RNA-mediated generation of integration-free iPS cell lines from late-outgrowth endothelial progenitor cells (L-EPCs) derived from human blood

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INTRODUCTION

In 2010, it was first published that repeated transfection of human dermal fibroblasts with a cocktail of reprogramming mRNAs can result in the generation of integration-free human iPS cells. While many advancements have been made to refine this process, to date no group has been able to demonstrate RNA-based reprogramming of a blood-derived cell type. This limitation has been primarily due to the inability to efficiently and repeatedly deliver mRNA to blood-derived cell types without inducing cellular toxicity.

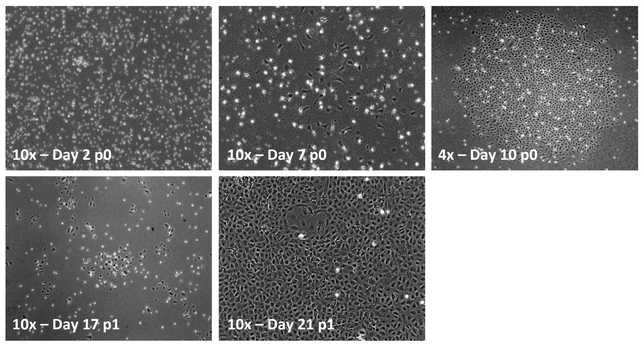
Peripheral blood provides easy access to adult human cell types for reprogramming purposes. Additionally, late-outgrowth endothelial progenitor cells (L-EPCs) can be clonally isolated from both human peripheral blood and cord blood. The L-EPC's adherent nature and high proliferative capacity while maintaining their cell identity makes them a highly desirable cell type for repeated transfection with RNA. Lastly, the ability to generate clinical-grade iPS cells from L-EPCs using RNA reprogramming technologies presents a unique therapeutic opportunity to treat myeloproliferative disorders in which the disease-causing somatic mutations are restricted to cells in the hematopoietic lineage.

In late 2012, two groups demonstrated the effective isolation, expansion, and subsequent generation of iPS cell lines from L-EPCs derived from human peripheral blood[2,3]. While circulating EPCs are a rare population in peripheral blood, we have effectively isolated and established multiple adherent and expandable L-EPC primary cultures, some from as little as 1 x 10^7 mononuclear cells (MNCs) (Figure 1).

Subsequently we have been able to generate iPS cell lines from multiple primary L-EPC cultures using two novel non-modified RNA reprogramming technologies. Successful application of the **polycistronic self-replicative RNA (srRNA)**[4] technology for reprogramming L-EPCs required optimization of mRNA delivery, culture media composition and transitions, as well as incorporation of miRNAs. These adjustments result in a minimal two transfection protocol (Figure 2) that yields TRA-1-60 positive iPS cell colonies in 3-4 weeks. The second novel mRNA reprogramming technology requires repeated transfection with a **cocktail of non-modified reprogramming mRNAs**, non-modified interferon ablating mRNAs, and microRNAs. An optimized protocol (Figure 4) requiring 9-10 daily transfection results in the efficient (< 0.25%) generation of TRA-1-60 positive iPS cell colonies in as few as 12 days. Both technologies result in the easy establishment of integration-free, wholly pluripotent, and genetically stable human iPS cell lines (Figures 3 and 5) from L-EPCs derived from human peripheral blood.

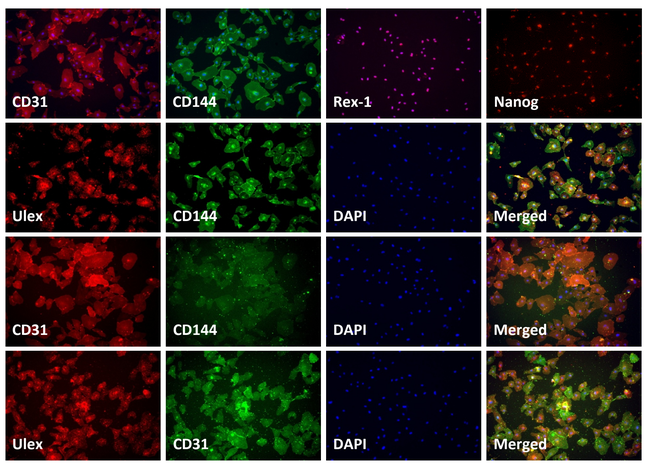
Figure 1. Establishment and immunocytochemical characterization of L-EPCs derived from peripheral blood.

A.



A. Derivation timeline of adherent L-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. From Day 1 to Day 13, the culture medium was changed every other day. **Day 2 →** Primary (p0) suspension culture of human mononuclear cells. **Day 7 →** Emerging L-EPC colonies. **Day 10 →** Maturation of L-EPC colony. **Day 17 →** p1 L-EPC culture 3 days after passaging. **Day 21 →** Confluent p1 L-EPC culture ready to be passaged.

