Miltenyi Biotec

New cell culture medium maintains phenotype and functional pluripotency of human iPSC lines

StemMACS™ PSC-Brew XF, human

Constantin Berger[a], Alevtina Cubukova[b], Dr. Daniela Zdzieblo[b], and Dr. Antje Appelt-Menzel[a]
[a]University Hospital Würzburg, Germany
[b]Fraunhofer Institute for Silicate Research (ISC), Würzburg, Germany

Background

Induced pluripotent stem cells (iPSCs) are invaluable resources for generating tissue-specific cells for disease modeling, regenerative therapy, and drug discovery. As achieving success with these applications depends heavily on the quality of the starting iPSC population, adhering to the best practice for culture and maintenance of such cells is a vital step.

This requires a culturing media produced under stringent quality control that ensures the maintenance of typical morphology, high expression of pluripotency markers, and the ability to differentiate into varying cell types.

This application note describes a detailed phenotypical characterization of a human iPSC line first maintained in StemMACS PSC-Brew XF, human and then differentiated into endothelial cells from the blood-brain barrier and pancreatic-like cells (hormone-secreting cells of the endocrine pancreas).

The human iPSCs showed all key pluripotent stem cell characteristics during culture, cryopreservation and thawing, in addition to having full terminal fate differentiation capacity as demonstrated by endothelial and pancreatic differentiation.

Materials and methods

- StemMACS PSC-Brew XF, human (130-127-865)
- FcR Blocking Reagent, human (130-059-901)
- iMR 90-4 human iPSCs
- Matrigel coated plates
- Gentle Cell Dissociation Reagent (STEMCELL Technologies)
- Accutase (Sigma-Aldrich)
- Total RNA Isolation Kit (VWR Life Science)
- cDNA Synthesis Kit (Bio-Rad)
- SsoAdvanced Universal SYBR Green Supermix (Bio-Rad)
- Fixation and Permeabilization Solution (BD Biosciences)
- Perm/Wash Buffer (BD Biosciences)
- KnockOut Serum Replacement (ThermoFisher)
- Dimethyl sulfoxide (DMSO) (Sigma-Aldrich)

Human iPSC culture and maintenance

The iMR 90-4 human iPSCs were maintained either in StemMACS PSC-Brew XF, human or in another comparable commercially available medium (control medium). The cells were passaged at 60–80% confluency using a cell dissociation reagent, plated onto Matrigel®-coated tissue culture plastic at a 1:6 to 1:20 ratio and incubated with fresh medium supplemented with 10 µM Y-27632 (ROCK inhibitor) for 24 h (passage number 8). The pluripotency of the human iPSC line was confirmed after 8 passages by quantitative RT-PCR (RT-qPCR), immunofluorescence, and flow cytometry for pluripotency marker genes and proteins.

The human iPSCs were then harvested as cell clusters, resuspended in freezing medium (90% KnockOut™ Serum Replacement, 10% DMSO), and frozen in liquid nitrogen. After 8 weeks, the frozen human iPSCs were thawed, passaged (passage number 8+4), and analyzed again for pluripotency marker proteins by flow cytometry.

Directed differentiation of human iPSCs

Directed differentiation of the human iPSCs maintained in StemMACS PSC-Brew XF, human and the control medium into pancreatic-like cells, started as a monolayer culture based on established protocols[1,2]. The spheroids were generated on day 15 of differentiation and characterized on day 22 by immunofluorescence.

Additionally, both human iPSC populations were differentiated into endothelial cells from the blood-brain barrier according to the published protocol[3]. The human iPSC-derived endothelial cells were then characterized by immunofluorescence on day 10 of differentiation.

Reverse transcription and quantitative polymerase chain reaction (RT-qPCR)
Cells were lysed in 350 µl TRK buffer containing 0.286 M β-mercaptoethanol followed by total RNA isolation using the Total RNA Isolation Kit. cDNA was synthesized from 500 ng of isolated RNA using the cDNA Synthesis Kit. RT-qPCR reactions were set up in technical duplicates and performed with the respective primers and SsoAdvanced™ Universal SYBR® Green Supermix Green Supermix in 10 µl reaction volume using CFX96 Touch Real-Time PCR Detection System.

