A versatile and robust xeno- and serum-free cultivation system for human pluripotent stem cells

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Introduction

Pluripotent stem cells (PSC) have traditionally been cultured on mouse embryonic feeder (mEF) cells, which contribute to maintenance of pluripotency and deposit extracellular matrix components mediating cell attachment. However, the xenogeneic nature of mEF cells and commonly used media components is not compliant with current efforts to establish clinically compatible protocols for maintenance and differentiation of PSC. Different compositions have been devised in order to maintain pluripotency in feeder-free conditions, but most media require extensive adaption periods.

We have optimized a xeno- and serum-free media formulation that allows rapid adaption to feeder-free conditions and rapid culture initiation after cryopreservation. The formulation enables robust and efficient expansion of PSC and supports episomal reprogramming of human fibroblasts. Furthermore, the medium supports PSC expansion in suspension culture. The formulation will allow a rapid translation into a clinical-grade medium designed following the recommendations of USP <1043> on ancillary materials and will be suitable for clinical-grade PSC expansion.

Results

1 StemMACS™ iPS-Brew XF allows rapid adaption to feeder-free culture conditions and rapid culture initiation after cryopreservation

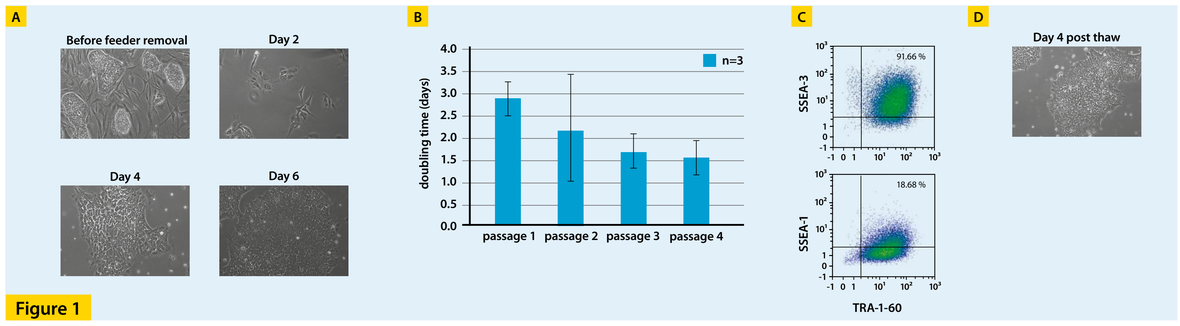

Figure 1

Using Feeder Removal MicroBeads two cell lines (PSC clone 1 (Miltenyi Biotec); PSC clone 2 (I-STEM)) were set to feeder-free culture conditions and cultivated for 4 passages in StemMACS™ iPS-Brew XF. Cells adapted rapidly to Matrigel® (**A**, PSC clone 1) or vitronectin (data not shown) and showed a homogenous morphology across all passages. A stable doubling time was reached after a minimum of 2 passages (**B**, PSC clone 1). To confirm the quality of the cells, marker expression was checked at every passage using flow cytometry. Both cell lines showed a high and persistent expression of TRA-1-60 and SSEA-3 and only low expression of SSEA-1 (**C**, PSC clone 1). Furthermore, we assessed the post-thaw recovery after cryopreservation. The cells reached a standard doubling time of 1–1.5 days directly at passage 1 after thawing and showed a normal morphology (**D**, PSC clone 1). In summary, StemMACS iPS-Brew XF enables a rapid adaption to feeder-free culture conditions and additionally ensures a fast recovery of the culture after cryopreservation.