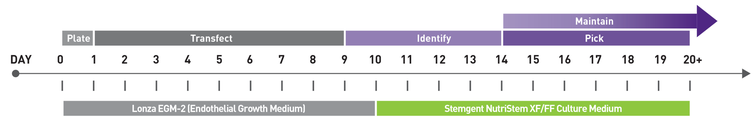
B.



B. Immunocytochemistry (ICC) of RJB-L-EPCs. L-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.

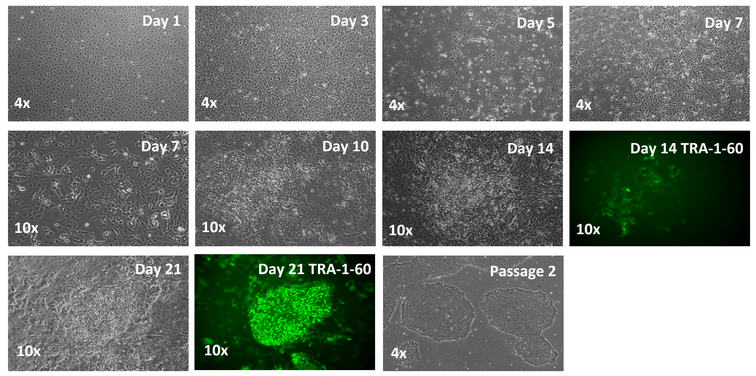
Figure 2. Protocol timeline and morphology progression for reprogramming L-EPCs with a non-modified, polycistronic self-replicative RNA (srRNA).

A.



A. Timeline for the reprogramming of human L-EPCs using non-modified srRNA (T7-OKSiM) and microRNA. RNA transfections were carried out in Lonza EGM-2 medium. Culture medium was transitioned to NutriStem® XF/FF Culture Medium on Day 17.

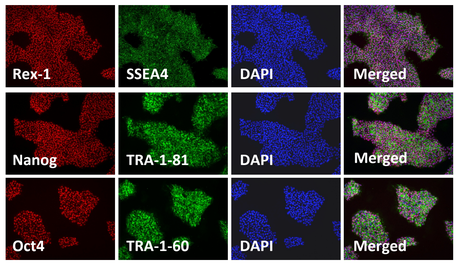
B.



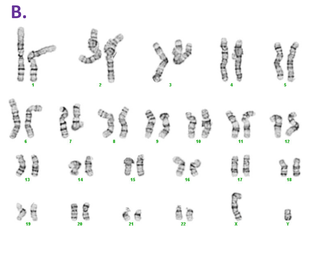
B. Primary reprogramming culture morphology progression, resulting from the reprogramming of RJB L-EPCs with srRNA. Day 0: L-EPCs (p6) were seeded at 2 x 10^5 cells per well of a 6-well Corning Matrigel® Matrix-coated plate. Day 1: L-EPCs were transfected with 70 pmol of microRNA. Day 2: L-EPCs were transfected with 1 µg of srRNA (T7-OKSiM). Days 0-16: culture was maintained at 37°C, 5% CO_2, and 5% O_2 in Lonza EGM-2 medium with puromycin (0.4 µg/ml) selection from Days 3-14. Day 17: culture medium was changed to NutriStem XF/FF Culture Medium. Recombinant B18-R protein was included in culture medium from Days 0-16. iPS cell morphologies emerge as early as Day 14 and are able to be isolated by Day 21. Day 14 and Day 21 primary iPS cell colonies were identified using Stemgent StainAlive™ TRA-1-60 antibody.

Figure 3. Characterization of RJB L-EPC iPS cell line derived using non-modified, polycistronic self-replicative RNA (srRNA).

A.

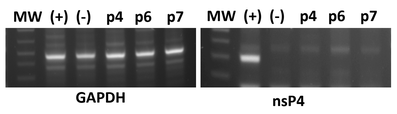


B.



A. Pluripotency immunocytochemistry (ICC) of RJB L-EPC iPS cell line (p13) derived from human L-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.
B. Normal karyotype of RJB L-EPC iPS cell line (p9) expanded on Corning Matrigel with NutriStem XF/FF Culture Medium.

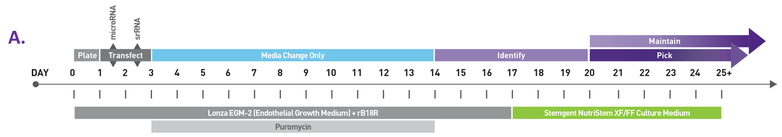
C.



C. RT-PCR analysis of RJB L-EPC iPS cell line for cytoplasmic retention of polycistronic srRNA. RJB L-EPC iPS cell line was expanded on Corning Matrigel with NutriStem XF/FF Culture Medium. Total RNA was isolated from: p4, p6, and p7 of the iPS cell line; non-transfected RJB L-EPCs [(-) control] and RJB L-EPCs transfected with 1 µg of polycistronic srRNA [(+) control]. Load control primers = GAPDH. Polycistronic srRNA specific primers = nsP4 (non-structural protein 4).

