A novel four transfection protocol for deriving iPS cell lines from human blood-derived endothelial progenitor cells (EPCs) and adult human dermal fibroblasts using a cocktail of non-modified reprogramming and immune evasion mRNAs

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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]. While circulating EPCs are a rare population of cells in blood, we have effectively isolated and established multiple adherent and expandable primary EPC from fresh and frozen human peripheral and cord blood samples, some from as little as 1 x 10^7 mononuclear cells (MNCs) (Figure 1). The EPCs adherent nature and high proliferative capacity while maintaining their cell identity makes them highly desirable for transfection, and ultimately reprogramming with RNA.

Here we present data demonstrating the unique combined application of non-modified reprogramming mRNAs (Oct4, Sox2, Klf4, cMyc, Nanog and Lin28) and immune evasion mRNAs (E3, K3, and B18) derived from Vaccinia virus with reprogramming-associated mature double stranded microRNAs (302/367 cluster) for the cellular reprogramming of human EPC lines derived from peripheral blood and cord blood into stable, pluripotent and clinically relevant iPS cells (Figure 4). Inclusion of the E3, K3, and B18 immune evasion mRNAs in the RNA transfection cocktail eliminates the need to supplement cell culture medium with recombinant B18 protein during the reprogramming process. Additionally, adjusting reprogramming factor mRNA molar stoichiometries to elevate Oct4 transcript levels in the RNA cocktail resulted in a four transfection protocol (Figure 3) that efficiently generated TRA-1-81 positive iPS cell colonies from both human blood-derived EPCs (≤0.02%) and HUVECs (≤0.40%) within 10 days. Moreover by increasing the total number of RNA transfections from 4 to 6, the reprogramming efficiency for EPCs increased to ≤0.25% while HUVECs increased to ≤0.60%. Equivalent application of the non-modified reprogramming RNA technology to human fibroblasts resulted in a four transfection protocol (Figure 5) where lower fibroblast seeding densities, exclusive use of NutriStem™ Culture Medium, and elevated Oct4 transcript levels proved beneficial for increasing the reprogramming efficiencies of both neonatal (0.5%) and adult dermal fibroblasts (1.6%) (Table 1). The effective application of this non-modified RNA platform for efficient cellular reprogramming of multiple human cell types with only 4-6 repeat transfections demonstrates both the flexibility and robustness of the system, potentially opening the door to the use of non-modified RNA in cell fate conversions to non-proliferative populations of cells which appear refractory to existing modified RNA technologies.

Establishment and immunocytochemical characterization of EPCs derived from peripheral blood

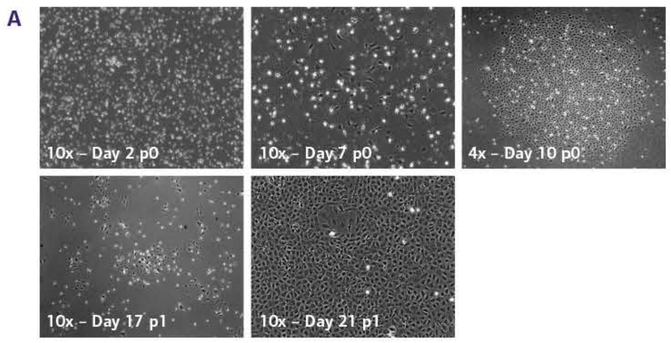

FIGURE 1A: Derivation timeline of adherent EPCs from peripheral blood.
A minimum of 1x10^7 human mononuclear cells (MNCs) were seeded into a single T75 flask coated with collagen I and cultured at 37°C, 5% CO_2, and 21% O_2 in Lonza EGM-2 medium, where supplied FBS was replaced with HyClone™ ES-qualified, defined FBS to 20% v/v final.
Day 2 → Primary (p0) suspension culture of human mononuclear cells.
Day 7 → Emerging EPC colonies.
Day 10 → Maturation of EPC colony.
Day 17 → p1 EPC culture 3 days after passaging.
Day 21 → Confluent p1 EPC culture ready to be passaged.

