such as CD34 and an endothelial marker protein such as VEGF R2.[7] Because CD34 is not exclusively expressed on haematopoietic stem cells but, at a lower level, also on

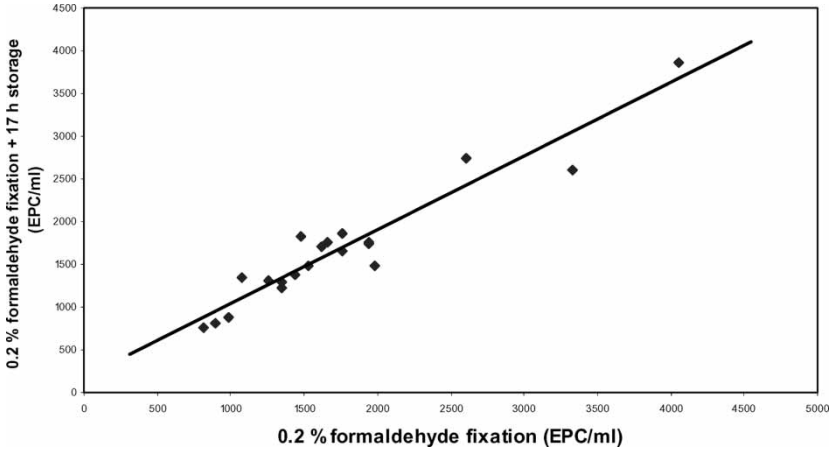

Figure 3. Comparison of the number of EPCs from peripheral blood measured by FACS-analysis immediately after staining and 17 h later, after storage at 4°C. (Correlation coefficient: r = 0.96, n = 20).

mature endothelial cells, further studies used the more immature haematopoietic stem cell marker CD133[3] and demonstrated that purified CD133+ cells can differentiate to endothelial cells in vitro.[15] CD133, also known as prominin or AC133, is a highly conserved antigen with unknown biological activity, which is expressed on haematopoietic stem cells but is absent on mature endothelial cells and monocytic cells.[16] Thus, CD133+/VEGF R2+ cells more likely reflect immature progenitor cells, whereas CD34+/VEGF R2+ may also represent shredded cells of the vessel wall.

A possible mix of both early progenitor and endothelial phenotype are CD133+/CD34+/VEGF R2+ cells, which do not express vascular endothelial (VE) cadherin and von Willebrand factor. Cells with these characteristics are localized predominantly in the bone marrow.[4] In the peripheral circulation of adults, more mature EPCs are found that obviously have lost CD133, but are positive for CD34 and VEGF R2. Mature ECs show a high expression of VEGF R2, VE-cadherin and von Willebrand factor. CD133 is not detectable on the surface of human umbilical vein ECs.[17] It seems that the loss of CD133 reflects the transformation of circulating EPCs into more mature endothelial-like cells. However, it is unclear at which time point the EPCs begin to lose CD133 during their transmigration from the bone marrow into the systemic circulation, or later during their circulation.

On the background of these observations and considerations, we decided to count the VEGF R2+/CD34+ cells because the EPC, anyway, should be contained in this fraction. To exclude a major portion of shredded cells from the vessel wall, we measured the number of CD133+/CD34+ cells, in addition. The CD133+/CD34+ cell-fraction should contain the immature progenitor cells. We have seen a correlation between the numbers of VEGF R2+/CD34+ cells and the number of CD133+/CD34+ cells, which was between 3:1 and 2:1 (data not shown). If the number of VEGF R2+/CD34+ cells exceeds the number of CD133+/CD34+ cells more than 3 times, this could be a sign of a major portion of shredded cells from the vessel wall. (In our data we did not observe such a case.)

Our presented protocol for the flow cytometric measurement of EPCs in peripheral blood can be used as a diagnostic or prognostic tool; we propose this protocol as the standard for the EPC quantification. The excellent correlation with the results from the endothelial progenitor cell culture could be an argument to replace the cell culture method by flow cytometry alone in future EPC-studies, because the cell culture is more susceptible to external influences and is more time consuming.

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