APPLICATION NOTE

CRISPR knockout and shRNA: Orthogonal tools to perturb gene expression in iPSC-cardiomyocyte differentiation.

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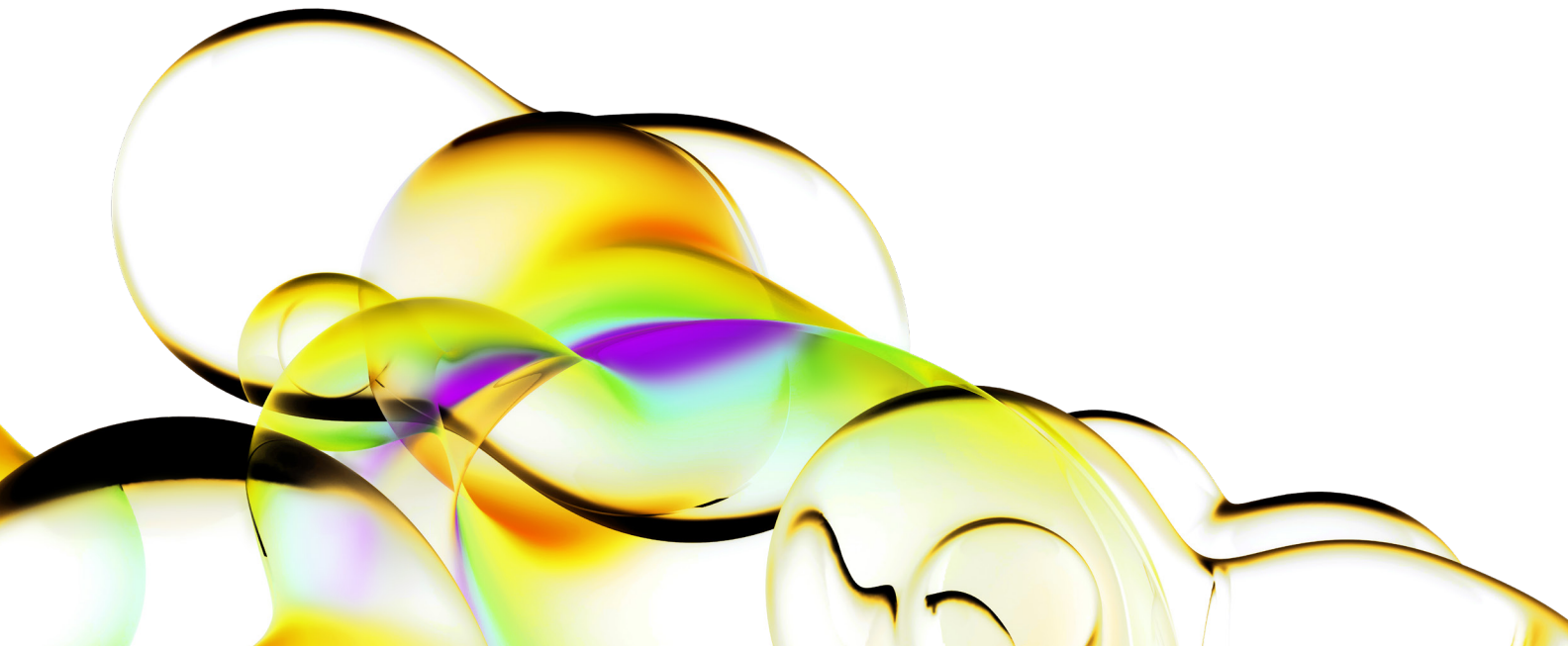
Abstract

Genetic loss-of-function (LOF) tools are critical to understanding gene function in both health and disease. In this study, we utilized CRISPR to knock out genes critical for cardiomyocyte differentiation and validated the resulting phenotypes using a secondary LOF tool, constitutive shRNA expression. Given the challenges associated with delivery to differentiating cardiomyocytes, we performed the genomic edits and constitutive shRNA delivery in the induced pluripotent stem cell (iPSC) state before differentiating transduced cell lines toward cardiomyocyte lineages. We found that the disruption of *T, EOMES*, or *MESP1* all strongly impaired differentiation efficiency. This study demonstrates the feasibility of engineering iPSC lines harboring specific genetic knockouts to study the effects of specific genes on cardiomyogenic potential. By utilizing shRNAs to target candidate genes that influence cardiomyogenesis, we also demonstrate the power of utilizing multiple LOF tools to validate phenotypes, mitigating the risk of identifying false positives or negatives or phenotypes produced by off-target rather than on-target effects.

Introduction

Loss-of-function (LOF) technologies are instrumental in establishing gene function under both normal and pathogenic circumstances and can aid in identifying novel therapeutic targets for the treatment of disease. Among these, RNA interference (RNAi) remains one of the most extensively used tools for disrupting gene expression across mammalian cell models.



RNAi leverages an endogenous pathway in which short hairpin RNAs (shRNAs), designed as active siRNA sequences embedded within a miRNA scaffold, are efficiently processed and incorporated into RNA-induced silencing complex (RISC), enabling sequence-specific binding to target mRNAs through complementary base pairing. Upon target recognition, the target mRNA is cleaved and degraded, depleting its levels and thereby preventing target protein synthesis. A distinct yet complementary LOF tool that has gained widespread popularity since its discovery is clustered regularly interspaced palindromic repeats (CRISPR)-based genome editing. CRISPR utilizes an RNA-guided nuclease, Cas9, to target specific genomic loci and introduce double-stranded breaks. These breaks are typically repaired by the error-prone repair pathway, non-homologous end joining (NHEJ), often resulting in insertion or deletion (indel) mutations that cause frameshifts in the protein sequence, resulting in non-functional proteins. Here, we apply both CRISPR and RNAi technologies to disrupt gene expression in a widely utilized model of human cardiomyogenesis.

Cardiovascular diseases are the leading cause of mortality worldwide, driven by both lifestyle and environmental factors as well as genetic predispositions[1]. In many cases, cardiac stress leads to a loss of cardiomyocytes, which negatively affects heart function. Understanding how genes govern cardiomyocyte differentiation, as well as how they contribute to pathological stresses leading to cardiovascular dysfunction, are both critical for the development of novel therapies for heart disease. However, studying these processes in primary human cardiac tissue is mired by extremely limited access to patient samples and the technical challenges of delivering reagents that influence gene expression to primary cardiomyocytes. The differentiation of human induced pluripotent stem cells (hiPSCs) into cardiomyocytes has gained immense popularity over the last decade, allowing for the study of human cardiac differentiation *in vitro*. Additionally, hiPSCs can be genetically manipulated with greater ease than primary cardiomyocytes, enabling the generation of pathogenic mutations or gene knockout lines to investigate gene function during cardiac differentiation or pathogenesis, alongside control lines with matched genetic backgrounds.