RPL4 was used as the housekeeping gene (internal control) and normalized fold changes (relative to the human iPSCs maintained in the control medium) were calculated by the ΔΔCT-method, assuming an amplification efficiency of 100%. The data shown is the avarage of three or two technical replicates of cells maintained in StemMACS PSC-Brew XF, human or the control medium, respectively.

Immunofluorescence staining
The human iPSCs and iPSC-derived endothelial cells were fixed for 20 minutes, permeabilized, blocked, and incubated first overnight with the primary antibody at 4 °C, and then the secondary antibody at RT for 1 hour. The samples were mounted in a mounting medium containing DAPI.

The differentiated pancreatic spheroids were fixed for 60 min, embedded in paraffin, cut into 5 µm thick sections, and mounted on superfrost or poly-l-lysine-coated glass slides. The samples were then dewaxed at 60 °C for 1 h followed by incubation in xylene and rehydrated in a descending EtOH series. After the heat-mediated antigen retrieval, samples were permeabilized, blocked, and incubated first overnight with the primary antibody at 4 °C , and then the secondary antibody at RT for 2 hours. The samples were mounted in a mounting medium containing DAPI.

Flow cytometry
Cells were harvested as single cells (using Accutase®) and washed twice with FACS Buffer (2mM EDTA, 1% BSA in DPBS (w/o) Ca, Mg)). For each staining 1 to 2×10^5 cells were used.

For the cell surface proteins (TRA1-60, TRA1-81, SSEA4), cells were incubated with an FcR-blocking reagent (15 min at 4 °C), washed (FACS buffer), and then stained with FACS antibody (diluted in FACS buffer, 30 min at 4 °C). For the intracellular proteins (NANOG, OCT4, SOX2), cells were incubated in Fixation/Permeabilization solution (20 min at 4 °C), washed (Perm/Wash™ Buffer, 2×), and then stained with FACS antibodies (diluted in Perm/Wash Buffer, 30 min at 4 °C).

After staining, cells were washed (3×), resuspended in 500 µl FACS buffer, and analyzed using BD Accuri C6 Plus Personal Flow Cytometer. In all flow cytometry analyses, an appropriate isotype control was used. Additionally, the positive population was defined where the fluorescent signal intensity exceeded that of the isotype control. Data from three (maintained in StemMACS PSC-Brew XF, human) or two (maintained in the control medium) technical replicates were averaged, and the percentage of positive cells among all cells were calculated and subjected to statistical analyses.

Results

Human iPSCs maintained in StemMACS PSC-Brew XF, human show appropriate gene and protein expression after continuous cultivation
IPSCs must show combined expression of key pluripotency-related markers, such as POU5F1 (POU Class 5 Homeobox 1, OCT4), SOX2 (SRY (sex determining region Y)-box 2), NANOG, TRA-1-60 (Podocalyxin), TRA-1-81, and SSEA4 (stage-specific embryonic antigen-4).

After the initial expansion (8 passages) of the human iPSCs, cells were analyzed to determine whether they retained their pluripotent properties both at gene and protein levels. Therefore, 3 key pluripotency markers (OCT4, SOX2, and NANOG) were assessed by RT-qPCR, immunofluorescence, and flow cytometry.

Gene expression analysis showed high expression of all three key pluripotency markers, for both conditions. Interestingly, expression levels of pluripotency marker *POU5F1 (OCT4)* were significantly higher in the human iPSCs maintained in StemMACS PSC-Brew XF, human, compared to the control medium. No statistically significant differences were detected between the two populations regarding the transcription of *SOX2* and *NANOG* (fig. 1).

From a qualitative point of view, the immunofluorescence data showed that the corresponding proteins as well as TRA-1-60, were expressed in all cells to a similar extent (fig. 2).

A further qualitative and quantitative analysis was performed using fluorescence-based flow cytometry for pluripotency and additional cell surface markers (TRA-1-60, TRA-1-81, and SSEA4). It was found that iPSCs cultured in both media showed expression of all evaluated pluripotency-associated markers (fig. 3A).

