Novel Regulatory Sequences Drive Persistent Transgene Expression During Directed Differentiation of iPSCs to Lymphocytes and Macrophages

Claire Aibel[1], Abigail Blatchford[1], Taeyun Kim[1], Christopher B. Rohde[1], Matthew Angel[1]

[1]Factor Bioscience Inc., Cambridge, MA

Summary

Cell therapies derived from knock-in human induced pluripotent stem cells (iPSCs) hold potential for increased scalability, broader accessibility, and superior production of genetically identical immune cells that express therapeutic transgenes. Such transgenes are often paired with synthetic promoters, which can drive high levels of protein expression but are often silenced during differentiation. To address this issue, we designed novel regulatory sequences paired with a reporter and electroporated them into mRNA-reprogrammed iPSCs. We demonstrate that incorporation of these sequences confers a 40-fold or 9-fold increase in the percentage of transgene-expressing iPSC-derived lymphocytes and macrophages, respectively, without introducing unintended phenotypic changes.

Conclusions

Here, we present novel synthetic regulatory sequences that mitigate silencing during differentiation of iPSCs into lymphocytes and macrophages. We demonstrate that iPSCs and iPSC-derived cells containing these sequences retain expression of expected markers (iPSCs express TRA-1-81, TRA-1-60, Sox2, Nanog, and Oct4; iLymphocytes express CD7 and CD56; and iMacrophages express CD14, CD45, and CD64). These sequence elements may prove useful in engineering cells with functional transgenes encoding therapeutic proteins and could contribute to the development of effective engineered iPSC-based therapies.

1 Generation of gene-edited mRNA-reprogrammed iPSCs

Development of iPS cell lines containing transgenes with enhanced regulatory features

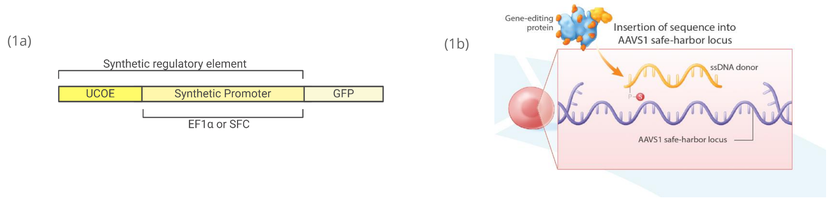
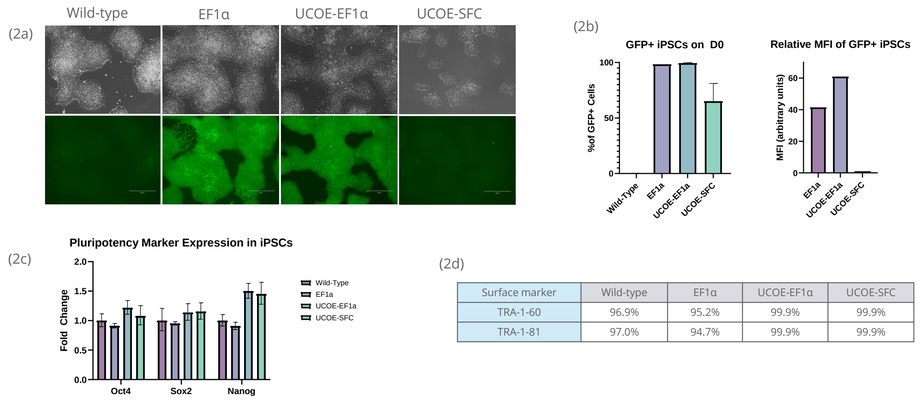


Figure 1. Development of gene-edited iPS cells. Figure 1a. Donor single-stranded DNA consisting of an ~800 base long universal chromatin opening element (UCOE) with 73% GC content, a synthetic promoter (EF1α or SFC), a GFP reporter, and AAVS1 homology arms were synthesized. Figure 1b. The donor DNA was electroporated into iPSCs at the AAVS1 safe-harbor locus. Single colonies were evaluated via PCR and those containing biallelic transgene insertions were expanded and used for downstream differentiations.

