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Genome-wide screen in hiPSC-EC differentiation

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Abstract

The goal is to identify genes that have impacts on endothelial cell differentiation by employing CRISPRi screen. WTC11Ci hiPSCs were infected with a genome-wide CRISPRi library and differentiated into endothelial cells. Differentiated cells at D3 were subjected to MACS sorting with CDH5 microbeads to collect CDH5-positive endothelial cells and CDH5-negative non-endothelial cells. Genomic DNA was extracted to amplify guides on each population in order to calculate enrichment or deletion of each guide compared to control guides.

Methods

WTC11Ci hiPSC cell culture

WTC11Ci cells (hiPSCs harboring doxycycline-inducible CRISPRi) were cultured on 6-well plates (Falcon) coated with Matrigel hESC-Qualified Matrix (Matrigel) (Corning). Plates were prepared by diluting 180 μ L matrigel with 12 mL of DMEM/F12 media (Gibco), adding 2 mL of the diluted matrigel to each plate, and placing the plates in an 37°C, 5% CO₂ humidified incubator for at least 1 hour. Cells were passaged using Accutase (STEMCELL). When cells reach 70-85% confluency, media from each well was aspirated and 1 mL of Accutase was added. Cells were then placed in the 37°C incubator for 3 minutes. After this time, 1 mL of mTeSR Plus media (STEMCELL) at room temperature was added. Cells were dissociated, collected, and centrifuged at 200 x g for 3 minutes. Then, cells were resuspended in 1 mL mTeSR Plus media containing 10 μ M ROCK Inhibitor-Y-27632 (RI) (STEMCELL) and counted using the Countess (Invitrogen). For cell maintenance, 100,000 cells were transferred to each well of a prepared plate with 2 mL of media with 10 μ M RI.

sgRNA library design and cloning

To build a genome-wide library, we first obtained the sgRNA protospacer sequences from the Dual sgRNA CRISPRi Libraries of the Weissman lab (Addgene #187246, #187247, #187248). This resulted in 123,168 sgRNAs targeting 20,528 genes (6 sgRNAs/promoter), 2680 safe-targeting and 3790 non-targeting sgRNAs, for a total of 129,638 total guides. Pool of sgRNA oligos containing the guide protospacer sequence flanked by partial human U6 promoter (TATCTTGTGGAAAGGACGAAACACCG) and partial scaffold (GTTTAAGAGCTATGCTGGAAACAGCATAG) sequences were synthesized by Agilent Technologies. The guide pool was amplified with a forward primer (GGCTTTATATATCTTGTGGAAAGGACGAAACACCG) and a reverse primer (CTTATTTAACTTGCTATGCTGTTTCCAGCATAGCTCTTAAAC), extending the Gibson arms for subsequent cloning step into Crop-Opti vector. Plasmid backbone was prepared by digesting with BsmBI and ClaI (New England Biolabs), followed by purification with 1X Ampure XP SPRI bead clean-up. Gibson assembly was performed for 1 hour at 50°C using Gibson Assembly Master Mix (New England Biolabs), 50 ng backbone, and 70 ng of purified PCR-amplified sgRNA pool in a final volume of 40 μ L. The cloned plasmid was purified with 0.7X Ampure XP SPRI, electroporated into Endura competent cells (Lucigen #602422), and cells were then expanded in liquid culture for 18 hours at 30°C. Plasmid library was then obtained with the Nucleobond Xtra Midi EF kit (MacheryNagel #740420.50). To sequence-validate sgRNAs and determine their relative abundance, we PCR amplified and sequenced the sgRNAs from the plasmid library on Illumina MiSeq standard v2 50-cycle kit. Analysis of the sequencing data showed that all designed sgRNAs were represented with relatively equal coverage of each (the difference in count frequency between the top and bottom 10th percentiles of sgRNAs was 3.3).

Lentivirus generation

550,000 HEK293T cells per well were seeded on 6-well plates and incubated for 24 hours. Then, a DNA mix was prepared containing 900 ng psPAX2 packaging vector (Addgene #12260), 360 ng pMD2.g VSV-G envelope vector (Addgene #12259), and 1.2 μ g of purified plasmid library. These were transfected using 192 μ L of OMEM and 5.8

uL of X-tremeGENE HPTM DNA Transfection Reagent (Millipore Sigma #06366236001). Viral supernatant was harvested 48 hours later with 0.45 uM filtration and immediately stored at -80°C.

