

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

Sarah Eminli-Meissner¹, Jung-Il Moon¹, Kevin Yi¹, Marco Poleganov², Tim Beißert², Ugur Sahin², Chris Huang³, Nick Morrell³, Amer Rana³, and *Brad Hamilton¹

1 Stemgent-Cambridge, MA, USA; 2 TRON-Translational Oncology at University Medical Center Mainz, Mainz, Germany; 3 Division of Respiratory Medicine, University of Cambridge, Cambridge, U.K.; *Corresponding Author: brad.hamilton@stemgent.com

INTRODUCTION

In 2010, it was first published that repeated transfection of 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 on fibroblasts, 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 10⁷ mononuclear cells (MNCs) (Figure 1). Additionally, transfection optimization studies revealed that mRNAs could be efficiently delivered to L-EPCs without inducing cellular toxicity (Figure 3). Subsequently, repeated daily transfection of L-EPCs with a cocktail of non-modified reprogramming mRNAs, non-modified interferon ablating mRNAs, and microRNA for only 9 days resulted in the efficient (≤ 0.25%) generation of TRA-1-60-positive iPS cell colonies in as few as 12 days (Figure 2). Characterization of L-EPC-derived iPS cell lines (≥ 13 passages) demonstrated a stable karyotype with uniform expression of key pluripotency markers (Figure 4). Ongoing studies are focused on *in vitro* and *in vivo* pluripotential as well as the genomic integrity of these expanded EPC-iPS cell lines.