Assessment of key pluripotency markers and transgene expression



(2d)

Surface marker	Wild-type	EF1α	UCOE-EF1α	UCOE-SFC
TRA-1-60	96.9%	95.2%	99.9%	99.9%
TRA-1-81	97.0%	94.7%	99.9%	99.9%

Figure 2. Expression of pluripotency markers and GFP in gene-edited iPS cell lines. Figure 2a. Brightfield (top) and GFP (bottom) images of wild-type and gene-edited iPSCs prior to differentiation. Figure 2b. Left: Percentage of GFP+ iPSCs determined using flow cytometry (n=3). Right: Relative median fluorescence intensity (MFI) of GFP+ iPSCs. Figure 2C. Relative mRNA expression levels of Oct4, Sox2, and Nanog determined using RT-PCR. Figure 2D. Percentage of iPSCs expressing pluripotency markers TRA-1-60 and TRA-1-81 determined using flow cytometry.

2 Differentiation of transgenic iPSCs to lymphocytes

iPSC → iLymphocyte differentiation overview

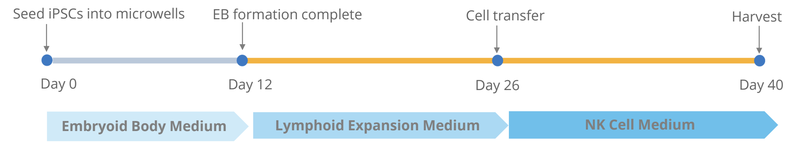
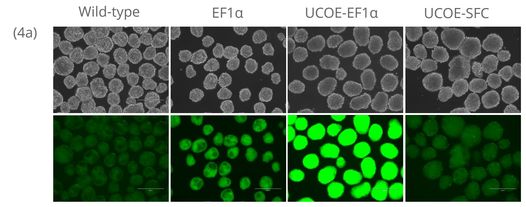


Figure 3. Differentiation of iPSCs into lymphocytes. Gene-edited iPSCs were seeded into microwells on day 0 in Embryoid Body (EB) Formation Medium (STEMCELL Technologies™) and transferred to a non-tissue culture treated plate on day 5. On day 12, EBs were enzymatically dissociated into single cells and underwent magnetic bead selection to isolate CD34+ cells. Selected cells were seeded into Lymphoid Progenitor Expansion Medium (STEMCELL Technologies™), followed by NK Cell Differentiation Medium (STEMCELL Technologies™), and then characterized.

Isolation of CD34+ cells from iPSC-derived embryoid bodies



(4b)

% of CD34+ cells on D12	
Wild-type	15.0%
EF1α	12.4%
UCOE-EF1α	9.4%
UCOE-SFC	11.9%

Figure 4. Dissociation of embryoid bodies and isolation of CD34+ cells. Figure 4a. Brightfield (top) and GFP (bottom) images of iPSC-derived embryoid bodies on day 5 of differentiation. Figure 4b. Percentage of CD34+ cells recovered after enzymatic dissociation of EBs on day 12, assessed using cell count after CD34 selection.

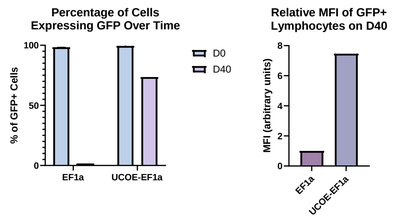
Assessment of lymphocyte and natural killer cell markers

Figure 5. Assessment of lymphocyte and natural killer (NK) cell markers on day 40 of differentiation. Expression of CD7 (early lymphocyte marker) and CD56 (classical NK cell marker) were evaluated using flow cytometry.

Surface marker	Wild-type	EF1α	UCOE-EF1α
CD7	77.7%	88.5%	54.5%
CD56	55.1%	28.9%	54.3%

iLymphocytes with UCOEs display higher transgene expression

Figure 6. Transgene expression in iLymphocytes on day 40. Left: Percentage of cells expressing GFP, assessed using flow cytometry, on days 0 and 40 of differentiation. Right: Relative median fluorescence intensity (MFI) of GFP+ cells on day 40 of differentiation.