Previous studies have shown that stem cell lines lacking expression of critical transcription factors—including *T*[2,3], *EOMES*[4,5], *MESP1*[6,7], *ISL1*[8,9], *and GATA4*[10,11]—exhibit markedly impaired differentiation toward mesoderm or cardiac lineages. In mouse models, deletion of any of these genes results in embryonic lethality. Importantly, these genes are not expressed in undifferentiated iPSCs, and therefore we reasoned that targeting them in iPSCs would not be associated with unintended growth deficiencies or toxicity. To target expression of these genes, we leveraged CRISPR to generate *T, EOMES, MESP1, ISL1,* and *GATA4* knockout (KO) iPSC lines, which were then differentiated to evaluate the efficiency of mesoderm and cardiac lineage commitment. We then validated our CRISPR KO findings using RNAi. Use of multiple LOF technologies can increase the confidence in linking a gene to a phenotype. Moreover, targeting genes with multiple LOF technologies minimizes the risk of overlooking a gene that affects differentiation due to unpredicted inefficiencies of gene targeting with a singular technology. Given the difficulty of delivering reagents to cardiomyocytes or differentiating progenitor cells, we instead opted to deliver RNAi reagents to hiPSCs prior to differentiation. As none of the gene targets are expressed in iPSCs, we chose to use constitutive lentiviral shRNA expression to ensure that, as soon as the genes became transcriptionally active during differentiation, the mRNA would be targeted by the shRNA for degradation. This approach ensured that we would not miss the targeting window to effectively perturb expression of these transiently expressed, critical differentiation factors. Together, these data serve as a proof of concept that gene function during cardiac differentiation can be interrogated by delivering both RNAi and CRISPR reagents to hiPSCs prior to inducing differentiation.

Results

Utilizing CRISPR to knock out critical genes for cardiomyocyte differentiation

Delivering genetic manipulation reagents to differentiating cardiac progenitor cells is challenging. Conventional strategies such as transfection or electroporation will likely disrupt the differentiation process, as precise temporal signaling cues and iPSC confluence are key factors in promoting efficient differentiation. Moreover, the target genes we identified via literature review are only transiently expressed in distinct mesoderm or cardiac progenitor states. For example, *T* and *EOMES* are among the earliest mesodermal transcription factors upregulated during cardiac differentiation, with expression peaking on differentiation Day 2 and returning to baseline by Days 3-5. *MESP1* expression is upregulated starting on differentiation Day 2, peaks on Day 3, and returns to baseline by Day 5. In contrast, *GATA4* and *ISL1* are upregulated later, with peak expression occurring between differentiation Days 5 and 7 (Figure 1, genes listed in red).

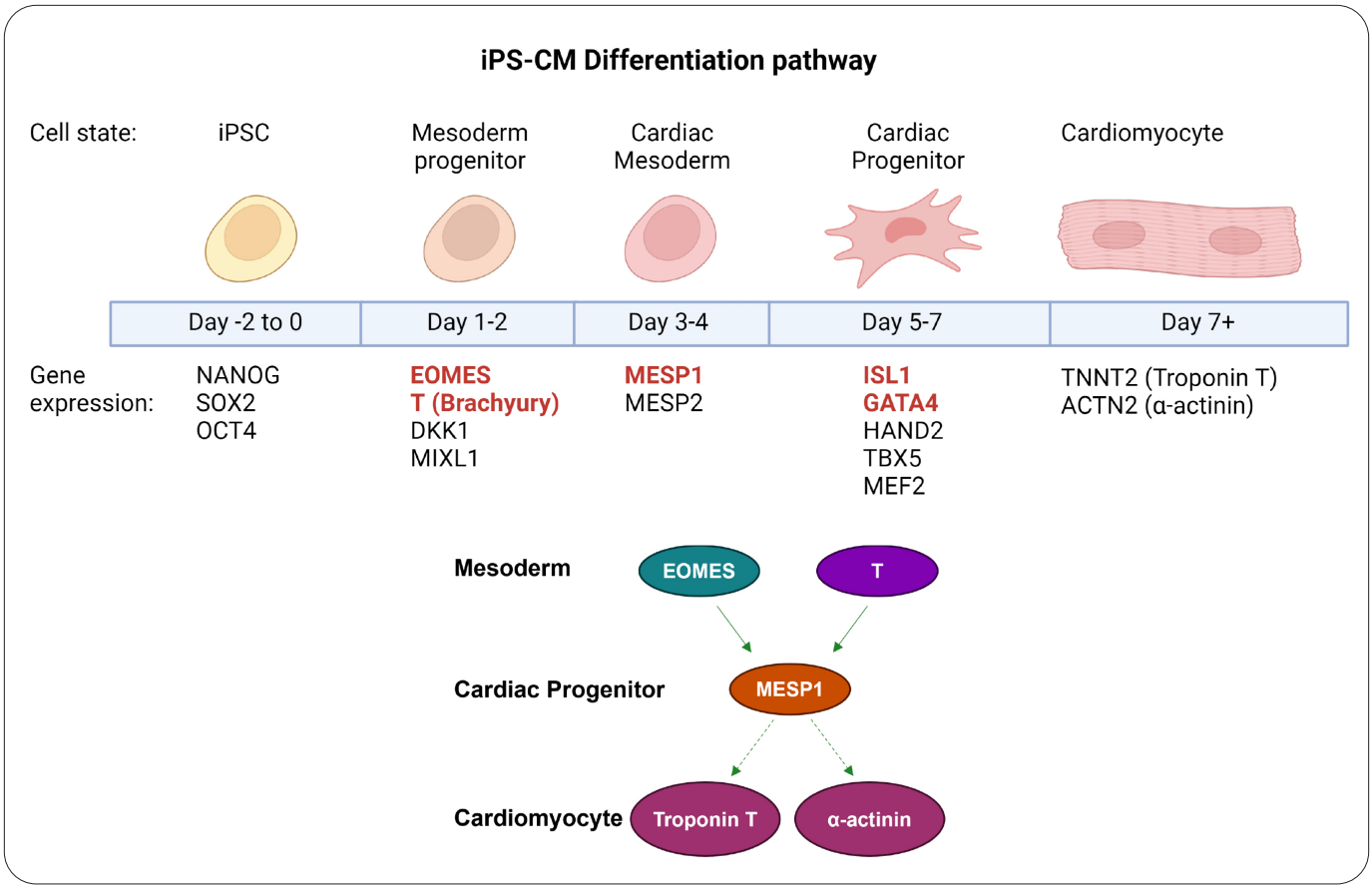


Figure 1: A) Schematic of iPSC differentiation to cardiomyocytes. As cells progress through differentiation, they pass through discrete intermediate cell states marked by distinct transcriptomic signatures before arriving at terminally differentiated cardiomyocytes. Red text denotes gene targets used in this study. B) Simplified schematic of transcription factor cascade. *T* (Brachyury) and *EOMES* directly upregulate *MESP1*, which in turn upregulates downstream transcription factors ultimately leading to cardiomyocyte lineage commitment, marked by cardiomyocyte specific genes including *TNNT2* and *ACTN2*. Created with BioRender.com

