Evaluation of STAT3 Signaling in Macrophages Using a Lentiviral Reporter System

Schwertfeger Laboratory
Emily Hartsough
Breast Cancer Prevalence

<table>
<thead>
<tr>
<th></th>
<th>Males</th>
<th>Females</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Estimated New Cases</strong></td>
<td></td>
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<tr>
<td>Prostate</td>
<td>160,890</td>
<td>21%</td>
</tr>
<tr>
<td>Lung &amp; bronchus</td>
<td>117,920</td>
<td>14%</td>
</tr>
<tr>
<td>Colon &amp; rectum</td>
<td>70,820</td>
<td>8%</td>
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<tr>
<td>Urinary bladder</td>
<td>58,950</td>
<td>7%</td>
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<tr>
<td>Melanoma of the skin</td>
<td>46,870</td>
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<tr>
<td>Non-Hodgkin lymphoma</td>
<td>40,170</td>
<td>5%</td>
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<tr>
<td>Kidney &amp; renal pelvis</td>
<td>39,650</td>
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<tr>
<td>Oral cavity &amp; pharynx</td>
<td>34,780</td>
<td>4%</td>
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<tr>
<td>Leukemia</td>
<td>34,030</td>
<td>4%</td>
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<tr>
<td>Liver &amp; intrahepatic bile duct</td>
<td>28,410</td>
<td>3%</td>
</tr>
<tr>
<td>All Sites</td>
<td>841,390</td>
<td>100%</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Males</th>
<th>Females</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Estimated Deaths</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lung &amp; bronchus</td>
<td>85,920</td>
<td>27%</td>
</tr>
<tr>
<td>Prostate</td>
<td>26,120</td>
<td>8%</td>
</tr>
<tr>
<td>Pancreas</td>
<td>21,450</td>
<td>7%</td>
</tr>
<tr>
<td>Liver &amp; intrahepatic bile duct</td>
<td>18,280</td>
<td>6%</td>
</tr>
<tr>
<td>Leukemia</td>
<td>14,130</td>
<td>4%</td>
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<tr>
<td>Esophagus</td>
<td>12,720</td>
<td>4%</td>
</tr>
<tr>
<td>Urinary bladder</td>
<td>11,520</td>
<td>4%</td>
</tr>
<tr>
<td>Non-Hodgkin lymphoma</td>
<td>11,520</td>
<td>4%</td>
</tr>
<tr>
<td>Brain &amp; other nervous system</td>
<td>9,440</td>
<td>3%</td>
</tr>
<tr>
<td>All Sites</td>
<td>314,290</td>
<td>100%</td>
</tr>
</tbody>
</table>

Adapted from Siegel et. al Cancer Statistics. 2016
Tumor Microenvironment and Macrophages

Coussens and Werb, 2002

Medrek et al., BMC Cancer, 2012.
STAT3 Signaling in Cancer Cells

- STAT3 is active in up to 70% of human breast cancers
- Induced by Growth Factors and Cytokines (IL-6)
- Increased STAT3 signaling leads to...
  - Tumor proliferation and survival
  - Angiogenesis
  - Invasion and metastasis
  - Tumor-promoting inflammation
  - Immune evasion
STAT3 signaling in Tumor Associated Macrophages – Previous Lab Data

• Loss of STAT3 in mammary tumor cells...
  • Leads to reduced tumor cell proliferation and decreased tumor burden

• Loss of STAT3 in myeloid cells...
  • Leads to worse prognosis in mice as well as faster tumor growth and proliferation
STAT3-GFP Reporter – My Project

- Dynamic reporter that can be used to evaluate STAT3 activity in live cells by reporting downstream transcription events instead of using cell-surface marker identification

- Short term goal:
  - Ensure the STAT3 reporter is functional and specific

- Long term goal:
  - Visualize tumor associated macrophages expressing GFP in vivo and further characterize their expression profiles
Specific Aims

• Aim 1. Optimize transduction of the STAT3-GFP, TATA-GFP (negative control), and EFS-GFP (positive control) reporters
  • RAW (mouse immortalized macrophage cell line)

• Aim 2. Measure STAT3-GFP reporter expression response to cancer cell secreted cytokines
  • +IL-6 (canonical ligand) to validate reporter
  • HS578T (triple negative breast cancer cell line) conditioned media
Transformation and Growing up Plasmid Preps.

Transform Original Plasmids into Competent *E. coli*

1. **STAT3-GFP** (experimental)
   - STAT3 binding region
   - TATA box
   - GFP

2. **TATA-GFP** (neg. control)
   - TATA box
   - GFP

3. **EFS-GFP** (pos. control)
   - EF-1 alpha promoter
   - GFP

Grow up Bacteria and Isolate Plasmids

Confirm with Sanger Sequencing
Naked DNA Transfection into HEK 293T cells

+ Prepped DNA sample (STAT3-GFP, TATA-GFP, EFS-GFP)
+ Transfection Reagent

48 hour incubation
Naked DNA Transfection into HEK 293T cells

Conclusion: The EFS-GFP and TATA-GFP transfected HEK 293T cells appear to have appropriate functionality.
Conclusion: The HEK 293T cells with the new STAT3-GFP plasmids express GFP at similar levels compared to the TATA-GFP negative control plasmid.
Lentivirus Transfection

