LIVE-CELL CHARACTERIZATION OF THE JNK SIGNALING CASCADE

by
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Abstract

Signaling cascades process extracellular information about environmental changes into spatiotemporally regulated signaling events, through a series of signal relays as well as feedback loops and crosstalk mechanisms. The JNK signaling cascade is a major mediator of vital cellular decision making processes, including cellular differentiation and inflammatory response. The cascade transduces a variety of extracellular signals into these functional responses, but its response to TNF, an important cytokine in immune system communications, has not been well characterized, especially at the single mammalian cell level. To characterize JNK quantitatively in single living cells, we used a FRET-based JNK activity reporter to visualize JNK activity in HEK cells. We found that JNK produces a graded response to varying concentrations of TNF, suggesting that JNK relays a graded input signal to its downstream components, which is subsequently converted into a digital response for executing important cellular decisions such as programmed cell death. Using the reporter targeted to the plasma membrane, we also discovered that JNK activation kinetics is slower at the plasma membrane compared to the cytosol, suggesting that JNK activities are regulated spatially. Moreover, JNK in HEK cells exhibited increased sensitivity to TNF when prestimulated with a low dose of the stimulus.

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CHAPTER 1: INTRODUCTION

1.1 Signaling Pathways

Our bodies are constantly receiving, interpreting, and responding to external signals such as sounds and odors, and similarly, cells receive countless external cues with information about environmental changes, which are carried to different subcellular compartments through a series of intracellular signaling pathways. Signaling cascades process the information through a series of feedback loops, crosstalk mechanisms, and delays, which ultimately allows the cell to make important decisions. For instance, in response to external cues such as inflammatory cytokines, the information carried by these inflammatory cues gets processed to make the ultimate decision of whether the cell will undergo apoptosis. Recent human genome sequencing efforts have identified that approximately half of the twenty-five largest protein families encoded by the human genome primarily function in information processing and signal transduction [1]. Evidently, signal transduction is a widely relevant topic due to its overarching involvement in virtually all cellular processes, and its further understanding may eventually lead to the development of novel therapeutics against different diseases.

Protein kinases play a major role in regulating signal transduction through phosphorylation of downstream targets, and ultimately cascading the signal from the extracellular environment to the targeted subcellular location. Specifically, the kinase in a tiered cascade modifies its substrates by chemically adding a phosphate group, thus producing a functional change in the substrate.

1.2 c-Jun N-terminal kinase (JNK) Signaling Cascade

A major player of signal transduction, and more specifically, a critical component of the cell's apoptosis decision-making mechanism, is the c-Jun N-terminal kinase (JNK)

signaling cascade. JNK was identified in 1993 as an activating kinase of the c-Jun transcription factor, which spurred further characterization of the cascade. The JNK cascade is heavily involved in a variety of cellular events, including cell differentiation and cell inflammatory response due to its pivotal role in controlling apoptosis, making it an essential player in cellular decision making. JNKs have often been referred to as stress-activated protein kinases, as they respond to stress-inducing stimuli, including cytokines, UV radiation, ischemia, and growth factor deprivation [2].

1.3 JNK Isoforms

JNK consists of ten different isoforms generated through alternative splicing and derived from the three genes, JNK1, JNK2, and JNK3. JNK1 and JNK2 are ubiquitously expressed throughout the body, while JNK3 is tissue specific and is primarily present in the brain [3]. Knockouts of various combinations of the three JNK genes further elucidate specific functions of each gene and the importance of JNK to the cell. JNK1 knockout mice display decreased differentiation of helper T cells [4]. JNK1 has also been shown to play a major role in obesity and insulin resistance, as the absence of JNK1 resulted in increased resistance to high fat diet induced obesity and increased adiposity [5]. JNK2 knockout mice also show deficiencies in helper T cell differentiation and have also been implicated to play a protective role for mice with Type I diabetes [4]. JNK1 and JNK2 play similar roles in T cell differentiation, but only JNK1 has been implicated in obesity and insulin sensitivity. Lastly, JNK3 plays a role in neuronal cell death and brain development [5]. Kainate, which activates JNK in neuronal cultures, was used to induce apoptosis in JNK3 knockout mice, demonstrating that JNK3 knockout mice had decreased kainate-induced damage and higher survival rates compared to normal mice

[5]. Overall, although the three JNK isoforms have similar homology, they perform different, but very vital functions for the cell.