Therefore, to achieve robust reagent delivery without impairing differentiation efficiency and to mitigate the risk of a genomic edit not occurring before temporal target gene expression, we opted to deliver CRISPR knockout reagents to iPSCs prior to differentiation. We delivered Dharmacon™ Edit-R™ All-in-one lentiviral sgRNA particles targeting *T, ISL1, MESP1*, or *GATA4*, alongside a positive control, *PPIB*, and a non-targeting control (NTC) to wildtype iPSCs. Transduced iPSCs were then selected with puromycin for five days and allowed to recover into stable edited cell populations (Figure 2A). We then confirmed high indel formation efficiency using a T7 endonuclease I (T7EI) mismatch assay within the *T, ISL1*, and *GATA4* loci, all of which were comparable in editing efficiency to the PPIB positive control locus (Figure 2B). We were unsuccessful in determining *MESP1* editing efficiency, likely due to the high GC content of the targeted genomic locus, which prevented successful PCR amplification (Figure 2B). Notably, none of the transduced iPSC lines exhibited any apparent phenotypes as the targeted genes are not expressed in native iPSCs, suggesting that constitutive Cas9 expression resulted in negligible effects in iPSCs. Upon differentiation of these iPSC lines into cardiomyocytes, knockout of T or *MESP1* significantly reduced the fraction of cells expressing Troponin T protein, thereby reducing cardiomyocyte differentiation efficiency (Figures 2C and D).

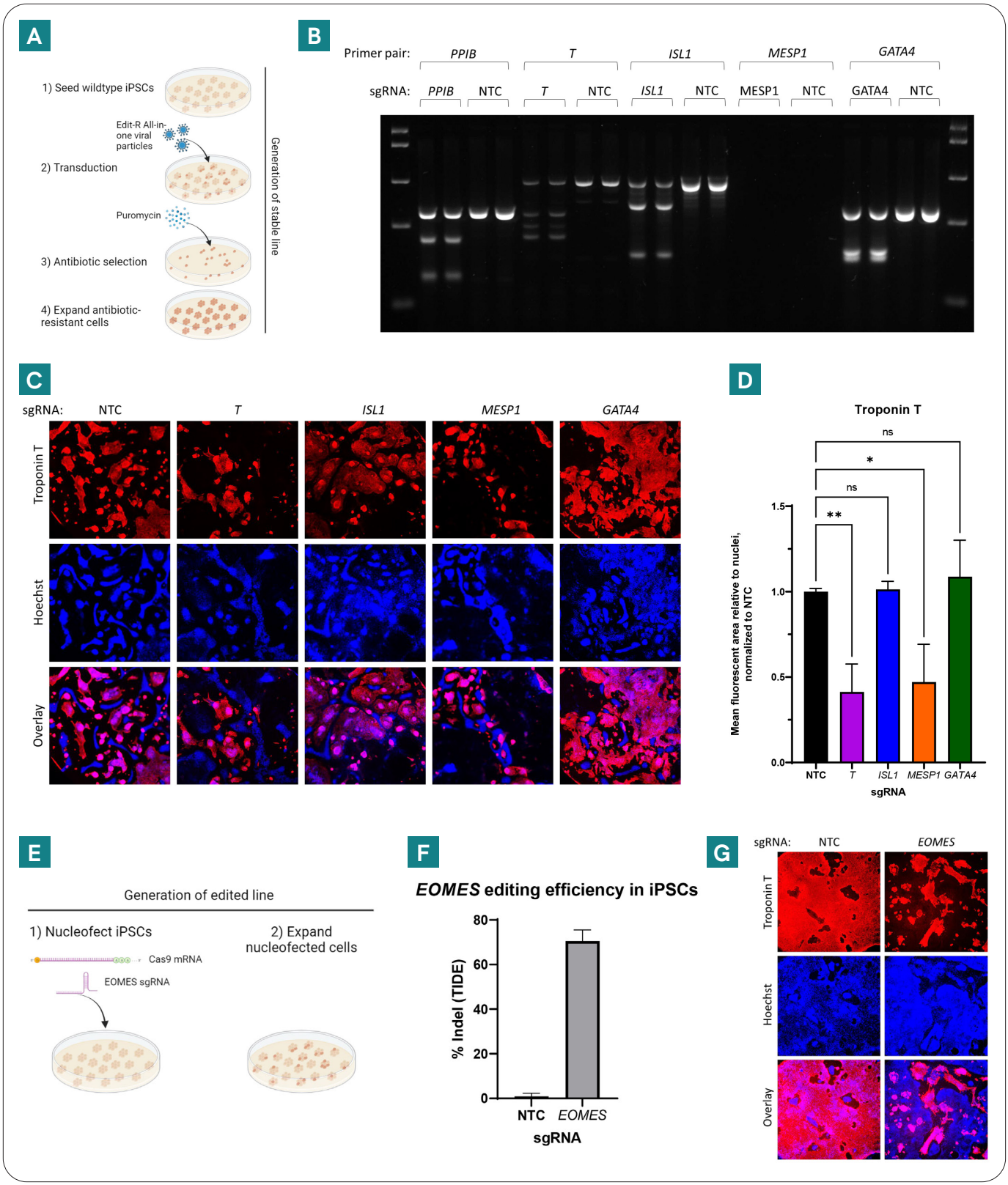


Figure 2: A) Schematic of Edit-R All-in-one viral particle transduction and selection in iPSCs. B) Agarose gel depicting CRISPR editing efficiency via T7 endonuclease I mismatch detection and cleavage at the targeted loci *PPIB, T, ISL1*, and *GATA4*. No bands were detected at the *MESP1* locus indicating PCR failure, likely due to high GC content at this genomic locus. C) Immunostaining for cardiac troponin T (cTnT; red) in Edit-R All-in-one lentiviral sgRNA-transduced iPS-CMs at differentiation Day 15. Nuclei are stained with Hoechst (blue). D) Quantification of cTnT mean fluorescent area relative to Hoechst staining from images depicted in C. *, ** $p < 0.05$, 0.01, respectively by one-way ANOVA followed by Tukey's *post hoc* test for multiple comparisons vs the NTC group. E) Schematic of Edit-R Cas9 mRNA and synthetic sgRNA nucleofection in iPSCs. F) Editing efficiency at the *EOMES* locus 72 hours post-nucleofection, assessed by TIDE. G) Immunostaining for Troponin T (red) in iPS-CMs at differentiation Day 15. Nuclei are stained with Hoechst (blue). Figures A and E created with BioRender.com

In a parallel experiment, we electroporated wildtype iPSCs with Edit-R Cas9 nuclease mRNA and predesigned Edit-R synthetic sgRNA targeting *EOMES* to compare knockout efficiency and cell phenotypes resulting from transient Cas9 expression rather than the constitutive expression strategy used above (Figure 2E). Using this approach, we observed ~70% editing efficiency, assayed by Sanger sequencing PCR amplicons and performing Tracking of Indels by Decomposition (TIDE) analysis (Figure 2F). Moreover, upon differentiation, *EOMES* knockout resulted in a reduced number of cells expressing Troponin T, indicating that like with constitutive Cas9 expression, transient delivery models are also suitable for generating a knockout population to characterize a phenotype (Figure 2G).

