Generation of clinically compatible and genetically stable iPS cell lines from human peripheral and cord blood using microRNA-facilitated srRNA reprogramming

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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 iPS cell lines from endothelial progenitor cells (EPCs) derived from human peripheral blood[1,2]. Notably, while circulating EPCs are a rare population of cells we have effectively clonally isolated and expanded multiple adherent EPC lines from only 10 mL or 1 x 10^7 fresh or cryopreserved mononuclear cell (MNC) preparations from both human peripheral and cord blood (Figure 2 and Table 1). The EPC's adherent nature and high proliferative capacity, makes them highly desirable for transfection, and ultimately reprogramming into iPS cells using RNA.

In 2013, published results demonstrated the reprogramming of human neonatal fibroblasts into iPS cells using self-replicative RNA (srRNA)[3], with as few as one transfection. Subsequently, we extended the application of srRNA for cellular reprogramming to peripheral and cord blood derived EPCs. Development of the protocol for generating EPC-srRNA-iPS cells required optimization of mRNA delivery, culture media composition and transitions, as well as incorporation of reprogramming associated microRNAs allowing us to develop a singular EPC reprogramming protocol. Using this protocol, we have generated integration-free, wholly pluripotent human RNA-EPC-iPS cell lines from 45 out of 57 different primary patient blood samples (79% reprogramming efficiency) on the first pass (Table 1). Subsequent improvements have resulted in a simple and robust two transfection, no-split protocol (Figures 3A and 3B) using only GMP-compatible substrates (vitronectin and laminin-511) and media (human serum-supplemented endothelial cell media and NutriStem), which enhance reprogramming efficiency (Figure 1, Figure 3C). Additionally, these integration-free RNA-EPC-iPS cells exhibit superior genetic stability when compared to fibroblast-derived RNA-iPS cells, lines derived using integrating reprogramming technologies (Table 2), and previously published results of lines derived from fibroblasts[4,5], making them an exceptional choice for cell fate manipulations and applications requiring clinical grade cells. Additionally, these iPS cell lines demonstrate highly consistent tri-lineage differentiation potential (Figure 4). The unique combined application of microRNA and srRNA, using GMP-compliant reagents, for the cellular reprogramming of human EPC lines derived from peripheral and cord blood results in genetically stable, clinically relevant iPS cells that are well suited for consistent application of *in vitro* differentiation protocols.

Blood Reprogramming: Protocol Improvements

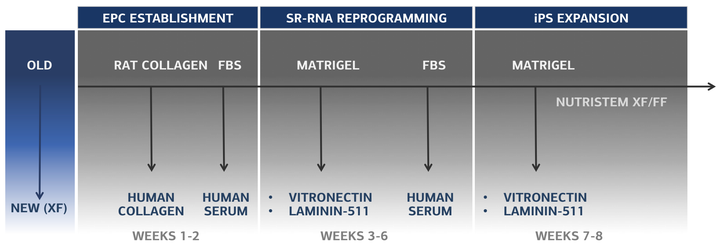
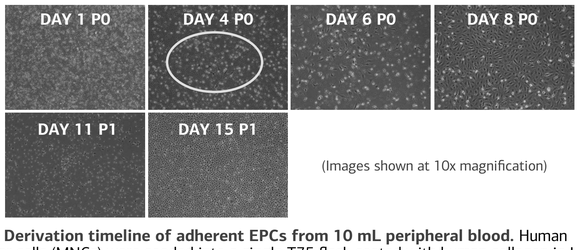


FIGURE 1: Xeno-free Protocol Optimization. EPC establishment from blood from only 10 mL of human blood by using human collagen and human serum. srRNA reprogramming protocol converted to xeno-free substrates (laminin-511 and vitronectin) and human serum. Generated EPC-srRNA-iPS cells expanded in Stemgent NutriStem™ XF/FF Culture medium.

Establishment of EPCs Derived from 10 mL Peripheral Blood



(Images shown at 10x magnification)

FIGURE 2: Derivation timeline of adherent EPCs from 10 mL peripheral blood. Human mononuclear cells (MNCs) were seeded into a single T75 flask coated with human collagen in Lonza EGM-2 medium, where supplied FBS was replaced with human serum (20% v/v final).

