Application Note

f.sight™ | Streamlined Workflow for the Generation of CRISPR-edited Mesenchymal Stem Cell Clones for Regenerative Medicine Applications

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Abstract

We demonstrate an efficient, high-throughput workflow that incorporates the f.sight™ for both selection and cloning of CRISPR/Cas9-edited mesenchymal stem cells (MSCs). Here, MSCs underwent gene editing to knockout the RANKL protein to further enhance the therapeutic capacity of MSCs in regenerative medicine. The f.sight's high sensitivity was able to detect the low fluorescent signal of the edited MSCs and enrich for this population. The high single-cell isolation efficiency (>93%) and gentle isolation of the f.sight enabled the generation of 182 monoclonal cell lines. Of these, 17 were selected for further characterization. We were able to generate two successfully edited clones that exhibited the capacity for osteogenic differentiation.

Introduction

Regenerative medicine is an expansive field dedicated to the repair of damaged cells, tissues or organs to restore healthy functionality. In this field, stem cells are prevalent because of their ability to differentiate into various cell types and are often used to replace the diseased cells. Although promising, the degree of success of stem cell therapy can widely vary. To increase the success rate of these therapies, CRISPR-mediated gene editing can enhance the performance of the stem cells[1].

CRISPR-mediated gene editing has provided researchers a faster and more efficient method to edit key genes of interest in cells. While more efficient than conventional methods, the genome editing process often compromises cell viability, and thus the scale up from single cell clones to colonies is a difficult and tedious process. A primary bottleneck in the CRISPR workflow is single-cell cloning. After transfection, the resulting cell population is heterogeneous with various degrees of edits. This population must be enriched from untransfected cells and cloned to create uniform, monoclonal cell lines for further characterization to ensure correct edits occurred. Additionally, the safety and traceability of the genetic changes must be guaranteed, especially for medical applications when using genetically modified cells. This is a barrier to meeting the requirements of both the European Medicines Agency (EMA) and the Food and Drug Administration (FDA)[2,3]. Current methods of single-cell cloning have several disadvantages including low throughput and no enrichment for positive cells as seen with limiting dilution (LD) or harsh isolation and incompatibility with small sample volumes in the case of fluorescence-activated cell sorting (FACS). Additionally, both methods do not provide visual confirmation of single-cell isolation.

Here, we introduce a streamlined workflow **(Figure 1)** for the generation of monoclonal CRISPR-edited cell lines with a traceable origin. The f.sight enables gentle, high-throughput single-cell isolation by dispensing cells encapsulated in single droplets. High-resolution optics and image recognition are used to identify droplets containing a single cell[4,5]. Single-use cartridges contain the cell samples during isolation to eliminate cross contamination between cell lines. Additionally, with fluorescent imaging, it is possible to enrich for positively transfected fluorescent cells. Here, we demonstrate a workflow where MSCs were CRISPR-edited to stop the expression of the RANKL protein for enhanced bone formation, which could be used as a therapy for diseases such as osteoporosis[6,7]. Despite the low editing efficiency, the f.sight was able to isolate transfected cells with low fluorescence. Gentle isolation enabled further expansion of 182 viable clones generated. Ultimately, 17 clones were selected for further characterization via 5 different validation methods, including qPCR. It was determined that 2 clones