2 StemMACS™ iPS-Brew XF enables robust and efficient expansion of PSC as single cells or cell clusters

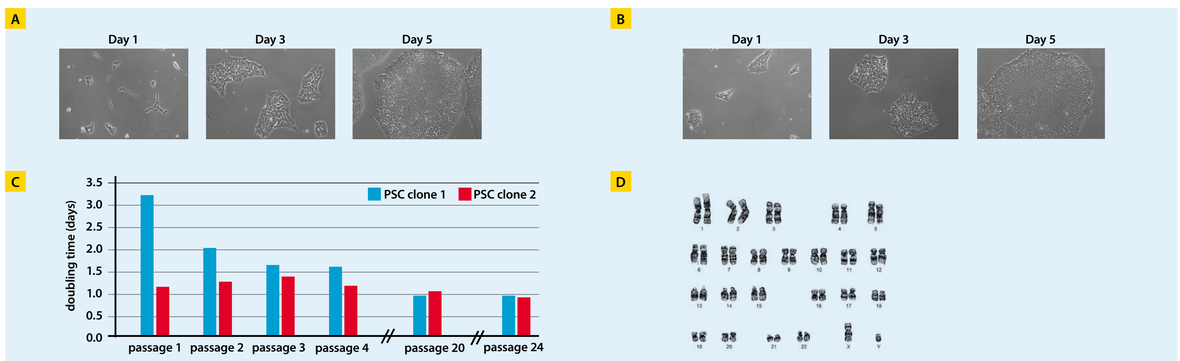

Figure 2

In order to evaluate the robustness of the culture conditions, we cultivated both cell lines for more than 20 passages in StemMACS™ iPS-Brew XF utilizing two different culture techniques: i) single-cell passaging with TrypLE™ (**A**) and ii) cell-cluster passaging using StemMACS Passaging Solution XF (**B**). Both cell lines constantly showed a homogenous morphology, regardless of which culture technique was used (**A** and **B**, PSC clone 1). Additionally, doubling time for single–cell passaging stabilized between 22 and 24 h (**C**). Using StemMACS Passaging Solution a split ratio of up to 1:10 was adequate for long-term passaging. To determine the genomic stability of PSC cultivated in our medium, we analyzed the karyotype of both cell lines at passages 11 (data not shown) and 19 (**D**, PSC clone 1). Both cell lines showed a normal karyotype. Thus, StemMACS iPS-Brew XF allows the robust and efficient expansion of PSC both as single cells and cell clusters, while retaining a normal karyotype.