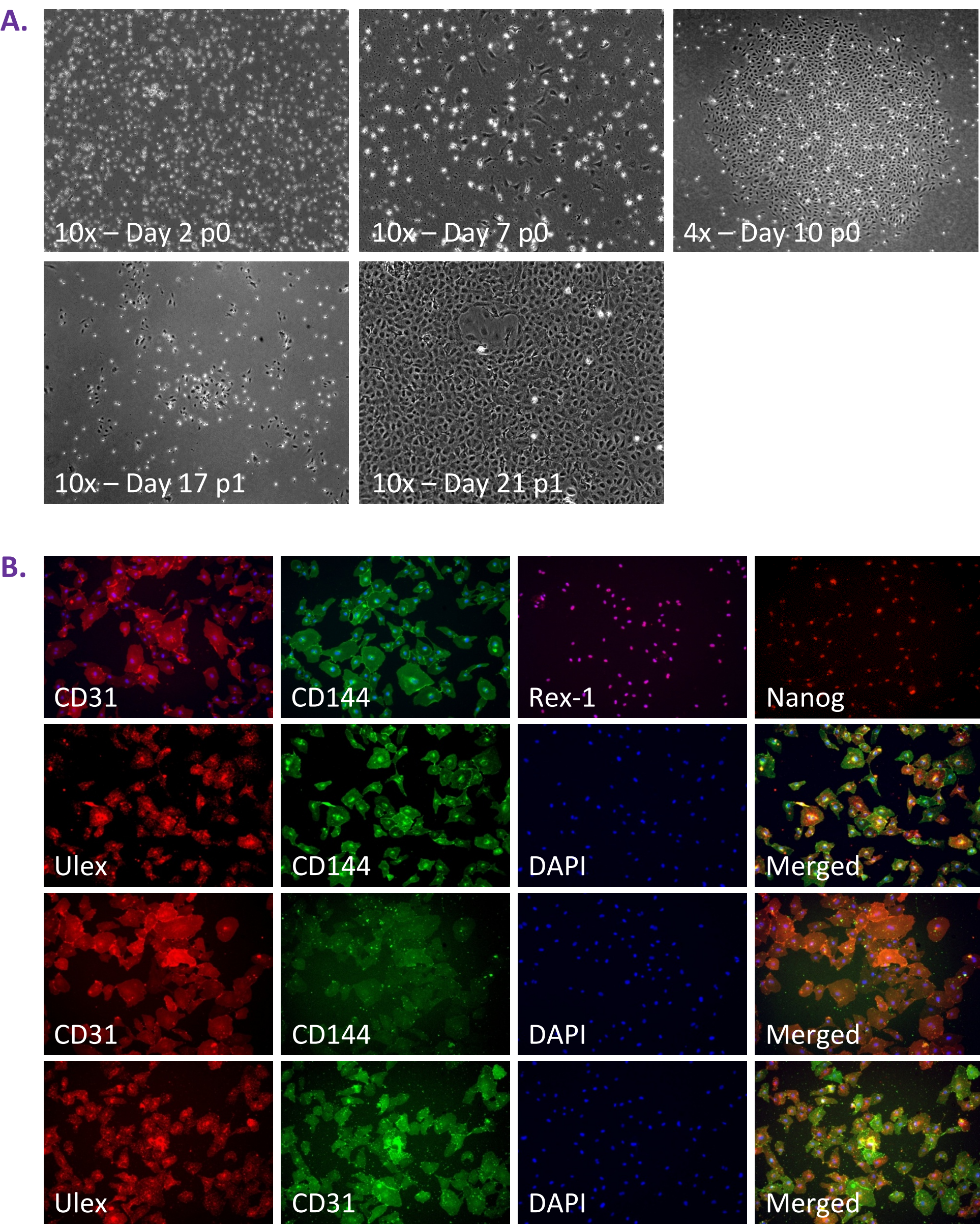


Figure 1. Establishment and immunocytochemical characterization of L-EPCs derived from peripheral blood.

A. Derivation timeline of adherent L-EPCs from peripheral blood. A minimum of 1x10⁷ human mononuclear cells (MNCs) were seeded into a single T75 flask coated with collagen I and cultured at 37°C, 5% CO₂, and 21% O₂ 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 7 →** 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. Immunocytochemistry (ICC) of RGB-L-EPCs. L-EPCs at p5 were seeded at a density of 5 x 10⁴ 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.

