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Quantitative assessment of human pluripotent stem cell differentiation potential

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Introduction

Human pluripotent stem cells (hPSCs) play an important role in disease modeling, drug discovery, and cell therapy applications. Nowadays, generation of hiPSCs is a standard procedure. Resulting cell lines need to be characterized after reprogramming and maintenance culture and must fulfill certain criteria, such as characteristic morphology, long-term self-renewal, karyotypic stability, expression of a specific marker profile, and differentiation capacity into all three germ layers. Differentiation capacity can be assessed *in vivo* using teratoma assay or *in vitro* using spontaneous or directed differentiation assays.

To assess the potential of PSCs, we developed a standardized, quantifiable differentiation assay based on lineage-specific, complete media which support directed 2D differentiation into all three germ layers within 7 days. The assay allows quantitative flow cytometry analysis as well as immunocytochemistry assessment. As proof of principle, four hiPSC lines were differentiated repeatedly and analyzed by flow cytometry and immunocytochemistry. The quantitative, flow cytometry–based analysis of all cell lines confirmed reproducible properties for their differentiation into ectoderm, mesoderm, and endoderm. Importantly, the assay revealed subtle differences in their intrinsic propensity to give rise to cells of the three germ layers, illustrating a convenient way to assess the differentiation potential of hPSC lines.