The percentage of OCT4, NANOG, and SSEA4 positive cells among all cells were comparable in the two populations (no significant differences), whereas the percentages of TRA-1-60 and TRA-1-80 positive cells were significantly higher in human iPSCs maintained in StemMACS PSC-Brew XF, human. Additionally, the percentage of SOX2 positive cells was significantly lower in the cells maintained in StemMACS PSC-Brew XF, human, compared to the control medium (fig. 3B).

Human iPSCs maintained in StemMACS PSC-Brew XF, human show appropriate protein expression after cryopreservation
A crucial step in the stem cell culture workflow is the cryopreservation of human iPSCs after they are derived and expanded. If not performed correctly, spontaneous differentiation or sample loss is possible. To facilitate the procedure and support the cells to retain their pluripotent characteristics, a complete and rich culture medium is needed. To this end, the expression of pluripotency markers was assessed again after the cells were subjected to cryopreservation and passaging (passage number 8+4) using flow cytometry. Cells in both conditions showed expression of pluripotency markers (fig. 4A).

The percentages of the positive cells were calculated and no statistically significant difference was detected between the two populations (fig. 4B).

Human iPSCs maintained in StemMACS PSC-Brew XF, human maintain full pluripotency and can differentiate into blood-brain barrier endothelial and pancreatic-like cells

The key characteristic of iPSCs is their ability to differentiate into any cell type. To evaluate the effect of the culturing media on the functional pluripotency of the cells, iPSCs were differentiated into endothelial cells from the blood-brain barrier or pancreatic-like cells (hormone-secreting cells of the endocrine pancreas) under defined conditions.

The identity of the generated cells was determined through the detection of marker proteins for each cell type. These proteins were detected by immunofluorescence on day 10 of the differentiation protocol for the blood-brain barrier endothelial cells (fig. 5) and day 22 of the differentiation protocol for the pancreatic-like cells (fig. 6). The immunofluorescence data showed that the corresponding proteins in both cell types were expressed in the two populations to a similar extent. Therefore, the human iPSCs maintained in StemMACS PSC-Brew XF, human are capable of differentiating under defined culture conditions into blood-brain barrier endothelial cells and pancreatic-like cells.

Conclusions

Human iPSC lines maintained in StemMACS PSC-Brew XF, human show:

- high and consistent expression of pluripotency markers in continuous cultivation;
- high and consistent expression of pluripotency markers after cryopreservation; and
- an ability to maintain pluripotency and differentiation potential into various cell types.

Together, these results indicate the high quality of human iPSCs maintained in StemMACS PSC-Brew XF, human medium.

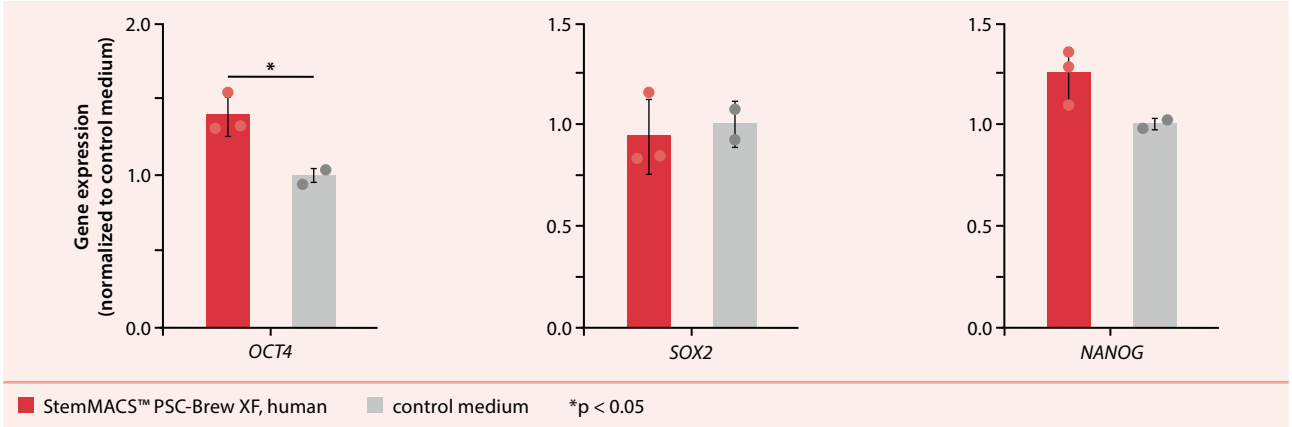

Figure 1: Gene expression analysis showed high expression of all three key pluripotency markers, for human iPSCs maintained in StemMACS PSC-Brew XF, human or the control medium after 8 passages. Presented data is the average of three or two technical replicates of cells maintained in StemMACS PSC-Brew XF, human or the control medium, respectively.

