iPSC: Basic Science and Pre-Clinical Studies

Thermo Fisher SCIENTIFIC

# Efficient Non-Invasive Delivery of RNA For Somatic Cell Reprogramming

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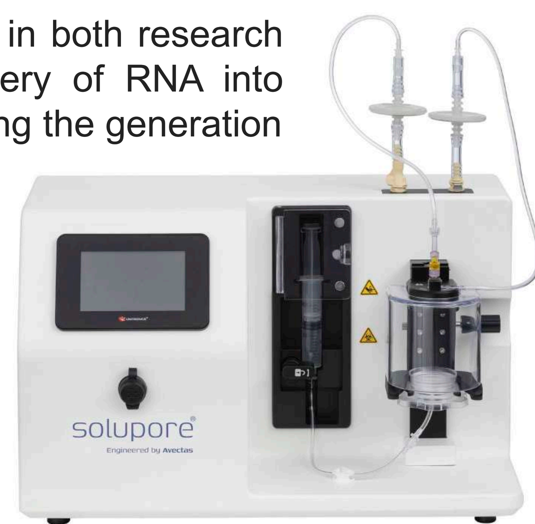
## Introduction

Induced pluripotent stem cell (iPSC)-based therapies are a promising area of regenerative medicine for treatment of degenerative diseases and as a renewable cell source for allogeneic cell therapies addressing broad indications, including oncology. A key component of the workflow is the generation of iPSC, with the critical requirement for efficient systems that are footprint free. Non-viral, RNA-based reprogramming is particularly appealing given its transient nature, eliminating the need to screen for the clearance of residual reprogramming factors. However, broader adoption of this technology has been hindered due to challenges in consistent and successful delivery of RNA into accessible starting materials such as blood cells. Solupore®, a delivery platform for non-viral modification approaches, utilizes physicochemical methods to reversibly permeabilize the cell membrane and deliver cargo. In contrast to traditional electroporation, the non-invasive nature of this method ensures better cell health and survival of modified cells with proven success in delivery of cargo to blood cell subtypes.

The Solupore system was used to evaluate efficacy of reprogramming Peripheral Blood Mononuclear Cells (PBMC) and CD34+ cells using self-replicating RNA (srRNA). High cell viability and recovery were observed across repeated transfections and clonal outgrowth of iPSCs was monitored. Clones exhibiting typical iPSC morphology were manually picked and cultured for a minimum of 5 passages prior to characterization. Pluripotency was assessed using flow phenotyping and immunocytochemistry (ICC). Embryoid bodies (EBs) were formed to analyze the tri-lineage differentiation potential of generated clones. Select clones were also genetically engineered on the Solupore using a CRISPR-Cas9 delivery method to knock-out the MHC Class I molecule, beta-2 microglobulin (B2M). This is the first study to successfully demonstrate the generation of iPSCs starting with blood cell types using RNA and a novel delivery platform, expanding the choice of starting cells, reprogramming systems and delivery methods, showing promise for cell therapy applications.

## Solupore® Transfection Instrument

Solupore® is a novel transfection system, available in both research and GMP-grade formats. It enables efficient delivery of RNA into blood cells for cell reprogramming, uniquely supporting the generation of induced pluripotent stem cells (iPSCs). Its gentle mechanism preserves cell viability and phenotype, minimizing cellular perturbation during this critical process. By eliminating the need for electroporation or viral vectors, the Solupore streamlines the iPSC manufacturing workflow, reduces costs, and offers a novel delivery technology for cell line developers and regenerative medicine applications.




## Materials and Methods

### RNA Reprogramming of Somatic Cells

Peripheral Blood Mononuclear Cells (PBMC) and CD34+ Cells were isolated from fresh apheresis material sourced by AllCells and cryopreserved prior to reprogramming. Cells were thawed and cultured to recover prior to transfection. Self Replicating RNA (srRNA) containing puromycin resistance was used to conduct single transfection of CD34 and PBMCs within the Solupore system. Transfected cells were plated on rhLaminin-521 (LN-521) in StemPro-34 medium, treated with Puromycin for antibiotic selection, and supplemented with B18R for sustained srRNA expression. Cells were maintained in hypoxic conditions (5% $O_2$). After a week, plated cells were transitioned to ReproTeSR medium with added Human iPS Reprogramming Boost Supplement to aid in reprogramming.

### PSC Selection, Culture, and Banking

Cells exhibiting a clonal morphology on the source plate 3 to 4 weeks after transfection were manually selected and seeded onto vitronectin (VTN) for subsequent culture in Essential 8™ Medium. These clones were cultured for more than 10 passages and then cryopreserved in PSC Cryopreservation Medium for subsequent processing and analytics.

