A versatile base medium to maintain a stable and xeno-free cell culture environment during PSC differentiation

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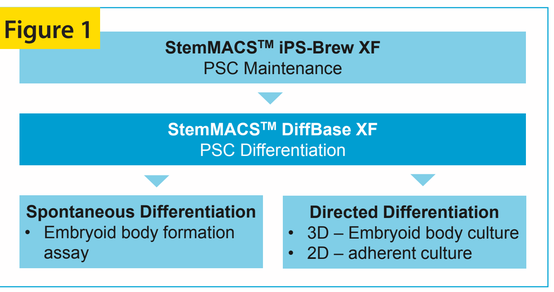
Introduction

Human pluripotent stem cells (hPSCs) play an important role in disease modeling, drug discovery, and cell therapy applications. Their ability to differentiate into cell types of all three germ layers makes them attractive to study specific cell development, cellular functions, or cellular behavior of certain cell types in response to different treatments.
hPSCs are sensitive to changes in their culture system in terms of medium composition, in particular with respect to nutrient, biosynthetic precursor and vitamin supply, pH, and osmolality. Therefore, PSCs benefit from a well-defined, steady medium environment to remain viable and functional during maintenance cultivation and subsequent differentiation experiments. Here, we developed a xeno-free and cytokine-free base medium which can be used for various differentiation purposes. The formulation is based on our xeno-free PSC maintenance medium and minimizes the risk of adaption stress when changing from maintenance to differentiation culture.
The medium efficiently supports spontaneous EB formation and can also be used as basis for several 2D or 3D directed differentiation assays.
As proof of principle, hPSCs were spontaneously differentiated using the cytokine-free base medium, first as embryoid bodies (EBs) in 3D suspension culture, followed by 2D adherent maturation. Subsequently, immunocytochemistry was used to successfully identify cells of the mesoderm, ectoderm, and endoderm lineage. Additionally, directed EB differentiation and directed 2D differentiation was performed by adding lineage-specific cytokines and small molecules to the base medium. The quantitative flow cytometry analysis confirmed a high differentiation efficiency both in 2D and 3D into all three germ layers. Therefore, the medium provides a good basis for a variety of differentiation protocols in 2D and 3D culture conditions.

Methods

1 Experimental design

Figure 1



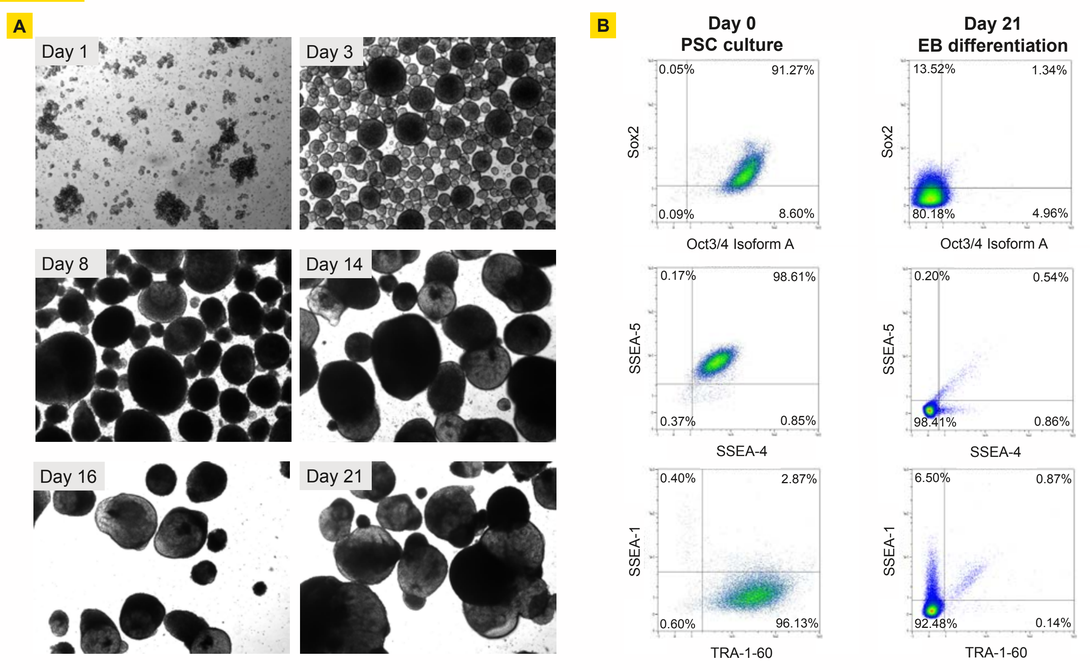
After cultivating hPSCs in our maintenance medium StemMACS iPS-Brew XF cells can be easily further differentiated using the xeno-free and cytokine-free base medium StemMACS DiffBase XF. Due to its similarity to the maintenance medium in terms of nutrients, biosynthetic precursor, vitamins, pH, and osmolality StemMACS DiffBase XF allows a smooth and gentle transition of hPSCs into various 2D or 3D differentiation protocols. Directed 2D or 3D differentiation can be done by adding lineage-specific cytokines and small molecules. 3D spontaneous differentiation can be achieved by simply using the base medium on its own.