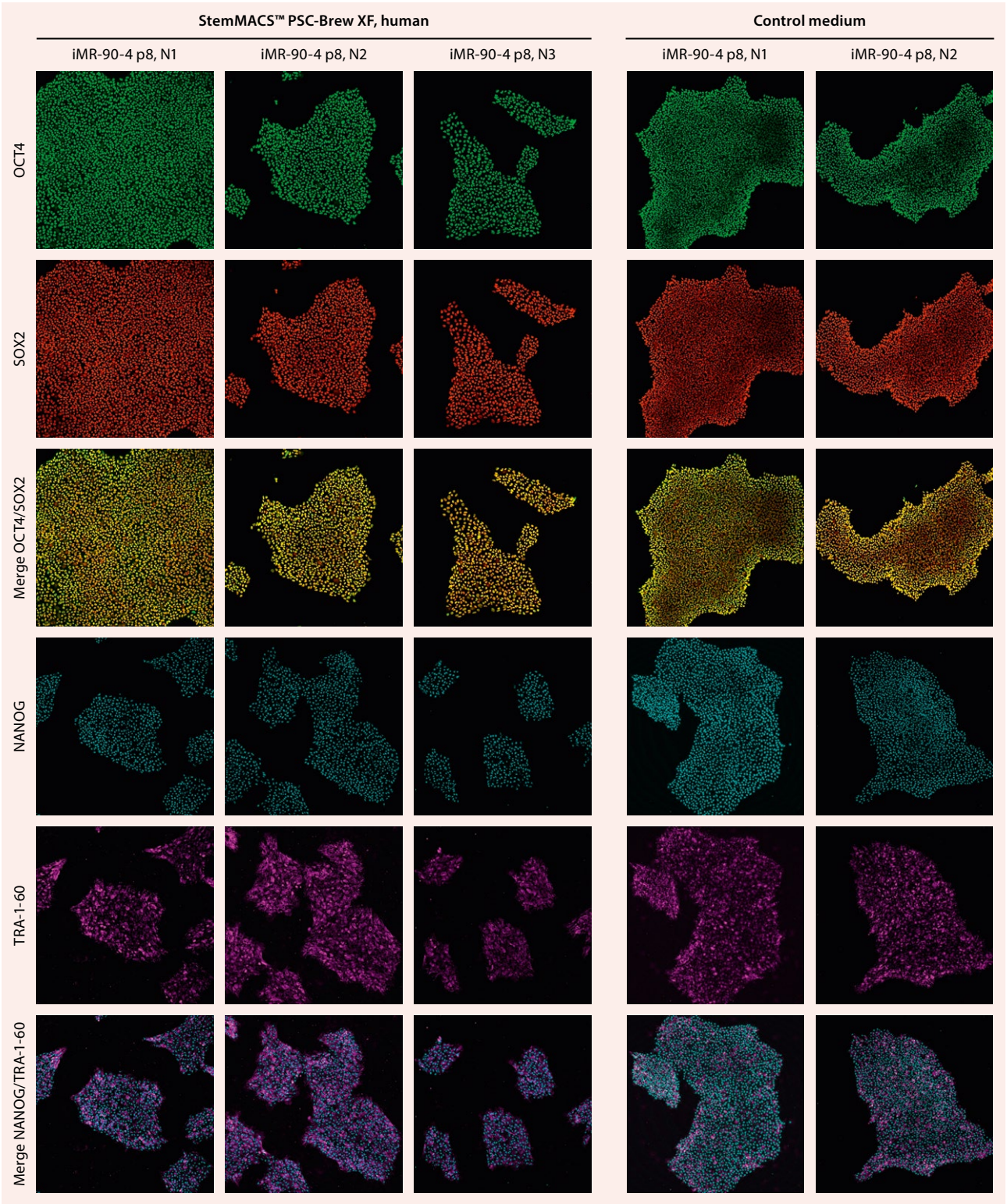

Figure 2: Protein expression analysis, assessed by immunofluorescence, showed high expression of all key pluripotency markers for human iPSCs maintained in StemMACS PSC-Brew XF, human or the control medium after 8 passages. Presented data is from three or two technical replicates of cells maintained in StemMACS PSC-Brew XF, human or the control medium, respectively.