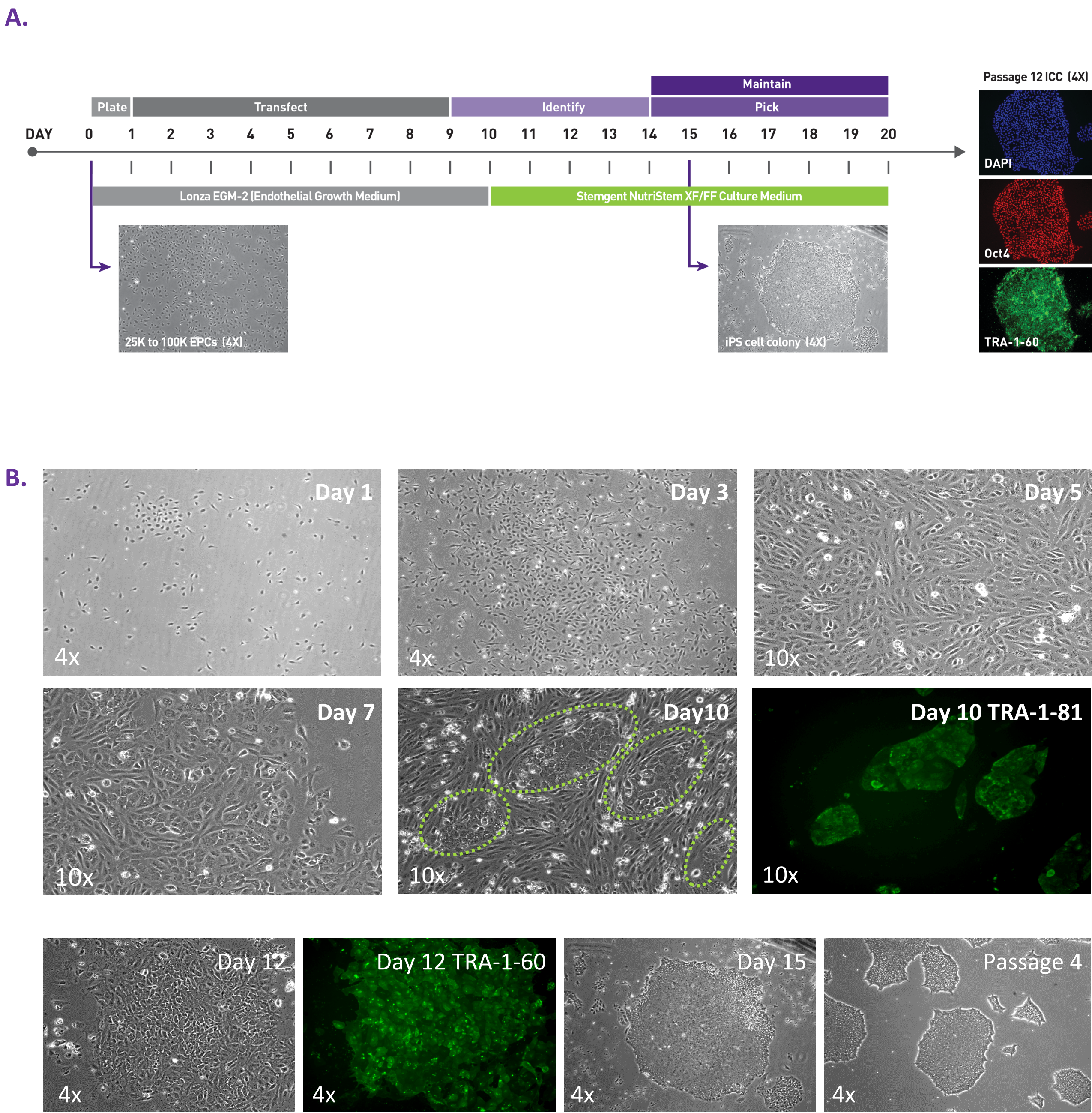


Figure 2. Human L-EPC RNA reprogramming timeline and morphology progression

A. Timeline for the reprogramming of human L-EPCs using non-modified reprogramming mRNA (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 Stemgent NutriStem™ XF/FF Culture Medium on Day10.

B. Primary reprogramming culture morphology progression, resulting from the RNA reprogramming of human peripheral blood-derived L-EPCs to iPS cell colonies. Day 0: L-EPCs at p6 were seeded at 2.5 x 10⁴ cells per well of a 6-well Matrigel-coated plate. Days 1 – 9: L-EPCs were transfected with non-modified reprogramming mRNA (OSKMNL), non-modified interferon-ablating mRNAs, and microRNA reprogramming cocktail. Culture was maintained at 37°C, 5% CO₂, and 5% O₂ in Lonza EGM-2 medium. Day 10: Culture medium was changed to Stemgent 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.

