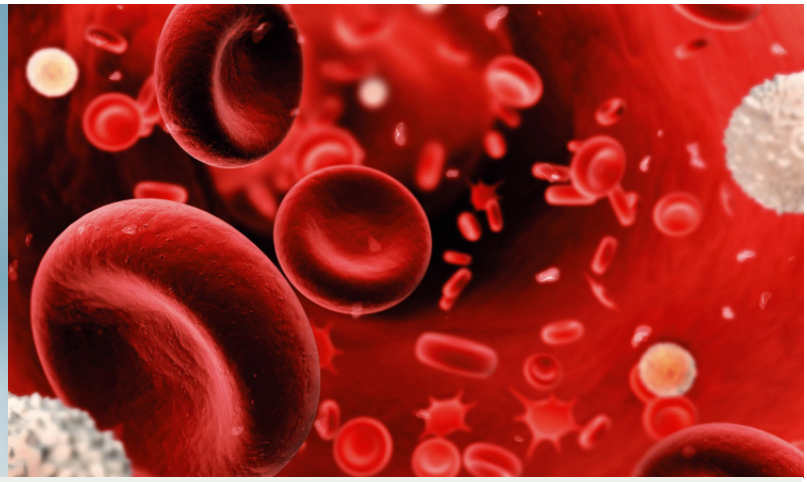

Stem cell research: Creating blood for high throughput screening

Automated culturing and differentiation of pluripotent stem cells into hematopoietic cells

Introduction

Many different research fields – including investigation of early human development processes, disease research, drug discovery and cellular therapy applications – require appropriate *in vitro* models which can accurately mimic *in vivo* processes to provide biologically relevant results. Pluripotent stem cells (PSCs) offer a powerful system for creating *in vitro* models to address these questions. PSCs can be proliferated indefinitely in an undifferentiated state and, when induced with appropriate growth factors and cytokines, can mature into virtually any of the cell types found in the human body.

Currently, differentiation of either human embryonic stem cells (hESCs) or induced PSCs (iPSCs) relies on the use of serum or co-culturing on feeder cell lines. Manual maintenance and differentiation of hESCs or iPSCs is technically challenging, labor-intensive and subject to inherent process variability, and is therefore not a viable long-term solution for scaling up the production of stem cells.

Cellular Dynamics International (CDI), based in Wisconsin, USA, has developed an automated method to produce industrial quantities of stem cells, and their derivatives, to address the needs of the market. This study describes a highly efficient, serum-free, feeder-free automated system for production of iPSCs, and their differentiation into hematopoietic precursor cells (HPCs).

Materials and Methods

The automated system, a Cellerity™500, is based on a Freedom EVO® 200 liquid handling platform, and includes an STX 500 automated incubator with a capacity for 500 plates (LiCONiC); a media storage fridge; an AutoLoader™ for loading flasks; a Cedex™ cell counter (Innovatis); spinner flasks for expansion and seeding of suspension cells; a Robotic Manipulator (RoMa) Arm to handle plates; Multichannel Arm (MCA) and an eight-channel Liquid Handling (LiHa) Arm using fixed tips.

The Cellerity also features a positive pressure, HEPA filtered environment in a BSL2-compliant class 100 sterile room, for operation under GMP guidelines.
iPSCs were generated from various adult cells using retroviral transduction transfection of OCT4/SOX2/KLF4/MYC or OCT4/SOX2/Lin28/Nanog [1]. CDI has also successfully generated iPSCs via a footprint-free oriP/EBNA1-based episomal approach that does not require integration into the genome [2].

Maintenance

The maintenance of pluripotent stem cells was carried out under sterile conditions in automation-friendly BD Matrigel-coated RoboFlasks® (Corning) with pierced septum caps, in mTeSR®1 medium (Stemcell Technologies). Individualizing and lifting of cells was performed using TrypLE™ (Invitrogen), after which cells were plated without a centrifugation step using soybean trypsin inhibitor (Invitrogen) and ROCK inhibitor H 1152 (Calbiochem), which improves the cell viability on individualized cells (patent pending technique developed at CDI). The daughter flasks were fed with TeSR media every 24 hrs after passaging, until they were ready to be split. The system was disinfected using a sporicidal solution at the end of each run, to prevent cross-contamination between cell lines.