Methods

1 StemMACS™ Trilineage Differentiation Kit protocol schematic

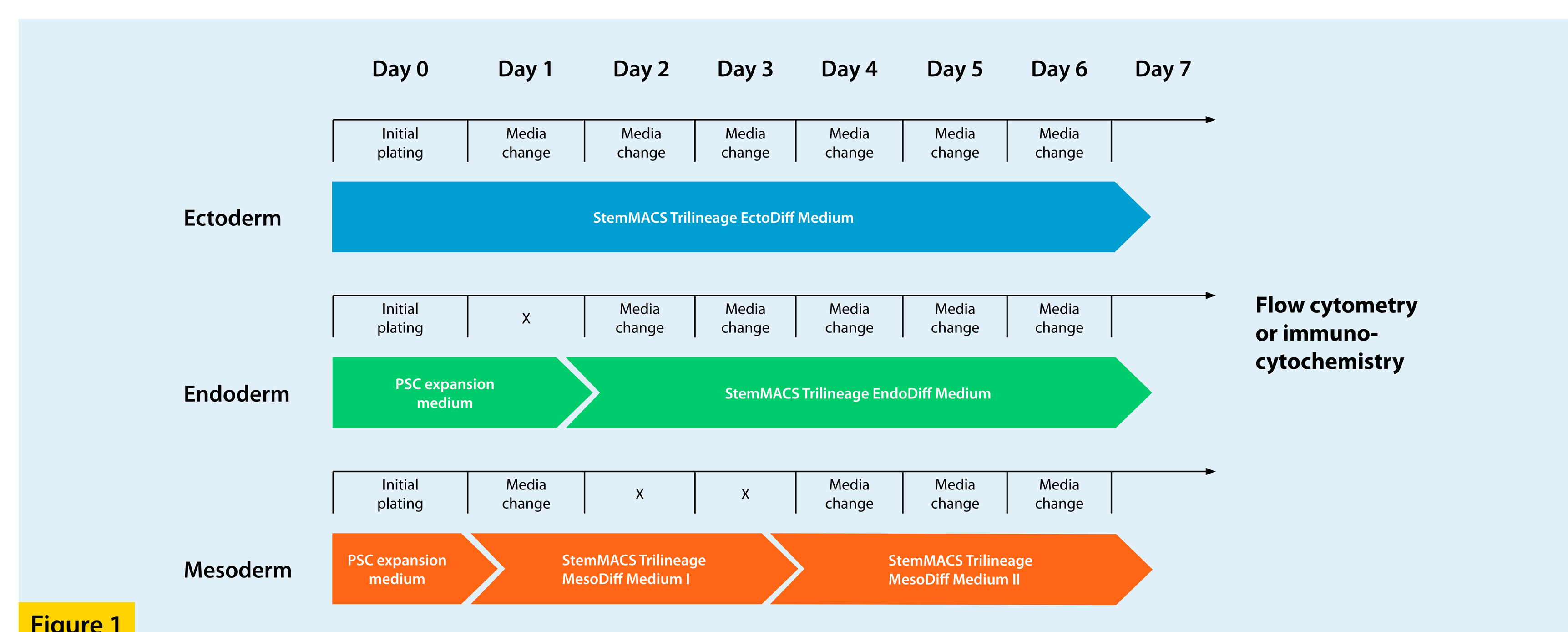

Figure 1

Lineage-specific differentiation was started on day 0 by seeding defined numbers of hPSCs into 12-well plates. The use of 24-well plates is recommended when final analysis is done by immunocytochemistry.

For differentiation into ectoderm, cells were seeded directly using Trilineage EctoDiff Medium supplemented with ROCK inhibitor; from day 1 to day 6 media changes were performed daily. For differentiation into endo- or mesoderm, cells were seeded using the PSC expansion medium supplemented with ROCK inhibitor. Differentiation into endoderm started on day 2 using Trilineage EndoDiff Medium, with daily media change until day 6. Mesoderm induction began on day 1 using Trilineage MesoDiff Medium I for 3 days; from day 4 to day 6 media changes were performed daily using Trilineage MesoDiff Medium II. Analyses of cells from all three differentiation pathways were done on day 7 using either flow cytometry or immunocytochemistry.