Moreover, JNK double knockout studies demonstrated compensatory roles of the JNK isoforms. JNK1/3 and JNK2/3 knockouts were viable and functional, but JNK1/2 were embryonically lethal and had dysregulation of apoptosis in the brain. Thus, JNK1 and JNK2 plays a major role in the development of the fetal brain by controlling apoptosis [5].

1.4 JNK Regulation

JNK is a subset of mitogen-activated protein kinases (MAPK), which are activated through cytokines and various stress stimuli through a three-tiered phosphorylation cascade (Figure 1-1). A stimulus instigates the activation of the upstream regulator, MAPKKK. Of the 20 MAPKKKs identified, 14 have been shown to activate JNK. The MAPKKK then proceeds to activate a MAPKK isoform through phosphorylation. For JNK, only two MAPKK isoforms, MAP kinase kinase 4 (MKK4) and MAPK kinase kinase 7 (MKK7), have been reported. Subsequently, these MAPKKs activate JNK by phosphorylating the Thr and Tyr residues of the TXY motif of the JNK activation loop. Upon activation, JNK translocates to the nucleus and modulates the activity of a variety of proteins, such as activating transcription factors c-Jun and activating transcription factor 2 (ATF2) or inhibiting signal transducer and activator of transcription 3 (STAT3). JNK's ability to activate or inhibit a myriad of transcription factors allows JNK to regulate important cellular functions.

1.5 JNK Activation

The JNK cascade can be activated by several different stimuli, such as growth

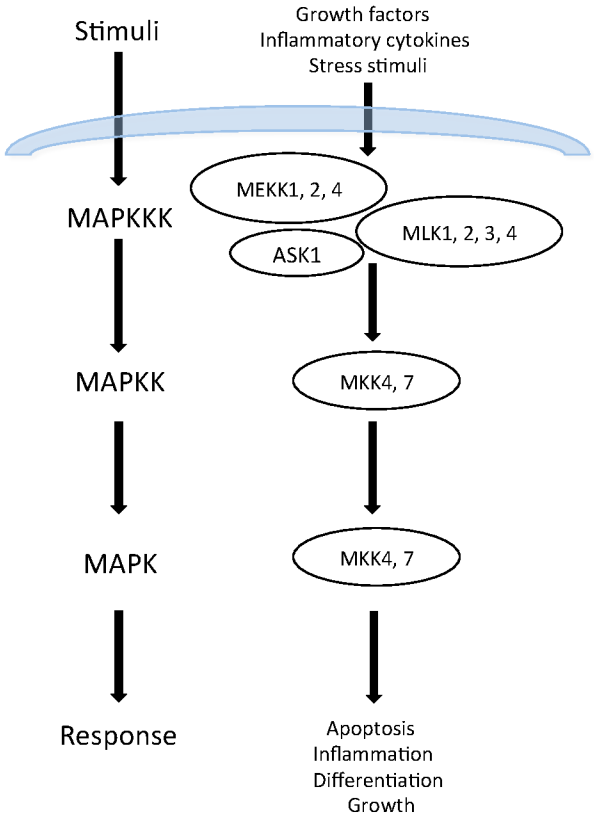

Figure 1-1. Schematic of JNK Signaling Components

factors, cytokines, and stress factors. Each of the many MAPKKKs is responsible for activation through different stimuli. For instance, the MAPKKK TAK1 is critical for activation in response to inflammatory cytokines, such as tumor necrosis factor, Toll-like receptors, B-cell receptor, and T-cell receptor [6-8].