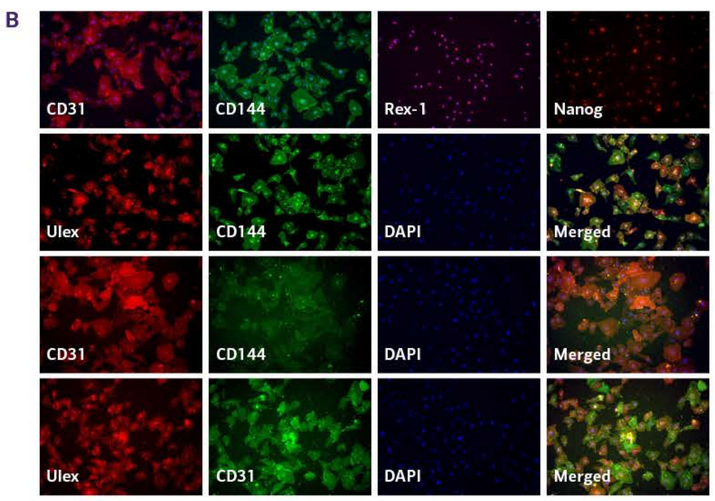

FIGURE 1B: Immunocytochemistry (ICC) of EPCs. EPCs at p5 were seeded at a density of 5 x 10^4 cells per well of a 12-well plate. Cells were fixed the following day with 4% paraformaldehyde (PFA) and stained with the appropriate antibodies and DAPI for visualization. Merged images are shown.

Efficient delivery of non-modified GFP mRNA to EPCs

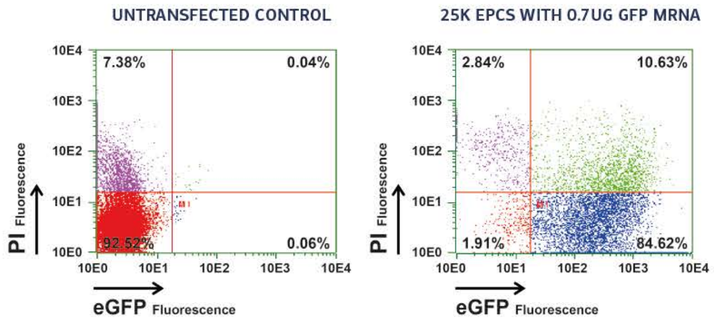

FIGURE 2: Efficient delivery of non-modified GFP mRNA to EPCs. Graph of mean fluorescence intensity versus cell viability as determined by flow cytometry. EPCs were plated at 2.5 x 10^4 per well of a 12-well plate and cultured at 37°C and 5% CO_2. The next day, the cells were transfected with 0.7 µg of non-modified GFP mRNA. 18 to 24h post-transfection, Propidium Iodide (PI) was added and samples analyzed by flow cytometry for GFP expression and cell viability. Overnight transfection resulted in >90 % transfection efficiency (right panel) with high cell viability as compared to the untransfected control (left panel).

Protocol timeline and morphology progression for reprogramming EPCs into iPS cells using a cocktail of non-modified RNAs

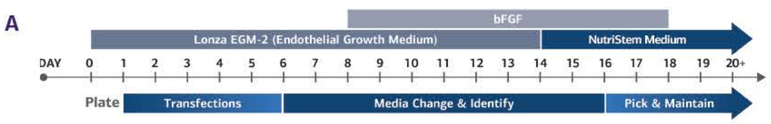

FIGURE 3A: Timeline for the reprogramming of human EPCs using non-modified reprogramming mRNAs (OSKMNL), non-modified interferon-ablating mRNAs (EKB), and microRNA (302/367) reprogramming cocktail. Daily RNA transfections were carried out in Lonza EGM-2 medium. Culture medium was transitioned to NutriStem XF/FF Culture Medium on Day14.

