Incorporating Flow Cytometry into the CRISPR/Cas-9 Gene Editing Workflow Speeds Time to Results

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Abstract

The power of gene editing provides the ability to change the biology of a cell or whole organism, enabling the investigation of a host of difficult or previously impossible to interrogate biological processes. As more and more gene editing tools are discovered and developed, the applications for gene editing will only continue to expand. Here we use CRISPR/Cas-9 editing of intracellular adhesion molecule-1 in a prostate cancer cell line to illustrate a method that incorporates enrichment and single-cell sorting to allow monocultures of edited cells to be generated in a reliable and more rapid manner.

Introduction

To investigate the effect of decreased intracellular adhesion molecule-1 (ICAM-1) expression on various cell signaling pathways, we analyzed gene and protein expression in a cellular knockout of *ICAM-1*. In addition, we demonstrate a streamlined method for CRISPR gene editing that includes enrichment and single-cell sorting flow cytometry.

ICAM-1 plays an important role in stabilizing cell-cell interactions and in mediating cell signaling during the immune response. It is a cell surface glycoprotein typically expressed in immune and endothelial cells and binds to integrins. ICAM-1 expression can be stimulated by interleukin-1 and tumor necrosis factor alpha (TNF-α), which facilitates leukocyte extravasation through endothelial cells into underlying tissue. ICAM-1 expressed by respiratory epithelial cells is also the binding site for rhinovirus, the causative agent of most common colds. Additionally, ICAM-1 is associated with a variety of cancer cell types and has been shown to play both a positive and a negative role in cancer cell invasion and metastasis. We found that a reduction of ICAM-1 expression through CRISPR-mediated gene editing altered gene and protein expression in the prostate cancer cell line PC3.

Materials and Methods

The Gene Pulser Xcell™ Electroporation System (Bio-Rad) was used to cotransfect PC3 cells with CRISPR/CAS9 vectors (OriGene) targeting *ICAM-1* and donor vectors containing templates for GFP and puromycin resistance. Transfection was confirmed by observing GFP expression after 24 hours (Figure 1). Fifty percent of viable cells that excluded propidium iodide were enriched for GFP expression 14 days post-transfection using the S3e™ Cell Sorter (Bio-Rad) and replated for continued growth (Figure 2). Following a further 14 days of culture, cells were subjected to selection with puromycin for 7 days before again using the S3e Cell Sorter to sort single cells based on viability staining by propidium iodide and GFP expression. For comparison, the remaining 50% of unenriched transfected cells were treated using a traditional protocol. Cells were cultured and split for seven generations before being subjected to puromycin selection. Selected cells were then grown to 2 x 10^6 cells/ml concentration.

Following 7–10 days of culture post–single cell sorting, colonies from the accelerated workflow were subjected to next generation sequencing to confirm gene editing. A colony positive for an *ICAM-1* edit, designated G1-31, was then used for protein and gene expression analysis.

The expression of GFP and the loss of ICAM-1 protein expression were confirmed by western blot and fluorescence cell imaging. For western blotting, control and edited cells were lysed on ice in RIPA buffer (VWR) containing protease inhibitor cocktail (VWR), sonicated, and spun down to remove particulates. Lysates were diluted in 4x loading buffer containing β-mercaptoethanol and run on a 12% precast acrylamide stain-free gel. Proteins were transferred to nitrocellulose using the Trans-Blot® Turbo™ Transfer System (Bio-Rad). The blot was probed and labeled with anti-CD54 PrecisionAb™ Primary Antibody recognizing ICAM-1 (Bio-Rad) and Goat Anti-Rabbit IgG StarBright™ Blue 700 Rabbit (Bio-Rad) (blue), and Anti-GAPDH hFAB™ Rhodamine Labeled Primary Antibody (Bio-Rad) (red), and Anti-turboGFP Primary Antibody (OriGene) (green). The blot was imaged on the ChemiDoc™ MP Imaging System (Bio-Rad).