EPC Derivation and Reprogramming Efficiency from 10 mL Blood

BLOOD SOURCE	PERIPHERAL BLOOD			CORD BLOOD
EPC Derivation Condition	50 mL FBS Rat Collagen	40 mL Human Serum Human Collagen (donor #1-8)	**10 mL Human Serum Human Collagen (donor #1-8)**	Frozen MNCs FBS Rat Collagen
Primary EPC Establishment Efficiency	18/22 = **82%**	8/8 = **100%**	7/8 = **87.5%**	46/53 = **87%**
Reprogramming Efficiency (Patient-to-Patient)	9/13 = **70%**	**N.D.**	3/3 = **100%**	33/41 = **81%**

TABLE 1. Primary EPC establishment from 10 mL and 50 mL blood and subsequent reprogramming efficiencies. EPC derived from 50 mL peripheral blood and a minimum of 5x10^7 cord blood MNCs using standard FBS protocol. EPCs from 10 mL and 40 mL peripheral blood samples were derived in parallel from the same donors by using human serum instead of FBS. All reprogramming efficiencies (patient-to-patient) generated using two transfection protocol.

Timeline for Reprogramming EPCs using srRNA

A

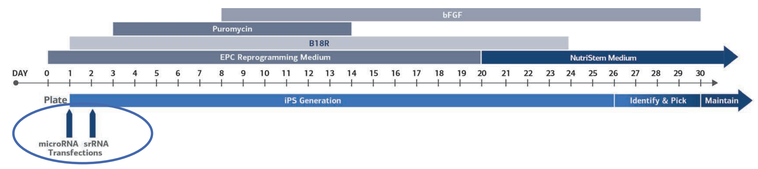


FIGURE 3A: Timeline for the reprogramming of human EPCs using **Stemgent StemRNA-SR Reprogramming Kit (Cat. No. 00-0075)** with non-modified srRNA and microRNA. RNA transfections and puromycin selection were carried out in EPC Reprogramming Medium (Lonza EGM-2 medium).

Morphology Progression and Reprogramming Efficiency of EPCs using Xeno-free Reagents

B

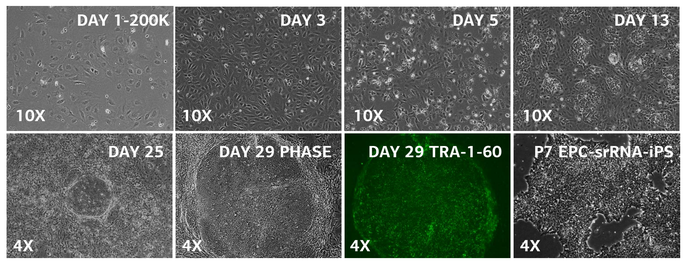


FIGURE 3B: Primary reprogramming culture morphology progression, resulting from the reprogramming of a 10 mL peripheral blood derived EPCs with **Stemgent StemRNA-SR Reprogramming Kit (Cat. No. 00-0075)**. **Day 0:** EPCs (p3) were seeded at 2 x 10^5 cells per well in a 6-well plate coated with laminin-511. **Day 1:** EPCs were transfected with 70 pmol of microRNA. **Day 2:** EPCs were transfected with 1 µg of srRNA (OKSiM). iPS cell morphologies emerge as early as **Day 13** and are able to be isolated between **Day 26-29. Day 29:** Primary iPS cell colonies were identified using Stemgent StainAlive™ TRA-1-60 antibody. **P7** EPC-srRNA-iPS colony was picked and expanded on laminin-511 and NutriStem XF/FF Culture Medium.

C

MATRIX	SERUM	# EPC-srRNA-iPS COLONIES
Corning® Matrigel®	FBS	3
Corning Matrigel	Human serum	80
Laminin-511	**Human serum**	**204**
Vitronectin	Human serum	92

D

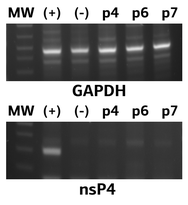


FIGURE 3C,D: (C) Enhanced reprogramming efficiency using **Stemgent StemRNA-SR Reprogramming Kit** by converting protocol to xeno-free substrate and human serum. **(D)** RT-PCR analysis of EPC-srRNA-iPS cell line for cytoplasmic retention of polycistronic srRNA. Total RNA was isolated from: p4, p6, and p7 of the RNA-iPS cell line; non-transfected EPCs [(-) control] and EPCs transfected with 1 µg of polycistronic srRNA [(+) control]. Load control primers = GAPDH. Polycistronic srRNA specific primers = nsP4 (non-structural protein 4).

