Highly efficient differentiation of hPSC into hepatocyte-like cells by selection of CXCR4 (CD184)+ definitive endoderm (DE) cells

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Introduction

Hepatocytes fulfill numerous central metabolic functions of the body. The loss of active hepatocytes during inherited or acute liver disease or intoxication leads to severe pathophysiological effects. Therefore, the etiology of liver disease is of major interest. Lack of healthy primary hepatocytes necessitates alternative cell sources for *in vitro* studies. Hepatocyte-like cells (HLC) derived from *in vitro* differentiated pluripotent stem cells (PSC) constitute a potential source for disease-in-a-dish modeling, drug screening, and future cell replacement strategies. Generation of HLC *in vitro* is usually achieved by exposing PSC to high doses of activin A in combination with FGF and Wnt pathway activation. This leads to induction of nascent definitive endoderm (DE) that can be subsequently specified to hepatic endoderm (HE), hepatoblasts, and finally metabolically active HLC. However, varying efficiencies of PSC differentiation are well documented and caused, e.g., by cell line predisposition, lot-to-lot variances of differentiation media components, and experimental inconsistencies. To overcome these limitations, we sought to develop an immunomagnetic selection strategy for the isolation of DE cells, thereby standardizing procedures for the differentiation of the endodermal lineage.

Results

1 CXCR4 identifies FoxA2- and Sox17-positive definitive endoderm (DE) cells

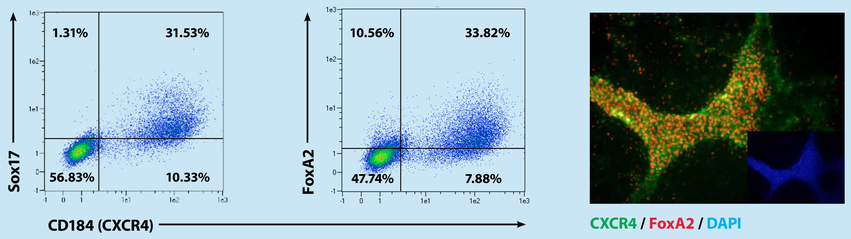

Figure 1

Co-expression of the transcription factors FoxA2 and Sox17 is characteristic for PSC-derived definitive endoderm (DE) cells. Likewise, the cell surface marker CXCR4 was found to be present on DE and was previously used to identify and isolate DE cells [1,2].
We modified the differentiation protocol from Bone *et al.*[3], using high-dose activin A (100 ng/mL) in combination with the GSK3 inhibitor CHIR99021 to derive DE cells from hESCs (SA001) as well as hiPSCs [4] under feeder-free conditions (fig. 4). After 5 days of differentiation, we analyzed DE marker expression and found a strong correlation between CXCR4 and FoxA2 as well as CXCR4 and Sox17. Flow cytometric and immunocytochemical analysis showed that the majority of differentiated cells was CXCR4/FoxA2- and CXCR4/Sox17-double positive, confirming previously published results [1,2]. Consequently, we were reassured that CXCR4 is a reliable marker for immunomagnetic isolation of DE cells by MACS Technology.