While both transduction of Edit-R All-in-one lentiviral sgRNA particles and transient delivery of Edit-R Cas9 mRNA alongside a synthetic sgRNA led to high indel formation at the targeted loci and resulted in impaired differentiation efficiency when the genes *T, MESP1*, or *EOMES* were knocked out, we were surprised at the fraction of cells that still differentiated into cardiomyocytes given the phenotypes reported in the scientific literature[2-6].

We reasoned that these mixed cell populations likely contained a fraction of unedited cells or heterozygously edited cells, which may have contributed to the weaker phenotype observed in our study compared to those reported in the literature. To test this hypothesis, we performed clonal isolation on our edited cell populations to generate homogeneous knockout populations. To this end, we sparsely seeded *T*-edited iPSCs into Matrigel-coated plates to ensure that a resulting colony originated from a single cell (Figure 3A). Clonal lines were expanded, banked, and seeded for differentiation. Cells were then fixed and stained for Brachyury (encoded by the *T* gene) at differentiation Day 3. While the mixed population of *T*-edited iPSCs still resulted in significant expression of Brachyury protein at differentiation Day 3, we were able to generate two lines with near-complete loss of Brachyury protein expression through clonal isolation (Figure 3B). Differentiation of these clonal lines resulted in a dramatic reduction in *MESP1* mRNA levels by Day 3 and nearly undetectable levels of *TNNT2* (Troponin T) mRNA by Day 7 (Figures 3C and D). Additionally, by Day 15, clonal *T* knockout lines expressed very few Troponin T- or α-actinin-positive cells compared to the NTC line (Figure 3E-F).

