INVESTIGATING DYNAMICS OF THE STAT3 AND C/EBPβ PATHWAYS UPON STIMULATION BY INFLAMMATORY CYTOKINES

A Senior Scholars Thesis

by

PENG CHENG

Submitted to the Office of Undergraduate Research
Texas A&M University
in partial fulfillment of the requirements for the designation as

UNDERGRADUATE RESEARCH SCHOLAR

April 2009

Major: Chemical Engineering

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ABSTRACT

Investigating Dynamics of the STAT3 and C/EBPβ Pathways upon Stimulation by Inflammatory Cytokines. (April 2009)

Peng Cheng
Department of Chemical Engineering
Texas A&M University

Research Advisor: Dr. Arul Jayaraman
Department of Chemical Engineering

The purpose of this project was to investigate the dynamics of the Jak-STAT3 (STAT3) and Erk-C/EBPβ (C/EBPβ) pathways upon stimulation with inflammatory cytokines such as Interleukin 6 (IL-6) and oncostatin M (OSM), which is also a member of the IL-6 family. Green fluorescent protein (GFP) reporter plasmids with STAT3 and C/EBPβ transcription factor binding sequences were transfected into human hepatocarcinoma (HepG2) cells. Upon stimulation with inflammatory cytokines, the transcription factors STAT3 and C/EBPβ were activated and lead to the expression of GFP. The intensity of the observed fluorescence was proportional to the activation of the transcription factors and could be non-invasively monitored using fluorescence microscopy. HepG2 cells with stably integrated reporter plasmids responsive to STAT3 and C/EBPβ into their genome exhibited a high signal-to-noise ratio. Our data showed that the expression of GFP (and hence, the activation of transcription factors) correlated with the specificity of

the cytokine used. Future work will focus on investigating (i) dynamics of STAT3 and C/EBPβ clones in response to different concentrations and durations of OSM stimulation and (ii) dynamics of STAT3 and C/EBPβ clones upon exposure to additional cytokines such as IL-10 and IL-1 that are likely to be present along with IL-6.

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The graduate students, Colby Moya and Zuyi Huang, as well as my co-workers, have made great contributions to my research and the research paper. Also, the guidance of my research advisor, Dr. Arul Jayaraman is greatly appreciated. This project was sponsored by the National Science Foundation and the Dwight Look College of Engineering, Texas A&M University.

NOMENCLATURE

GFP	Green Fluorescence Protein
Hr	Hour
OSM	Oncostatin M
TF	Transcription Factor

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CHAPTER I

INTRODUCTION

Cytokines, such as interleukin-6 (IL-6), are important in the onset and sustenance of the acute phase inflammatory response of the liver upon injury or inflammation [1]. Binding of IL-6 to its cell surface receptor (glycoprotein 130, gp130) leads to a series of intracellular molecular events that are collectively known as signal transduction. The end product of this signal transduction is the activation of transcription factors (TF). These events are instrumental in regulating the expression of target genes involved in the acute phase response. IL-6 signal transduction proceeds mainly through two pathways –Jak-STAT3 and Erk- C/EBPβ [2, 3]. Examples of IL-6 regulated genes include plasma acute phase proteins (APPs) that are upregulated (e.g., C-reactive protein, haptoglobin, and serum amyloid A) as well as down-regulated (e.g., albumin and transferrin) [1]. Changes in the expression of these proteins are often used as indicators of the inflammatory response and its complications. In particular, the ratio of increase in C-reactive protein to decrease in albumin has been correlated to mortality of systematic inflammation [4]. Therefore, understanding the molecular mechanisms underlying the regulation of acute phase proteins could lead to novel approaches for the treatment of inflammatory diseases.

This thesis follows the style of *BMC Systems Biology*.