2 Definitive endoderm (DE) cells can be efficiently enriched by magnetic cell sorting based on CXCR4

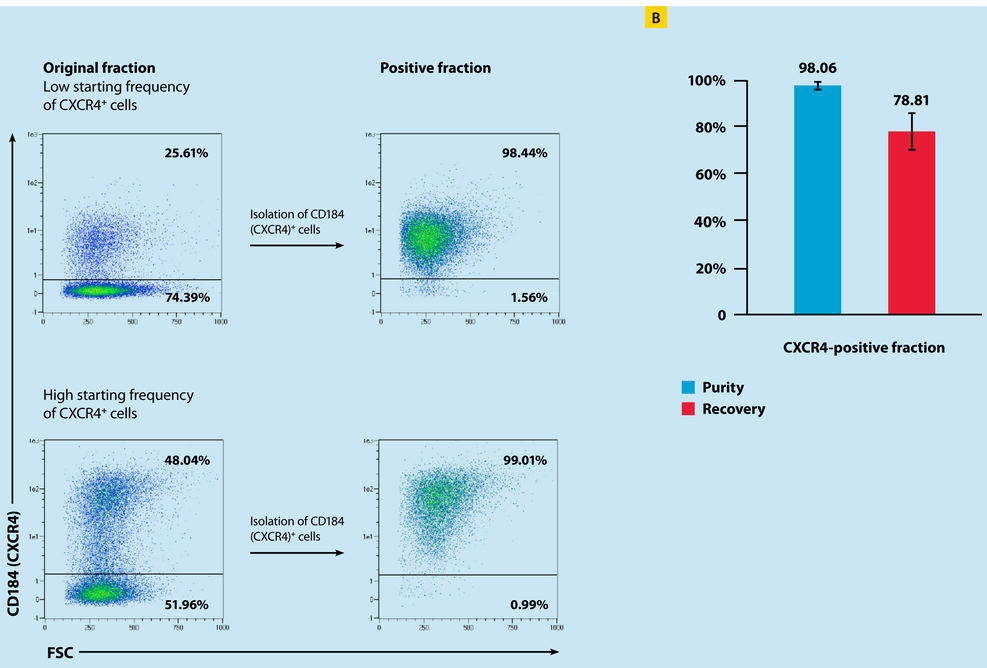

Figure 2

As CXCR4 was mostly co-expressed with the specific DE markers FoxA2 and Sox17, we sought to establish a dependable protocol for enrichment of DE cells based on CXCR4. First, hPSCs were differentiated to the DE stage (fig. 4). Subsequently, cells were harvested and labeled with CD184 (CXCR4)-APC and Anti-APC MicroBeads. Labeled DE cells were enriched by magnetic cell sorting using LS Columns. The amount and frequency of CXCR4+ DE cells before and after enrichment were determined by flow cytometry. Regardless of the starting frequency of CXCR4+ cells we were able to enrich CXCR4+ DE cells to purities of 98.06±1.67% (mean±sd, n=7) while ensuring high cell yields of 78.81±8.19% (mean±sd, n=7). Thus, enrichment based on CXCR4 reproducibly resulted in virtually pure DE cells, irrespective of the induction efficiency during DE differentiation.