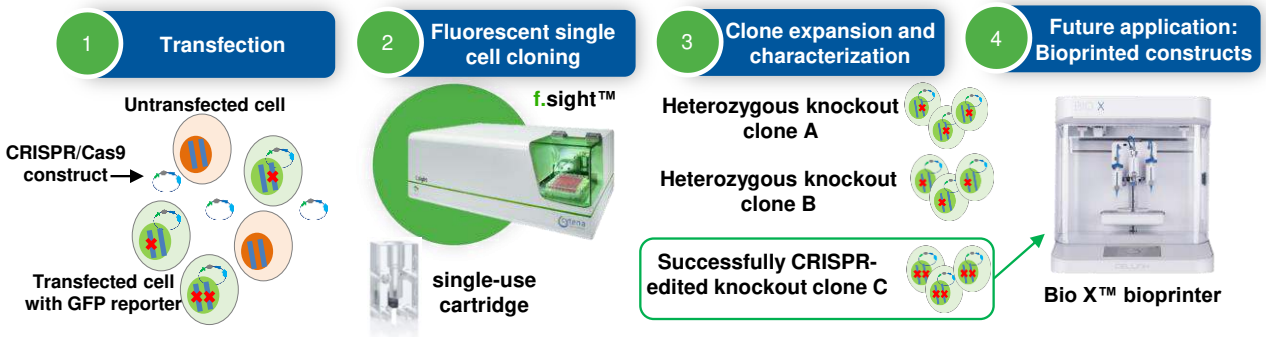

Figure 1. CRISPR/Cas9 gene-editing and cloning workflow. **1.** Cells are transfected with a CRISPR/Cas9 construct to integrate the intended gene edits. A fluorescent reporter is included to denote an edited cell. **2.** Transfected cells are cloned with the f.sight to generate cell lines with uniform gene edits. Single-use cartridges hold the cell suspension and prevent cross-contamination. **3.** Clones are expanded and analyzed to determine which cell lines have the gene edits on both alleles as represented by the 2 red Xs. Additional analysis is performed to confirm if the edits result in the desired effect on the cell behavior. **4.** Gene-edited cells are valuable in a variety of therapeutic areas. In regenerative medicine, a future application is the bioprinting of modified cells into 3D scaffolds (BIO X™ bioprinter) that can be used to repair damaged tissue.

successfully contained the knockout for RANKL and the genetic modification did not affect the differentiation and osteogenic properties of the MSCs.

Materials and methods

Samples and culture conditions

Immortalized MSCs were cultured in MEM alpha Medium (1x) + GlutaMax – I (Gibco) supplemented with 10% fetal bovine serum (Gibco) and 1% penicillin-streptomycin (Gibco) and incubated at 37°C and 5% CO_2.

Transfection of the immortalized MSCs

For the genetic modification, the gRNA CRISPR/Cas9 All-in-One Non-viral Vector - Human (abm) was used, subsequently referred to as pNV-RANKL/KO. Plasmids were amplified in competent *E.coli* cells (Mix&Go) and purified by ZymoPURE II Plasmid Miniprep kit (ZymoResearch). MSCs were transfected with both the pNV-RANKL/KO vector and a separate MSC group was transfected with the vector pmaxGFP as a transfection reference and control. Human MSC Nucleofector kit (Lonza) and Nucleofector 2b device (Lonza) were used to transfect the cells.

CRISPR-edited MSCs preparation and single-cell isolation

Prior to single-cell isolation with the f.sight, transfected MSCs were washed 1 to 2 times with PBS before being harvested to remove detached, nonviable cells. The MSCs were detached with trypsin and transferred to a Falcon tube and centrifuged at 300*g* for 5 minutes. The supernatant was discarded, and the cells were resuspended in 1 mL of PBS and counted. Cells were diluted to a concentration of 5 – 8 x 10^5 cells/mL and 30 µL of cell suspension was loaded into a cartridge for dispensing. Single cells were isolated into a 96-well plate prefilled with 290 µL of culture media. Plates were imaged with a NyONE imager (SynenTec) as second confirmation of single-cell isolation. Cells were incubated at 37°C and 5% CO_2.

Working principle of single-cell dispensing in f.sight

The f.sight houses a dispensing chip which rapidly generates picoliter-sized droplets on demand when the silicon membrane is deflected by a piezo-actuated piston. The f.sight can isolate both fluorescent and nonfluorescent cells with high efficiency and sensitivity. This is achieved with a novel dual-camera system that captures and saves brightfield and fluorescence information simultaneously at full resolution. The dispensing chip nozzle region is imaged continuously during operation. A cell detection algorithm analyzes each passing particle until a single cell is present in the nozzle. If the cell satisfies the selected criteria (cell size, roundness and fluorescent intensity), it is dispensed into a well of the 96- or 384-well plate. Unwanted droplets (i.e., empty droplets or droplets with multiple cells) are removed by vacuum prior to dispensing. A series of images of the nozzle region is

A

f.sight™ Settings	Range
Cell Size	15 – 30 µm
Cell Roundness	0.6 – 1
pmaxGFP Transfected MSCs	
Laser intensity	30 – 40%
Exposure time	15 – 30 ms
Fluorescence threshold	30 – 250 AFU
RANKL-KO Transfected MSCs	
Laser intensity	90 – 100%
Exposure time	80 – 120 ms
Fluorescence threshold	50 – 250 AFU