### Non-Viral Genetic Modification of PSCs

Cryopreserved iPSCs were thawed and cultured for three passages for recovery and scale-up. Control cells were passaged without being sprayed on the Solupore. Mock cells were sprayed on the Solupore without editing cargo (RNP) included. CRISPR/Cas9 knock-out (KO) condition was sprayed twice via the Solupore with a Cas9-sgRNA RNP targeting the B2M locus. For the knock-out condition, cells were given a minimum of 1 hour between sprays to recover.

### Analytics

Cell counts, flow cytometry, and image-based methods were used to collect results at critical stages of the biomanufacturing process (NucleoCounter® NC-3000™, Attune™, EVOS™ microscopes). Flow phenotyping was conducted to assess the quality of iPSC clones after more than 5 passages, for pluripotency (SSEA4 and TRA-60) and differentiation markers (SSEA-1). Immunocytochemistry (ICC) was also used to assess pluripotency (OCT4, SSEA4, SOX2, TRA1-60). Embryoid bodies (EBs) formed from modified and unmodified CD34- and PBMC-derived iPSC lines were cultured under differentiation conditions for 21-days to demonstrate the functionality of the clones to differentiate. These EBs were analyzed for differentiation via ICC to endoderm (alpha-fetoprotein), mesoderm (alpha-smooth muscle actin) and ectoderm (beta-III tubulin). Nuclei for both ICC assays were counterstained with DAPI. Isolated genomic DNA (gDNA) from EBs and paired iPSC controls was analyzed using the TaqMan™ hPSC ScoreCard™ Assay to determine patterns in self renewal and lineage specific gene expression.

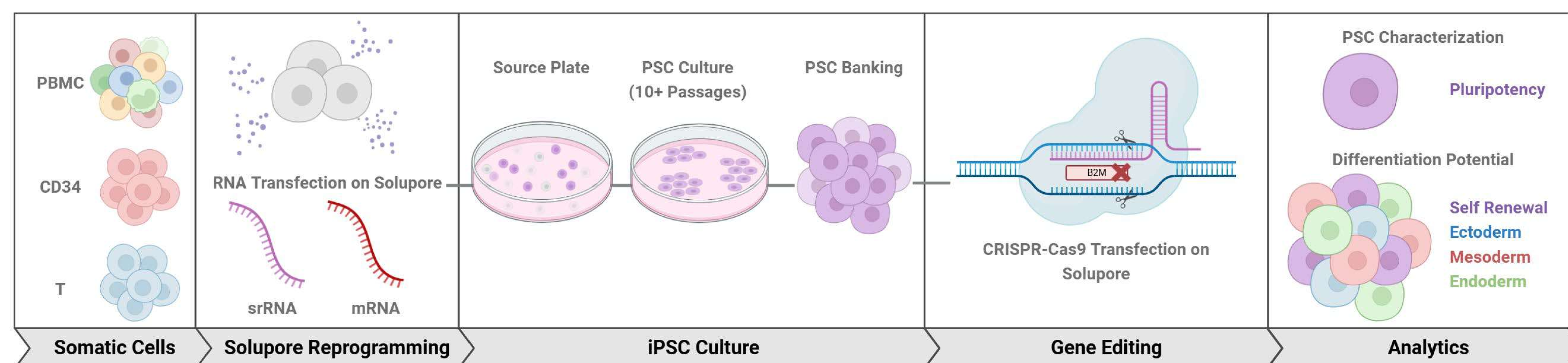



**Figure 1. Solupore Use Case for RNA Reprogramming and Modification of Blood Cells.** Blood cells, including PBMC, CD34+, and T-Cells are common starting materials for reprogramming in cell therapy and disease modelling. The Solupore can be used to transfect RNA, including both srRNA and mRNA, into somatic cells with high viability and recovery. Transfected cells are seeded into a source plate and clonal outgrowth is monitored. Clones are then manually selected, passaged and banked for subsequent use. The Solupore® instrument can also be used to modify cells via CRISPR-Cas9 transfection, in this case using the iPSCs previously generated. Clone pluripotency and differentiation potential can then be assessed via flow cytometry, ScoreCard™, and more.