3 The hepatogenic potential is restricted to CXCR4+ cells in DE differentiation cultures

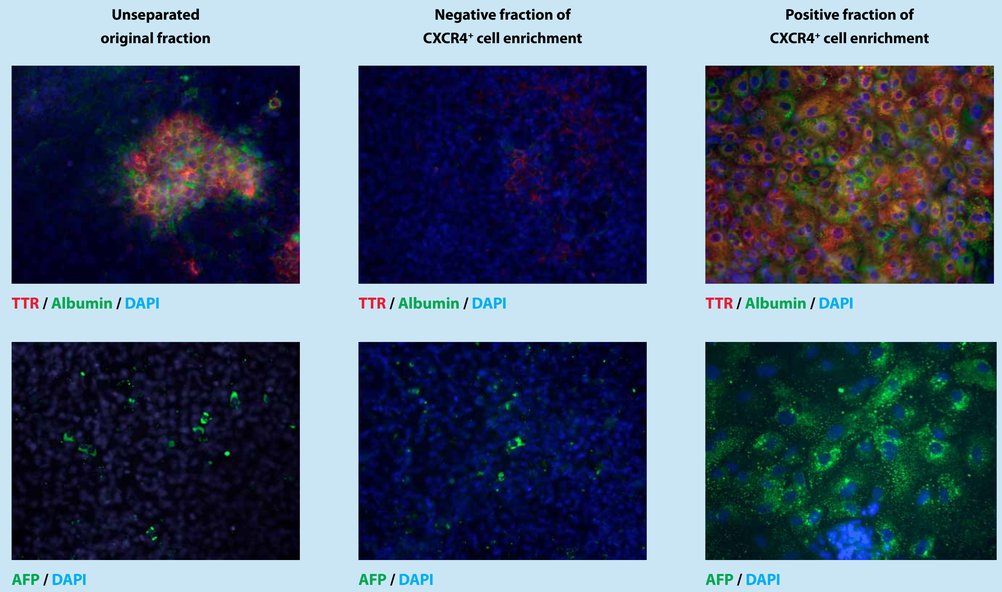

Figure 3

We next determined the hepatogenic potential of the differentiated DE cultures 5 days after switching to DE induction medium. We compared the unseparated original fraction, the CXCR4⁻ fraction, and the CXCR4+ fraction. Cells were plated on Matrigel (70.000 cells/cm²) and differentiated for another 14 days by exposing them to the hepatogenic factors HGF, FGF-4, oncostatin M, and dexamethasone (fig. 4). The hepatogenic potential was determined by immunocytochemical analysis of the hepatoblast markers α-fetoprotein (AFP) and transthyretin (TTR), and the hepatocyte marker albumin (ALB). Enrichment of CXCR4+ DE cells allowed us to reproducibly generate highly pure hepatocyte-like cells: After 14 days of differentiation virtually all cells of the CXCR4+ cell fraction expressed AFP, TTR, and ALB. In contrast, almost no AFP-, TTR-, or ALB-positive cells could be detected in the differentiated CXCR4⁻ cell fraction. In the unseparated original fraction, which initially contained between 15 and 50% CXCR4+ DE cells, AFP-, TTR-, and ALB-positive cells occurred at a much lower frequency than in the enriched CXCR4+ cell fraction.
These results show that the hepatogenic potential is limited to the CXCR4+ DE cells and that enrichment of CXCR4+ DE cells leads to consistently pure differentiation cultures of HLCs.

4 Experimental workflow

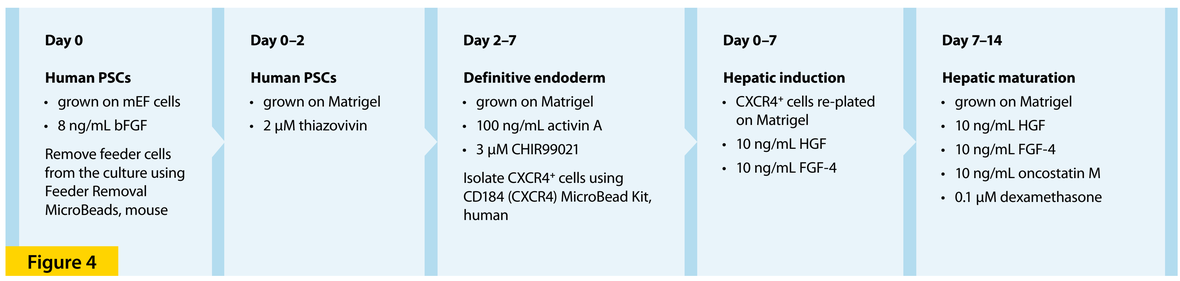

Figure 4

hPSCs cultivated on feeder cells were harvested, feeder cells were depleted using Feeder Removal MicroBeads, and the cells were re-plated on Matrigel in mTeSR-1 medium or NutriStem supplemented with 2 µM thiazovivin. At day 2 of cultivation, when cells normally reached a confluency of 95%, the medium was replaced with DE differentiation medium (mTeSR-1 or NutriStem + 100 ng/mL bFGF, both supplemented with 100 ng/mL activin A and 3 µM CHIR99021). After 5 days of differentiation with daily media changes, cells were harvested, and CXCR4+ cells were enriched using the CD184 (CXCR4) MicroBead Kit. CXCR4+ cells were re-plated on Matrigel at a density of 70.000 cells/cm² and cultivated for 14 days in differentiation medium (KnockOut-DMEM, 1 mM non-essential amino acids, 2 mM L-glutamine, 2% of KnockOut serum replacement), supplemented for 7 days with 10 ng/mL HGF and 10 ng/mL FGF-4 and for another 7 days with 10 ng/mL HGF, 10 ng/mL FGF-4, 10 ng/mL oncostatin M, and 0.1 µM dexamethasone.

Conclusion

- Using well-established DE induction protocols we were able to generate DE cells at frequencies between 30 and 80% at day 5 of differentiation. These cells co-expressed FoxA2, Sox17, and the cell surface marker CXCR4.
- The hepatogenic potential is limited to the CXCR4+ cell population at day 5 of DE differentiation.
- Immunomagnetic isolation based on CXCR4 (CD184) constitutes a robust method to obtain virtually pure DE populations (>98%)
- Isolation of DE cells enables reproducible generation of highly enriched HLC cultures and compensates for inherent variabilities during PSC differentiation, resulting in a more standardized differentiation procedure.
- The protocol may also serve to improve generation of other cell types derived from DE, such as pancreatic beta cells or lung epithelial cells.

References

1. Wang, P. *et al.* (2011) Cell Stem Cell 8: 335–346.
2. Cheng, X. *et al.* (2012) Cell Stem Cell 10: 371–384.
3. Bone, H. K. *et al.* (2011) J. Cell Sci. 124: 1992–2000.
4. Haase, A. *et al.* (2009) Cell Stem Cell 5: 434–441.