Results

1 StemMACS™ DiffBase XF assures efficient generation and long-term survival of embryoid bodies

In order to evaluate the robustness of the cytokine-free differentiation base medium, we performed a standard embryoid body (EB) formation assay and cultivated the EBs for 21 days in StemMACS DiffBase XF. To that end, hPSCs were harvested and re-plated as single cells in a 10 cm ultra-low attachment dish (ULA) using 5×10^6 cells/12 mL StemMACS DiffBase XF supplemented with ROCK inhibitor. After 48 h the medium was exchanged, and cells were further differentiated as EBs in StemMACS DiffBase XF only. Subsequently, the medium was exchanged every other day. EBs developed nicely within the first days, grew and survived for at least 21 days (A). hPSCs were highly pluripotent at day 0 (start of experiment) expressing TRA-1-60, Sox2, Oct4, SSEA-4, SSEA-5, and no SSEA-1 (differentiation marker). During spontaneous EB differentiation pluripotency markers were down-regulated over time, while SSEA-1 started to be up-regulated, as assessed by flow cytometric analysis (B).

Figure 2



2 Assessment of pluripotency using the spontaneous embryoid body differentiation assay and StemMACS DiffBase XF

StemMACS DiffBase XF was also used to assess the pluripotency of hPSCs using the standard and well-known spontaneous EB differentiation assay, where EBs are cultivated as described above (fig. 1) and re-plated after 14 days on Matrigel® to allow cell outgrowth from the EBs. 2–3 days after re-plating of the EBs in StemMACS DiffBase XF, cells started to grow out and displayed promptly the typical morphology of, e.g., neuronal or hepatocyte like cells (A). Plated EBs were cultivated in StemMACS DiffBase XF for 7 days in total; medium was replaced every other day. At day 7 plated EBs were fixed and analyzed by immunocytochemistry. We could clearly identify cells of all three germ layers: $TUJ1^+$ and $Sox2^+$ neuronal cells (ectoderm), SMA^+ smooth muscle cells (mesoderm) and TTR^+ and $ASGPR\text{-}1^+$ hepatocyte like cells (endoderm) (B).

Figure 3

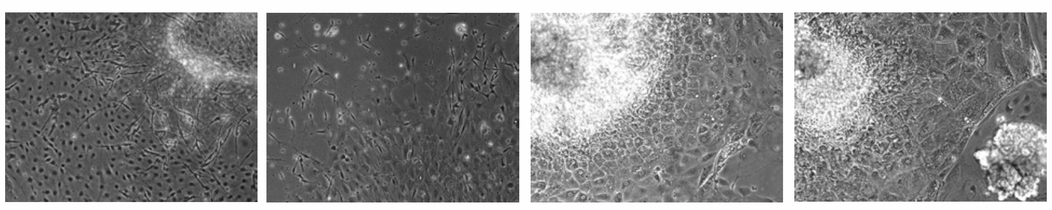
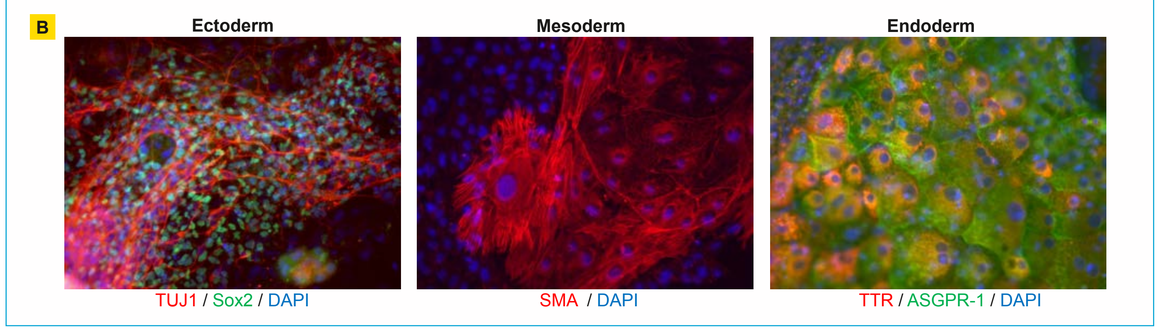