## Results

### Solupore® srRNA Reprogramming

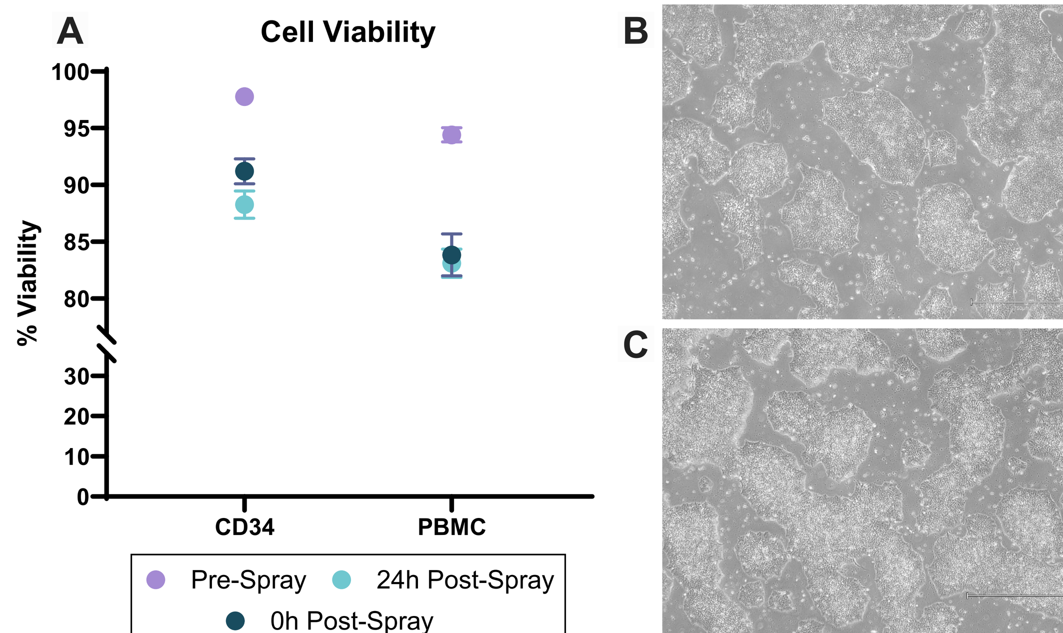



**Figure 2. High Cell Viability and Good Morphology Post srRNA Reprogramming of CD34 and PBMCs.** A. Cell viability remained greater than 80% after srRNA transfection. B. Morphology of reprogrammed cells was typical of healthy iPSCs for CD34 (B) and PBMC (C) derived iPSCs, with images taken at 4X magnification, scale bars shown are 750 µm.

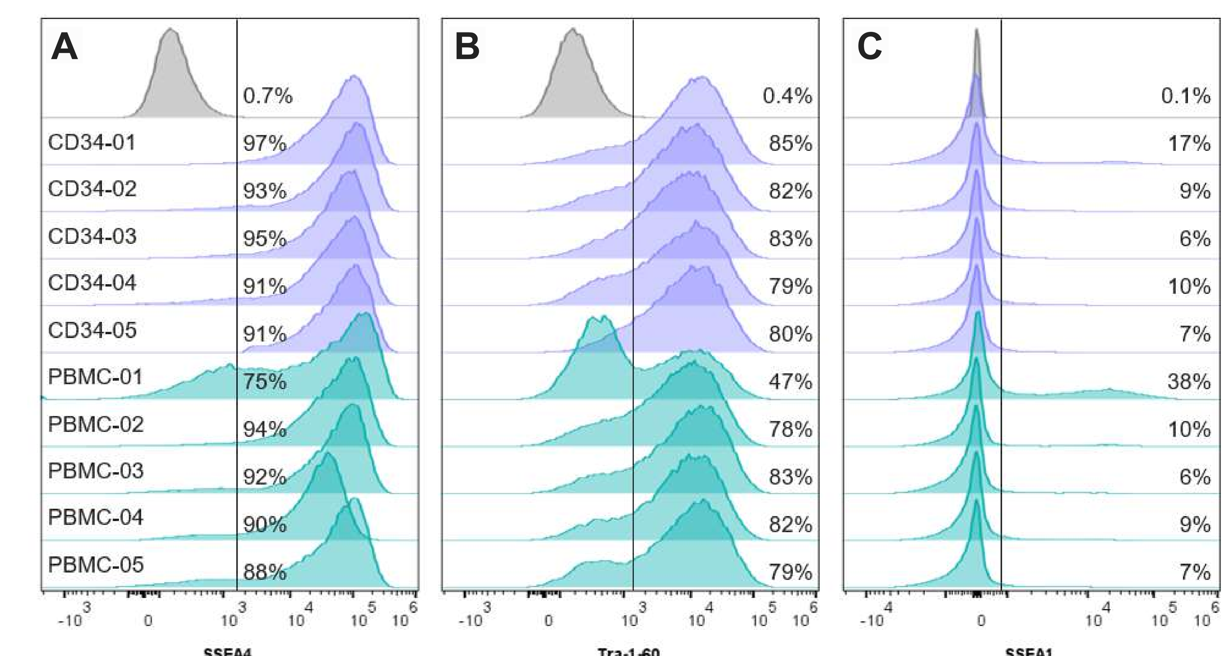



**Figure 3. Majority of iPSCs Generated Express Pluripotency Markers, with Low Levels of Differentiation Markers After Extended Culture.** Pluripotency and differentiation were assessed via flow cytometry phenotyping utilizing SSEA4 (A) and Tra-1-60 (B) as well as SSEA1 (C). Nine out of the ten clones (PBMC-01 excluded) were banked and utilized for subsequent culture and analytics.