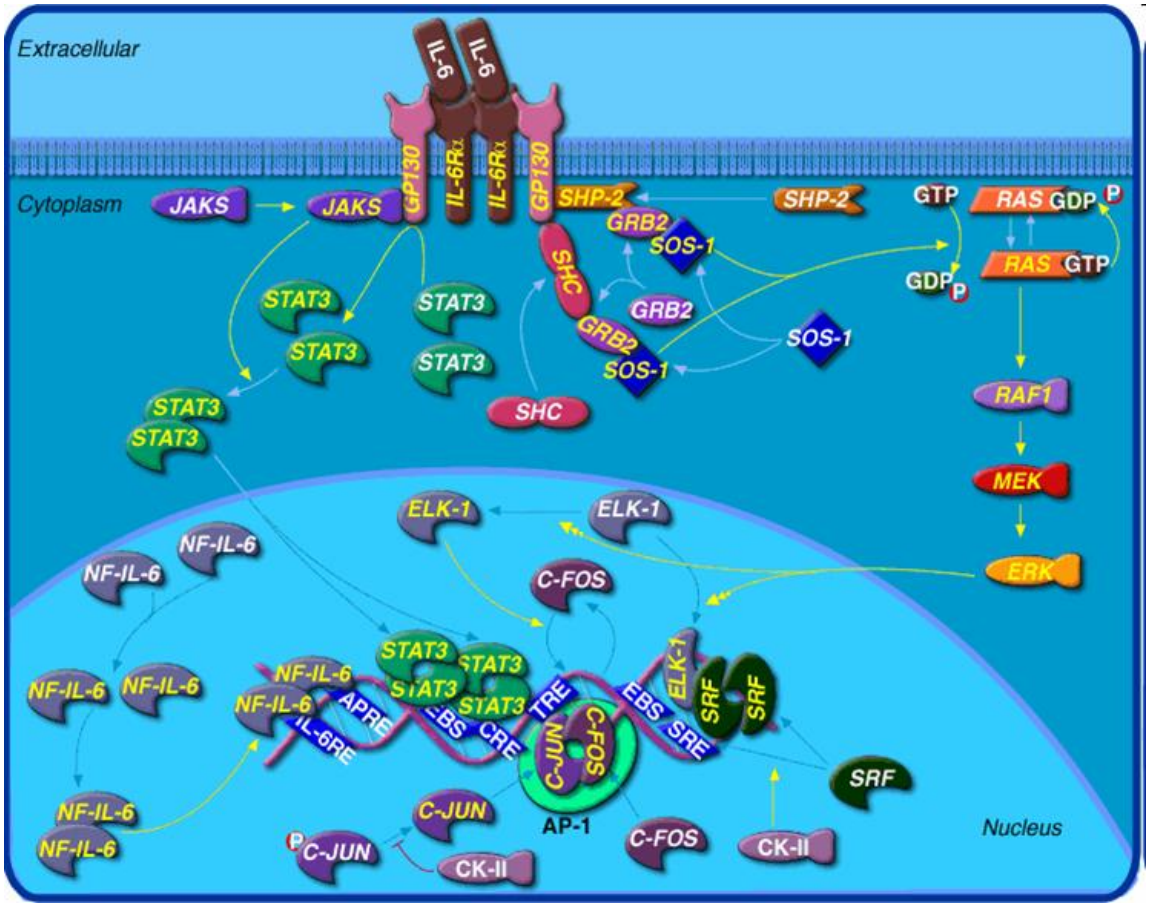

Figure 1. Schematic of IL-6 signal transduction pathways [5].

A detailed description of the STAT3 and C/EBPβ pathways is beyond the scope of this paper. Briefly, signaling through the STAT3 pathway upon IL-6 binding to gp130 leads to activation of the kinase JAK, which in turn activates (i.e., phosphorylates) the transcription factor STAT3 [1]. Activated STAT3 dimerizes and translocates into the nucleus to regulate gene expression. Given its important role in IL-6 signaling, STAT3 is chosen as representative molecule for monitoring activation through the Jak-STAT3 pathway. The Erk-C/EBPβ pathway is initiated by binding of an adaptor protein SHP-2 to a protein complex involving IL-6 and different receptors [1]. SHP-2 binding leads to

activation of kinases which eventually lead to activation of the transcription factor C/EBPβ (also known as NF-IL-6). Therefore, C/EBPβ is chosen as the indicator for the Erk-C/EBPβ pathway. Both pathways have negative feedback which serves to regulate activation of the two pathways. SOCS3, which is a protein up-regulated by STAT3 itself, acts as an inhibitor of signal transduction through the Jak-STAT3 pathway [2, 3, 6]. In addition, SOCS3 has also been speculated to impact signal transduction through the Erk-C/EBPβ pathway [7, 8]. Figure 1 is a brief demonstration of the mechanisms of the STAT3 and C/EBPβ pathways.

CHAPTER II

METHODS

Principle of GFP reporter system

As shown in Figure 2, a low half-life (2hr) GFP was cloned into a plasmid under the control of a minimal promoter (CMV_{min}). When a TF bound to its upstream recognition sequence, the promoter was activated and GFP was expressed.