Lentiviral infection of WTC11Ci hiPSCs

WTC11Ci cells were transduced at a multiplicity of infection (MOI) of 1 at a coverage of >500 transduced cells per gRNA. Transduction was performed by adding lentivirus and polybrene (Millipore Sigma #TR-1003-G) to a final concentration 8 ug/mL into the cell media for 24 hours. Then, media was aspirated and replaced with media containing 0.5 µg/mL puromycin (Sigma Aldrich) for 24 hours. Media was replaced again with media containing 0.5 µg/mL puromycin to continue selection and 1 µg/mL doxycycline (Selleck) to induce expression of dCas9-KRAB simultaneously and incubated for additional 24 hours, for a total of 48 hours of puromycin selection. All media after this contained doxycycline to maintain dCas9-KRAB expression.

Optimal differentiation seeding density test

Before conducting large-scale experiments, we performed test differentiations to determine the optimal seeding density for the batch of hiPSCs used at the time. We tested seeding densities ranging from 150,000 to 350,000 cells per well in 6-well plates and differentiated the cells into endothelial cells. Based on these results, we selected the seeding density that yielded the highest differentiation efficiency for the large-scale experiments. The cell numbers were then scaled proportionally to the surface area of the culture vessels used in the larger-scale setup.

Differentiation and cell harvest

Infected WTC11Ci hiPSCs were passaged using accutase and seeded at the optimal density as determined above in mTESR Plus media containing 10µM rock inhibitor and 1 µg/mL doxycycline. After 24 hours, cells were differentiated into arterial endothelial cells using a protocol published by [Loh et al.](#) On day 3, cells were ready to be harvested. To this end, we washed the cells with PBS, added 1mM EDTA and incubated at 37°C for 15 minutes to facilitate cell dissociation. EDTA was then aspirated and cells were physically dissociated by titrating in differentiation media. This cell suspension was processed for flow cytometry analysis and MACS sorting as described below.

Flow cytometry

One million cells were set aside for flow cytometry to confirm successful differentiation. Cells were centrifuged at 300 x g for 3 minutes, supernatant aspirated, and pellet resuspended in 270µL PBS + 1% BSA. Each sample was then equally split into three FACS tubes. In the first tube, an isotype antibody mix was added to 1:50 dilution for APC-iso (BioLegend) and 1:500 dilution for FITC-iso (BioLegend)). In the second tube, a targeting antibody mix was added to 1:100 dilution for APC-CD144 (BioLegend) and 1:200 dilution for FITC-CD31 (BioLegend)). In the third tube, PBS + 1% BSA was added to serve as an unstained control. All tubes were covered and incubated at 2-8°C for 30 minutes. Cells were centrifuged and washed with PBS + 1% BSA. This washing step was repeated again, and the final product was resuspended in 300µL PBS + 1% BSA. We also included hiPSCs as negative control and TeloHAEC as positive control for antibody staining. These samples were then analyzed using the

MA900 Multi-Application Cell Sorter (Sony) to determine the frequency of APC-CD144 and FITC-CD31 double positive population.

MACS sorting

Cells were magnetically labeled with CD144 MicroBeads (Miltenyi Biotec 130-097-857) according to the manufacturer's instructions. Briefly, the day 3 cell suspension was centrifuged at 300 x g for 5 minutes, then resuspended with 80µL MACS buffer per 10 million cells. Then 20µL of MicroBeads were added and incubated at 2-8°C for 15 minutes. After incubation, 1mL MACS buffer was added to wash and centrifuged again at 300 x g for 10 minutes. Cell pellet was resuspended in 500 µl MACS buffer and magnetic separation of cells was performed within LS Column (Miltenyi Biotec) on a MACS Separator and MultiStand (Miltenyi Biotec). CD144 labeled cells (endothelial cells) and non-labeled cells (non-endothelial cells) were collected and centrifuged at 300 x g for 5 minutes. Supernatant was carefully aspirated and cell pellets were frozen at -20°C for later genomic DNA extraction.

