Application Note

single-cell printer™ | Generating single-cell clones of immortalized red blood cell (RBC) precursors using single-cell dispensing

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Introduction

Currently, the blood supply required for patients undergoing surgeries, cancer treatment and treatment for traumatic injuries is dependent on volunteer donors. Due to high demand, there are efforts underway to develop a safer and more readily available source of blood supply. This challenge can potentially be solved through blood pharming; producing blood cells *in vitro* using embryonic, bone marrow or induced stem cells. However, more research is needed to understand the mechanisms involved in enabling high-efficiency *in vitro* generation of enucleated blood cells.

This study highlights a method to immortalize erythroid progenitor cells that produce two major blood cell types (megakaryocytes and erythrocytes). The overexpression of oncogenes (c-myc and BCL-XL) into erythroid precursors enables sustained exponential self-replication of erythroid progenitor cells. Although the cell line had robust growth in culture, the resulting cell population exhibited a heterogeneous phenotype. Thus, the single-cell printer™ (scp™) enabled high-throughput generation of single-cell clones to identify clones exhibiting ideal erythroid lineage marker expression. Leveraging this workflow, over 200 clones were developed and propagated for further expression marker analysis.

Results and Discussion

To achieve a continuous supply of differentiated enucleated blood cells, a workflow was developed from transfection to single-cell cloning to select clones for further differentiation into mature RBCs (Figure 1). First, various combinations of oncogenes were transfected into an erythroid progenitor cell line derived from CD34+ stem cells.

Oncogenes are genes that can induce cancer. Oncogenes' capacity to increase cell proliferation and reduce cell death were exploited to enhance cell growth of the erythroid progenitor cell line, which is typically difficult to cultivate continuously. An antibiotic-inducible lentiviral vector system was used to deliver five different oncogenes (c-myc, BCL-XL, SV40, geT, Bmi-1, LhX-2) to cells independently and in pairs. 48 hours post-transfection, oncogene expression was activated by adding doxycycline (dox). It was found that individual oncogene transduction did not lead to cell immortalization. Only combined transduction of c-myc and BCL-XL led to immortalization of erythroid precursor cells, and the cells could be kept in culture and proliferate (Figure 2) for more than one year. However, removing dox deactivated oncogene expression and enabled further differentiation (Figure 3).

In addition to the capacity for long-term culture, the immortalized cells exhibited typical markers of early hematopoietic progenitor cells, including $CD34^{very\ low}CD45^{+/-}CD133^{+/-}CD71^{+}CD44^{high}CD105^{high}CD235a^{very\ low}$. While the cell line contained basophilic erythroblasts, a further differentiated euthyroid precursor, the overall population was heterogenous. Thus, single-cell cloning was performed to screen for cells that exhibited RBC-like properties.

Compared to standard cloning techniques, the scp™ enables a high-throughput method of isolating single-cell clones for further downstream analysis and screening. The scp™ technology is based on an inkjet-like principle. First, cells are deposited into a disposable cartridge that contains a

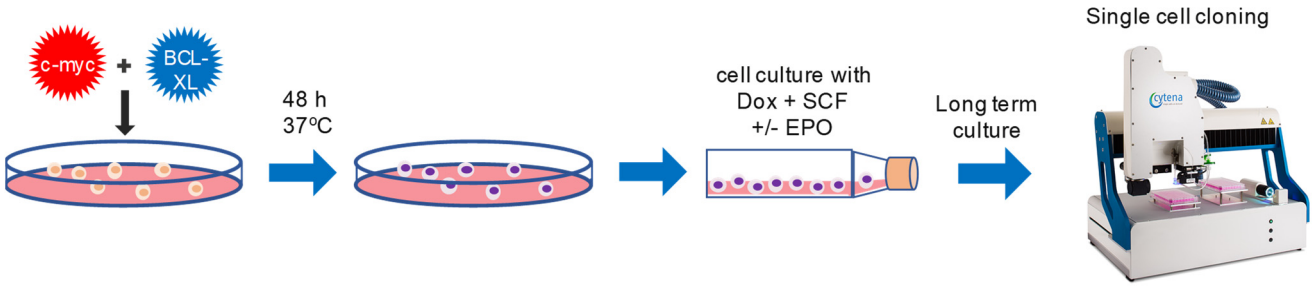

Figure 1. Workflow of erythroid cell clone generation. First, c-myc and BCL-XL oncogenes were transfected into an erythroid progenitor cell line. 48 hours post-transfection, oncogene expression was activated by adding doxy into the culture, enabling continuous culture. The cell culture produced a heterogeneous population of erythroid cells, and single-cell cloning was performed using the scp™ to select specific clones that exhibited surface markers for further differentiation into erythroid cells. Selected clones were further differentiated by removing dox and adding stem cell factor (SCF) and erythropoietin (EPO); they were then further characterized.