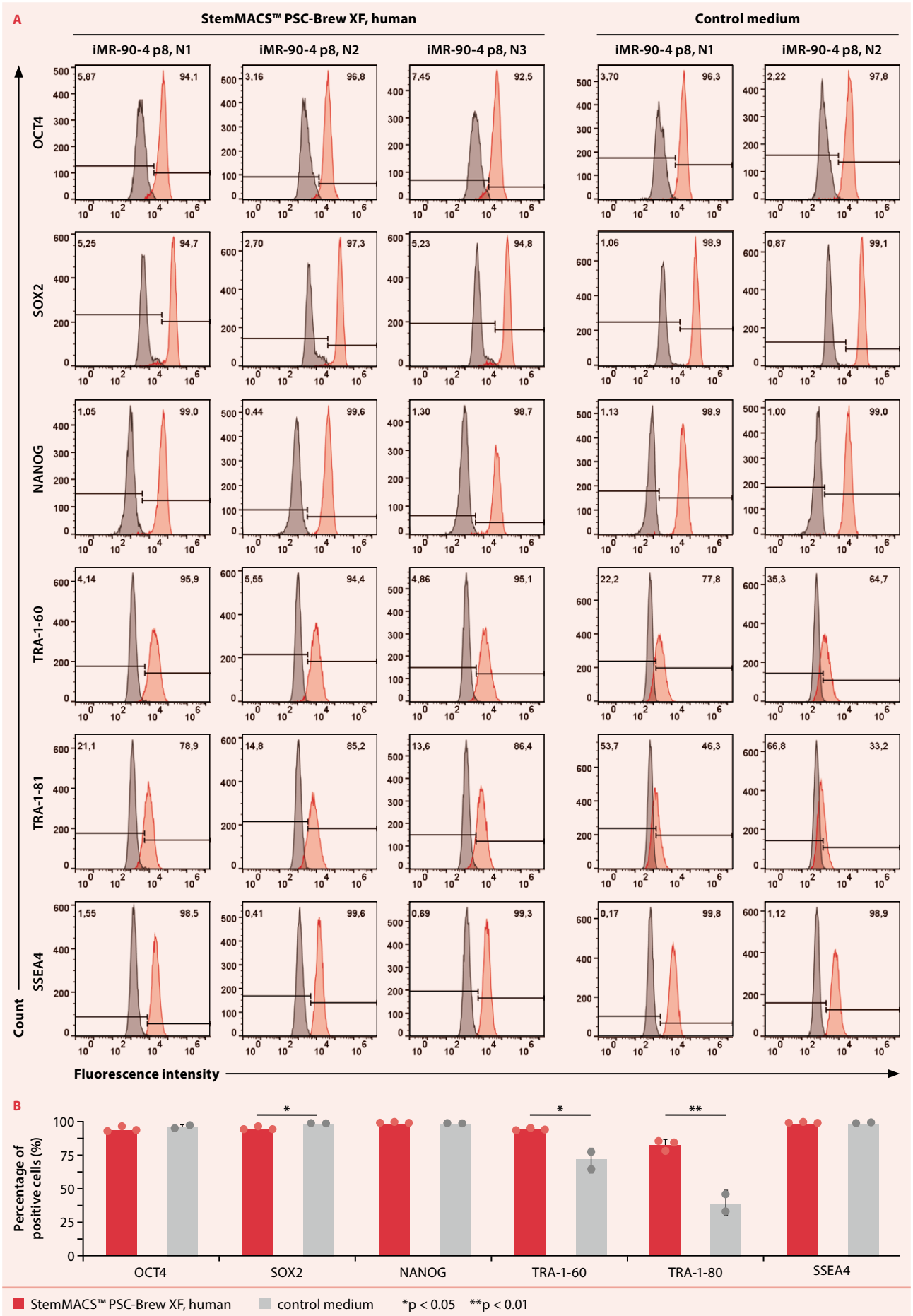

Figure 3: Protein expression analysis showed high expression of all key pluripotency markers for human iPSCs maintained in StemMACS PSC-Brew XF, human or the control medium after 8 passages. The positive population was defined where the fluorescent signal intensity exceeded that of the isotype control. Presented data is the average of three or two technical replicates of cells maintained in StemMACS PSC-Brew XF, human or the control medium, respectively.