CNV Analysis Shows Superior Genetic Stability of EPC-srRNA-iPS Cells

iPS CELL LINE	SAMPLE	AUTOSOMAL CNVS IN iPSC	CHROMOSOME	SIZE OF REGION COVERED BY CNVS	COPY GAIN OR LOSS	NUMBER OF iPS CELL LINES WITH CNVs
EPC-srRNA	patient 1	1	16	12806	single copy loss	2/8
	patient 2	0				
	patient 3	0				
	patient 4 (D)	0				
	patient 5	0				
	patient 6-1 (D)	0				
	patient 6-2 (D)	1	6	50160	single copy loss	
	patient 6-3 (D)	0				
EPC-retro	patient 6-1 (D)	3	6	105930	single copy loss	3/3
			6	233158	single copy loss	
			13	47059	single copy loss	
	patient 6-2 (D)	3	3	346679	double copy loss	
			5	64569	double copy loss	
			6	52655	single copy loss	
	patient 5	2	3	82105	double copy loss	
			3	148326	single copy loss	
Fibroblast-srRNA	patient 1 -1	3	5	190516	double copy loss	2/2
			20	159147	double copy loss	
			7	145891	double copy loss	
	patient 1 -2	1	2	3490	double copy loss	

TABLE 2. Copy number variation (CNV) comparison of EPC and fibroblast iPS cell lines generated using srRNA or retroviral-mediated reprogramming factor delivery. CNV data generated on the Illumina HumanCytoSNP-12 DNA Analysis BeadChip platform. For CNV calls, 5kb size cut-off value and a minimum of ten markers (SNPs) were used as analysis configurations.

Differentiation Capacity of iPS Cells Derived using Stemgent StemRNA-SR Reprogramming Kit

TERATOMA ANALYSIS (*in vivo*)

A

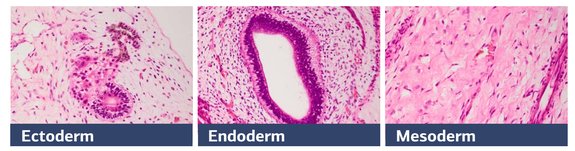


FIGURE 4A: Histological analysis of teratoma resulting from the injection of EPC-srRNA-iPS cells into the kidney capsule of NOD-SCID mice. Prior to injection, EPC-srRNA-iPS cells expanded on Corning Matrigel with NutriStem XF/FF Culture Medium.

MESODERM DIFFERENTIATION (*in vitro*)
TroponinT

B

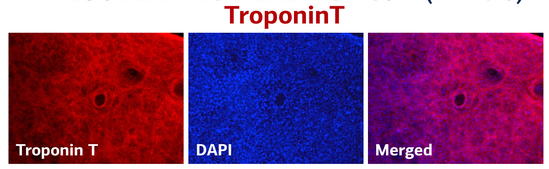


FIGURE 4B: EPC-srRNA-iPS cells were differentiated into cardiomyocytes and immunostained for Troponin T (red) and DAPI (blue).

ECTODERM DIFFERENTIATION (*in vitro*)
Nestin/b-tubulin

C

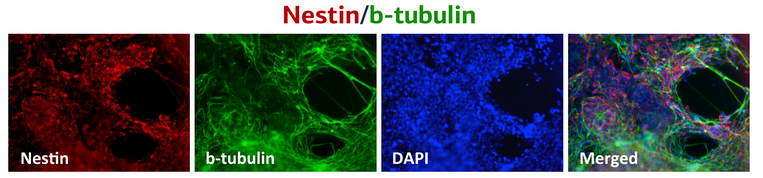


FIGURE 4C: EPC-srRNA-iPS cells were differentiated into neurons and immunostained for neuronal markers Nestin (red) , bIII-tubulin (green) and DAPI (blue).

ENDODERM DIFFERENTIATION (*in vitro*)
AFP

D

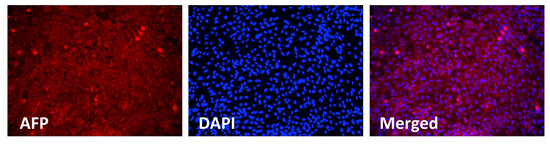


FIGURE 4D: EPC-srRNA-iPS cells were differentiated into early endoderm and immunostained for alpha-Fetoprotein (AFP) (red) and DAPI (blue).

Summary

- **Simplified reprogramming protocol using Stemgent StemRNA-SR Reprogramming Kit (Cat. No. 00-0075)**
  - 200,000 EPCs
  - Requires only laminin-511, vitronectin or Matrigel
  - No feeders, conditioned medium or FBS required
  - No reprogramming culture passaging/manipulation required
  - Approximately three weeks required for primary iPS cell colony establishment
  - Polycistronic srRNA cleared from isolated iPS cell lines in 3-4 passages (2 weeks)
- **Simple, efficient primary EPC line establishment from only 10 mL of human blood or cord blood**
  - Fresh or frozen samples can be used
  - More efficient and shorter derivation time line using human serum vs. FBS
  - Only two week primary culture establishment needed
- **EPCs are a more genetically stable target cell type than fibroblasts for cellular reprogramming**
- **srRNA-EPC-iPS cell lines exhibit greater genetic stability than retrovirus-EPC-iPS cell lines**

References

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