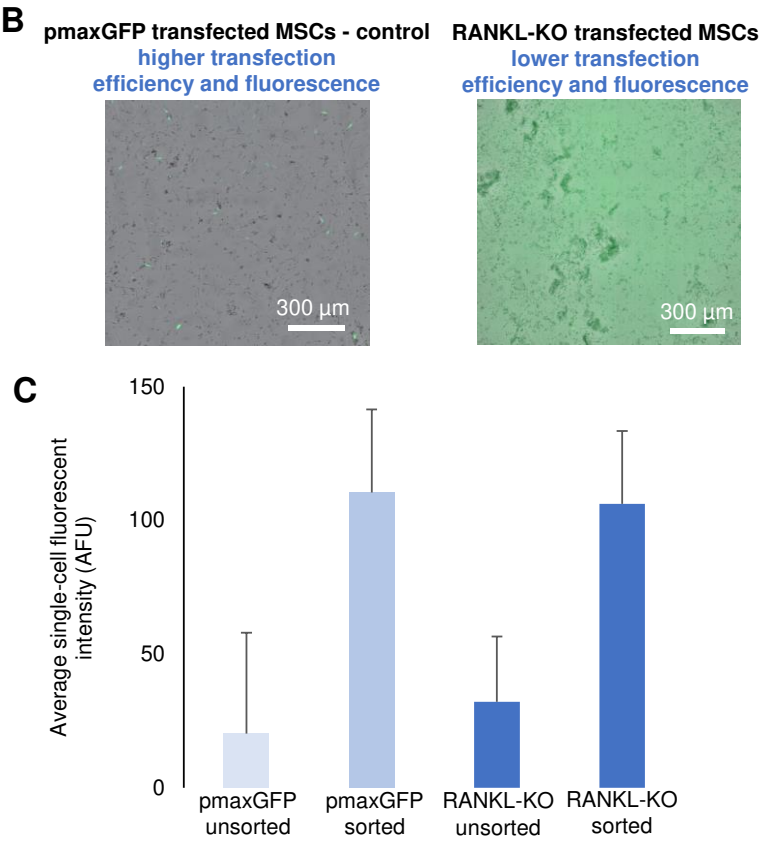

Figure 2. The f.sight can enrich and isolate both high and low fluorescing CRISPR-edited MSCs. **A.** Both pmaxGFP and RANKL-KO MSCs were assessed by the f.sight prior to cell dispensing to determine optimal dispensing parameters. The selected cell size and roundness were the same for both cell types. The fluorescent settings were adjusted to account for the differences in fluorescent signal between the two transfected cell populations with higher laser intensity and exposure time for RANKL-KO MSCs. **B.** Combined brightfield and fluorescent images of pmaxGFP and RANKL-KO transfected MSCs in culture. pmaxGFP exhibit a higher fluorescent signal and healthier morphology as compared to RANKL-KO transfected MSCs. **C.** The f.sight easily enriches for the fluorescent population. Here is a comparison of the average single-cell fluorescent intensities for RANKL-KO and pmaxGFP transfected cells before and after sorting with the f.sight. The error bars represent the standard deviation.