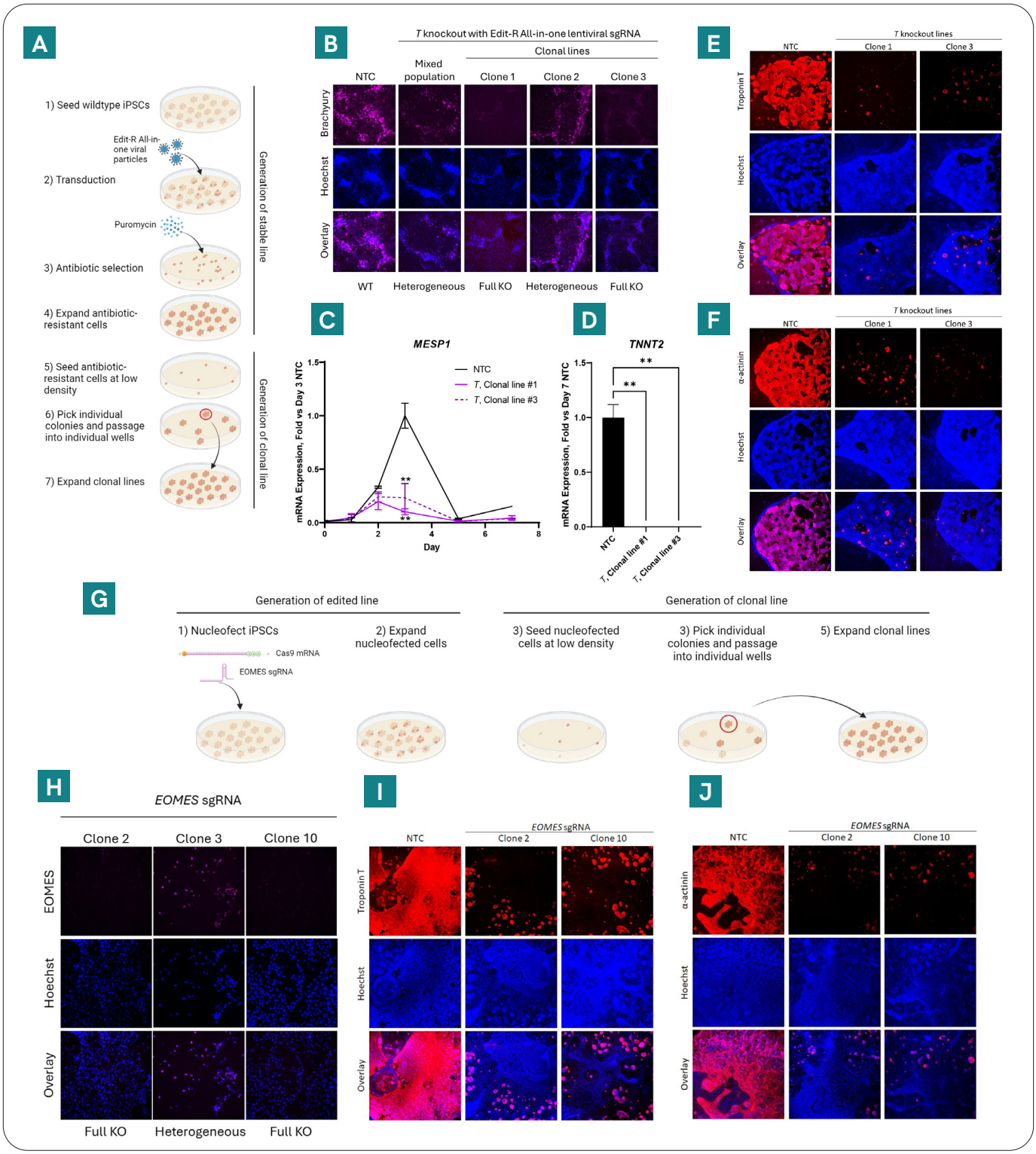


Figure 3: A) Schematic of Edit-R All-in-one viral particle transduction and selection in iPSCs, followed by clonal isolation and expansion of clonal lines. B) Immunostaining for Brachyury (encoded by *T*, magenta) in clonal *T* knockout (KO) iPS-CMs at differentiation Day 3. Nuclei are stained with Hoechst (blue). C) Messenger RNA expression of *MESP1* at various timepoints over the course of differentiation in clonal *T* KO lines and the non-targeting control (NTC) line. Expression is normalized to the NTC line at the denoted day of differentiation, corresponding to maximal expression over the course of differentiation. ** $p < 0.01$ by two-way ANOVA followed by Tukey's *post hoc* test for multiple comparisons vs the NTC group. D) Messenger RNA expression of *TNNT2* at differentiation Day 7 in clonal *T* KO lines and the non-targeting control (NTC) line. Expression is normalized to the NTC line. ** $p < 0.01$ by one-way ANOVA followed by Tukey's *post hoc* test for multiple comparisons vs the NTC group. E-F) Immunostaining for Troponin T (E) or cardiac α-actinin (F) in clonal *T* KO iPS-CMs at differentiation Day 15. Nuclei are stained with Hoechst (blue). G) Schematic of Edit-R Cas9 mRNA and synthetic sgRNA nucleofection in iPSCs followed by clonal isolation and expansion of clonal lines. H) Immunostaining for EOMES (magenta) in clonal *EOMES* KO iPS-CMs at differentiation Day 3. Nuclei are stained with Hoechst (blue). I-J) Immunostaining for Troponin T (I) or cardiac α-actinin (J) in clonal *EOMES* KO iPS-CMs at differentiation Day 15. Nuclei are stained with Hoechst (blue). Figures A and G created with BioRender.com

In parallel, we performed clonal isolation on the *EOMES*-edited cell population (Figure 3G). Upon differentiation, we successfully generated cell lines with homogeneous *EOMES* knockout, as evidenced by near-complete loss of EOMES protein expression at differentiation Day 3 (Figure 3H). Similar to the clonal *T* knockout lines, the clonal *EOMES* knockout lines had dramatically reduced numbers of Troponin T or α-actinin positive cells compared to the NTC line, indicating homogeneous knockout of these critical transcription factors potently impairs cardiomyogenesis (Figure 3I-J). Unfortunately, due to commercial antibody limitations, we were unsuccessful in characterizing a homogeneous *MESP1* knockout population.

Phenotypic confirmation via orthogonal genetic targeting with shRNA

Use of multiple LOF technologies to target a gene is a powerful approach to establish whether a gene plays a critical role in a cellular process. For example, observing the same phenotype with two LOF approaches more clearly rules out off-targeting due to the distinct gene-targeting mechanisms of each technology. CRISPR targets a genomic locus to introduce a short Indel mutation, causing a frame shift mutation that results in a non-functional protein. In contrast, shRNA targets a mature mRNA transcript and results in active degradation or translational repression of the mRNA transcript. Thus, the mechanisms of each technology differ dramatically, and therefore the likelihood of overlapping off-targets is minimal. Additionally, use of multiple LOF tools can mitigate the risk of false positives or negatives that can arise from inefficient or non-specific targeting by a single technology.

Therefore, to confirm our CRISPR knockout findings, we leveraged SMARTvector shRNA viral particles to constitutively express shRNAs targeting *T, EOMES*, or *MESP1*.

Given the delivery challenges during cardiac differentiation outlined above, we again opted to transduce wildtype iPSCs with viral particles prior to initiating differentiation. Following viral transduction, cells were selected with puromycin for five days, expanded, and banked as stable shRNA-expressing lines (Figure 4A). We then differentiated these lines and observed significant knockdown of all three targeted genes using two different shRNA sequences (Figure 4B-D). Notably, the ***T*** knockdown efficiency with both shRNAs exceeded 75% (Figure 4C). Similarly, the first shRNA sequence targeting *MESP1* exceeded 75% knockdown efficiency, while the second achieved over 60% (Figure 4D). However, the *EOMES* knockdown efficiency with either shRNA sequence, while statistically significant, only achieved a 40-60% knockdown efficiency (Figure 4B). Importantly, both *EOMES* and *T* induce the expression of *MESP1*. Indeed, *T* knockdown greatly reduced *MESP1* expression at both differentiation Day 2 and 3 (Figure 4D, purple lines). In contrast, *EOMES* knockdown attenuated *MESP1* expression at Day 2, but did not significantly affect levels at Day 3 (Figure 4D, orange lines). These results could be due to inefficient *EOMES* knockdown or due to compensation by other transcription factors including Brachyury *(T)*. Both *T* and *MESP1* knockdown resulted in dramatic reductions in the number of cells expressing Troponin T and α-actinin, confirming the findings observed with CRISPR knockout (Figure 4E and F). However, *EOMES* knockdown did not dramatically reduce the number of cells expressing Troponin T and α-actinin, potentially due to inefficient knockdown efficiency. Together, these results underscore the importance of using multiple genetic manipulation techniques to confirm a phenotype and highlight important shortcomings of each LOF approach, which should be carefully considered when choosing which techniques are best leveraged in a particular cell model.