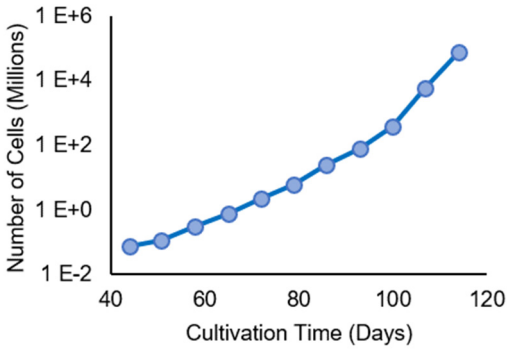

Figure 2. Proliferation rates of immortalized erythroid progenitor cells.

microfluidic chip. The bottom of the chip contains a nozzle where small, free-flying droplets are dispensed. The nozzle

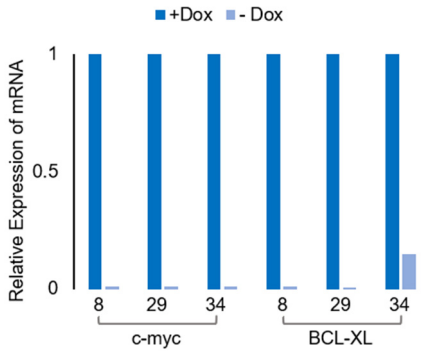

Figure 3. The withdrawal of dox from culture reduces mRNA expression of the c-myc and BCL-XL oncogenes for three different cell clones (Number 8, 29 and 34).

is continuously imaged to determine if the resulting droplet will have 0, 1 or multiple droplets. If a droplet contains a single cell, it is dispensed into the target well plate. If the droplet does not contain a cell, the droplet is disposed of as waste. The erythroid progenitor cells were dispensed into five 96-well plates (384-well plates are also compatible) filled with semisolid media. A series of five images (Figure 4A) serves as assurance of clonality and ensures that only a single cell was dispensed. According to the scp™ image series, 97.7% of the wells (469/480) could be addressed with single cells. Additionally, the dispensing process is gentle; cell growth was observed just one day post-dispensing.

From the dispensed cells, over 200 clones could be propagated. Clones that exhibited ideal morphology and surface marker expression (CD71, CD45, CD34, CD235a) were further analyzed. Dox, the component responsible for maintaining oncogene expression and continued proliferation, was removed from culture to further differentiate cells into hemoglobin-producing cells within 7 days (Figure 4B), highlighting the potential for possible development in to mature RBCs.

Conclusion

This study demonstrates that a typical nondividing progenitor RBC-precursor can be immortalized by transfecting the c-myc and BCL-XL oncogenes. The resulting cell line was heterogenous and required single-cell cloning to screen for ideal cell characteristics. Compared to standard methods, the scp™ enabled a high-throughput, robust method of developing single-cell clones while maintaining cell viability. The scp™ was able to produce over 200 clones that were later further differentiated and characterized for key progenitor markers.

Affiliations

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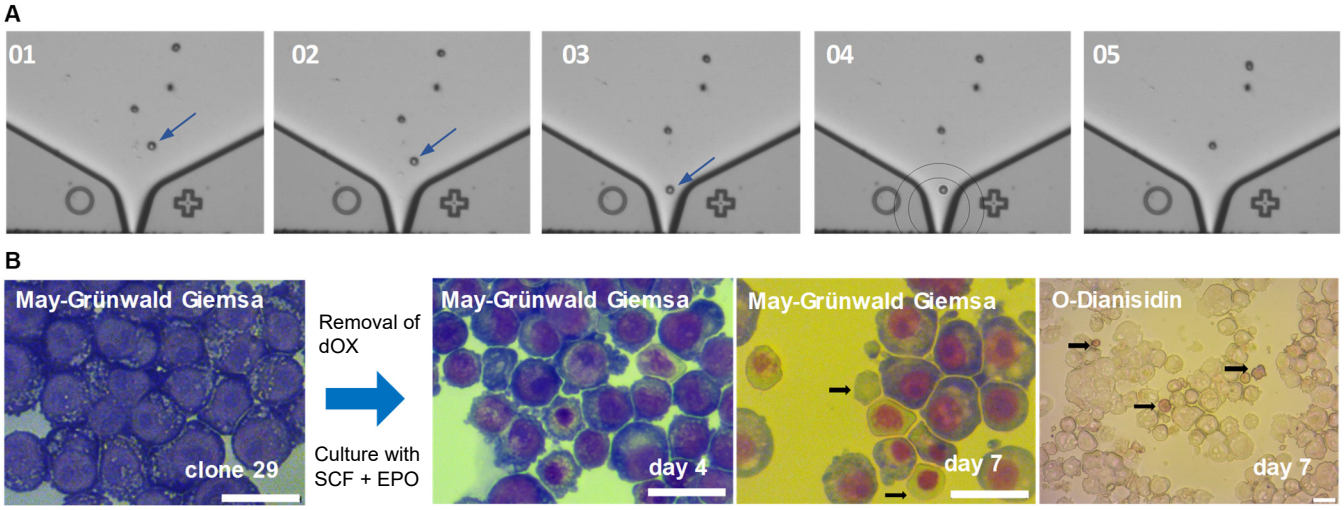

Figure 4. A. scp™ provides assurance of clonality by documenting that each cell that is deposited into the target plate. The scp™ achieved 97.7% single-cell deposition efficiency. **B.** One erythroid progenitor cell clone (29) was selected for further differentiation by removing dox and adding SCF and EPO. Hemoglobin-producing cells, as indicated through staining, were present in the culture.