Differentiation

CDI has developed a novel, defined, serum-free, automation-friendly three dimensional (3D) protocol to promote differentiation into multipotent HPCs. This method involves the formation of 3D structures called embryoid bodies (EBs) that, in the presence of cytokines, allow the development of various lineages, including HPCs.

Undifferentiated hESCs/iPSCs were harvested at confluence using TrypLE and neutralized in medium containing ROCK inhibitor. Following harvest, the cells were placed in serum-free EB formation media for 12 hrs in low attachment plates to facilitate aggregate formation. EB basal media was supplemented with an optimal concentration of growth factors and cytokines for the next 12-16 days under hypoxic conditions. The growth of hematopoietic cells from hESCs and iPSCs using this system is shown in Figure 1.

At the end of the differentiation process, the aggregates were individualized and the HPC content was quantified by flow cytometry. The efficiency of HPC differentiation (CD34+/CD43+) was determined as a ratio of the final viable cell number to the initial cell number of the hESC/iPSC population.

Screening

For the high throughput screen, the aggregates were made by placing 0.1 million hESCs in low attachment 96-well plates. The screen was performed using 2,200 known bioactive compounds at a final concentration of 10 µM in DMSO. The aggregates were treated with compound-containing media on days 5, 7 and 9 of hematopoietic differentiation. Cells differentiating in the absence of compounds, as well as cells treated with the vehicle control (DMSO), were also included in each plate as inbuilt controls.

The aggregates were harvested after 12 days of differentiation, and the HPCs were quantified by flow cytometry. The Hypercyt system (Intellicyt) was used to quantify the HPCs in a streamlined, automated manner. FlowJo data analysis software (Tree Star) was used for accurate exploration of HPC subsets and quantification of HPC content.

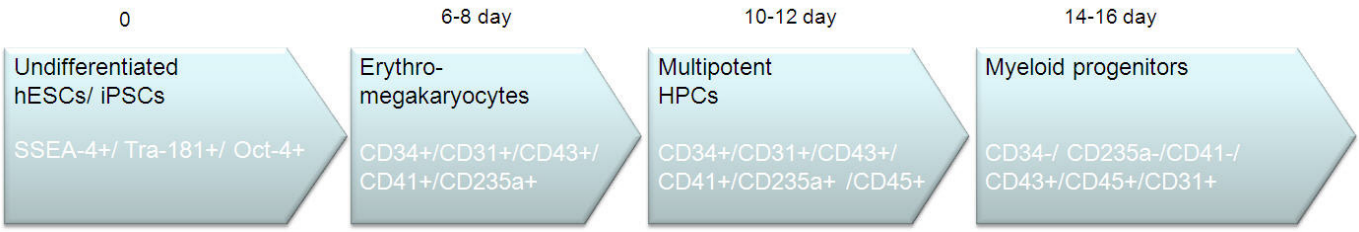

Figure 1 Differentiation steps from hESCs/iPSCs to PC

Results

Automated maintenance of pluripotent stem cells in an undifferentiated state

This study has shown that the Cellerity system can handle the feeder-free, defined culturing process for seeding, feeding and expansion of hESCs/iPSCs in Matrigel-coated RoboFlasks under hypoxic conditions, generating up to twentymillion hESCs in five days. The cells were continuously maintained on the platform for 10 consecutive passages, and tested for karyotype analysis at passages 0, 6 and 10, revealing a normal karyotype (Figure 2) and morphology (Figure 3) for undifferentiated hESCs.