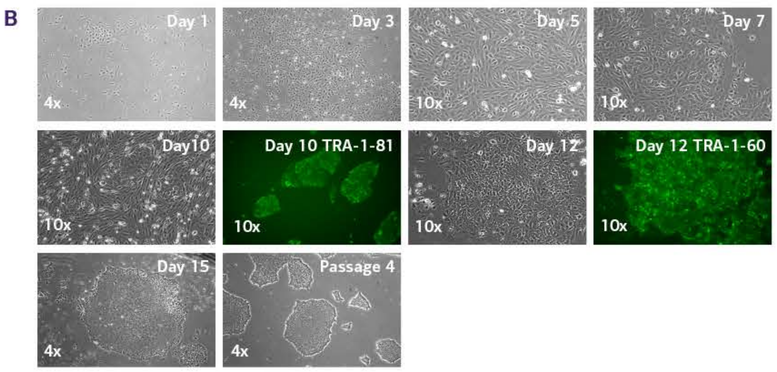

FIGURE 3B: Primary reprogramming culture morphology progression, resulting from the reprogramming of EPCs with non-modified RNA reprogramming cocktail. Day 0: EPCs (p6) were seeded at 2.5 x 10^4 cells per well of a 6-well Corning Matrigel-coated plate. Days 1 – 8: EPCs were transfected with non-modified reprogramming mRNAs (OSKMNL), non-modified interferon-ablating mRNAs (EKB), and microRNA (302/367) reprogramming cocktail. Culture was maintained at 37°C, 5% CO_2, and 5% O_2 in Lonza EGM-2 medium. Day 14: Culture medium was changed to NutriStem XF/FF Culture Medium. Days 10 – 15: EPC-iPS cell morphologies emerge as early as Day 10 and are able to be isolated from the primary culture by Day 15. Day 10 and Day 12 primary EPC-iPS cell colonies were identified using Stemgent StainAlive TRA-1-81 and TRA-1-60 antibodies, respectively.

References

1. Geti, I. *et al.* (2012) A practical and efficient cellular substrate for the generation of induced pluripotent stem cells from adults: blood-derived endothelial progenitor cells. *Stem Cells Transl Med.*; 1:855-65.
2. Chang, W.Y. *et al.* (2013) Feeder-independent derivation of induced-pluripotent stem cells from peripheral blood endothelial progenitor cells. *Stem Cell Res.*; 10:195-202.

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Characterization of EPC-iPS cell line derived using a cocktail of non-modified RNAs

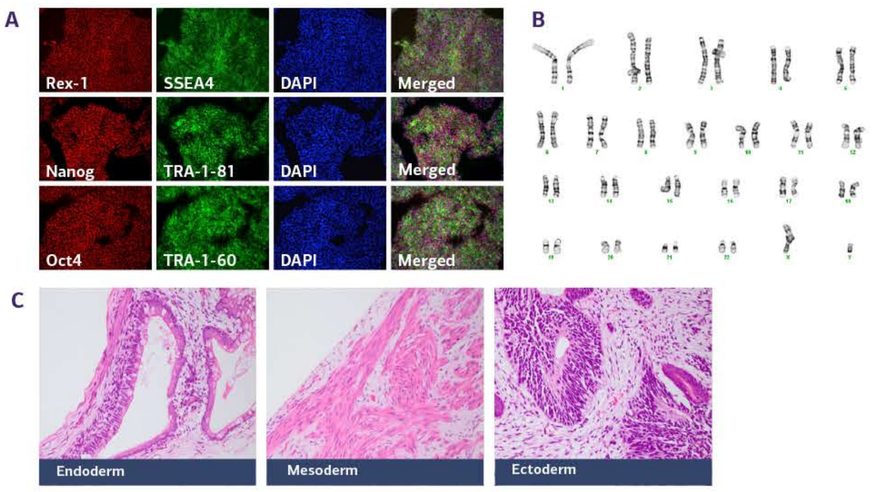

FIGURE 4A. Pluripotency immunocytochemistry (ICC) of EPC iPS cell line (p15) derived from human EPCs (p5) and expanded on Corning Matrigel with NutriStem XF/FF Culture Medium. Nuclear pluripotency staining with Rex1, Nanog, and Oct4 antibodies is seen in red. Cell surface pluripotency staining with SSEA4, TRA-1-81, and TRA-1-60 antibodies is seen in green. Merged images are shown.