Results

1 Quantitative analyses of trilineage differentiation using flow cytometry

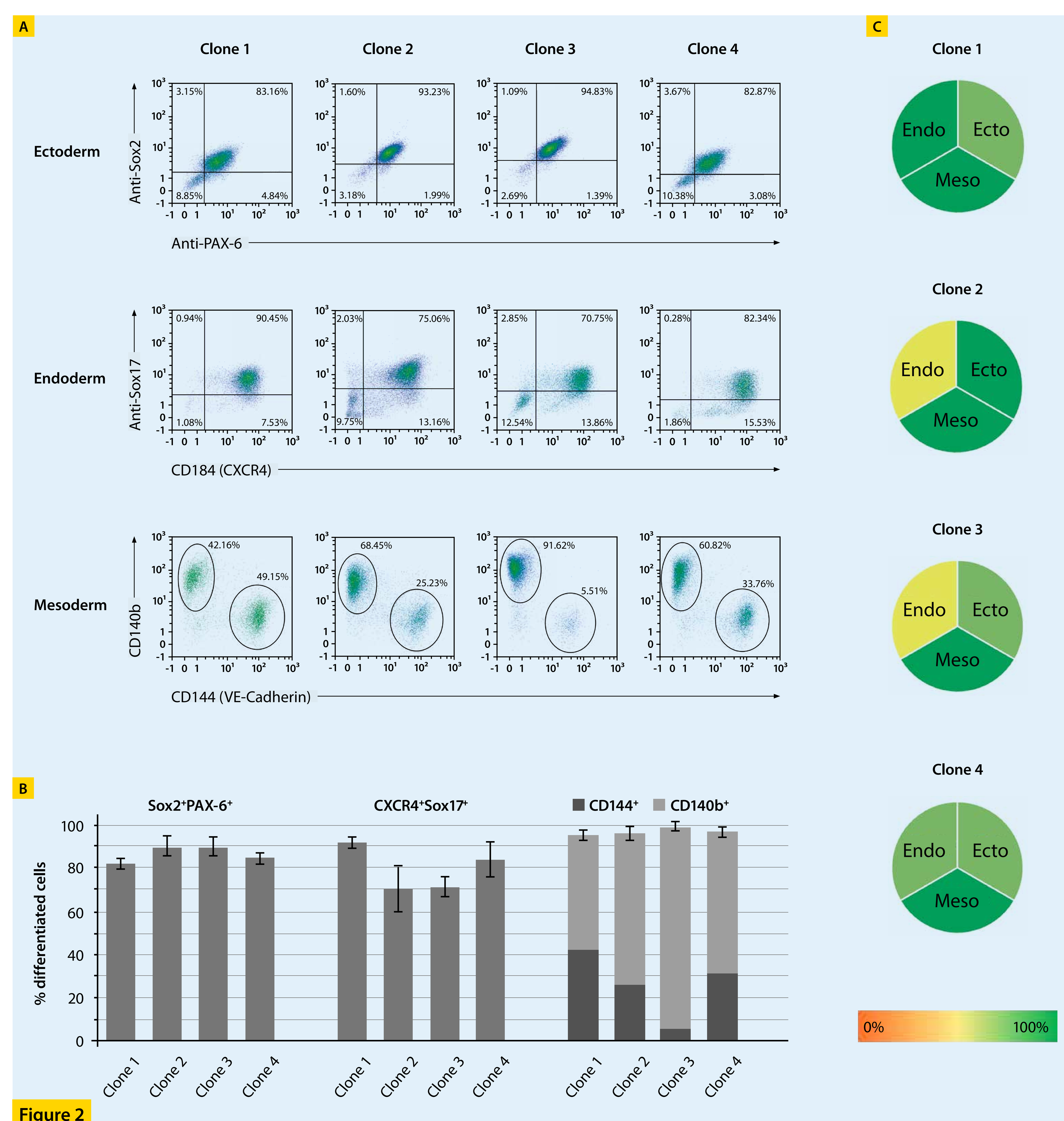

Figure 2

Four hiPSC lines were differentiated using the protocol described in figure 1. Their differentiation potential was assessed by quantitative flow cytometry. All four lines differentiated into Sox2$^+$PAX-6$^+$ neuroectoderm cells (ectoderm), CXCR4$^+$Sox17$^+$ definitive endoderm cells (endoderm), and CD140b$^+$ vascular smooth muscle cells or CD144$^+$ endothelial precursors (mesoderm) (A, representative examples). The assay revealed different propensities for each line to differentiate into each germ layer: Clone 2 and clone 3 showed a high capacity for differentiation into neuroectoderm (89–90%). Clone 1 and clone 4 had a slightly lower efficiency for differentiation into neuroectoderm with 81–84% Sox2$^+$PAX-6$^+$ cells. Endodermal differentiation capacity was higher for clone 1 (91%) and clone 4 (83%) compared to clones 2 and 3, which showed a lower tendency to differentiate into definitive endoderm cells (70–71%). The overall capacity to differentiate into mesodermal lineage was high for all four clones (94–99%), but differences could be detected in the numbers of CD140b$^+$ and CD144$^+$ cells. While clone 1 showed almost equal distribution of CD140b$^+$ (42%) and CD144$^+$ (53%) cells, clone 2 and clone 4 were more prone to differentiate into vascular smooth muscle cells (65–70%). Clone 3 differentiated almost exclusively into CD140b$^+$ cells (94%) (B; mean$^+$/-SD; n = 3).

In summary, the assay pointed out differences between the four tested hiPSC lines regarding their preference to differentiate into the three germ layers (C).