1.5.1 Tumor Necrosis Factor α (TNF) Signaling

TNF is a pleiotropic cytokine and a major mediator of apoptosis. Its dysregulation has been implicated in a variety of diseases, including Alzheimer's and various cancers. TNF primarily regulates the cell's immune response, but is involved in a variety of cellular functions and affects most of the organs in the body. TNF is produced by macrophages, fibroblasts, and lymphocytes in response to inflammation, and is often considered to be an anti-cancer agent. As a key intermediary in the cell's inflammatory response, TNF has been implicated in cell apoptosis and its pathological activity and involvement in many diseases, including diabetes, cancer, multiple sclerosis and sepsis, has attracted much interest. TNF in the cell produces varying affects. For instance, high levels of TNF have been correlated with increased mortality rates [9], and mice without TNF experience septic shock when infected with gram negative bacteria. As a result of TNF's pathological involvement, there is substantial interest in using TNF as a therapy against various cancers.

TNF signaling begins when TNF binds to its cell surface receptor, either TNF receptor type 1 (TNFR1, p55) or TNF receptor type 2 (TNFR2, p75). TNFR1, expressed by most cells, is thought to be the main mediator of TNF cytotoxicity. TNFR1 can be fully activated through the membrane bound and soluble forms of TNF. TNFR2 is only found in immune system cells and only responds to the membrane bound form of TNF.

After TNF binds to TNFR1, the signaling cascade begins through the recruitment of signal transducers that then go on to activate downstream effectors, ultimately resulting in the activation of downstream targets, including two major transcription factors, c-Jun and nuclear factor-kB. When TNF binds to the extracellular domain of TNFR1, the receptor forms a trimer and this conformational change in the receptor causes the inhibitory protein, Silencer of Death Domains (SODD), to dissociate from the receptor, which subsequently allows TNF Receptor-Associated Death Domain (TRADD) to bind to the intracellular death domain. TRADD then recruits three other adaptor proteins, Receptor-Interacting Protein (RIP), TNFR Associated Factor 2 (TRAF2), and Fas-Associated Death Domain (FADD). Subsequently, these three adaptor proteins recruit various enzymes that are responsible for initiating signaling cascades. Specifically, TRAF2 activates a MAPKKK, which then activates JNK cascade, resulting in the phosphorylation and increased activity of c-Jun. TNF produces the strongest activation of JNK, among the three major MAPK cascades of JNK, p38, and ERK.

Although the JNK pathway has been extensively studied, the JNK response to TNF, an important cytokine in immune system communications, has not been well characterized, especially at the single mammalian cell level. Understanding this response can help uncover the role of TNF and JNK in cell death and the mechanisms behind it.

1.6 Downstream JNK Targets

Once JNK is activated through the cascade described above, it can proceed to phosphorylate and activate multiple downstream targets. Many of JNK's nuclear substrates are transcription factors, allowing JNK to directly mediate cellular decision making through phosphorylation of these targets. In addition, the non-nuclear substrates

allow JNK to control other salient cellular behaviors, such as cellular movement and programmed cell death. JNK was first identified as an activator of transcription factor c-Jun and is currently the only MAPK known to phosphorylate the N-terminal end of c-Jun. c-Jun has been heavily associated with cancer development, as it was found to be overexpressed in 31% of primary and metastatic lung tumors [10]. Other studies of hepatocellular carcinomas have also indicated that c-Jun is required for early stage tumor development, whereas c-Jun deletion suppresses tumor formation [11].

1.7 JNK and Cancer

The JNK pathway plays a multifaceted role in tumorigenesis, as it has been suggested to be both tumor suppressing as well as tumorigenic. First, there is substantial evidence that JNK functions as a proapoptotic kinase, promoting tumor suppression [12]. Multiple tumor cell lines possess constitutively active JNK [13], and several JNK family kinases have been shown to be involved in the biological process of regulating apoptosis in response to cellular injury [14]. JNK1/2 knockouts in mouse embryonic fibroblasts (MEFs) were resistant to apoptosis induced by UV irradiation, DNA-alkylating agent methyl methanesulfate, and translation inhibitor anisomycin [15] . This study suggested that the phosphorylation of c-Jun by JNK1 and JNK2 is required for UV-induced apoptosis. Moreover, JNK3 knockout mice were resistant to glutamate-induced apoptosis of hippocampal neurons, suggesting the major role of JNK3's phosphorylation of c-Jun on excitotoxic cell death [15] . In all, all three JNK isoforms can play a pro-apoptotic role, but this role is often affected and determined by other cellular factors, including the parallel activation of anti-apoptotic pathways. Although many studies have identified JNK as a pro-apoptotic gene, JNK is not solely a pro-apoptotic kinase, as studies also