Day 1: Plate viral packaging cells (293T) at 5 x 10^6 cells

Day 2: Transfect cells
+ RK5-Rev vector (Rev Protein)
+ MD2C-VSVG vector (Envelope Vector)
+ pRK-PAO vector (Packaging Vector: Gag and Pol)
+ Lentiviral vector (STAT3-GFP, TATA-GFP, EFS-GFP)

Harvest virus over 48 hours and concentrate
Lentivirus Transduction

Plate RAW cells and evaluate response to IL-6/HS578T Conditioned Media

Successful Lentiviral Transduction

-/+/ IL-6 or HS578T Conditioned Media
Results – Lentiviral Transduction into RAW cells

**EFS-GFP Lentiviral Transduction**

Conclusion: The EFS-GFP plasmid was successfully transduced into RAW cells. There was no observable GFP expression with the STAT3-GFP or TATA-GFP plasmids. This may be due to the fact that they were not successfully transduced or the GFP levels were too low to be visualized.

Next Steps: FACS sorting of EFS-GFP RAW cells. Determine the MOI of the lentiviral vector.
Future Directions

• Optimize lentiviral vectors
  • Determine lentivirus multiplicity of infection (MOI)
    • Optimize amount of virus needed, amount of cells, and exposure time
  • Determine transduction efficiency
    • P24 ELISA or dual reporter
• Perform Flow Cytometry to quantify GFP expression levels
• Perform FACS to sort out GFP-positive cells
• Transduce lentiviral vectors into different cell types (bone marrow derived macrophages)
• Obtain plasmids with antibiotic resistance gene
Acknowledgements

• Schwertfeger lab
  • Kaylee Schwertfeger, Ph.D.
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  • Michael Lewis, Ph.D.
  • Mu Wang, Ph.D.

• Funding
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References


Macrophage are crucial participants in inflammation and wound healing

LPS, IFNγ

M1 (classically activated)

- infection control
  - IL-6, IL-1β, IL-12, Cxcl9, reactive oxygen species

IL-4, IL-13

M2 (alternatively activated)

- resolution of damage,
  - ECM remodeling, immune suppression: IL-10,
  - scavenging receptors, arginase pathway

Tumor-associated macrophages
IL-6, LIF

Ccl2
Ccl5
Cox-2
IL-12

IL-6, LIF

Dampens inflammatory signals; “status quo”

Inhibition of T cell responses

Increased production of pro-inflammatory cytokines and chemokines

Promotion of tumor growth

Polly Chuntova
Plasmids

EFS-GFP Plasmid (Positive control)

TATA-GFP Plasmid (Negative control)

STAT3-GFP Plasmid (Experimental)
Transfection of HEK 293T #2 – Non-infected control

STAT3-GFP

TATA-GFP

EFS-GFP

-IL-6

-IL-6

-IL-6

+IL-6

+IL-6

+IL-6

Conclusion: Not enough time allocated for transcription/translation of plasmid and STAT3 induction of GFP.
## Transfection of HEK 293T #2

<table>
<thead>
<tr>
<th></th>
<th>TATA-GFP (original prep)</th>
<th>EFS-GFP (original prep)</th>
</tr>
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<tbody>
<tr>
<td><strong>-IL-6</strong></td>
<td><img src="image" alt="6 hour -IL-6" /></td>
<td><img src="image" alt="6 hour -IL-6" /></td>
</tr>
<tr>
<td><strong>+IL-6</strong></td>
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<tr>
<td><strong>-IL-6</strong></td>
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<tr>
<td><strong>+IL-6</strong></td>
<td><img src="image" alt="24 hour +IL-6" /></td>
<td><img src="image" alt="24 hour +IL-6" /></td>
</tr>
<tr>
<td>STAT3-GFP A (new)</td>
<td>STAT3-GFP B (new)</td>
<td>STAT3-GFP (original prep)</td>
</tr>
<tr>
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<td>-------------------</td>
<td>--------------------------</td>
</tr>
<tr>
<td>6 hour</td>
<td>6 hour</td>
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<tr>
<td>-IL-6</td>
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<tr>
<td>+IL-6</td>
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<tr>
<td>24 hour</td>
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<tr>
<td>+IL-6</td>
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<td>+IL-6</td>
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Conclusion: The HEK cells are able to expel the plasmids within 2-3 passages. There is no apparent difference in expression levels of the new STAT3-GFP plasmids at the different time points (6 hour and 24 hours) or in response to stimulation by IL-6.
Sanger Sequencing

STAT3-GFP

EFS-GFP

TATA-GFP
Hypothesis

• We hypothesize that by using this STAT3 reporter, we will be able to accurately visualize activated STAT3 signaling in TAMs in vivo by monitoring GFP expression. This will allow us to characterize the expression profiles of these TAMs within the breast cancer tumor microenvironment. Ultimately, we anticipate that STAT3 signaling will be diminished in macrophages that are associated with the tumor cells.