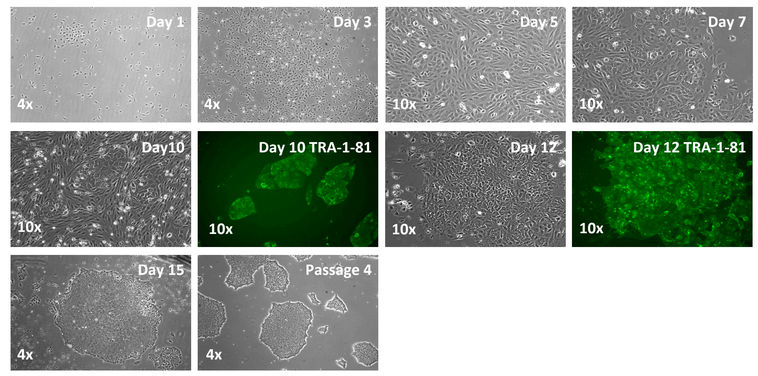
Figure 4. Protocol timeline and morphology progression for reprogramming L-EPCs with a cocktail of non-modified RNAs.

A.



A. Timeline for the reprogramming of human L-EPCs using non-modified reprogramming mRNAs (OSKMNL), non-modified interferon-ablating mRNAs, and microRNA reprogramming cocktail. Daily RNA transfections were carried out in Lonza EGM-2 medium. Culture medium was transitioned to NutriStem XF/FF Culture Medium on Day10.

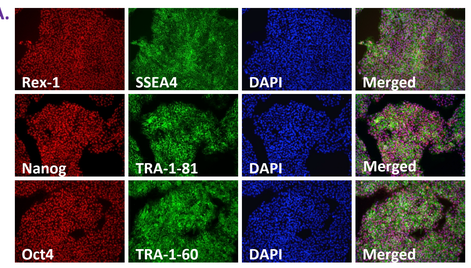
B.



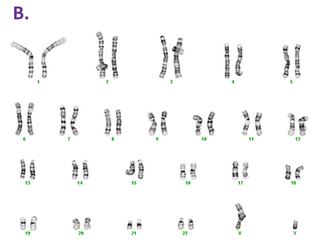
B. Primary reprogramming culture morphology progression, resulting from the reprogramming of RJB L-EPCs with non-modified RNA reprogramming cocktail. Day 0: L-EPCs (p6) were seeded at 2.5 x 10^4 cells per well of a 6-well Corning Matrigel-coated plate. Days 1 – 9: L-EPCs were transfected with non-modified reprogramming mRNAs (OSKMNL), non-modified interferon-ablating mRNAs, and microRNA reprogramming cocktail. Culture was maintained at 37°C, 5% CO_2, and 5% O_2 in Lonza EGM-2 medium. Day 10: Culture medium was changed to NutriStem XF/FF Culture Medium. Days 10 – 15: 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 iPS cell colonies were identified using Stemgent StainAlive TRA-1-81 and TRA-1-60 antibodies.

Figure 5. Characterization of RJB L-EPC iPS cell line derived using a cocktail of non-modified RNAs.

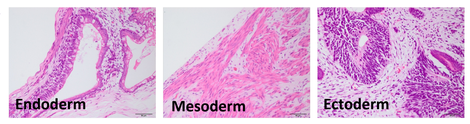
A.



B.



C.



A. Pluripotency immunocytochemistry (ICC) of RJB L-EPC iPS cell line (p15) derived from human L-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.
B. Normal karyotype of RJB L-EPC iPS cell line (p13) expanded on Corning Matrigel with NutriStem XF/FF Culture Medium.
C. Histological analysis of teratoma resulting from the injection of RJB L-EPC iPS cells (p13) into the kidney capsule of NOD-Scid mice. RJB L-EPC iPS cells expanded on Corning Matrigel with NutriStem XF/FF Culture Medium.

REFERENCES

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3. 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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SUMMARY

- Simple L-EPC line establishment from human peripheral blood in two weeks
- Generation of stable, pluripotent EPC-iPS cell lines using a non-modified, **polycistronic self-replicative RNA (srRNA)**
  - Requires only 2 transfections (microRNA + srRNA)
  - Four weeks for iPS cell colony establishment
  - Polycistronic srRNA cleared from isolated iPS cell lines in four passages (2 weeks)
  - Protocol requires rB18-R protein
- Generation of stable, pluripotent EPC-iPS cell lines using a **non-modified RNA cocktail**
  - Requires 9-10 RNA cocktail transfections (reprogramming mRNAs, interferon ablating mRNAs, and microRNA)
  - Two weeks for iPS cell colony establishment (No screening required)
  - Protocol does NOT require rB18-R protein
- Both non-modified RNA technologies allow for establishment of integration-free human iPS cell lines that are easily expandable in NutriStem XF/FF Culture Medium on Corning Matrigel