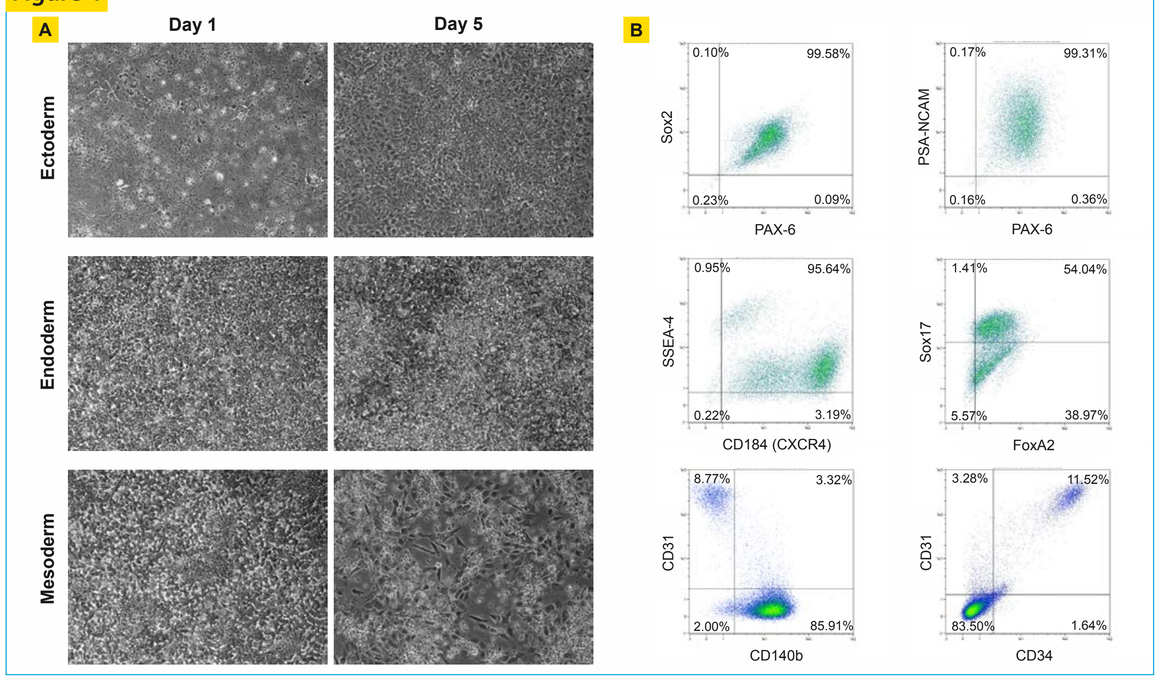
Figure 3 (continued)



3 StemMACS DiffBase XF supports various directed 2D differentiations

To further determine the potential of the medium, StemMACS DiffBase XF was tested in directed 2D differentiations. hPSCs were harvested as single cells and re-plated on Matrigel-coated plates at differentiation-specific cell densities. For ectoderm differentiation, cells were directly seeded in StemMACS DiffBase XF supplemented with lineage-specific small molecules and ROCK inhibitor. For meso- and endoderm differentiation, cells were first seeded in the maintenance medium supplemented with ROCK inhibitor. After the cultures had reached the needed confluency, the medium was changed to StemMACS DiffBase XF supplemented with the respective lineage-specific cytokines and small molecules. For all three differentiation pathways, cells were treated with StemMACS DiffBase XF supplemented with germ-layer specific patterning factors over the next days. Cell morphology changed over time as expected (A). Furthermore, differentiated cells expressed lineage-specific markers like 99% $Sox2^+Pax6^+PSA\text{-}NCAM^+$ (neuroectoderm), 54% $Sox17^+FoxA2^+$ (definitive endoderm), and 86% $CD140b^+$ (vascular smooth muscle cells, mesoderm) and 12% $CD31^+CD34^+$ (endothelial precursors, mesoderm), as assessed by quantitative flow cytometry at day 7 (B).