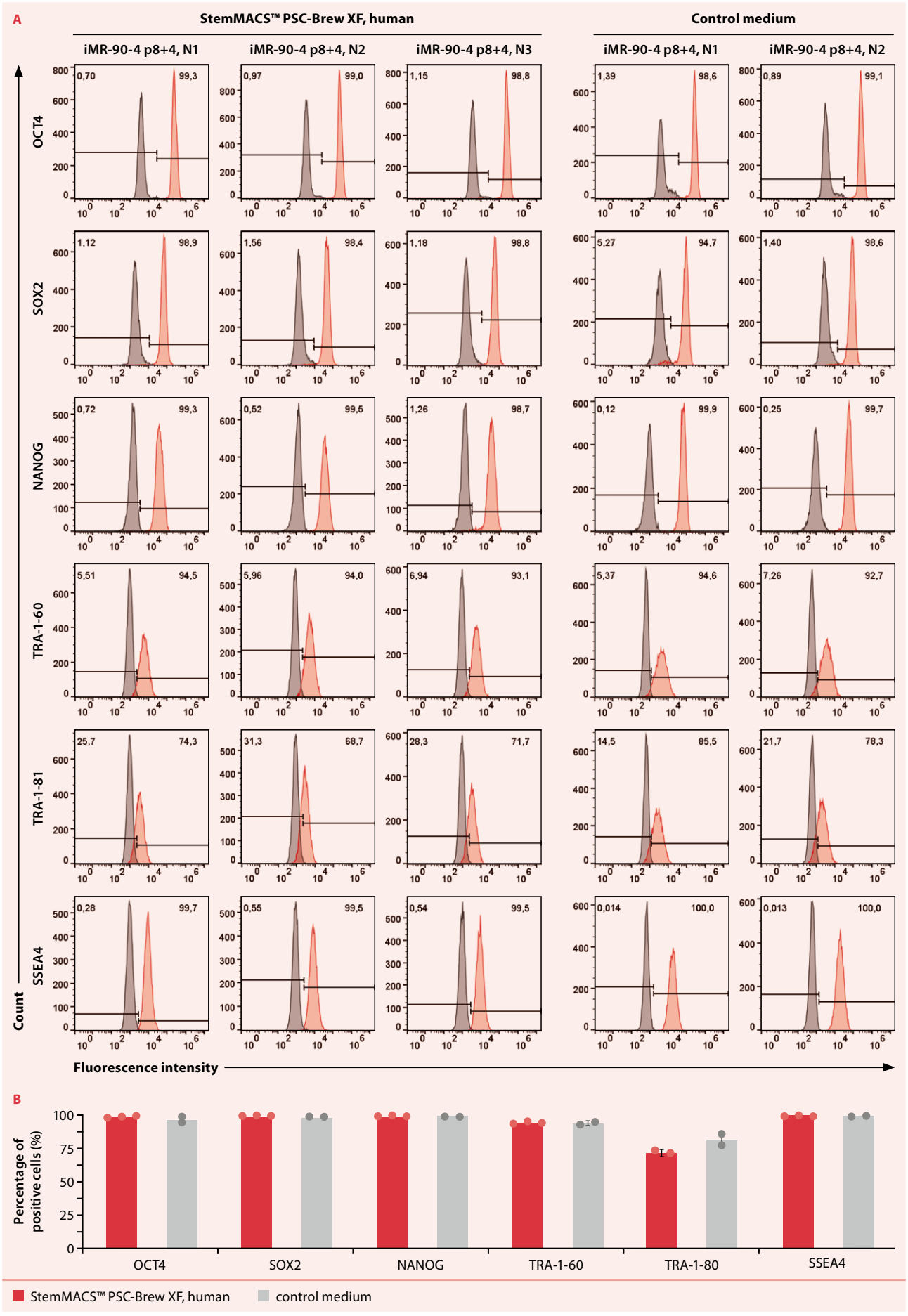

Figure 4: Protein expression analysis showed high expression of all key pluripotency markers for human iPSCs maintained in StemMACS PSC-Brew XF, human or the control medium (passage number 8+4). The positive population was defined where fluorescent signal intensity exceeded that of the isotype control. Presented data is the average