### Tri-Lineage Differentiation Potential of srRNA Derived iPSC

| Source Material | Cell Type | Self Renewal | Ectoderm | Mesoderm | Endoderm |
|---|---|---|---|---|---|
| CD34-Derived | iPSC | + | - | - | - |
| CD34-Derived + B2M KO | | + | - | - | - |
| PBMC-Derived | | + | - | - | - |
| PBMC-Derived + B2M KO | | + | - | - | - |
| CD34-Derived | EB | - | + | + | + |
| CD34-Derived + B2M KO | | - | + | + | + |
| PBMC-Derived | | - | + | + | + |
| PBMC-Derived + B2M KO | | - | + | + | + |

**Table 1. CD34+ and PBMC-Derived iPSCs Successfully Differentiated into Three Germ Layers.** Unmodified control iPSC lines were cultured in parallel with a homogenous population of modified iPSCs with B2M knock-out. Embryoid bodies (EBs) were then generated from each of these lines and cultured under differentiation conditions for 21-days and the ScoreCard™ Assay was used to demonstrate the differentiation capacity of the clones. Undifferentiated iPSCs were cultured as a control. Scores were determined by relating the gene expression profiles of each of the samples against an undifferentiated reference set. A positive (+) result indicates upregulation in comparison to the reference, while a negative (-) result indicates downregulation. All EB samples displayed increased levels of gene expression for the three germ layers when compared to their iPSC controls and the reference set. Together, these results indicate the ability of the iPSCs to successfully differentiate into the three lineages.

### Solupore® CRISPR-Cas9 Gene Editing

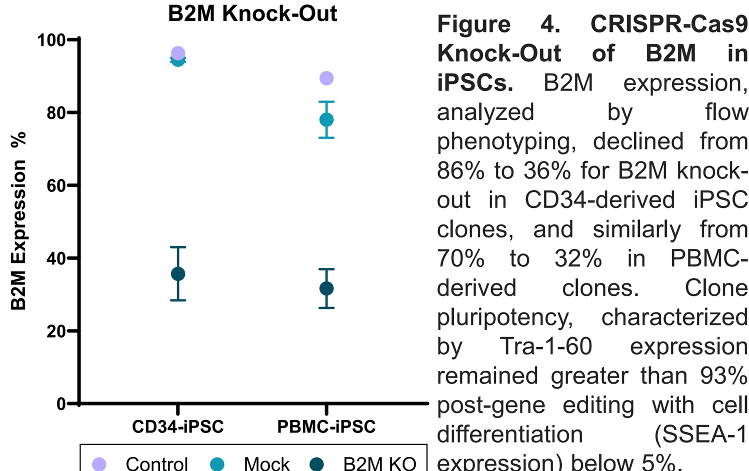



**Figure 4. CRISPR-Cas9 Knock-Out of B2M in iPSCs.** B2M expression, analyzed by flow phenotyping, declined from 86% to 36% for B2M knock-out in CD34-derived iPSC clones, and similarly from 70% to 32% in PBMC-derived clones. Clone pluripotency, characterized by Tra-1-60 expression remained greater than 93% post-gene editing with cell differentiation (SSEA-1 expression) below 5%.

