miRNA-facilitated self-replicative RNA (srRNA) reprogramming of endothelial progenitor cells (EPCs) derived from human peripheral and cord blood

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Introduction

Peripheral blood provides easy access to adult human cell types for reprogramming purposes. In late 2012, two groups demonstrated the effective isolation, expansion, and subsequent generation of retrovirally-induced iPSC lines from endothelial progenitor cells (EPCs) derived from human peripheral blood. While circulating EPCs are a rare population of cells, we effectively isolated and expanded multiple adherent EPC lines. These primary EPC cultures can be established from fresh and frozen human peripheral blood as well as cord blood samples, some from as little as 1 x 10^6 mononuclear cells (MNCs) (Figure 1). The EPCs adhere to a high proliferative capacity while maintaining their cell identity marker, making them highly desirable for transfusion, and ultimately reprogramming into iPSCs with RNA.

In 2013, published results demonstrated the reprogramming of human neonatal fibroblasts into iPSCs using a polyinosinic self-replicative RNA (siRNA) with as few as a total of 25 transfusions. Subsequently, we have extended and improved the application of siRNA for cellular reprogramming to peripheral blood derived EPCs, cord blood derived EPCs, as well as adult fibroblasts. The generation of siRNA-EPC-iPS cells requires optimal siRNA delivery, culture media composition and transitions, as well as incorporation of reprogramming associated microRNAs. These improvements resulted in a simple two-transfection, no-split protocol on extracellular matrix without the need for conditioned medium (Figure 2) for the establishment of integration-free, wholly pluripotent human iPSC cell lines (Figure 3) from multiple target cell types (Table 1). Additionally, ipsc cells derived from siRNA reprogramming of EPCs exhibit unique innate genetic stability (Tables 2 and 3) when compared to previously published results of lines derived from fibroblasts or lines derived using integrating reprogramming technologies, making them an exceptional choice for cell fate manipulations and applications requiring clinical grade cells. Lastly, generation of these clinically relevant EPC-iPS cells using this novel siRNA reprogramming technology presents a therapeutic opportunity to specifically treat myeloproliferative disorders in which the disease-causing somatic mutations are restricted to the cells in the hematopoietic lineage.

Establishment and immunochemical characterization of EPCs derived from peripheral blood

A. Derivation timeline of adherent EPCs from peripheral blood. A minimum of 10^6 human mononuclear cells (MNCs) were seeded into a single T75 flask coated with collagen and cultured at 37°C, 5% CO_2, and 7% O_2 in Lonza EGM-2 medium. When supplied FBS was replaced with OptiMEM in the absence of serum.

B. Characterization of EPC srRNA iPS cell line (Donor 6-1) derived using Stemgent StemRNA-SR Reprogramming Kit.

FIGURE 1A: Derivation timeline of adherent EPCs from peripheral blood. A minimum of 10^6 human mononuclear cells (MNCs) were seeded into a single T75 flask coated with collagen and cultured at 37°C, 5% CO_2, and 7% O_2 in Lonza EGM-2 medium. When supplied FBS was replaced with OptiMEM in the absence of serum.

FIGURE 1B: Characterization of EPC srRNA iPS cell line (Donor 6-1) derived using Stemgent StemRNA-SR Reprogramming Kit.

Protocol timeline and morphology progression for reprogramming EPCs using Stemgent microRNA and self-replicative RNA (OKSIM)

FIGURE 2A: Timeline for the reprogramming of human EPCs using Stemgent StemRNA-SR Reprogramming Kit (Cat. No. 00-0070) with non-modified siRNA and microRNA. Transfections and paraxial selection were carried out in Lonza EGM-2 medium. Culture medium was transitioned to Nutristem XF/IF Culture Medium on Day 20 prior to B81R removal on Day 24.