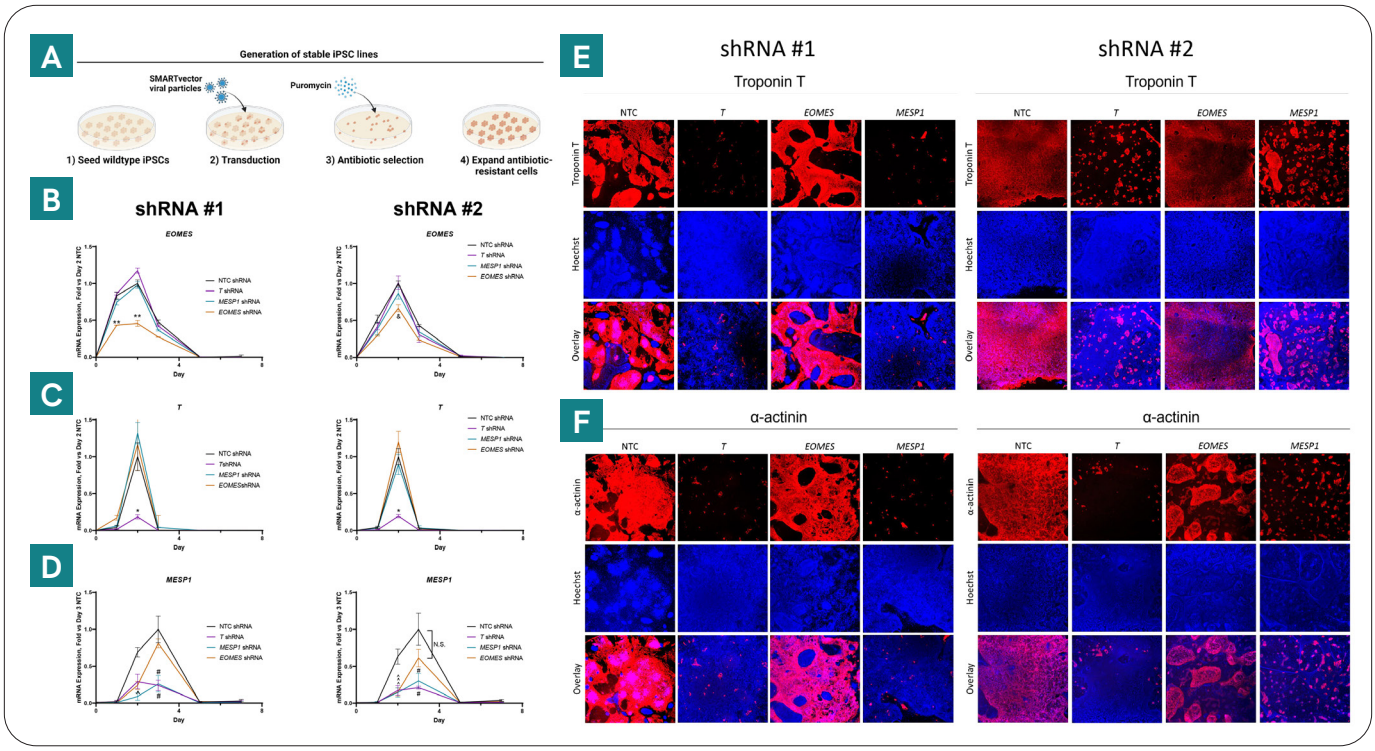


Figure 4: A) Schematic of SMARTvector viral particle transduction and selection in iPSCs. B-D) Messenger RNA expression of *EOMES* (B), *T* (C), and MESP1 (D) at various timepoints over the course of differentiation in SMARTvector shRNA-transduced iPS lines. Viral particles encoding two distinct shRNA sequences targeting each gene were used to create two distinct iPSC lines per gene: shRNA #1 (left panels) and shRNA #2 (right panels). Expression is normalized to the non-targeting control (NTC) line at the denoted day of differentiation, corresponding to maximal expression over the course of differentiation. *, ** $p < 0.05$, 0.01, respectively by one-way ANOVA followed by Tukey's *post hoc* test for multiple comparisons vs all other groups. #, &, ^ $p < 0.05$ by one-way ANOVA followed by Tukey's post hoc test for multiple comparisons vs NTC and *EOMES* shRNA groups, vs NTC and *T* shRNA groups, or vs the NTC group only, respectively. E-F) Immunostaining for Troponin T (E, red) or cardiac α-actinin (F, red) in SMARTvector-transduced iPS-CMs at differentiation Day 15. Nuclei are stained with Hoechst (blue). Figure A created with BioRender.com

Discussion

Cardiomyogenesis is a complex process that proceeds via a defined, temporally orchestrated cascade of transcriptional events. In this study, we show that perturbing expression of critical mesodermal or cardiac progenitor transcription factors dramatically reduces the number of cells that undergo complete differentiation to cardiomyocytes. A particularly important consideration when selecting tools for genetic manipulation is the choice between transient or constitutive expression. Here, we utilized both transient and constitutive delivery of Cas9 and achieved comparable results with either modality. In the case of Cas9, transient delivery enables the generation of a permanent genomic edit while minimizing the concern that continuous Cas9 expression leads to off-target effects. However, we did not notice any growth defects or unexpected differentiation phenotypes in iPSC lines transduced with Edit-R All-in-one Lentiviral sgRNA viral particles. Thus, as long as proper controls are included in every experiment, the choice to utilize transient or constitutive expression should be informed by the characteristics of the cell model, as some models are more amenable to lentiviral transduction than transfection or electroporation. Additionally, we chose to generate iPSC lines constitutively expressing shRNAs because the targets we were interested in are not expressed in iPSCs. Transient delivery methods (e.g., transfection or electroporation) could impact differentiation efficiency. Additionally, inducible shRNA expression would likely be arduous to optimize for every target, given the distinct temporal expression patterns of each target evaluated throughout differentiation. Nevertheless, other models may be more suited to transient or inducible shRNA expression instead.

Notably, we did not observe an obvious phenotype in the CRISPR-edited mixed populations of *GATA4* or *ISL1* KO lines, despite the literature indicating these targets play key roles in the differentiation process. This lack of phenotype could be attributed to several factors: 1) the differentiation phenotype being more mild than *T*, *EOMES*, or *MESP1* disruption, and thus not detectable without clonal isolation; 2) there may be inherent differences between human cardiac

differentiation in 2D and *in vivo* animal models or murine 2D differentiation models; or 3) *ISL1* and *GATA4* play larger roles in cardiomyocyte subtype specification (e.g., atrial, ventricular, pacemaker), maturation, proliferation, or electrophysiology properties and less of a role in upregulation of sarcomere proteins such as Troponin T or α-actinin, which were used as surrogate readouts of differentiation efficiency in this study. While we observed a stark reduction in differentiation efficiency by perturbing expression of *T*, *EOMES*, or *MESP1*, small numbers of cells expressing Troponin T or α-actinin persisted. This could be due to redundancy in the cardiomyogenic cascade of transcription factor expression such that single gene knockout is insufficient for fully disrupting differentiation[12] or is compensated for by additional non-targeted cardiomyogenic transcription factors[13].

Additionally, we note that while we observed strong disruption of cardiomyocyte differentiation in clonal *EOMES* KO lines, and mild disruption of differentiation in the mixed population KO line, we did not observe any disruption in the iPSC lines expressing *EOMES* shRNAs. This may be due to the relatively mild level (~40-60%) of *EOMES* knockdown we were able to achieve with constitutive shRNA expression. This level of knockdown may simply not be sufficient to produce a noticeable phenotype, especially given the redundancy in the cardiomyogenic transcription factor cascade[12]. In contrast, we observed strong knockdown efficiency (>75%) in iPSC lines expressing shRNAs targeting *MESP1* or *T*, which also resulted in strong disruption of cardiomyocyte differentiation. Thus, identifying highly active shRNAs in a particular model may enable robust phenotypic characterization.

Mechanistically, shRNAs act to degrade mRNA transcripts or repress translation, both of which reduce—but do not fully deplete—the pool of EOMES protein. Thus, the degree of knockdown is likely more consistent throughout the population, with ~40-60% transcript depletion occurring in most cells. In contrast, CRISPR editing produces a heterogeneous population, with some cells undergoing editing at both alleles, a single allele, or neither allele. Moreover, within the edited populations, Indels vary, and some edits may still produce in-frame mutations. In a mixed population, CRISPR editing produces a mosaic of cells, with many expressing no functional protein and others retaining near-wildtype expression levels. Thus, differentiation propensity can vary greatly from cell to cell, which likely explains why we observed mild disruption in CRISPR mixed population KO lines, but much stronger disruption in clonal KO lines, which arise from a single cell and therefore contain the same single genomic edit.