3 Cells cultivated in StemMACS™ iPS-Brew XF stay highly pluripotent

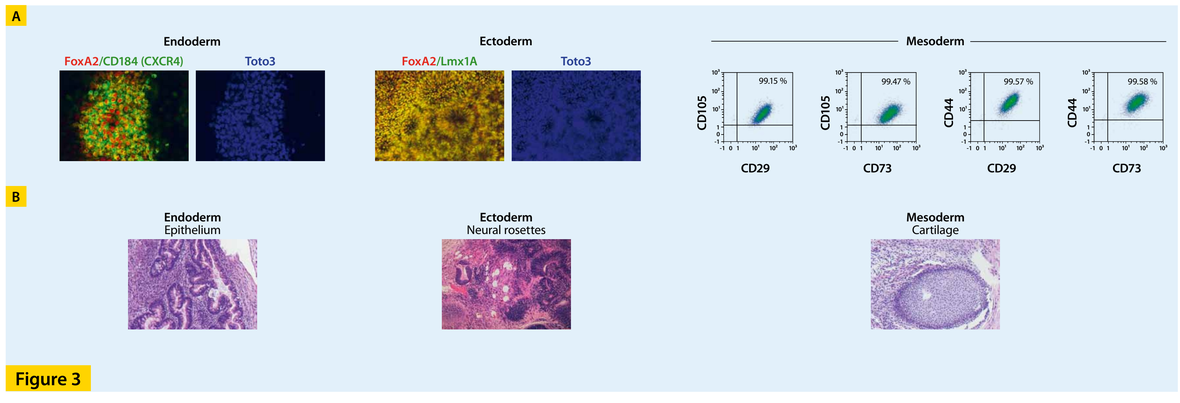

Figure 3

We next determined the differentiation potential of both cell lines after 10 passages of cultivation in StemMACS™ iPS-Brew XF (examples shown for PSC clone 1). *In vitro* differentiation: i) endodermal differentiation was done using high-dose activin A combined with GSK3 inhibition to derive FoxA2$^+$CXCR4$^+$ definitive endoderm cells (modified from Bone *et al.*[1] (**A**, top left), ii) for ectodermal differentiation FoxA2$^+$Lmx1A$^+$ dopaminergic progenitors were generated using dual SMAD inhibition for rapid neural conversion, and GSK3 inhibition in addition to SHH activation for regionalization of the cells (modified from Kirkeby *et al.*[2] (**A**, bottom left), iii) mesodermal differentiation was done using a KO-DMEM–based medium supplemented with FCS/Aa2-P/FGF-2 to derive CD105$^+$CD44$^+$CD29$^+$CD73$^+$ MSC[3] (**A**, right). *In vivo* differentiation: For teratoma formation PSC were mixed with Matrigel and injected subcutaneously into NOD/SCID-mice. After 8 weeks animals were sacrificed, the teratomas were explanted, sectioned, and stained with hematoxylin/eosin. Representative tissue from all three embryonic germ layers could be detected in each teratoma (**B**). Hence, cell lines cultivated in StemMACS iPS-Brew XF retain their *in vitro* and *in vivo* differentiation potential.

4 Scalable expansion of PSC in suspension culture using StemMACS™ iPS-Brew XF

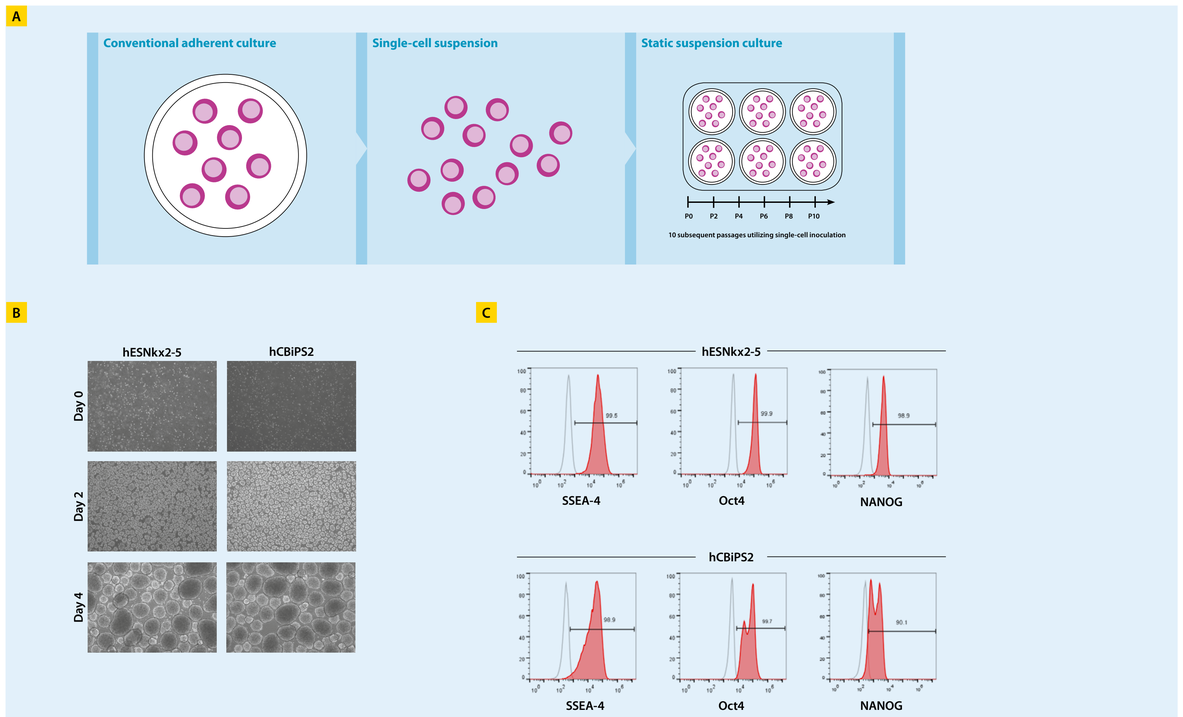

Figure 4

Utilizing the PSC lines hCBiPS2[5] and hES Nkx2-5[6] undifferentiated PSC were seeded as single-cell suspensions in Costar® 6-well ultra-low attachment plates at each passage[4] (**A**). During expansion of PSC in static suspension culture, the cells formed aggregates (**B**). Flow cytometry analysis revealed that the majority of PSC grown for 10 passages in StemMACS™ iPS-Brew XF in static suspension culture expressed the pluripotency markers NANOG, Oct4, and SSEA-4 (**C**, isotype control in grey). Therefore, StemMACS iPS-Brew XF supports expansion of undifferentiated PSC over 10 passages in static suspension culture.