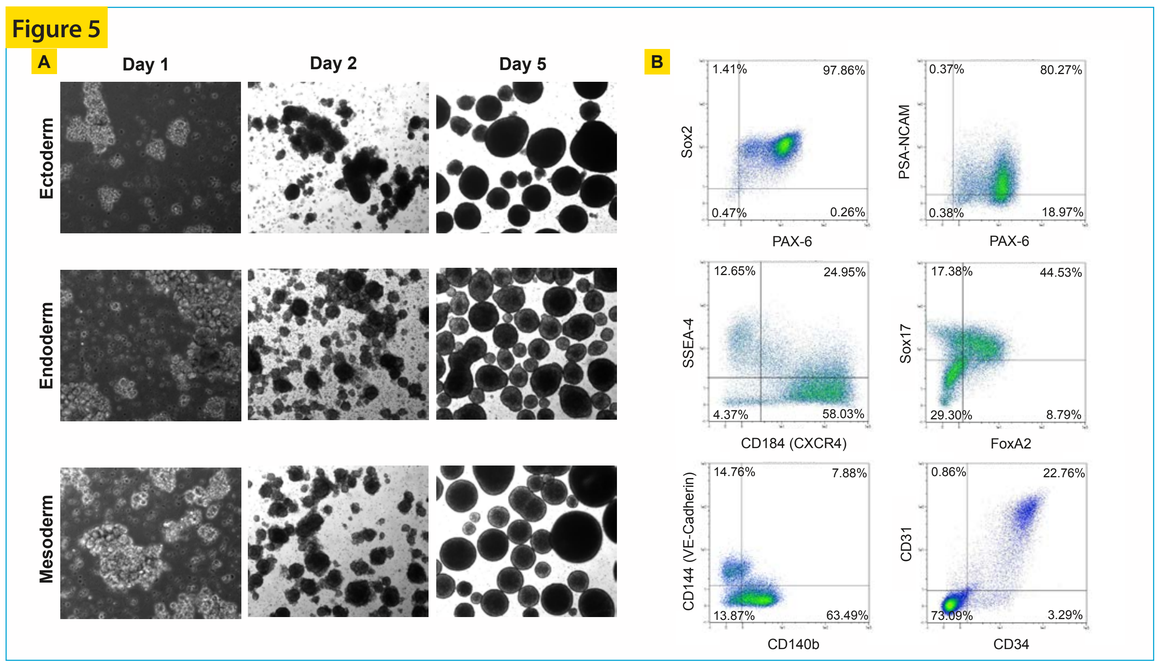
Figure 4



4 StemMACS DiffBase XF is suitable for diverse directed 3D differentiation protocols

Next, we assessed the efficacy of StemMACS DiffBase XF as base medium in directed 3D differentiation. EBs were generated as described above (fig. 1). 24 h after initiating EB formation the medium was changed to StemMACS DiffBase XF supplemented with lineage-specific patterning factors for ectoderm, endoderm, or mesoderm. EBs were treated with those StemMACS DiffBase XF-based differentiation media over 5 days with a daily medium change. EBs developed nicely over time for all three germ layer–specific differentiations (A). Quantitative flow cytometric analysis revealed their differentiation status after dissociating the EBs into single cells at day 6. To avoid any proteolytic degradation of marker epitopes, appropriate dissociation reagents were chosen depending on the selection of analysis markers. Directed ectoderm differentiation led to 80–97% $Sox2^+Pax6^+PSA\text{-}NCAM^+$ neuroectoderm cells. A percentage of 45–58% $Sox17^+FoxA2^+CXCR4^+$ cells showed a successful definitive endoderm differentiation. Directed mesoderm differentiation led to 63% $CD140b^+$ vascular smooth muscle cells and 23% $CD31^+CD34^+$ endothelial precursors.

Figure 5



Conclusion

Here we developed a xeno-free and cytokine-free base medium which:

- efficiently supports spontaneous EB formation and long-term survival of EBs,
- promotes the standard spontaneous EB assay to assess pluripotency of PSCs,
- can be used as differentiation base medium for various 2D and 3D directed, differentiation protocols,
- minimizes the risk of adaption stress when changing from maintenance to differentiation culture, because media formulations are well matched.