Here, we present robust phenotypic characterization following perturbation of critical differentiation factors following lentiviral delivery of CRISPR- or shRNA-based genetic manipulation tools. Together, these data highlight important considerations when selecting among distinct LOF technologies to target a gene of interest in a complex cell culture model—specifically, the differentiation of iPSCs into cardiomyocytes.

Methods

Cell culture and maintenance

Prior to initiating experiments, plates were coated with Matrigel (Corning; cat# 354277) at a 1:75 dilution in DMEM/F12 (Cytiva; cat# SH30023.03). WTC-11 iPSCs (Coriell Institute; cat# GM25256) were maintained in mTeSR™ plus basal medium (STEMCELL; cat# 100-0276). Upon reaching 75-80% confluence, culture medium was aspirated, and cells were washed once with 1X Dulbecco's phosphate buffered saline (DPBS, GIBCO; (cat# 14190-144) and detached from cell culture plates with pre-warmed StemPro® Accutase® (Gibco; cat# A11105-01). Cells were then transferred to a 15 mL conical tube, diluted 5:1 with 1X DPBS, and pelleted at 210 × *g* for 5 minutes. Supernatant was carefully aspirated, and cells were washed once in 1X DPBS, pelleted a second time, and supernatant was again carefully aspirated. Cells were then resuspended in mTeSR plus supplemented with 10 µM Y-27632 (STEMCELL; cat# 72308) and counted on an automated cell counter (e.g., Revvity Cellometer K2). Cells were then seeded into Matrigel-coated 6-well plates containing 2 mL mTeSR plus supplemented with 10 µM Y-27632 at a density of 1.25×10^5 cells per well (reaches confluence in 3 days) or 6×10^4 cells per well (reaches confluence in 4 days). 24 hours after seeding cells, culture medium was aspirated and replenished with fresh mTeSR plus without Y-27632. Transduced iPS cell lines were banked in freezing medium containing 60% mTeSR plus, 30% Knock Out Serum Replacement (Gibco; cat# 10828-028), and 10% DMSO (Sigma; cat# D2650-5x10mL). For clonal line generation, CRISPR-edited iPSCs were plated at a density of 100-500 cells per well in 6-well plates in mTeSR plus medium containing 10 µM Y-27632. 24 hours after seeding cells, culture medium was aspirated and replenished with fresh mTeSR plus without Y-27632, and subsequent medium changes were performed every 48 hours. Once colonies grew large enough to passage, individual colonies were scraped and carefully transferred by micropipette to separate wells of a 96-well plate pre-coated with Matrigel. Clonal lines were expanded and banked as above.

Supplier	Catalog number	Product name
Revvity	VSGH11936-247493450	Dharmacon Edit-R All-in-one Lentiviral sgRNA hEF1a T
	VSGH11936-247506766	Dharmacon Edit-R All-in-one Lentiviral sgRNA hEF1a ISL1
	VSGH11936-247488158	Dharmacon Edit-R All-in-one Lentiviral sgRNA hEF1a MESP1
	VSGH11936-247516288	Dharmacon Edit-R All-in-one Lentiviral sgRNA hEF1a GATA4
	VSGH11980	Dharmacon Edit-R All-in-one Lentiviral sgRNA hEF1a PPIB
	VSGC11964	Dharmacon Edit-R All-in-one Lentiviral sgRNA hEF1a Non-targeting Control #1
	S02-005000-01	Dharmacon SMARTvector Non-targeting hEF1a-TurboGFP Control Particles
	V3SH7590-226352887	Dharmacon SMARTvector Lentiviral Human T hEF1a-TurboGFP shRNA
	V3SH7590-225083905	Dharmacon SMARTvector Lentiviral Human ISL1 hEF1a-TurboGFP shRNA
	V3SH7590-224764730	Dharmacon SMARTvector Lentiviral Human EOMES hEF1a-TurboGFP shRNA
	V3SH7590-226394830	Dharmacon SMARTvector Lentiviral Human T hEF1a-TurboGFP shRNAT, shRNA #2 pSMART hEF1a/TurboGFP
	V3SH7590-225142282	Dharmacon SMARTvector Lentiviral Human MESP1 hEF1a-TurboGFP shRNA
	V3SH7590-225243130	Dharmacon SMARTvector Lentiviral Human EOMES hEF1a-TurboGFP shRNA

Viral transduction

WTC-11 iPSCs were seeded into Matrigel-coated 24-well plates into mTeSR plus supplemented with 10 µM Y-27632 at a density of 15,000 cells per well. 24 hours after seeding cells, medium was aspirated and replenished with fresh mTeSR plus without Y-27632. Dharmacon™ Edit-R™ All-in-one Lentiviral sgRNA viral particles and Dharmacon SMARTvector™ viral particles were added to iPSCs at 0.3, 1, 3, and 10 Transducing Units (TU) per mL. 48 hours post-transduction, cells were selected with 0.4 µg/mL puromycin, and the functional Multiplicity of Infection (MOI) was determined by estimating the fraction of surviving cells after selection. 3 TU/mL was sufficient to transduce ~25-30% of iPSCs (~0.3 MOI) with Edit-R All-in-one Lentiviral sgRNA viral particles while 10 TU/mL was used for SMARTvector viral particles to achieve a similar functional MOI. Viral particles used in this study are listed in the table below.

Electroporation

Prior to electroporation, cells were treated with 10 µM Y-27632 for 2 hours to promote viability post-electroporation. Cells were dissociated into a single cell suspension using StemPro Accutase, washed with 1X DPBS to dilute residual Accutase, and resuspended in mTeSR plus medium supplemented with 10 µM Y-27632. Cells were counted using an automated cell counter and resuspended in P3 primary cell buffer (Lonza; Cat# V4SP-3096). 8×10^4 cells were added to 20 µL reactions containing 2 µg Dharmacon Edit-R Cas9 nuclease mRNA (CAS11195) and 9 µM Edit-R synthetic sgRNA (Dharmacon; NTC1 - cat# U-009501-01-05; *EOMES* - cat# SG-017483-03-0005) per reaction. Cells were electroporated on a Lonza 4D nucleofector using program DC-100. After nucleofection, cells were allowed to rest for 5 minutes at room temperature. 80 µL mTeSR plus medium was then added to each reaction, gently mixed by pipette, and electroporated cells were incubated for another 5 minutes at room temperature. 50 µL of each reaction was then transferred to a Matrigel-coated 96-well plate containing 200 µL mTeSR plus medium supplemented with 10 µM Y-27632. 24 hours after seeding electroporated iPSCs, medium was aspirated and replenished with fresh mTeSR plus medium without Y-27632. Fresh mTeSR plus medium was replenished every 48 hours.