5 StemMACS™ iPS-Brew XF supports episomal reprogramming of human fibroblasts

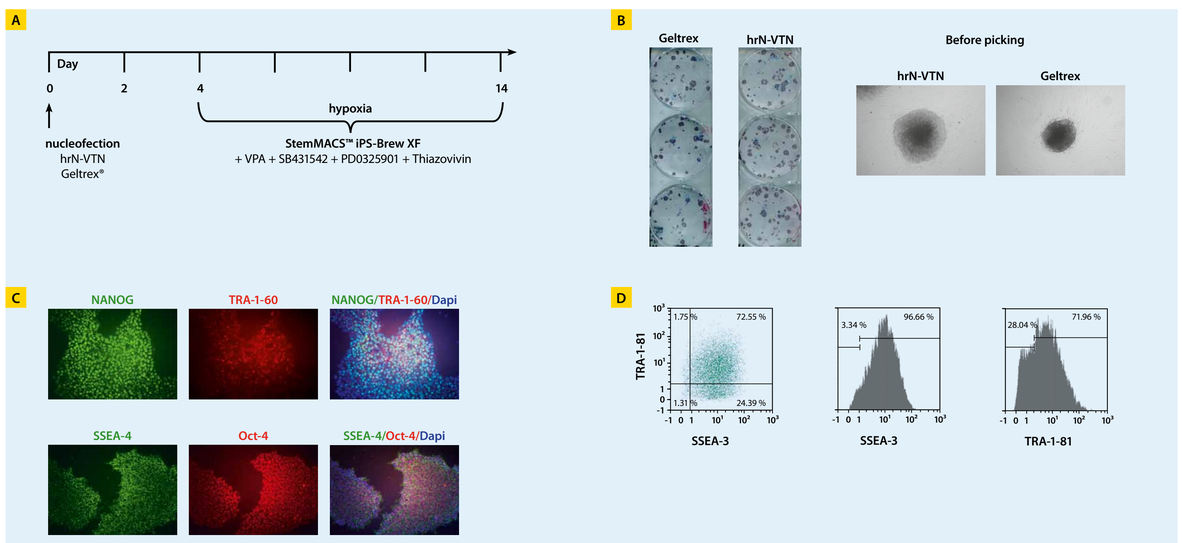

Figure 5

Human iPSC were generated from human dermal fibroblasts (CRL2097, ATCC) using episomal plasmid vectors[7]. At day 0, cells were transfected and kept in fibroblast medium for 4 days. Then the medium was changed to StemMACS iPS-Brew XF supplemented with small molecules, and the cells were cultivated for another 10 days under hypoxia (**A**). The efficiency of reprogramming was assessed by alkaline phosphatase staining (**B**). The generated iPSC were maintained on hrN-VTN and grown in StemMACS™ iPS-Brew XF for several passages. To confirm the pluripotency of the generated iPSC, immunocytochemistry was done at passage 11. The iPSC expressed the pluripotency markers NANOG, TRA-1-60, SSEA-4, and Oct-4 (**C**). Additionally, flow cytometry analysis at passage 12 demonstrated that the iPSC were also positive for the pluripotency markers TRA-1-81 and SSEA-3 (**D**). Consequently, StemMACS iPS-Brew XF can be used as basal medium for episomal reprogramming of dermal fibroblasts.

Conclusion and outlook

We have optimized a xeno- and serum-free medium that:
- enables rapid adaption from feeder to feeder-free culture conditions
- ensures fast recovery of the cells after cryopreservation
- allows robust and efficient expansion of PSC passaged as single cells or cell clusters
- keeps the cells in a highly pluripotent state for more than 20 passages while retaining a normal karyotype

Additionally, StemMACS iPS-Brew XF supports expansion of undifferentiated PSC in static suspension culture and can be used as basal medium for episomal reprogramming of dermal fibroblasts. The formulation allows a rapid translation into a clinical-grade medium.

Acknowledgment
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