Targeted integrations of fluorescent proteins into STAT3 and STAT1 loci via ZFN mediated homologous recombination in human cancer cell lines provides a model system for cell-based testing of new candidate drugs for human tumor treatment.

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Abstract

STAT3 is a member of the family of signal transducers and activators of transcription (STAT), and is constitutively activated in human cancer cell lines and human cancers including breast, gastric, lung, and pancreatic. Another member of the STAT family, STAT1, possesses cancer-inhibitory properties and once activated may promote apoptosis in tumor cells. Thus, STAT3 has been identified as a promising drug target for many cancers. Indeed, the potential inhibition of STAT3 may be very selective and thus minimally toxic (1).

To target these cancer research important genes in small groups, STAT3 and STAT1 loci with fluorescent protein (FP) sequences (AAAGSGSG) and respective ZFNs (3) were generated. Thus, it is possible to achieve targeted integration.

Living cell imaging studies indicated that both STAT3 and STAT1 protein expression and preservation of protein function are maintained following STAT3 and STAT1 gene knock-in (1). STAT3 and STAT1 genes endogenously tagged with FP enable the visualization of STAT3 and STAT1 through fluorescence microscopy. This facilitates the study of STAT3 and STAT1 activities and can be useful in testing of potential STAT3 inhibitors while ensuring that the STAT3 pathway is not affected. This approach of tagging endogenous genes could be applied to other cancer targets.

Introduction

Signal Transducers and Activators of Transcription (STATs) are transcription factors that mediate signaling in cytokines. Following type I IFN (IFN-α/β) and TNF type I (TNF-α/β) or other cytokines binding to cell surface receptors, Jak kinases become activated, leading to phosphorylation of STATs. The phosphorylated STATs dimerize, associate with SOCS2/SOCS3 to form complete STAT signaling transcription factor (3). The STATs are then transported to the nucleus in order to target DNA. The STATs can also be inhibited by ZFNs to achieve the transcription of introduced oligonucleotides that drive the cell to an antithetical oncogenic pathway (4). It was shown that STATC is constitutively active in numerous cancer cells and nearly 70% of all human cancer includes multiple myeloma, sarcomas and lymphomas, breast cancer head and neck cancers, colon, ovarian cancer, melanoma, renal cell cancer, colorectal cancers and thymic epithelial tumors. Therefore, STAT3 is considered to be an oncogene (5). The inhibition of STAT3 induces apoptosis in melanoma cells and suppresses the growth of breast and lung tumors in nude mice (6). On the contrary another STAT family member – STAT1 is an oncogene and promotes apoptosis in tumor by inducing the expression of cell surface death receptors and their ligands (7). STAT1 is also known to act as a negative regulator of tumor growth and metastasis by presence a key role in the inhibition of angiogenesis (8).

It means that potential anti-cancer drug candidate should selectively inhibit STAT3 while not affecting STAT1. STAT1 inhibition usually involves participation in the binding to the phosphorylated receptor and oxidative stress as well in STAT activation (9). A small molecule that can penetrate the cell membrane, directly and selectively bind the STAT3 (or STAT1) domain to prevent STAT3 (or STAT1) transactivation, and does not interfere with STAT3 signal transduction by major STAT3 inhibitors is desirable.

Our goal was to design a cell model system where activities of both STAT3 and STAT1 could be investigated. Mouse embryonic fibroblast (MEF) cell lines where the STAT3 genes are endogenously tagged with RFP (10), the cytokine-induced transcription factor that dimerizes and makes a double strand break (DSB) between the binding sites. DSBs are repaired by either an NHEJ or HDR pathway (1). Figure 1: ZFN targeting mechanism. A. ZFNs bind to the target site. Then the FokI endonuclease domains dimerize and make a double strand break (DSB) between the binding sites. DSBs are repaired by either an NHEJ or HDR pathway. B. HDR results in the integration of the homologous recombination (HR) or HDR of the ZFN site due to the targeted fluorophore reporter (AAAGSGSG).