T7EI & TIDE

Cellular genomic DNA was extracted 13 days post-transduction (Edit-R All-in-one lentiviral sgRNA transduced iPSC lines) or 96 hours post-electroporation (Edit-R Cas9 mRNA/synthetic sgRNA electroporated iPSC lines) by lysing cultures in 1X HF buffer (Thermo; cat# F-518L) containing 1:20 proteinase K (Thermo cat# FEREO0492) at 56 °C for 30 minutes. Following lysis, proteinase K was inactivated by heating lysates at 95 °C for 5 minutes.

Amplicons spanning the edit window were amplified from genomic DNA extracts using touchdown PCR and predesigned detection primers (Primer sequences listed below). Following amplification, the PCR products were heated to 95°C for 10 minutes and cooled to 25 °C over 20 minutes. 10 µL PCR product was then incubated with T7 endonuclease I (NEB; cat# M0302L) in NEBuffer 2 (NEB cat# B7002S) and incubated at 37 °C for 25 minutes. Following T7EI incubation, reactions were immediately run on a 2% agarose gel and analyzed. Detailed protocol can be found here.

TIDE analysis was performed as above, but following touchdown PCR amplification, PCR reactions were purified and sequenced by Sanger sequencing. Sequencing data was then run through the webtool http://shinyapps.datacurators.nl/tide/ to determine editing efficiency.

Primer	Sequence (5' → 3')
T T7EI/TIDE forward primer	CCTGGCGAAGGGTTTCAGTG
T T7EI/TIDE reverse primer	CCGCGGCTCTATTTATGGGG
EOMES T7EI/TIDE forward primer	CACCACCAAGTCCATCTGCA
EOMES T7EI/TIDE reverse primer	CGGAGCCCTTTGTCAACACT
MESP1 T7EI/TIDE forward primer	GAAGGGGACACTAACCGGGG
MESP1 T7EI/TIDE reverse primer	TCTCGTCCCCAGACTCATGG
GATA4 T7EI/TIDE forward primer	CTCTTCTCGTGCTCAGGGGA
GATA4 T7EI/TIDE reverse primer	TAATCCCCGATGCACACCCT
ISL1 T7EI/TIDE forward primer	AAACCTCCCAGAGTACGCCC
ISL1 T7EI/TIDE reverse primer	GGAGCAATACCTGTACTCGCT

iPSC differentiation

WTC-11 iPSCs were dissociated into a single cell suspension using StemPro Accutase, washed with 1X DPBS to dilute residual Accutase, and resuspended in mTeSR plus medium supplemented with 10 µM Y-27632. Cells were counted using an automated cell counter and plated into Matrigel-coated 96-well plates at a density of 2×10^4 cells per well. 24 hours after seeding cells, medium was aspirated and replenished with fresh mTeSR plus medium without Y-27632. After an additional 24 hours, cells were differentiated with the STEMdiff™ Ventricular Cardiomyocyte Differentiation Kit (STEMCELL; cat# 05010) according to the manufacturer's recommendations. Briefly, supplements A, B, and C (all 10X stocks) were each added to individual aliquots of cardiomyocyte differentiation basal medium. Additionally, Matrigel was added to the aliquot of cardiomyocyte differentiation basal medium containing supplement A, at a 1 in 100 dilution. Cardiomyocyte maintenance supplement (50X stock) was added to cardiomyocyte maintenance basal medium. 48 hours after seeding iPSCs (Day 0), mTeSR plus medium was aspirated and replenished with differentiation medium containing supplement A. After an additional 48 hours (Day 2), differentiation medium containing supplement A was aspirated and replenished with differentiation medium containing supplement B. After an additional 48 hours (Day 4), differentiation medium containing supplement B was aspirated and replenished with differentiation medium containing supplement C. After an additional 48 hours (Day 6), medium was aspirated and replenished with differentiation medium containing supplement C. After an additional 48 hours (Day 8), medium was aspirated and replenished with maintenance medium. Maintenance medium was aspirated and replenished every 48 hours for the duration of the experiment.

RT-qPCR

Total RNA was isolated from iPS-CM cell lysates at various time points using an SV 96 Total RNA Isolation System (Promega, Z3500). cDNA was synthesized from extracted RNA using a Maxima First Strand cDNA Synthesis Kit (Thermo, K1672). RT-qPCR master mix containing 6 µL TaqMan Master Mix (Thermo, 4304437), 0.6 µL of appropriate gene-specific FAM-labeled TaqMan probe (All 20X; *EOMES*, Hs00172872_m1; *T*, Hs00610080_m1; *MESP1*, Hs00251489_m1; *TNNT2*, Hs00943911_m1), 0.2 µL of ACTB VIC-labeled TaqMan probe (60X, Thermo, beta-actin, Hs03023943_g1), and 3.6 µL nuclease free water, per reaction. 10.4 µL master mix was added to 1.6 µL undiluted cDNA in 384-well plates and loaded onto a Roche LightCycler 480 II for thermal cycling and analysis. Each cDNA sample was run in duplicate. The relative expression of each target gene was calculated with the ΔΔCq method using beta-actin as the reference gene and normalized to a non-targeting control.

Immunocytochemistry

On Day 3 (EOMES/Brachyury staining) or Day 15 (Troponin T/cardiac α-actinin staining) differentiating iPS-CMs were rinsed once in 1X PBS and fixed in 2% paraformaldehyde for 15 min at room temperature. Cells were washed three times in 1X PBS and permeabilized with 0.2% (v/v) Triton X-100 for 15 min at room temperature. Cells were again washed three times in 1X PBS and then blocked in 10% (v/v) fetal bovine serum (Cytiva; cat# SH30071.03). Primary antibodies (EOMES; BioLegend cat# 157702 – 1:200; Brachyury; Cell Signaling cat# NC1543793 – 1:500, Cardiac Troponin T; Thermo Scientific, cat# ms-295-p - 1:400; α-actinin; Sigma, cat# A7811-100UL - 1:400) were diluted in 10% (v/v) fetal bovine serum and added to fixed cells for 1 h at room temperature. Cells were washed three times in 1X PBS and then incubated with diluted secondary antibodies (goat-anti-rat Alexa 647; BioLegend, cat# 405416 – 1:1000; goat-anti-rabbit Alexa 647; Thermo, cat# A-21245 – 1:1000, goat-anti-mouse Alexa 555; Thermo, cat# A-21422 – 1:1000) and Hoechst 33342 (1:10,000) for 1 h at room temperature in the dark. Cells were then washed three times in PBS and fluorescent images were captured using a Nikon Eclipse Ti.

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