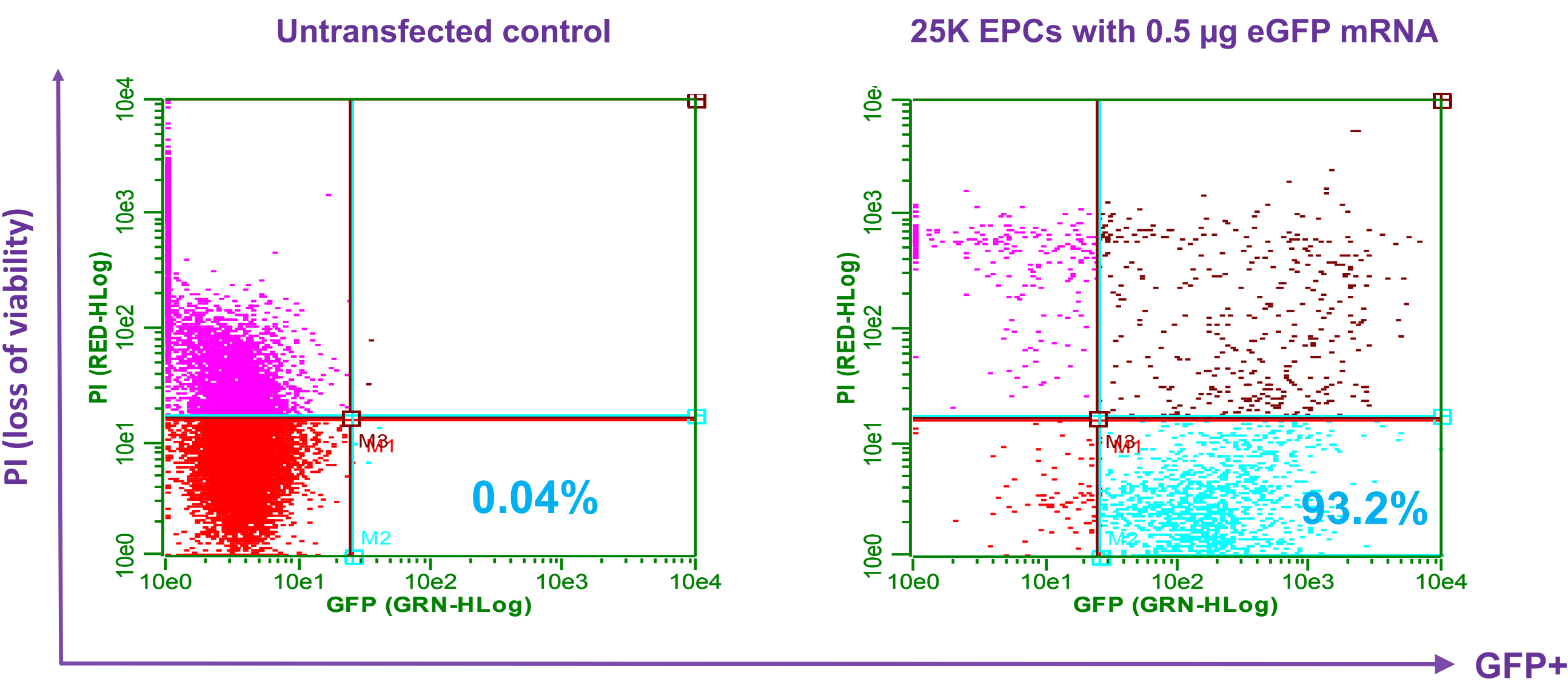


Figure 3. Efficient delivery of mRNA to L-EPCs. Graph of mean fluorescence intensity versus cell viability as determined by flow cytometry. L-EPCs were plated at 2.0 x 10⁴ cells per well of a 24-well plate and cultured at 37°C and 5% CO₂. The next day, the cells were transfected with 0.5 µg of eGFP mRNA. The L-EPCs were incubated with Propidium Iodide (PI) 18 to 24 hours post-transfection and analyzed by flow cytometry for GFP expression and cell viability. Transfection resulted in >90% transfection efficiency with high cell viability as compared to the un-transfected control (left panel).