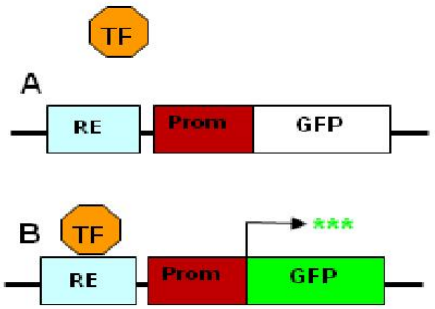

Figure 2. Principle of GFP reporter systems.

Reporter cell line generation

Reporter plasmids for monitoring activation of STAT3 and C/EBPβ were introduced into HepG2 cells by electroporation. Approximately 5 x 10^6 cells were mixed with 10 µg of linearized plasmid (pSTAT3-d2egfp or pC/EBPβ-d2egfp) in a pre-chilled sterile 4-mm electroporation cuvette and electroporated at 240V and 950 µF. Growth media (600 µL) was immediately added to the cells after electropration and allowed to sit for 10 min at room temperature. The mixture was transferred to a 10 cm tissue culture dish and

incubated for 2 days prior to addition of 800 μg/mL of G418 as selection pressure. Surviving colonies (i.e., cells with the STAT3 or C/EBPβ reporter plasmid integrated into their genome) were selected and screened for induction of STAT3 or C/EBPβ activity after 3 weeks.

Fluorescence microscopy

HepG2 reporter cell lines for STAT3 and C/EBPβ were seeded in 24-well plates and exposed to 100 ng/mL of the cytokines IL-6 or OSM, respectively. The cell culture plate was placed on the incubated stage of a Zeiss Axiovert 200M fluorescence microscope where constant temperature and CO_2 levels were maintained. Transmitted light and fluorescence images were taken every 45 minutes for 21 hrs. Fluorescence images were analyzed by MATLAB [9] to obtain the dynamic profiles of STAT3 and C/EBPβ activation and then compared with a model derived from the TF calculation. The scheme to calculate the TF data from the GFP data is listed below (Figure 3).

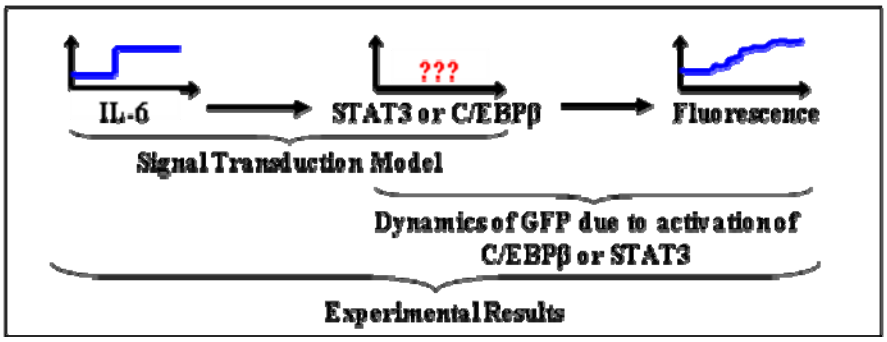

Figure 3. Scheme to calculate TF data from GFP data. Relationship between input, output, and concentration of transcription factors with GFP-reporter systems.

CHAPTER III

RESULTS

HepG2 reporter cells were cultured as described in Methods and exposed to different inflammatory cytokines. Figure 4 is representative of the HepG2 STAT3 cell line. As seen in Figure 4, the HepG2 STAT3 cell line exhibited fluorescence even without stimulation (baseline fluorescence); however, the level of GFP fluorescence remained constant throughout the duration of the experiment (~ 22 hr). The fluorescence intensity of the HepG2 STAT3 cell line stimulated with 100 ng/mL of IL-6 increased after 9 hr. A similar trend was observed when HepG2 STAT3 cells were treated with 100 ng/mL of OSM.

Figure 5 shows the quantified fluorescence temporal profile of STAT3 clones treated with IL-6 (100 ng/mL) and OSM (100 ng/mL). The data show that fluorescence rapidly increases after 1 hr of exposure and reaches a maximum after 6 hr. Subsequently, the fluorescence levels decrease slightly and reach a steady state value beyond 12 hr of stimulation. A similar temporal profile was observed with OSM stimulation; however, the GFP levels remained constant after 8 hr without exhibiting any decrease. The maximum fluorescence obtained with OSM stimulation was also higher than that observed with IL-6 (~ 16-fold vs. 6-fold, respectively).