FIGURE 4B. Normal karyotype of EPC-iPS cell line (p13) expanded on Corning Matrigel with NutriStem XF/FF Culture Medium.

FIGURE 4C. Histological analysis of teratoma resulting from the injection of EPC-iPS cells (p13) into the kidney capsule of NOD-SCID mice. Prior to injection, EPC-iPS cells expanded on Corning Matrigel with NutriStem XF/FF Culture Medium.

Protocol timeline for reprogramming human fibroblasts with a cocktail of non-modified RNAs

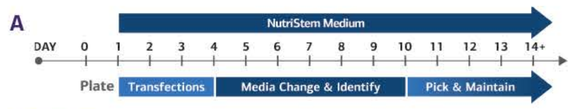

FIGURE 5A: Timeline for the reprogramming of human fibroblasts using non-modified reprogramming mRNAs (OSKMNL), non-modified interferon-ablating mRNAs (EKB), and microRNA (302/367) reprogramming cocktail. Daily RNA transfections (1.8 µg total). Reprogramming culture as well as maintenance of iPS cells were carried out in NutriStem XF/FF Culture Medium.

CELL LINE	CELL # (K)	STOICHIOMETRY (OSKMNL)	MEDIA	TRA-1-60+ COLONY COUNT	EFFICIENCY IN %
Nuffs	50	1:1:1:1:1:1	NutriStem to NutritStem-CM	0	0
Nuffs	100	1:1:1:1:1:1	NutriStem to NutritStem-CM	0	0
Nuffs	50	3:1:1:1:1:1	NutriStem to NutritStem-CM	81	0.162
Nuffs	100	3:1:1:1:1:1	NutriStem to NutritStem-CM	7	0.007
Nuffs	50	3:1:1:1:1:1	NutriStem Only	262	0.524
Nuffs	100	3:1:1:1:1:1	NutriStem Only	54	0.054
HDFa	50	1:1:1:1:1:1	NutriStem to NutritStem-CM	95	0.19
HDFa	100	1:1:1:1:1:1	NutriStem to NutritStem-CM	0	0
HDFa	50	3:1:1:1:1:1	NutriStem to NutritStem-CM	>500	>1
HDFa	100	3:1:1:1:1:1	NutriStem to NutritStem-CM	>500	>0.5
HDFa	25	3:1:1:1:1:1	NutriStem Only	393	1.6
HDFa	50	3:1:1:1:1:1	NutriStem Only	713	1.4
HDFa	100	3:1:1:1:1:1	NutriStem Only	121	0.1

TABLE 1. Reprogramming efficiencies for non-modified RNA reprogramming of human fibroblasts into iPS cells. Adult human HDFa and neonatal newborn foreskin fibroblasts (NuFFs) were plated at 25k , 50k or 100k on Corning Matrigel-coated 6-well plates. Each reprogramming well was transfected for 4 consecutive days with non-modified reprogramming mRNAs (OSKMNL), non-modified interferon-ablating mRNAs (EKB), and microRNA (302/367) reprogramming cocktail. Cells were either cultured exclusively in NutriStem XF/FF Culture Medium or transitioned from NutriStem to human foreskin fibroblast conditioned NutriStem on day 5.

Summary

- **Generation of stable, pluripotent EPC-iPS cell lines using a non-modified RNA**
  - Only 4-6 RNA cocktail transfections
  - Two weeks for iPS cell colony establishment **(No screening required)**
  - Protocol does not require rB18 protein
  - Protocol does not require conditioned medium or feeder cells
- **Generation of stable, pluripotent adult and neonatal fibroblast-iPS cell lines using a non-modified RNA**
  - Only 4 RNA cocktail transfections
  - Ten days for iPS cell colony establishment **(No screening required)**
  - Protocol does NOT require rB18 protein
  - Establishment of fibroblasts iPS cells in Xeno-free NutriStem XF/FF Culture Medium
- **Simple, efficient primary EPC line establishment**
  - Human peripheral and cord blood samples
  - Fresh or frozen samples
  - Two week primary culture establishment