suggest JNK's involvement in anti-apoptosis and cell survival. For instance, mice fibroblasts with a mutated c-Jun allele that lacks a JNK phosphorylation site is resistant to transformation induced by Ras, an oncogene that is mutationally activated in approximately 30% of human cancers, and Fos [13]. Furthermore, a mouse model of intestinal cancer with the c-Jun gene ablated, showed reduced tumor size and number and a prolonged lifespan. Furthermore, the hindbrain and forebrain regions of JNK1/2 knockout mice showed enhanced apoptosis at E10.5 [16, 17], suggesting JNK's involvement in cell survival of those regions of the brain during development. The anti-apoptotic involvement may also result in enhancement of tumorigenesis. When JNK activation is inhibited by anti-sense oligonucleotides, the growth of several tumor cells was also inhibited [18]. Not only JNK, but also its directly upstream pathway component, Mitogen-Activated Protein Kinase Kinase 4, has also been involved in several cancer studies, and demonstrated a similarly complex role [6, 19-24]. Overall, JNK and its pathway components have been heavily implicated in cancer, but it's role and mechanism in apoptosis and tumorigenesis is unclear.

These findings do not demonstrate whether JNK is an intrinsic component of the cell's apoptosis machinery, or whether it solely modulates the process [12]. Studying the molecular mechanism of JNK in regulating and modulating apoptosis will allow clearer understanding of JNKs role in apoptosis and cellular decision making. Several drugs that target JNK have been developed and have shown to be effective at protecting mice from Type 2 diabetes and tissue injury from ischemic disease. Through developing a deeper understanding of the JNK pathway mechanism, JNK may be used towards the development of novel targeted therapies for the prevention or treatment of human

diseases due to JNKs widespread role in programmed cell death and cancer.

1.8 Spatial Compartmentalization of Signaling Proteins

1.8.1 Introduction

Protein kinases, including JNKs, are often referred to as the intermediate nodes that connect upstream stimuli to produce downstream cellular responses. The mechanism by which the kinase processes the signal information results in vital cellular decision making downstream, such as whether to undergo apoptosis or to induce gene expression. Since these kinases are often responsible for diverse functional outputs, modulating various signaling events often requires tight spatiotemporal control of kinase activities to elicit specific functional outputs. For instance, in many signaling cascades, scaffolding proteins are heavily involved in signal transduction process, often by enhancing the specificity of the relay process and increasing the signal efficiency by concentrating the signaling components locally. Thus, to accurately determine dynamic signaling behavior, the cascade must be analyzed with all its cellular regulatory components intact. Specifically, for example, the Western blot technique is commonly used to analyze the activity and presence of protein kinases subject to various stimuli, but this approach only provides snapshots of averaged signal from lysed cells and cannot capture dynamic kinase behavior in live cells. As a result, there is substantial need for an approach to study protein kinases with all of the cell's regulatory mechanisms intact, and without altering the spatial compartmentalization of the kinases or its mechanism of action within the cell. In addition, the protein kinetics should also be investigated at the single cell level. Population level dynamics often average out important signal transduction events, such as oscillatory activities or bistable responses.

1.8.2 Novel Method to Study the Spatiotemporal Regulation of Signaling Proteins: Fluorescence Resonance Energy Transfer (FRET)-Based Biosensors

Although several fluorescent biosensors have been created for various signal transduction events, such as changes in membrane potential [25], up until recently, it has been difficult to obtain quantitative live cell data for the activity of protein kinases. An innovative method to examine the single cell, quantitative dynamics of protein kinases with high spatial and temporal resolution is based on fluorescence resonance energy transfer (FRET). FRET is the transfer of energy from an excited donor fluorophore to an acceptor fluorophore. Due to recent significant progress in improving fluorescent proteins and the increased availability of fluorescent protein color variants, fluorescent protein FRET pairs can now be coupled with different sensing modules to create FRET-based reporters that can sense dynamic activity changes in single, living cells.