For gene expression analysis, cells were cultured ±10 ng/ml TNF-α for 24 hours to stimulate ICAM-1 expression. Cells were lysed using the SingleShot™ Cell Lysis Kit (Bio-Rad), allowing preparation of gDNA-free RNA directly from cell culture. SingleShot Cell Lysates were added to three different PrimePCR™ Predesigned 384-Well Pathway Plates: Male Urogenital Diseases Tier 1, Extracellular Matrix, and Neoplasm Metastasis (Bio-Rad). We screened 962 targets by real-time PCR to look for changes in gene expression using a CFX384 Touch™ Real-Time PCR Detection System (Bio-Rad) and analyzed resulting data using CFX Maestro™ Software (Bio-Rad). Targets showing gene expression changes compared to controls were then further investigated for protein expression changes using Bio-Rad's Bio-Plex® Multiplex Immunoassay System.

Results

Compared to traditional protocols, enriching for GFP-positive cells increased the number of viable cells available for single-cell sorting and colony growth following puromycin selection (Figure 3). This enrichment step brought cells to the single-cell sort stage approximately 30 days earlier than a protocol without enrichment.

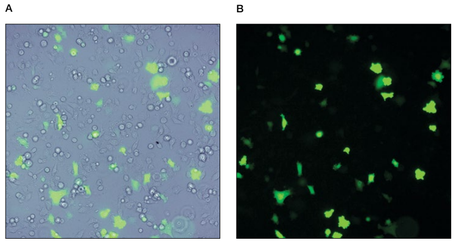

Fig. 1. PC3 cells imaged 24 hours after transfection with OriGene ICAM1 gRNA vector 1. Merged (**A**) and GFP channel (**B**) view of PC3 cells 24 hours after transfection using the Gene Pulser Xcell System captured using the ZOE™ Fluorescent Cell Imager.

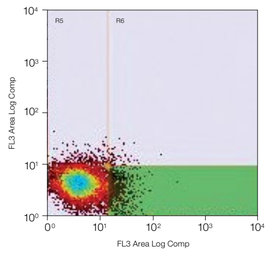

Fig. 2. Gating for live (PI-negative) GFP-positive transfected PC3 cells using the S3e Cell Sorter. Cells were enriched for GFP, resulting in selection of potentially edited cells for further culture.

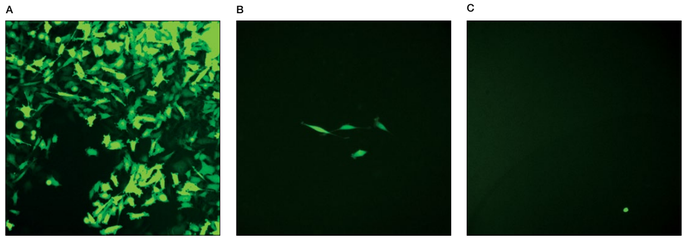

Fig. 3. Comparison of enriched and nonenriched cultures. Transfected cells were cultured for 14 days. The live GFP-positive cell population was enriched from the mixed cell culture using the S3e Cell Sorter. Puromycin selection 14 days later further selected for edited cells. Significantly more puromycin resistant cells were available earlier for single-cell sorting from the enriched population (**A**) than the nonenriched cultures (**B**). Live GFP-positive cells were sorted using the S3e Cell Sorter and single-cell isolation and GFP fluorescence were confirmed using the ZOE Cell Imager (**C**). Cells were then passaged for 2 weeks until 80% confluent for further analysis.

Protein analysis by western blot (Figure 4) and immunocytochemistry (ICC) (Figure 5) showed a complete loss of ICAM-1 expression in our edited cells. However, using PrimePCR Real-Time PCR Assays and Bio-Plex Multiplex Immunoassays, we were able to show gene and protein expression of *ICAM-1* in our knockout cell lines upon stimulation with TNF-α, albeit to a lesser extent than in control unedited PC3 cells.

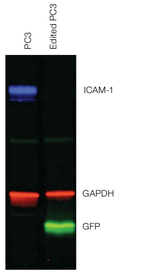

Fig. 4. Confirmation of successful gene editing by western blot. Edited PC3 cells show no detectable expression of ICAM-1 protein, but demonstrate strong GFP expression.