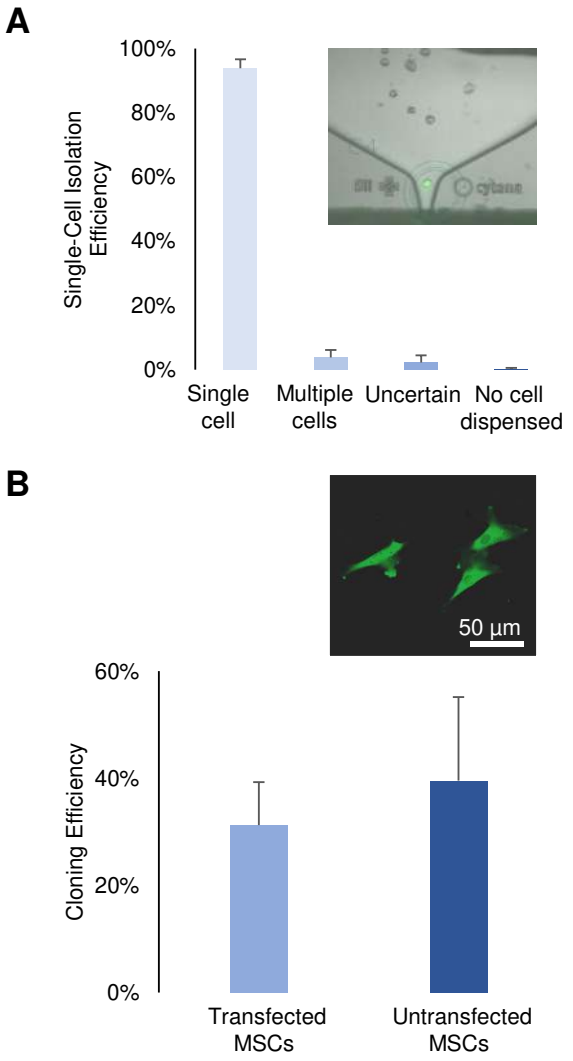

Figure 3. Single-cell isolation efficiency and cloning efficiency. **A.** The f.sight exhibited a single-cell isolation efficiency of over 93%. The image shows a single fluorescent cell in the nozzle that was selected for dispensing. **B.** The percentage of single cells that developed into colonies for the sensitive transfected MSCs was 31% as compared to 40% of untransfected MSCs. Here is an image of an early MSC colony after cloning. The error bars represent the standard deviation.

automatically stored for each deposited droplet in both brightfield and fluorescent channels, and single-cell ejection can be verified by reviewing the image series.

Results and discussion

High-throughput CRISPR-edited clone generation

The first step in the workflow is to design a CRISPR/Cas9 system to target a specific gene of interest. Since the goal is to cease the expression of RANKL in the MSCs for improved bone formation, RANKL's gene (*TNFSF11*) was the targeted sequence for removal by the CRISPR/Cas9 construct, RANKL-KO. RANKL-KO was then transfected into the MSCs. Another construct (pmaxGFP) was transfected into a separate group of MSCs as transfection reference and control. Both constructs contained a fluorescent reporter to indicate the presence of an edit. **Figure 2B** shows images of the cells 48 hours post transfection. The transfection efficiency of pmaxGFP was around 15% and, as shown in the image, transfected cells had significant fluorescent signal. The transfection efficiency of RANKL-KO was lower at around 5% with a reduced fluorescent signal for the transfected cells. Additionally, the viability of the RANKL-KO transfected cells was compromised.

Despite the low transfection efficiencies, the f.sight successfully distinguished the viable, gene-edited subpopulation of both RANKL-KO and pmaxGFP transfected cells. Before dispensing into the target plate, the f.sight performs an assessment of the cell size, roundness and fluorescent intensity of a given cell suspension. These values are then used as selection criteria to enrich for the target population **(Figure 2A)**. For both populations of transfected cells, the cell size selected was 15 to 30 µm. A roundness of 0.6 to 1 was selected to exclude irregularly shaped, nonviable cells. The fluorescence criteria were tailored for each group. For the pmaxGFP transfected cells, the fluorescent signal was strong and thus the laser intensity was set around 30% to 40%, with an exposure time of 15 to 30 ms. The threshold intensity ranged from 30 to 250 AFU. For the RANKL-KO transfected cells, the fluorescence intensity was lower and only a few cells were fluorescent, thus the fluorescence settings were adjusted accordingly with the laser power set around 90% to 100% with an exposure time of 80 to 120 ms. **Figure 2C** shows for both transfected cell groups the average single-cell fluorescent intensity before and after enrichment with the f.sight. This demonstrates that the f.sight can readily distinguish even low fluorescing transfected cells for the generation of monoclonal cell lines.

Single-cell isolation efficiency and cloning efficiency

For single-cell cloning, six 96-well plates were filled with both RANKL-KO and pmaxGFP transfected cells. **Figure 3A** shows the classification of dispensed cells and an example of a single fluorescent cell selected for dispensing. The overall single-cell isolation efficiency was 93.9%. A benefit of the f.sight is the ability to exclude wells without a single cell by reviewing the image series generated for each dispensing cycle. After single-cell dispensing, the cells were incubated for 20 to 25 days and the cloning efficiency was assessed. The NyONE imager was used to monitor colony growth. The transfected cells were benchmarked against cloned untransfected cells to assess changes in viability. **Figure 3B** shows that 31% of the transfected cells and 40% of the untransfected cells developed into monoclonal colonies. While there was a reduction in the clone recovery because of the harsh treatment of transfection, especially with the knockout, the f.sight's gentle method of isolation still enabled the generation of sufficient clones for further scale up and analysis. Seventeen clones were selected for further characterization.