Methods

We used CompuSyn technology to develop KnockOut nutlin-3a’s expression in the STAT3 gene (11). The cytokine-induced transcription factor that dimerizes and enters the nucleus in response to stimuli is invivo (12). The STAT3 and STAT1 genes were constructed using cell lines which were transfected with lentivirus containing STAT3 or STAT1 constructs. Flow cytometry analysis indicated that both STAT3 and STAT1 gene expression is conserved in in vitro differentiated chondrocyte and primary mesenchymal stromal cells (13). This indicates that STAT3 and STAT1 expression was similar in the rodent and human models. The STAT3 and STAT1 genes were engineered with SAD 3/6-7 and 487 UFP1 and SAD 3/6-7 and 487 UFP1, respectively. The STAT3 and STAT1 FP-tagged promoters were used in chondrocytes. For the injection of STAT3/GFP and STAT1-GFP, the cells were transfected with STAT3/GFP and STAT1-GFP constructs for 24 hours.

Table 1: Current FP-tagged Cell Lines.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>FP Tag</th>
<th>Gene Tag</th>
<th>Cancer Type</th>
<th>Signaling Pathway</th>
<th>Protein Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>CLL1140</td>
<td>A549</td>
<td>RFP-STAT3</td>
<td>Lung cancer</td>
<td>STAT3 (N) Signaling pathway protein</td>
<td></td>
</tr>
<tr>
<td>CLL1097</td>
<td>A549</td>
<td>CELLS EGFR BIOSENSOR</td>
<td>Lung cancer</td>
<td>GFP-tagged Grb2 SH2 binding domain for activated EGFR Receptor binding domain (activated EGFR)</td>
<td></td>
</tr>
<tr>
<td>CLL1039</td>
<td>MCF10A</td>
<td>RFP-TUBA1B</td>
<td>Breast epithelium</td>
<td>TUBA1B (N) Cytoskeletal protein</td>
<td></td>
</tr>
<tr>
<td>CLL1218</td>
<td>U2OS</td>
<td>LMNB1-TUBA1B-ACTB</td>
<td>Osteosarcoma</td>
<td>LMNB1 (N), TUBA1B (N), and ACTB (N) Nuclear envelope protein and Cytoskeletal proteins</td>
<td></td>
</tr>
<tr>
<td>CLL1037</td>
<td>U2OS</td>
<td>GFP-ACTB</td>
<td>Osteosarcoma</td>
<td>ACTB (N) and TUBA1B (N) Cytoskeletal proteins</td>
<td></td>
</tr>
<tr>
<td>CLL1035</td>
<td>U2OS</td>
<td>RFP-ACTB</td>
<td>Osteosarcoma</td>
<td>ACTB (N) Cytoskeletal protein</td>
<td></td>
</tr>
<tr>
<td>CLL1033</td>
<td>U2OS</td>
<td>GFP-LMNB1</td>
<td>Osteosarcoma</td>
<td>LMNB1 (N) Nuclear envelope protein</td>
<td></td>
</tr>
<tr>
<td>CLL1031</td>
<td>U2OS</td>
<td>GFP-TUBA1B</td>
<td>Osteosarcoma</td>
<td>TUBA1B (N) Cytoskeletal protein</td>
<td></td>
</tr>
</tbody>
</table>

The STAT3/GFP and STAT1-GFP cell lines were exposed to a mixture of 100 ng/mL of IL-6 and INF-γ (A549 cell line). The STAT3 and STAT1 images were taken 40 minutes after addition of a mixture of 100 ng/mL of IL-6 and INF-γ. The STAT3 and STAT1 images were taken 25 minutes after addition of a mixture of 100 ng/mL of IL-6 and INF-γ. The STAT3 and STAT1 images were taken 20 minutes after addition of a mixture of 100 ng/mL of IL-6 and INF-γ. The STAT3 and STAT1 images were taken 25 minutes after addition of a mixture of 100 ng/mL of IL-6 and INF-γ. The STAT3 and STAT1 images were taken 25 minutes after addition of a mixture of 100 ng/mL of IL-6 and INF-γ. The STAT3 and STAT1 images were taken 25 minutes after addition of a mixture of 100 ng/mL of IL-6 and INF-γ.