2 Assessment of trilineage differentiation using immunocytochemistry

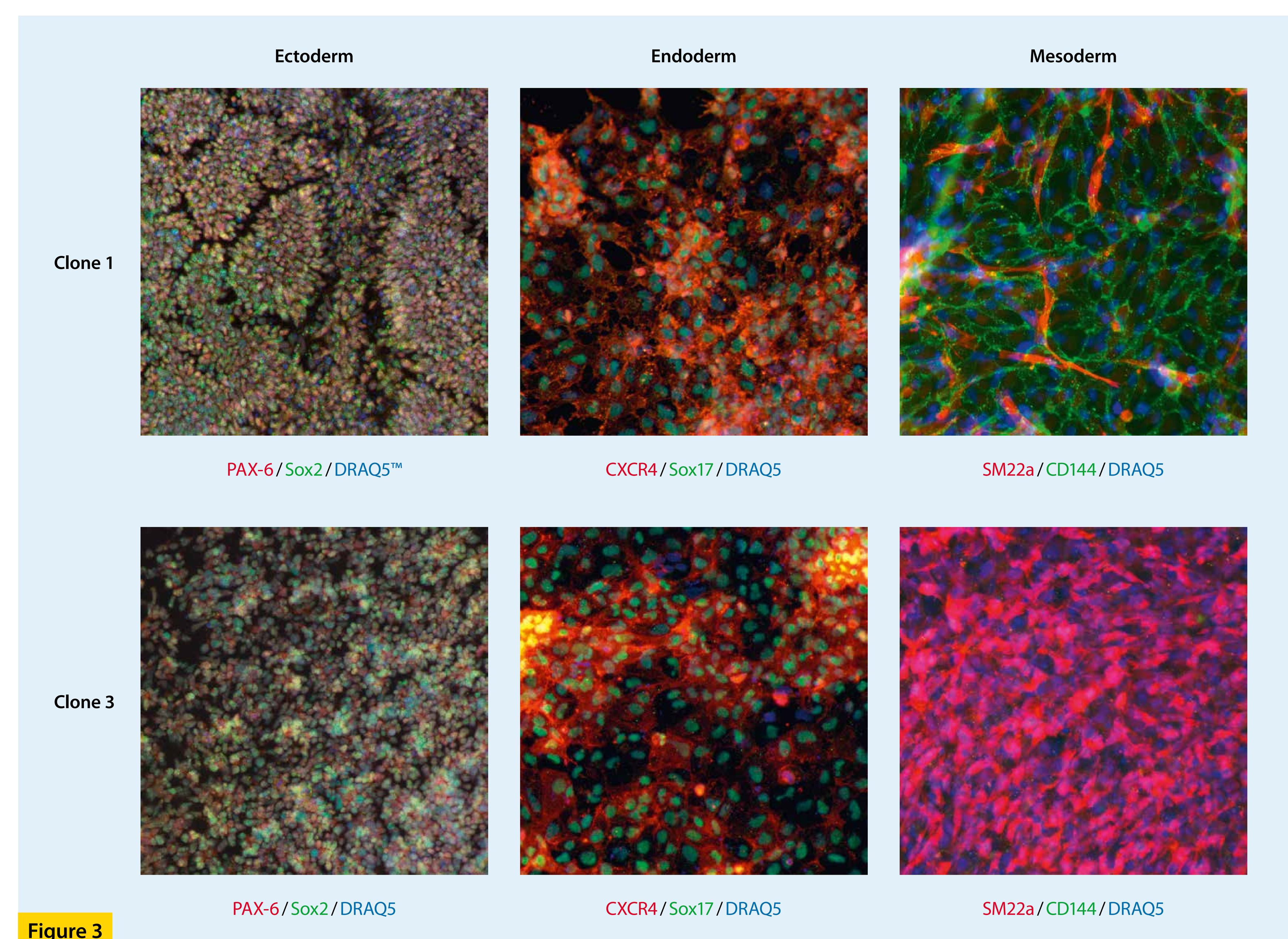

Figure 3

Qualitative analysis by immunocytochemistry was performed additionally to assess the differentiation capacity of all four hiPSC lines. To this end, cells were fixed on day 7 and stained for lineage-specific markers, i.e., Sox2 and PAX-6 (ectoderm), CXCR4 and Sox17 (endoderm), VE-cadherin (CD144) and smooth muscle 22α (mesoderm). All clones showed positive cells for each germ layer, allowing a rough estimation of the individual differentiation propensity.