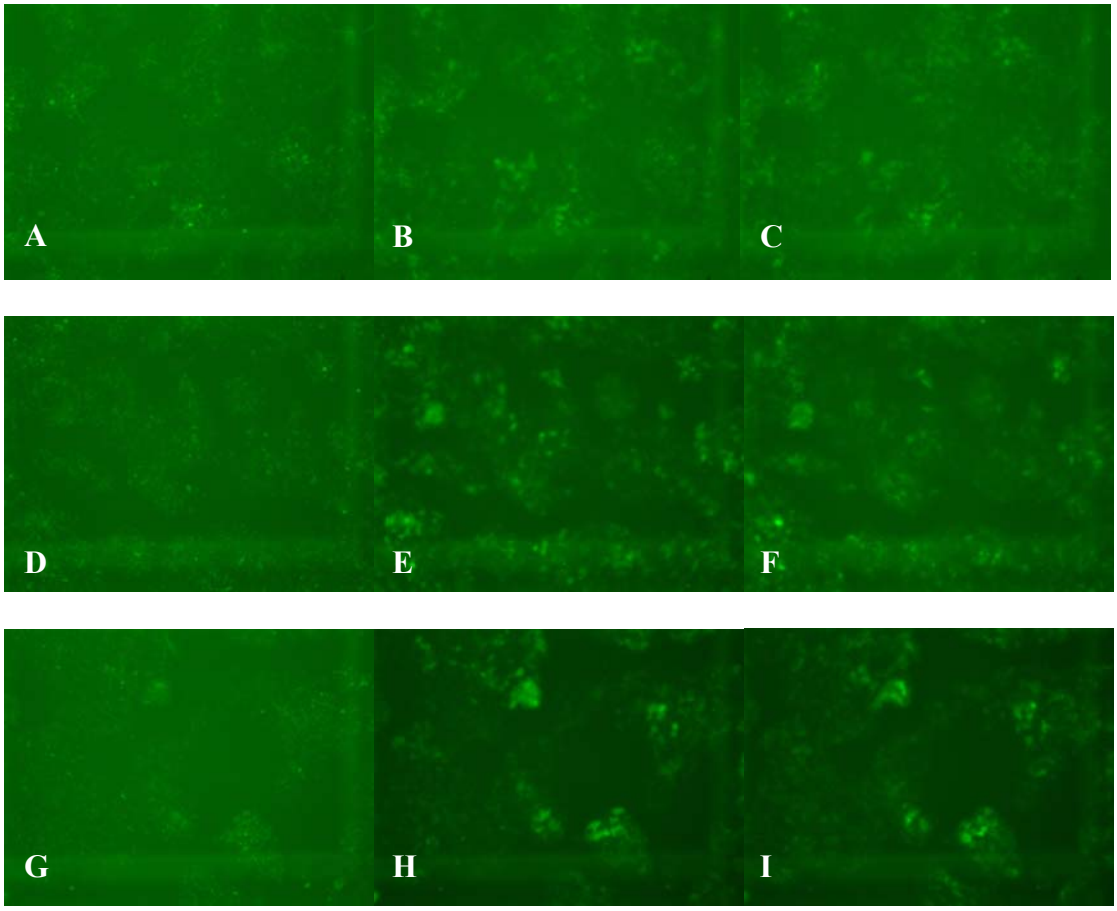

Figure 4. Dynamics of fluorescence intensity with HepG2 STAT3 clone. Images A through I show the dynamics of fluorescence intensity with HepG2 cells stably integrated with the STAT3 reporter plasmid for 21.75 hrs. Image A: control at 0 hr; Image B: control at 9 hr; Image C: control at 21.75 hr; Image D: IL-6(100 ng/mL) at 0 hr; Image E: IL-6 (100 ng/mL) at 9 hr; Image F: IL-6 (100 ng/mL) at 21.75 hr; Image G: OSM (100 ng/mL) at 0 hr; Image H: OSM (100 ng/mL) at 9 hr; Image I: OSM (100 ng/mL) at 21.75 hr.