The efficiency of the energy transfer is inversely proportional to the sixth power of the distance between the fluorophore pair, as FRET is extremely sensitive to the distance and orientation of the donor and acceptor fluorophores, and the energy transfer will only occur when the two fluorophores are 1-10 nm apart. The efficiency of FRET is also determined by spectral overlap of donor emission and acceptor excitation peaks, and acts as a sensitive measurement of molecular events such as protein-protein interactions, or changes in protein conformation. The fluorophore pair selected for FRET must also have separate excitation and emission in order to prevent signal bleedthrough and fluorophore cross excitation. The most commonly used FRET pair is yellow fluorescent protein (YFP) and cyan fluorescent protein (CFP) due to their high level of spectral overlap. Provided that the two fluorescent proteins are in close proximity, excitation of

the donor fluorophore, CFP, with a wavelength of 430 nm, will results in the emission of the acceptor fluorophore, YFP, at a wavelength of 515 nm, due to energy transfer.

The general design of genetically encoded kinase activity reporters is essentially a kinase activity-dependent molecular switch sandwiched between two fluorescent proteins that can undergo FRET (Figure 1-2). The switch consists of a substrate domain, and a phosphoaminoacid binding domain. Biosensors that report on kinase activity are constructed based on three major concepts. First, the substrate domain must be specific to the kinase to be studied. Second, the phosphoaminoacid binding domain must be compatible with the substrate sequences, and may need to be modified with point mutations to fit the requirements. Third, sufficient linker regions between each of the components must be present, and can also be optimized to enhance the dynamic range and stability of the reporter [26]. Upon satisfying these conditions, when a specific kinase of interest phosphorylates the substrate domain, the phosphoaminoacid binding domain can bind the phosphorylated substrate, changing the distance and/ or orientation of the two fluorophores from the FRET pair, resulting in a FRET change. The FRET based reporter can easily be introduced into living cells and can even be fused with localization signals to target a specific, subcellular location.

1.8.3 JNK Activity Reporter 2 (JNKAR2) Probe

JNK Activity Reporter 1 (JNKAR1), a live cell JNK activity reporter, has been created using these criteria [27]. In the design of JNKAR, the docking domain from a known JNK substrate JDP2 recruits JNK, which phosphorylates the substrate domain, also a sequence from JDP2, which causes the phosphoaminoacid binding domain to intramolecularly bind the phosphorylated JDP2 domain, resulting in the conformational

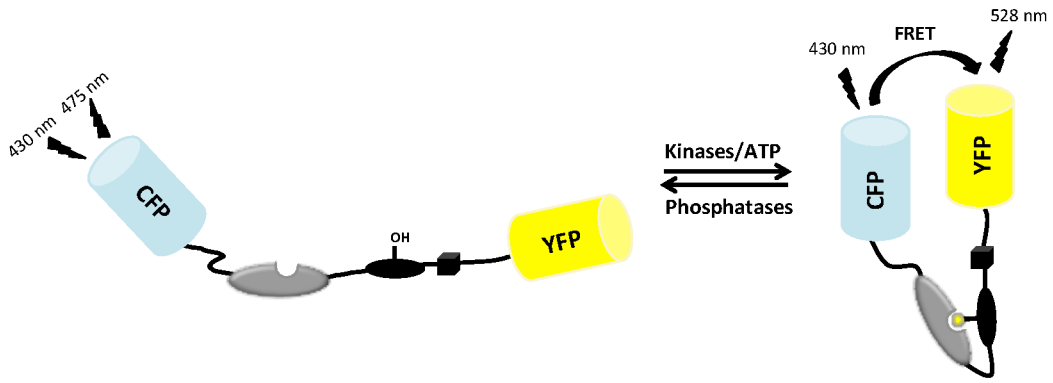

Figure 1-2. FRET-based biosensor general schematic

Excitation of the CFP fluorophore using a wavelength of 430 nm will result in emission at a wavelength of 475 nm. If the two fluorophores are in close proximity, such as after phosphorylation by a kinase, excitation of CFP will lead to emission of the acceptor fluorophore, YFP, at a wavelength of 515 nm. Subsequently, this energy transfer ultimately results in an emission wavelength of 528 nm due to energy transfer.