of three or two technical replicates of cells maintained in StemMACS PSC-Brew XF, human or the control medium, respectively.

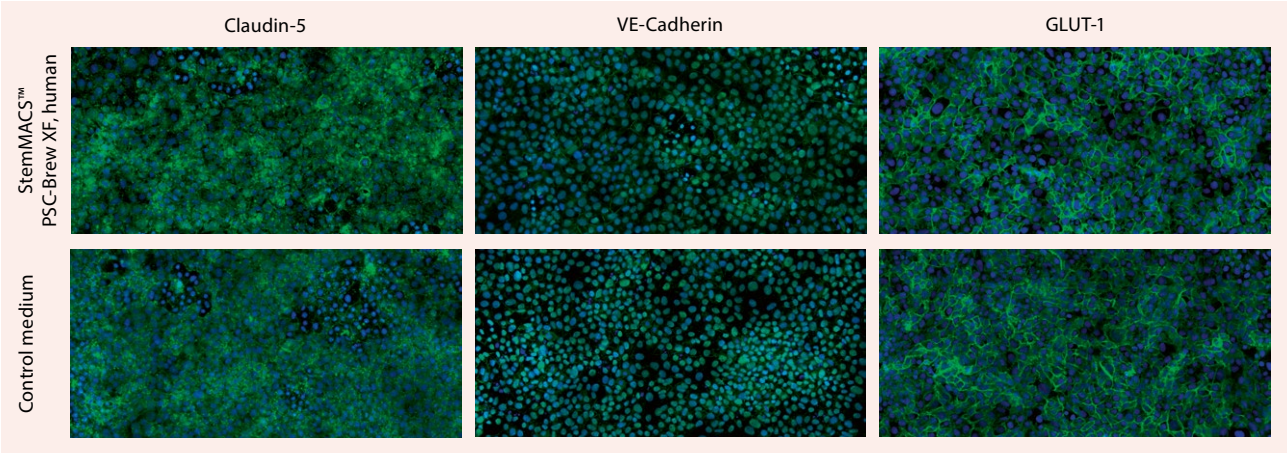

Figure 5: Expression of blood-brain barrier endothelial cell markers, assessed by immunofluorescence, is similar in human iPSC-derived cells maintained in StemMACS PSC-Brew XF, human and control medium.