Genomic DNA extraction and amplicon sequencing

Extraction of genomic DNA for each sorted cell sample was performed using the **QIAamp Blood Maxi Kit** according to the manufacturer's spin protocol with a maximum of 20 million cells per spin column. Following extraction, each genomic DNA sample was split into separate PCR replicates in a 96-well plate (Eppendorf) with each PCR reaction containing 6 µg of genomic DNA. Genomic region containing the gRNA was amplified using Q5 High-Fidelity 2X Master Mix (New England Biolabs, no M0492L) and 0.5 uM of each amplification primer containing 8bp sample index and Illumina adapters:

AATGATACGGCGACCACCGAGATCTACACNNNNNNNNCGATTTCTTGGCTTTATATATCTTGTG;

CAAGCAGAAGACGGCATACGAGATNNNNNNNNCGGTGCCACTTTTTCAAGTTG. Genomic amplicon PCR was carried out with the following thermocycling parameters: 98°C for 3 min and then 28 cycles of 98°C for 10 s, 62°C for 20 s, 72°C for 25 s, followed by a final 72°C extension for 2 min. Equal volumes of PCR replicates were pooled together and DNA was purified by a double-sided SPRI bead cleanup (Beckman) (first, a left-handed cleanup at 0.5X, then a right-handed cleanup at 1.2X). 5µL of each sample was then run on a 2% agarose gel (Invitrogen) to confirm a clear band of 210bp. DNA concentration was measured using the Qubit 1X dsDNA HS Assay Kit (Invitrogen, no Q33231). DNA amplicon libraries were sequenced on an Illumina Nextseq 550 System (High Output v2.5, 75-cycle kit) using a custom sequencing primer:

CGATTTCTTGGCTTTATATATCTTGTGGAAAGGACGAAACACCG for Read 1 and

AAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCG for Index 1.

Data Analysis

Raw sequencing reads were demultiplexed using the bcl2fastq program (Illumina) to generate FASTQ files and trimmed to contain the unique gRNA sequences. These processed reads were then aligned to the designed gRNA sequences from the library using **bowtie2** to obtain read counts for each gRNA in each sorted sample.

Two approaches were used to identify and rank hits. In the first approach, we used the **MAGECK** robust ranking aggregation (RRA) algorithm to identify screening hits (mageck test -k sgrna_count.txt -t positive_sample -c

negative_sample --control-sgrna non_targeting_safe_targeting_gRNAs.txt).

In the second approach, we calculated the gene fold change for each gene based on the frequency of gRNA reads in the CDH5+ versus CDH5- populations. This was calculated as follows:

1. Calculate the fold change of control guides between the CDH5+ and CDH5- populations.

These guides should have not affected growth or differentiation, so their frequency in these populations should theoretically be the same. However, other guides will affect growth, leading to cell death and a lower frequency of guide in the CDH5+ population. Because of this decrease, the frequency of control guides will seem to have increased in the CDH5+ population. To counteract this, we will normalize our calculations by this factor.

For each bio-replicate, the fold change of all (1) safe and (2) non-targeting guides between CDH5+ vs CDH5- was calculated. Then, the average between these two values was recorded. This will be used to normalize our next calculation.

$$\begin{aligned}\text{Safe_FC} &= [\text{freq. safe guides, CDH5+}] / [\text{freq. safe guides, CDH5-}] \\ \text{NonTargeting_FC} &= [\text{freq. nontargeting guides, CDH5+}] / [\text{freq. nontargeting guides, CDH5-}] \\ \text{Control_FC} &= (\text{Safe_FC} + \text{NonTargeting_FC}) / 2\end{aligned}$$

1. Calculate the fold change for all target genes between D3+ and D3- populations.

1. For each gene, the frequency of guides targeting that gene in the CDH5+ population was divided by the frequency within the CDH5- population.
2. To normalize this value to control guides, the value in (a) was then divided by the control guide fold change in Step 1.
3. The log was then taken for the value found in (b).

$$\begin{aligned}\text{Gene_FC} &= [\text{frequency of gene in CDH5+}] / [\text{frequency of gene in CDH5-}] \\ \text{Normalized_Gene_FC} &= \text{Gene_FC} / \text{Control_FC} \\ \text{Log2_Gene_FC} &= \log_2(\text{Normalized_Gene_FC})\end{aligned}$$

To calculate the p-value and adjusted p-value for each gene between CDH5+ vs CDH5-, a Mann-Whitney U-test with B-H correction was performed on the ratios of guide frequencies for all guides targeting each gene, versus the ratios of guide frequencies for all negative control guides.