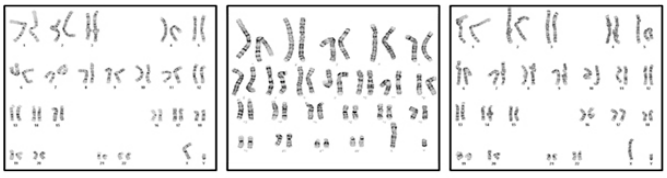

Figure 2 Karyotype of hESCs maintained on the Cellerity at different passages

Differentiation of hESCs/iPSCs into hematopoietic cells

Using hESCs which have been automatically maintained on the Cellerity platform, the 3D stem cell differentiation process developed by CDI closely mimics the kinetics of HPC differentiation from hESCs or iPSCs reported by Vodyanik et al [3]. One million hESCs or iPSCs are capable of generating between 60 - 80,000 HPCs.

Hematopoietic and endothelial cells develop from early CD34+Flk1+CD45- HPCs in a stage-specific manner. Erythro-megakaryocytic progenitors emerge between days 6 and 8, multipotent HPC cells emerge after 8 to 10 days, and cells with lymphoid-myeloid differentiation potential peak at 15-25 % of the population on day 12 (Figure 4).

High throughput screening and identification of small molecules that augment generation of HPC

Using the optimized 3D differentiation process, a small molecule screen was performed with hESCs with 2,200 known bioactive compounds from Microsource Spectrum libraries,

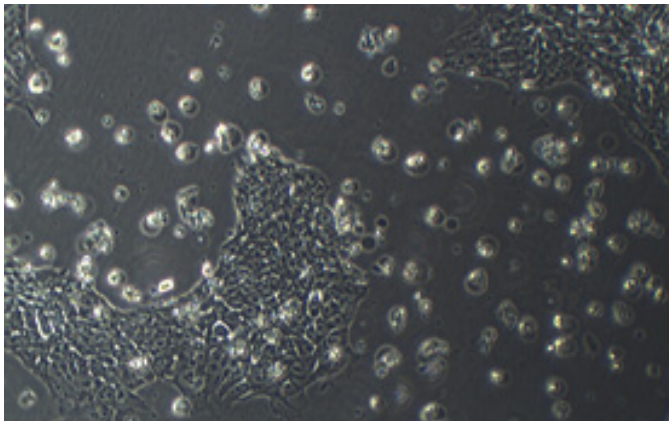

Figure 3 Morphology of hESCs cultured in RoboFlasks at the third passage

using a multichannel arm to provide high throughput. The purpose of the screen was to search for compounds which augment the generation of HPCs, and the various sub populations were quantified by flow cytometry.

The preliminary round of screening revealed ~25 hits. These compounds were cherry-picked and the screen was repeated to confirm the hits. The second screen revealed five reproducible hits, with all five compounds resulting in a two-fold increase in hematopoietic lineages compared to standard differentiation (Figure 5). These molecules were identified as known potent modulators of hematopoietic cells, as reported in the literature, and included GSK-3 beta inhibitors, PKC inhibitors and anti-fungal agents.