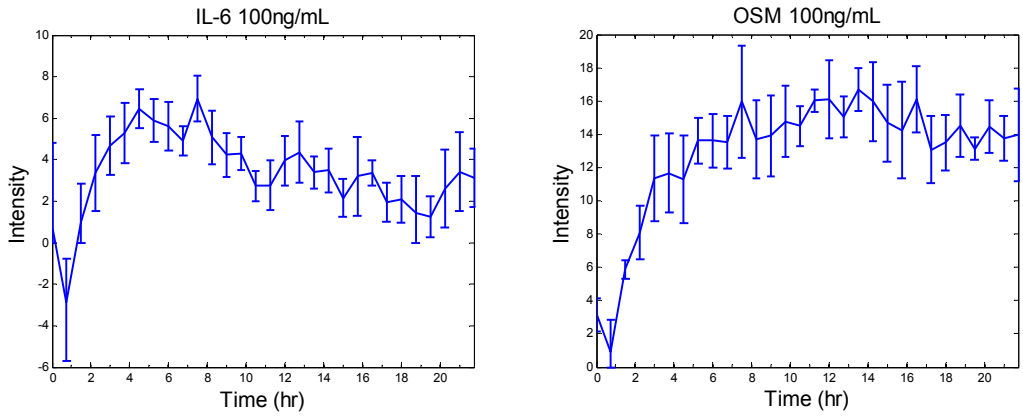

Figure 5. Dynamics of STAT3 pathway. The dynamics of fluorescence intensity with HepG2 cells stably integrated with the STAT3 reporter plasmid and exposed to IL-6 (100 ng/mL) or OSM (100 ng/mL) for 21.75 h. Left: IL-6 (100 ng/mL); right: OSM (100 ng/mL). Data shown are the average of 4 experiments.

As shown in Figures 6 and 7, the HepG2 C/EBPβ cell line also exhibited baseline GFP expression and its fluorescence levels also remained relatively constant throughout the experiment. The fluorescence intensity of HepG2 C/EBPβ reporter cells treated with 100 ng/mL of OSM increased in a manner similar to that seen with the STAT3 reporter cell line. However, the maximum fluorescence observed with the C/EBPβ reporter cell line upon OSM exposure (~8-fold) was less than that observed with the STAT3 reporter cell line (~16-fold).

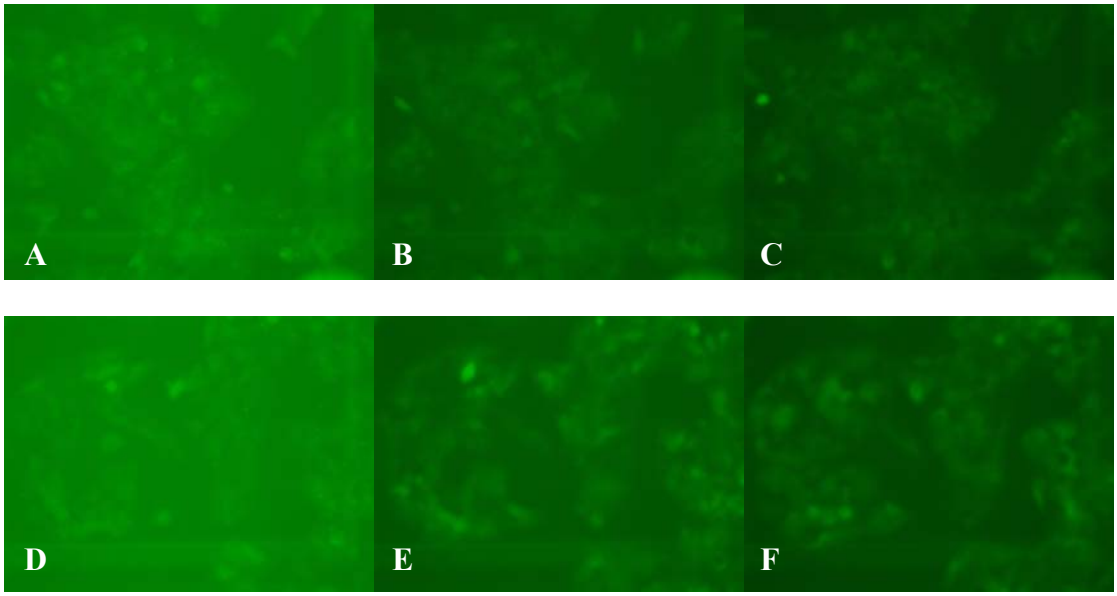

Figure 6. Dynamics of fluorescence intensity with a HepG2 C/EBPβ clone. Images A through F show the dynamics of fluorescence intensity with HepG2 cells stably integrated with the C/EBPβ reporter plasmid for 21.75 hrs. Image A: control at 0 hr; Image B: control at 9 hr; Image C: control at 21.75 hr; Image D: OSM (100 ng/mL) at 0 hr; Image E: OSM (100 ng/mL) at 9 hr; Image F: OSM (100 ng/mL) at 21.75 hr.