Characterization of CRISPR-edited clones

Although CRISPR-mediated gene editing is a fast method, the process can still result in inaccurate or partial editing where only one of the alleles has received the edit. Thus, each clone must be characterized to determine the clones that have a successful knockout of RANKL. Various methods including the Surveyor Assay, qPCR, Emulsion Coupling technology[8] and ELISA were used to determine the successful knockout. After analysis, 2 clones were determined to have edits on both alleles. A final validation test was performed to ensure the differentiation capacity of the knockout clones were still

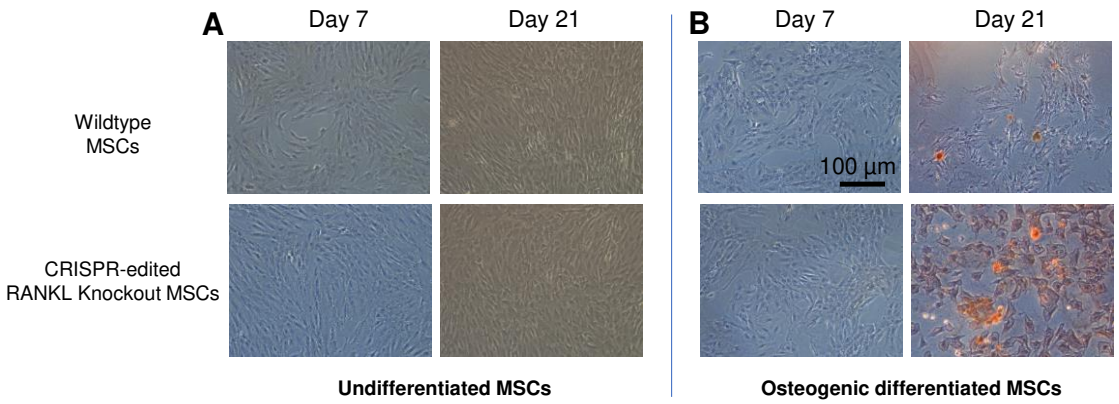

Figure 4. CRISPR-edited RANKL-KO MSCs still retained their differentiation capacity. **A.** Images of undifferentiated wildtype MSCs and RANKL-KO MSCs at Day 7 and Day 21. Both exhibit similar morphology. **B.** Images of differentiated wildtype MSCs and RANKL-KO MSCs at Day 7 and Day 21. At Day 21, the edited MSCs also exhibit calcifications as visualized with Alizarin red staining that is indicative of osteogenic differentiation.

maintained. **Figure 4** compares the morphology and phenotypic properties of wildtype MSCs to one of the CRISPR-edited RANKL knockout MSCs both in undifferentiated and differentiated states. **Figure 4A** shows that the gene-edited MSCs maintain the normal morphology as compared to the wildtype when cultivated in standard media. However, when cultured in differentiation media **(Figure 4B)**, the cells begin to differentiate similarly to the wildtype untransfected cells. At Day 21, the cells were fixed and stained with Alizarin red dye to visualize the calcifications that are normally exhibited in bone cells. The presence of calcifications shows that the RANKL knockout MSCs still maintained their capacity to differentiate with osteogenic properties.

Conclusion

The f.sight provides a fast and gentle method for cloning single cells post transfection in a gene editing workflow while maintaining the traceability of the individual clones at all times. The flexibility the fluorescent settings enables the detection and isolation of low fluorescing transfected MSCs from the bulk population. With high single-cell isolation efficiencies over 93%, the f.sight can generate many single-cell clones in a high-throughput manner. Gentle handling is key because the viability of cells post transfection is often compromised. Here, we show that even with compromised viability, the percentage of colony formation for transfected MSCs was still comparable to untransfected MSCs. Lastly, other properties of the f.sight, including minimal sample volume and ease of use, make it readily compatible into similar cloning workflows used in gene editing and cell therapy. Thus, the f.sight can be used to ultimately meet the requirements of medical agencies for using genetically modified cells in many medical applications.

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