Targeted Integration of Fluorescent Reporter Genes Utilizing Zinc Finger Nucleases

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Introduction

Engineering of the mammalian genome is an area of active research that also impacts drug discovery and cell-based assays. This can be accomplished using a new generation of ZFNs – synthetic, chimeric proteins engineered to cleave DNA at a sequence-specific location and leave across both strands. The cell's natural machinery repairs the break in one of two ways: non-homologous end joining (NHEJ) or homologous recombination. Implementation of the homologous recombination pathway was used to insert a donor template into a specific, desired location in mammalian cells. ZFNs were custom-designed to cleave near the desired site of integration for four separate specific genes. These ZFNs were delivered along with a donor construct containing a fluorescent reporter gene flanked by homologous arms to the target integration site into U-2 OS cells. Integration resulted in expression of fluorescent fusion proteins that labeled a particular organelle.

Four gene loci were tagged: TUBA1B (α-tubulin 1b, microtubule), ACTB (β-actin, actin stress fibers), LMNB1 (lamin B1, nuclear envelope) and HMGA1 (high mobility group AT-hook 1, nucleus). Fluorescent proteins expressed in the ACTB, LMNB1 and HMGA1 loci localized to the nucleus. Preservation of protein function and gene regulation was observed. Additionally, due to the specificity of the targeted integration process, multiple reporter genes were successfully integrated into the U-2 OS genome.

Methods

U-2 OS (Cat. No. HTB-167) cells were obtained from ACC and cultured according to the product manual. Nucleofections were performed with the Amaxa® Nucleofector® device (Cat. No. A136-1038) and the cell line was electroporated with a pCX-E12-ZFN-TUBA1B plasmid. The expression of Gfp and Rfp was monitored by fluorescence microscopy. All experiments were performed with U-2 OS cells in a 6-well plate culture dish. Cells were transfected with ZFNs and incubated for 24 hours prior to analysis. For Southern hybridizations, genomic DNA was isolated from U-2 OS cells using the QIAamp DNA Mini Kit (Qiagen). To analyze integrant bands, PCR digestion with PstI overnight was performed.

Results

Successful Tagged Loci

<table>
<thead>
<tr>
<th>NM_number</th>
<th>Humanen</th>
<th>Distance Between Integration</th>
<th>Expression</th>
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<td>NM_006082</td>
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<td>13</td>
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Discussion/Conclusion

Until now, fluorescent labeling of proteins relies on external promoters and techniques requiring cell fixation. With ZFNs it is now possible to create stable integration of reporter genes into a genome. Small fusion proteins generated with an external promoter is the fusion proteins contain a fluorescent protein fused at the N-terminus of the protein, which can be used to study the protein's localization and behavior.

Acknowledgments

We would like to thank Dhian D'Grac and D. Munkisi for their assistance and encouragement.

References/Endnotes

3. Phenotypic A, Gurova A, Trapp A, Stempfle, H., Hooftveld, G., Steenbergen, C. 1979. Inhibition of migration: a simple system obtained using a ZFNs expressed in the N-terminus of the protein, which can be used to study the protein's localization and behavior.
4. http://www.sciencemag.org/content/320/5880/1203.full
5. http://www.sciencemag.org/content/320/5880/1203.full
6. Vincristine is a mitotic inhibitor used in cancer chemotherapy. It blocks the action of tubulin subunits thereby inhibiting the assembly of microtubule structures. 2D-Tagged: TUBA1B U-2 OS cells were exposed to 21 µM Vincristine for 6 h. At this time, tubulin is reassembled into a crystalline structure.

Figure 1: ZFN targeting mechanism. A. ZFNs bind to the target site. Then the FokI endonuclease domain cleaves both strands of DNA forming a site-specific DNA double-strand break (DSB). The human repair machinery repairs the DSB in one of two ways: non-homologous end joining (NHEJ) or homologous recombination. NHEJ introduces deletions or insertions, and cell-based assays. This can be accomplished using zinc finger nucleases (ZFNs) – synthetic, chimeric proteins engineered to cleave DNA at a sequence-specific location and leave across both strands. The cell's natural machinery repairs the break in one of two ways: non-homologous end joining (NHEJ) or homologous recombination. Implementation of the homologous recombination pathway was used to insert a donor template into a specific, desired location in mammalian cells.

Figure 2: Successfully Tagged Loci. A. GFP integrated into the LMNB1 locus in U-2 OS cells localized in the nucleus.

Figure 3: Targeted Integration of TUBA1B in U-2 OS Cells. A. Schematic of the TUBA1B/ZFN-1 construct with GFP ACTB in U-2 OS cells. B. Schematic of the TUBA1B/ZFN-1 construct with RFP ACTB in U-2 OS cells. C. Schematic of the TUBA1B/ZFN-1 construct with RFP ACTB in U-2 OS cells. D. Schematic of the TUBA1B/ZFN-1 construct with RFP ACTB in U-2 OS cells. E. Schematic of the TUBA1B/ZFN-1 construct with RFP ACTB in U-2 OS cells. F. Schematic of the TUBA1B/ZFN-1 construct with RFP ACTB in U-2 OS cells.

Figure 4: Cytochalasin B Time Course. Cytochalasin B is a mycotoxin. It blocks the formation of contractile microfilaments thus inhibiting cytoskeletal dynamics. By blocking microtubule assembly, actin filaments are shortened. GFP tagged TUBA1B U-2 OS cells were exposed to 21 µM Cytochalasin B. Over time, shortening of actin filaments can be observed.

Figure 5: Vincristine time course. Vincristine is a mitotic inhibitor used in cancer chemotherapy. It blocks the action of tubulin subunits thereby inhibiting the assembly of microtubule structures. 2D-Tagged: TUBA1B U-2 OS cells were exposed to 21 µM Vincristine for 6 h. At this time, tubulin is reassembled into a crystalline structure.

Figure 6: Cytochalasin B Time Course. Cytochalasin B is a mycotoxin. It blocks the formation of contractile microfilaments thus inhibiting cytoskeletal dynamics. By blocking microtubule assembly, actin filaments are shortened. GFP tagged TUBA1B U-2 OS cells were exposed to 21 µM Cytochalasin B. Over time, shortening of actin filaments can be observed.

Figure 7: Targeted Integration of TUBA1B in U-2 OS Cells. A. Schematic of the TUBA1B/ZFN-1 construct with GFP ACTB in U-2 OS cells. B. Schematic of the TUBA1B/ZFN-2 construct with GFP ACTB in U-2 OS cells. C. Schematic of the TUBA1B/ZFN-3 construct with GFP ACTB in U-2 OS cells. D. Schematic of the TUBA1B/ZFN-4 construct with GFP ACTB in U-2 OS cells. E. Schematic of the TUBA1B/ZFN-1 construct with GFP ACTB in U-2 OS cells. F. Schematic of the TUBA1B/ZFN-1 construct with GFP ACTB in U-2 OS cells.