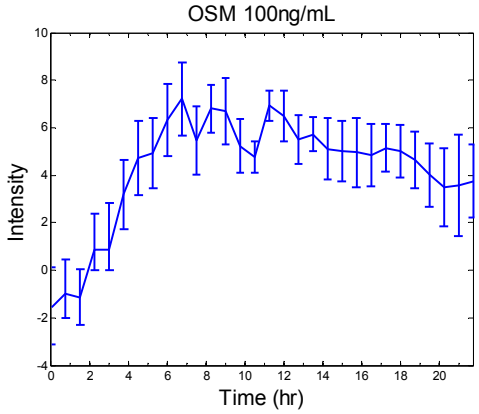

Figure 7. Dynamic of C/EBPβ pathway. The dynamics of fluorescence intensity with HepG2 cells stably integrated with the CEBP reporter plasmid and exposed to OSM (100 ng/mL). Data shown are the average of 4 experiments.

The fluorescence intensity obtained with the HepG2 STAT3 and C/EBPβ cell lines were used to determine the validity of a previously developed IL-6 signal transduction model. The model predicted profiles of STAT3 and C/EBPβ were used to simulate the temporal profile of GFP production under control of an inducible-expression system. The simulated profiles were compared to the profiles obtained from the experiments. As shown in Figure 8, our data show excellent agreement between the experimental data and model predictions, and demonstrate the validity of the previously developed IL-6 signal transduction model.

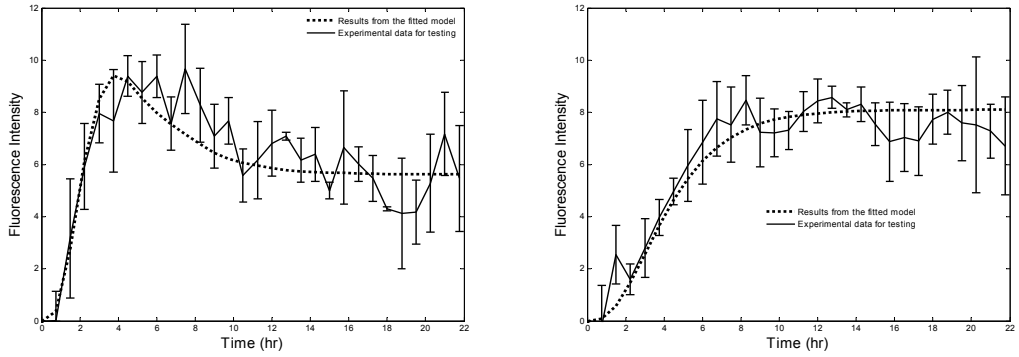

Figure 8. Comparison of model predictions and experimental data. Solid line is the experimental data and dash line is the model prediction. Left: STAT3; right: C/EBPβ.

CHAPTER IV

CONCLUSIONS

The result of the fluorescence microscopy showed that both the HepG2 STAT3 and C/EBPβ cell lines expressed GFP in response to the activation of IL-6 and/ or OSM through the Jak-STAT3 or Erk-C/EBPβ pathways, respectively. The expression of GFP (and hence, the activation of transcription factors) correlated with the specificity of the cytokine used. The dynamic profiles of the STAT3 and C/EBPβ activations obtained from the fluorescence images were in excellent agreement with the model derived from the TF calculation.

Future work will focus on investigating (i) dynamics of the STAT3 and C/EBPβ pathways in response to different concentrations and durations of OSM stimulation and (ii) dynamics of STAT3 and C/EBPβ upon exposure to additional cytokines such as IL-10 and IL-1 that are likely to be present along with IL-6.

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CONTACT INFORMATION

Name:	Peng Cheng
Professional Address:	c/o: Arul Jayaraman, Department of Chemical Engineering, Texas A&M University, College Station, TX 77843-3122
Email Address:	pengcheng@neo.tamu.edu
Education:	B.S., Chemical Engineering, Texas A&M University, August 2010 Undergraduate Research Scholar