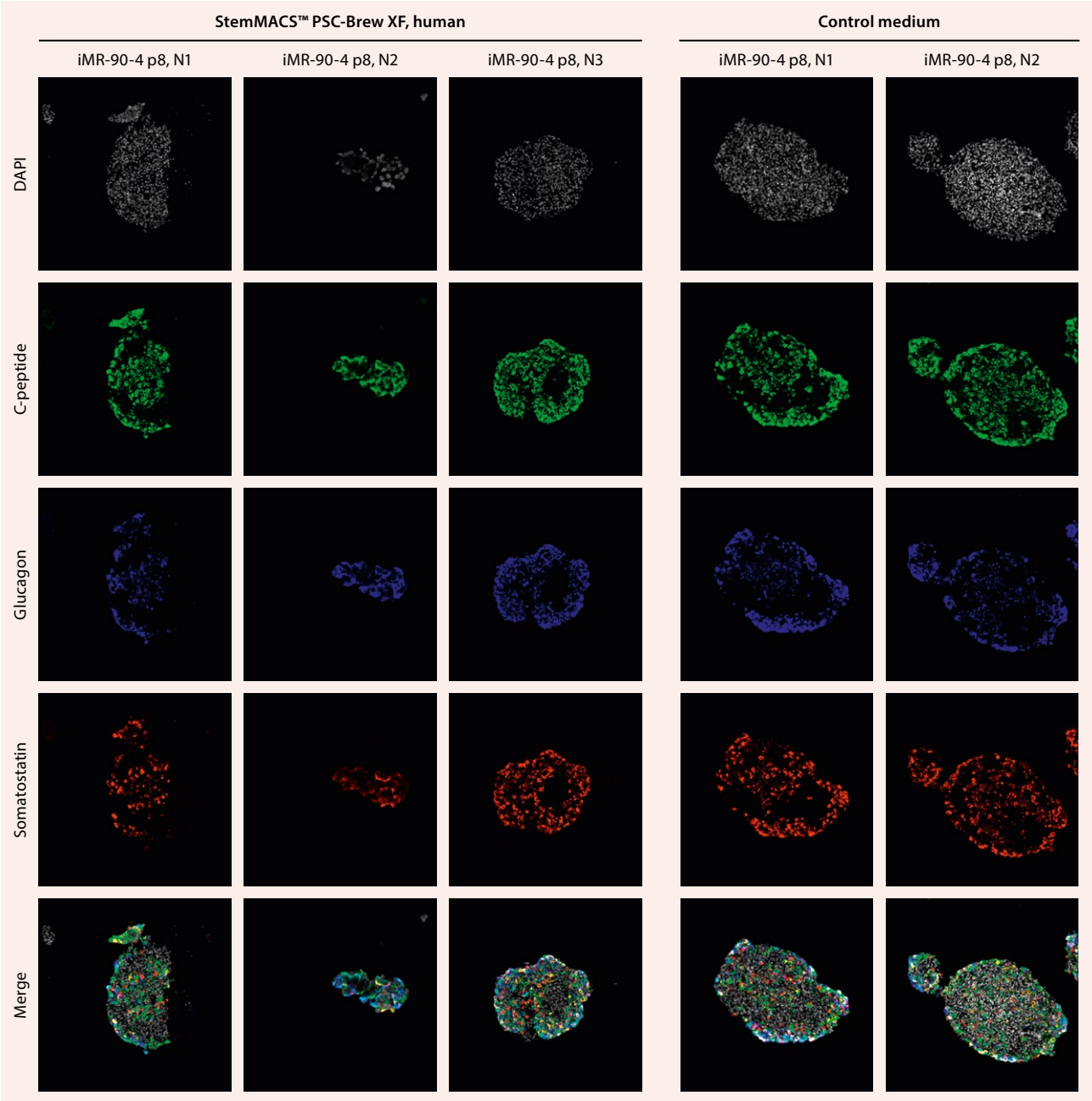

Figure 6: Expression of pancreatic-like cell markers, assessed by immunofluorescence, is similar in human iPSC-derived cells maintained in StemMACS PSC-Brew XF, human and control medium. Presented data is from three or two technical replicates of cells maintained in StemMACS PSC-Brew XF, human or the control medium, respectively.

References

1. Rezania, A. *et al.* (2012) Maturation of human embryonic stem cell-derived pancreatic progenitors into functional islets capable of treating pre-existing diabetes in mice. Diabetes. 61:2016-2029.
2. Rezania, A. *et al.* (2014) Reversed of diabetes with insulin-producing cells derived *in vitro* from human pluripotent stem cells. Nature Biotechnology. 32:1121-1133.
3. Appelt-Menzel, A. *et al.* (2017) Establishment of a Human Blood-Brain Barrier Co-culture Model Mimicking the Neurovascular Unit Using Induced Pluri- and Multipotent Stem Cells. Stem Cell Reports. 8:894-906.

Miltenyi Biotec

Miltenyi Biotec B.V. & Co. KG | Phone +49 2204 8306-0 | Fax +49 2204 85197 | macsde@miltenyi.com | **www.miltenyibiotec.com**

Miltenyi Biotec provides products and services worldwide. Visit **www.miltenyibiotec.com/local** to find your nearest Miltenyi Biotec contact.

130-132-532