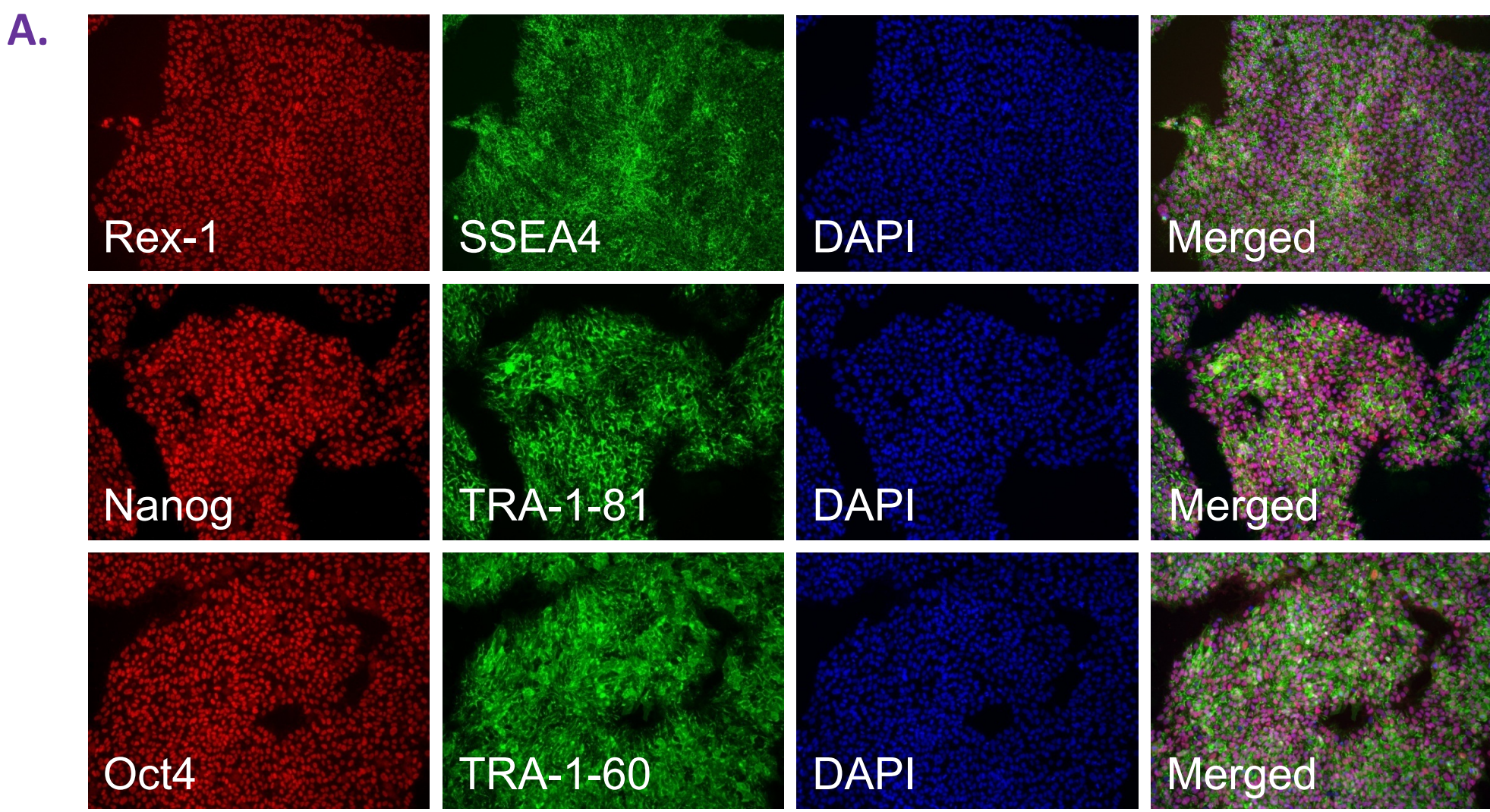


Figure 4. Characterization of RGB L-EPC iPS cells .

A. Pluripotency immunocytochemistry (ICC) of RGB-iPS cell line (p15) derived from human L-EPCs (p5) and expanded on Matrigel with NutriStem XF/FF Culture Medium. Nuclear pluripotency staining with Rex1, Oct4, and Nanog 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 RGB-iPS cell line (p13) derived from human L-EPCs and expanded on Matrigel with NutriStem XF/FF Culture Medium.

SUMMARY

- Simple L-EPC line establishment from human peripheral blood in two weeks
- Highly efficient, non-toxic delivery of mRNAs into L-EPCs
- Efficient generation of iPS cell colonies from human L-EPCs via RNA transfection in 12 days
 - RNA Cocktail: non-modified reprogramming mRNAs, non-modified interferon-ablating mRNAs, and microRNA
 - Complete reprogramming in 9-10 RNA transfections
 - No recombinant B18-R protein required
- Stable, normal karyotype and uniform pluripotency marker expression upon extended culture of human L-EPC-iPS cell lines in NutriStem XF/FF Culture Medium on Matrigel

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.