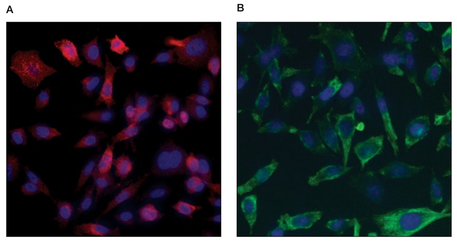

Fig. 5. Confirmation of successful gene edits by immunocytochemistry. Control and edited cells were plated onto microscope slides 48 hours prior to staining with anti-ICAM-1 antibody (red) and DAPI (blue)-containing mounting medium (Vector Laboratories). Slides were imaged at the bench using the ZOE Cell Imager. Edited cells show expression of GFP (green) but no ICAM-1 expression. **A**, Immunostaining of control PC3 cells reveals abundant ICAM-1 expression. **B**, CRISPR-edited PC3 cells have lost ICAM-1 expression and gained stable GFP expression.

Results (continued)

We found expression changes in a number of genes and proteins related to the extracellular matrix and other cell signaling pathways (Figures 6 and 7). Particularly significant were alterations to MMP13 and phosphorylated AKT and sTNFr1.

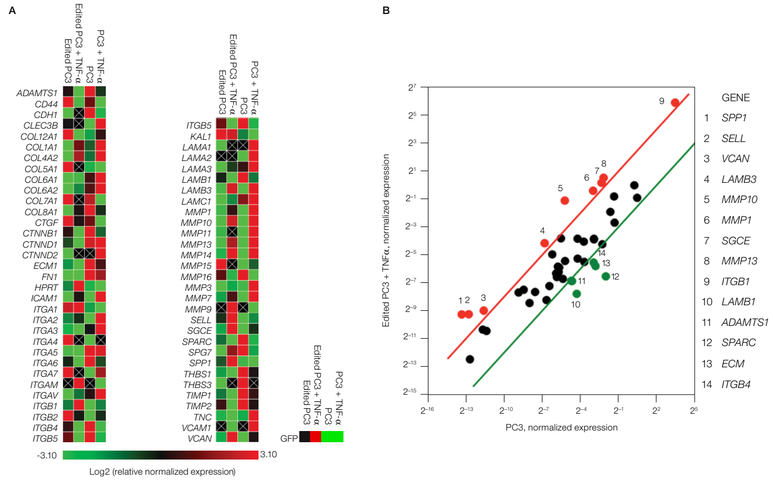

Fig. 6. Gene expression analysis in control and edited cells in response to TNF-α treatment. Control and edited cells were treated with TNF-α to stimulate ICAM-1 expression. **A**, relative to control cells (unstimulated PC3 cells), the unstimulated edited cells show little or no *ICAM-1* expression; **B**, a scatter plot provides an alternate view of changes in expression. The red line represents a fourfold increase and the green line a fourfold decrease in expression compared to control cells; **C**, several genes were found to have an altered expression in our *ICAM-1* knockout clone at basal level and upon stimulation with *TNF-α*; these include *COL1A*, *ITGB1*, *SELL*, *SPP1*, and a number of matrix metalloproteinases.

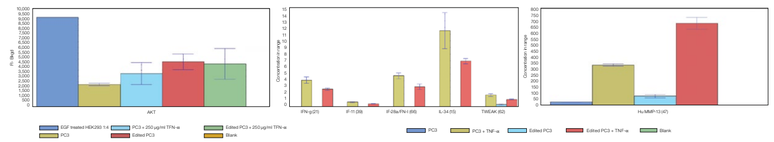

Fig. 7. Analysis of changes in protein expression. To investigate the effect of ICAM-1 reduction on other cellular functions we screened a range of protein targets involved in extracellular metabolism, cellular signaling, and inflammation using Bio-Plex Multiplex Immunoassays. Significant changes were seen in our edited cells compared to controls. For example, pAKT, MMP13, and a number of inflammatory cytokines had an attenuated expression to TNF-α treatment.

Conclusions

Enrichment of transfected cells by flow cytometry saves time, reduces cell culture waste, and increases the chances of selecting and growing successfully edited colonies. Reducing the level of ICAM-1 expression in PC3 cells affects other cell signaling pathways involved in extracellular matrix remodeling and cell signaling under both basal and stimulated conditions.

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