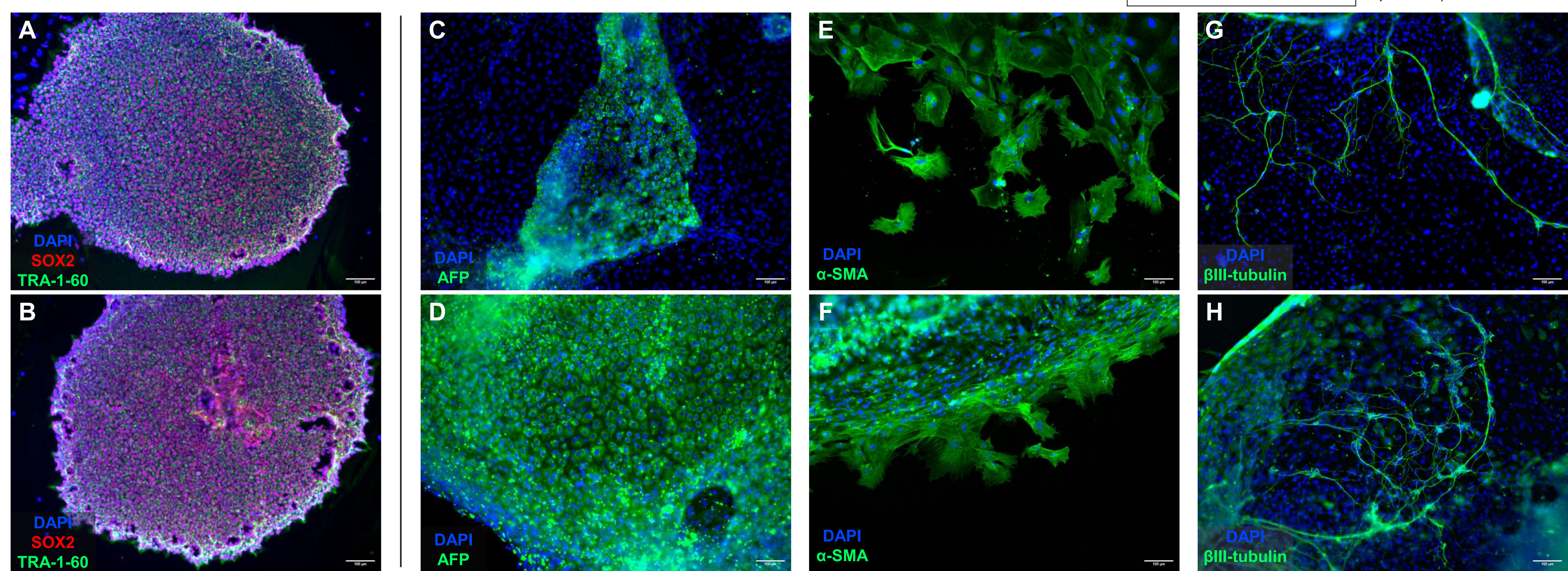



**Figure 5. Immunocytochemistry of Pluripotency Markers in CD34- and PBMC-derived iPSCs Generated by srRNA Reprogramming and Subsequent Differentiation Markers of Generated Embryoid Bodies.** A-B. Representative images of srRNA-reprogrammed induced pluripotent stem cells (iPSCs), derived from CD34 cells and PBMCs, respectively, and cultured under feeder-free conditions for greater than 6 passages. The clones exhibit robust expression of pluripotency markers SOX2 (red), a key transcription factor regulating stemness which localizes in the nucleus, and TRA-1-60 (green), a surface glycoprotein on undifferentiated cells. Nuclei were counterstained with DAPI (blue). C-H. Embryoid bodies formed from both iPSC lines were cultured under differentiation conditions for 21 days to demonstrate the functionality of the clones to differentiate. Lineage specific marker expression corresponding to the three embryonic germ layers included alpha-fetoprotein (C-D, AFP, green), a definitive endoderm marker, alpha-smooth muscle actin (E-F, α-SMA, green), indicative of mesodermal commitment, and beta-III tubulin (G-H, βIII-tubulin, green), an ectoderm marker associated with neuronal differentiation. Nuclei are counterstained with DAPI (blue) to visualize tissue architecture. Scale bars are 100 µm for A-H.

## Conclusions

This is the first study to successfully demonstrate the reprogramming of blood cell types using srRNA to produce functional, pluripotent iPSCs. This work also highlights RNA reprogramming and the support the Solupore® system can provide to this cell therapy workflow.

- srRNA Transfection of both CD34+ and PBMCs on the Solupore® produced highly viable cells with cell recovery consistent with other reprogramming methods
- iPSC clones were observed 4-weeks post transfection and were selected and cultured for greater than 10 passages
- Gene editing of iPSC clones produced cells with significantly decreased expression of B2M for the knock-out condition
- Generated iPSCs, with and without further genetic modification, were capable of differentiation into the three-germ layers

## Future Work

Preliminary work delivering srRNA to T-Cells, and mRNA into a range of blood cell types is ongoing. Current work has highlighted the importance of high-quality, tunable reprogramming RNAs to increase reprogramming efficiency. Next steps will include sourcing additional mRNA for reprogramming to optimize efficiency and exploring end to end solutions for this workflow.

## Acknowledgements

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Learn more about our services at patheon.com and the Solupore® at avectas.com/technology.

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