FIGURE 2B: Primary reprogramming culture morphology progression, resulting from the reprogramming of peripheral blood derived EPCs (Donor 6-1) with Stemgent StemRNA-SR Reprogramming Kit (Cat. No. 00-0070). Day 0 EPCs (p6) were seeded at 2 x 10^5 cells per well of a 6-well Corning Matrigel coated plate. Day 1 EPCs were transfected with 10 pmol of siRNA. Day 2 EPCs were transfected with 1 pmol of siRNA. OKSIM. Days 0-19: culture was maintained at 37°C, 5% CO_2, and 7% O_2 in Lonza EGM-2 medium with penicillin (50 U/ ml) and streptomycin (50 U/ml) selection from Days 3-14. Day 20 culture medium was changed to Nutristem XF/IF Culture Medium. Recombinant B81R protein was included in culture medium from Days 0-24. iPSC cell morphology emerges as early as Day 14 and are able to be tanned by Day 26. Day 14 and Day 21 primary iPSC cell colonies were stained using Stemgent SolarBlue® TRA-1-60 antibody.

TABLE 1. Primary EPC establishment and subsequent reprogramming efficiencies for siRNA reprogramming of EPCs from human blood samples. Primary EPC establishment efficiency for peripheral blood is comparable of both fresh and cryopreserved MNCs. Cord blood efficiency data is from cryopreserved MNCs only. All samples had a minimum of 5 x 10^6 MNCs for seeding onto collagen coated T-75 flask. Reprogramming efficiencies (patient to patient or well) generated using non-integrated reprogramming protocol.

<table>
<thead>
<tr>
<th>BLOOD SOURCE</th>
<th>PRIMARY EPC ESTABLISHMENT EFFICIENCY (*)</th>
<th>PRIMARY EPC ESTABLISHMENT EFFICIENCY (PATIENT TO PATIENT)</th>
<th>REPROGRAMMING EFFICIENCY (PER WELL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peripheral Blood</td>
<td>18/22 (2-82%)</td>
<td>9/13 (70%)</td>
<td>0.0015-0.0052%</td>
</tr>
<tr>
<td>Cord Blood</td>
<td>9/65 (14-87%)</td>
<td>3/3 (81%)</td>
<td>0.0005-0.013%</td>
</tr>
<tr>
<td>TOTAL</td>
<td>67/54 (7-85%)</td>
<td>32/14 (81%)</td>
<td>0.0005-0.013%</td>
</tr>
</tbody>
</table>

TABLE 2. Copy number variation (CNV) comparison of EPC and fibroblast iPSC lines generated using siRNA or retroviral-mediated reprogramming factor delivery. CNV data generated on the Illumina HumanCytoSNP-12 DNA Analysis BeadChip platform. CNV: copy number variation.

<table>
<thead>
<tr>
<th>NUMBER OF IPS CELL LINES ANALYZED</th>
<th>TOTAL CNVs</th>
<th>CNV (% of genome)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EPC-siRNA-iPSCs</td>
<td>8</td>
<td>2.25</td>
</tr>
<tr>
<td>EPC-retro-iPSCs</td>
<td>3</td>
<td>2.67</td>
</tr>
<tr>
<td>srRNA-Fibroblast-iPSCs</td>
<td>2</td>
<td>4.2</td>
</tr>
</tbody>
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TABLE 3. Summary of CNV data generated on the Illumina HumanCytoSNP-12 DNA Analysis BeadChip platform.

Summary

- EPCs are a more genetically stable target cell type than fibroblasts for cell reprogramming
- siRNA-EPC-iPS cells exhibit greater genetic stability than retrovirus-EPC-iPS cells
- Simplified reprogramming protocol using Stemgent StemRNA-SR Reprogramming Kit (Cat. No. 00-0070) - 200,000 primary EPCs seeded into Corning Matrigel
- Requires only 2 transfections (siRNA + srRNA)
- No B81R mRNA contamination or conditioning required - uses B81R protein
- No reprogramming culture passaging/manipulation
- 3-wks for iPSC cell colony establishment
- Polymeric IF/IF culture added to iPSC lines in 3-4 passages (2 weeks)
- Single, efficient primary EPC line establishment
- Human peripheral and cord blood samples
- Fresh or frozen samples
- Two week primary cell establishment.

References