3 StemMACS™ Trilineage Differentiation Kit is compatible with a variety of PSC cultivation systems

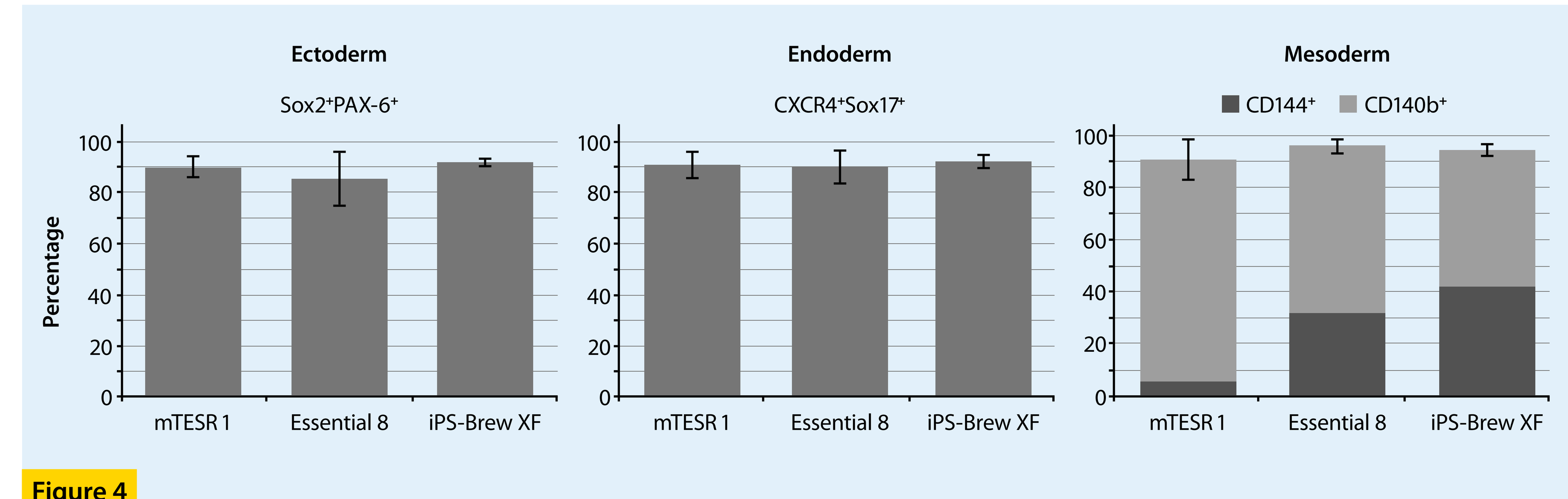

Figure 4

Clone 1 was cultivated for >10 passages in either StemMACS™ iPS-Brew XF, mTeSR™ 1, or Essential 8™. Subsequently, cells were differentiated using the StemMACS Trilineage Differentiation Kit and analyzed on day 7 by quantitative flow cytometry. Regardless of the PSC expansion media used, differentiation efficiency was the same for each germ layer. Sox2$^+$PAX-6$^+$ neuroectoderm cells: 92% (StemMACS iPS-Brew XF), 90% (mTeSR 1), 85% (Essential 8). CXCR4$^+$Sox17$^+$ definitive endoderm cells: 91% (StemMACS iPS-Brew XF), 90% (mTeSR 1), 89% (Essential 8). Overall differentiation capacity mesodermal lineage: 95% (StemMACS iPS-Brew XF), 91% (mTeSR 1), 96% (Essential 8). The percentage of CD144$^+$ cells was lower in mTeSR 1 compared to Essential 8 and StemMACS iPS-Brew XF. Thus, the StemMACS Trilineage Differentiation Kit can be used in combination with various PSC cultivation media.

Conclusion

Based on lineage-specific, complete media, we developed a directed, 2D differentiation assay which:

- supports differentiation into all three germ layers,
- reduces analysis efforts due to temporally harmonized 7-day differentiation protocols,
- is standardized and gives reproducible results regardless of the PSC cultivation medium used for initial expansion,
- can be used in combination with immunocytochemistry or quantitative flow cytometry,
- facilitates parallel assessment of multiple hPSC lines.

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