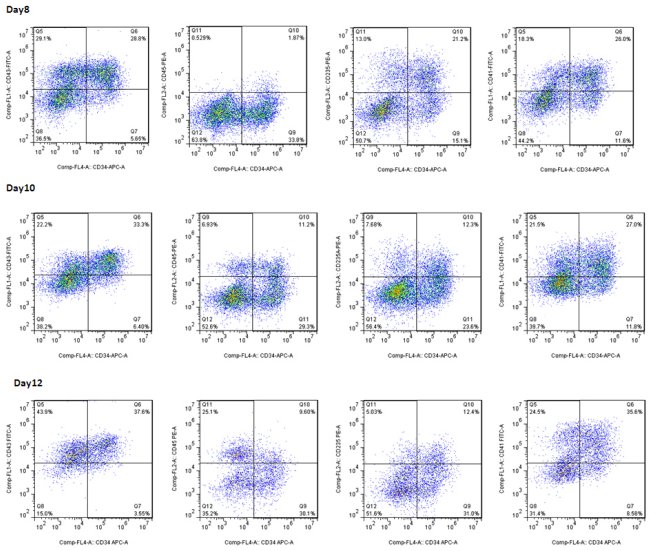

Figure 4 Emergence of HPCs shown by FACS analysis

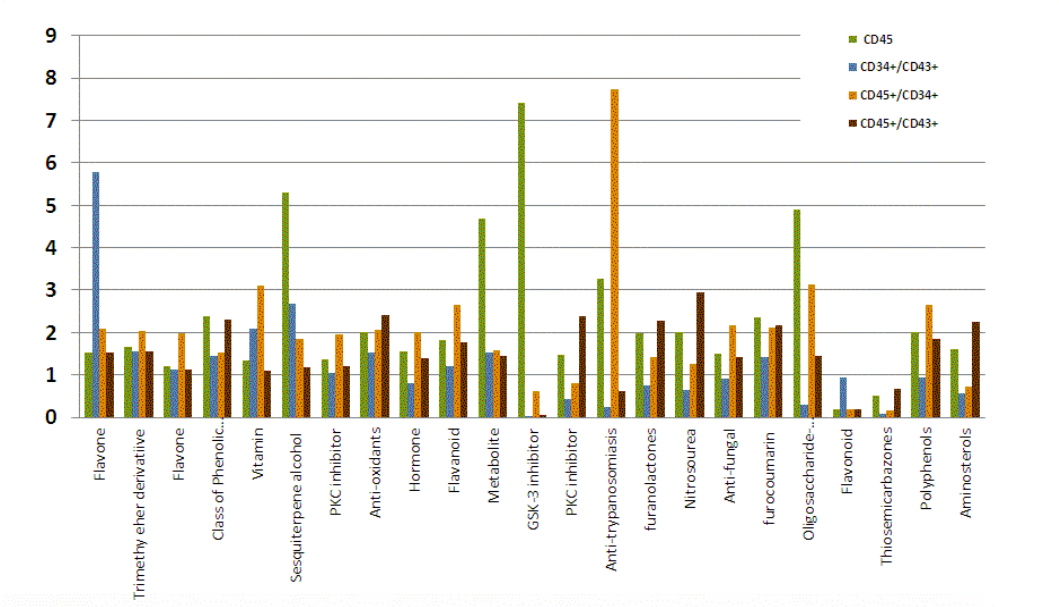

Figure 5 High throughput screen: compounds augmenting generation of HPCs

Conclusion

This study demonstrated the capacity of the Cellerity system to maintain hESCs/iPSCs through 10 passages, with no alteration to genetic stability. Using the differentiation protocol developed by CDI, the system can differentiate hESCs/iPSCs into multipotent HPCs within 12 days. The study also shows successful implementation of a high throughput small molecule screen, correctly identifying molecules that augment the generation of HPCs.

Automation of this differentiation procedure enables increased production of undifferentiated hESCs/iPSCs, multipotent HPCs and mature blood cell lineages, providing a large scale production system for disease studies, regenerative medicine research and drug discovery.

Acknowledgements

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References

1. Yu J, Vodyanik MA, Smuga-Otto K, Antosiewicz-Bourget J, Frane JL, Tian S, Nie J, Jonsdottir GA, Ruotti V, Stewart R, Slukvin, II, Thomson JA: Induced pluripotent stem cell lines derived from human somatic cells. Science 318: 1917-1920, 2007
2. Yu J, Hu K, Smuga-Otto K, Tian S, Stewart R, Slukvin, II, Thomson JA: Human induced pluripotent stem cells free of vector and transgene sequences. Science 324: 797-801, 2009
3. Vodyanik MA, Thomson JA, Slukvin, II: Leukosialin (CD43) defines hematopoietic progenitors in human embryonic stem cell differentiation cultures. Blood 108: 2095-2105, 2006

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