3 Differentiation of transgenic iPSCs to macrophages

iPSC → iMacrophage differentiation overview

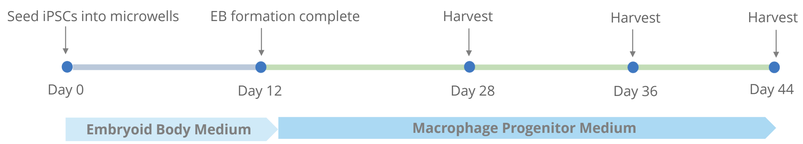


Figure 7. Differentiation of iPSCs into macrophages. iPSCs were differentiated into EBs following the steps outlined in Figure 3. On day 12, EBs were transferred to macrophage progenitor expansion medium in Matrigel®-coated cultureware. Macrophages that developed from adherent EBs were evaluated via flow cytometry on days 28, 36, 44, 54, and 69.

Assessment of key macrophage markers

(9a)

Surface marker	Expected	Wild-type	EF1α	UCOE-EF1α	UCOE-SFC
CD14	+	87.7%	97.2%	99.6%	99.6%
CD45	+	99.6%	98.9%	99.6%	99.6%
CD64	+	98.9%	88.6%	96.7%	92.3%

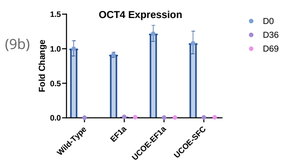


Figure 9. Assessment of key macrophage and iPSC markers. Figure 9a. Expression of CD14 (lipopolysaccharide binding receptor), CD45 (leukocyte common antigen), and CD64 (Fc gamma receptor) on day 36 of differentiation was evaluated using flow cytometry. Figure 9b. Relative mRNA expression levels of Oct4 on days 0, 36, and 69 were assessed using RT-PCR. (Note: wild-type cells were not evaluated on day 69.)

iMacrophages with UCOEs display higher transgene expression

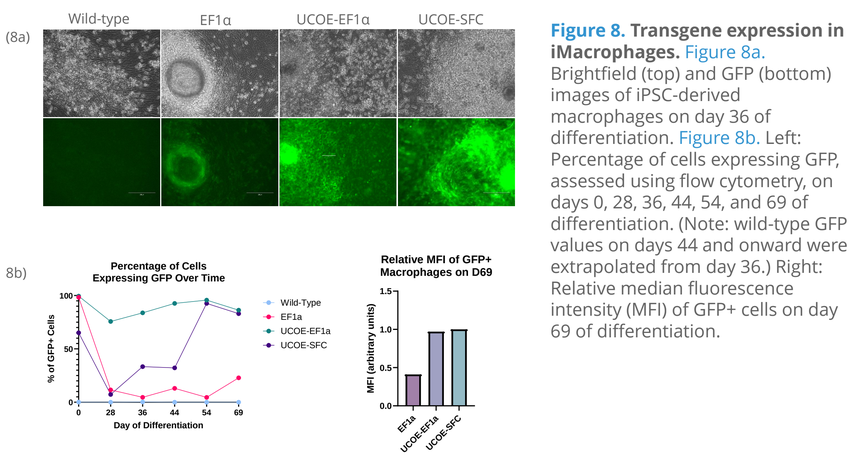


Figure 8. Transgene expression in iMacrophages. Figure 8a. Brightfield (top) and GFP (bottom) images of iPSC-derived macrophages on day 36 of differentiation. Figure 8b. Left: Percentage of cells expressing GFP, assessed using flow cytometry, on days 0, 28, 36, 44, 54, and 69 of differentiation. (Note: wild-type GFP values on days 44 and onward were extrapolated from day 36.) Right: Relative median fluorescence intensity (MFI) of GFP+ cells on day 69 of differentiation.