change of the reporter. This subsequently causes a change in the FRET emission from the two sandwiching fluorescent proteins, eCFP (a CFP) and Citrine (a YFP), and this change in fluorescence can easily be detected with fluorescence microscopy of cells expressing the probe. JNKAR1 generated a relatively modest 20.0% FRET change upon stimulation with a ribotoxic drug, anisomycin. A larger dynamic range should increasd probe sensitivity. Therefore, an improved reporter with a higher dynamic range, JNKAR2 (JNK Activity Reporter 2), was generated. The FRET pair used in the design of the JNKAR2 probe is Venus, a monomeric YFP variant, and Cerulean, a more photostable and brighter variant of CFP (Figure 1-3A) [28]. JNKAR2 can also be targeted to track JNK activity at subcellular locations, such as the mitochondria, plasma membrane, and nucleus, by fusing the probe with specific targeting motifs. The plasma membrane targeted JNKAR2 construct is shown in Figure 1-3B.

Previous findings demonstrate that JNK responds to stimuli in an all or none manner in oocytes, but these studies were conducted at the population level, and single cell data especially in mammalian cells has not been attainable until now. HeLa cells expressing the JNKAR1 probe were treated with varying concentrations of anisomycin stimulus and the normalized emission ratio, which shows the JNK activity, suggest that JNK activation is all or none in HeLa cells with anisomycin stimulation [27]. The improved probe, JNKAR2, now allows us to study the spatiotemporal changes of JNK signaling in more physiological settings.

Figure 1-3. Domain structures of JNKAR2 reporter design.

(A) The domain structure for whole cell JNKAR2. The reporter used Cerulean and Venus, cpVE182, as the FRET pair, and the phosphoaminoacid binding domain was Forkhead-Associated 1. The substrate domain incorporated was a truncated version of Jun Dimerization Protein 2. **(B)** The domain structure for plasma membrane targeted JNKAR2.

CHAPTER 2: JNK RESPONSE

2.1 Introduction

As a cell receives signals from the external environment, it is able to process information and execute many vital cellular decisions. The cell essentially takes a continuous input signal, such as the concentration of a stimulus, and converts it into a discrete response. The cell's response varies based on the type and concentration of stimulus, but typical responses include the production of secondary messengers or activation of protein kinases, both of which further relay the signal to downstream targets, to ultimately make vital decisions such as whether to proliferate or undergo apoptosis. A major question today concerns where that "switch" occurs during signal processing, as modulation of signal transduction activity has proven to be a common initiation point for switch-like behavior [29, 30]. The JNK signaling cascade transmits a vast variety of extracellular information that results in important cellular responses. JNK's ability to differentiate between the various external stimuli and induce the appropriate response is vital; otherwise, cells will indiscriminately respond to different stimuli, preventing the correct execution of crucial cellular processes. Many of the details of the JNK pathway are unknown, such as whether the JNK cascade converts a continuous input signal into a discrete response. As a result, understanding whether the JNK cascade processes these inputs by producing graded or bistable responses may elucidate the role of JNK in apoptotic signaling. If the JNK cascade transmits graded information from upstream activators, then the apoptosis decision is likely made downstream of JNK, since apoptosis is an all-or-none event. In contrast, if the JNK cascade exhibits bistable character, then JNK may likely be converting a graded input

into a switch like response, filtering out noise and generating an ultrasensitive response, and relaying this all or none signal to downstream effectors.

Previous experiments to study JNK's response were conducted in *Xenopus* oocytes because of their large size, allowing the signal transduction events to be assessed, because oocytes can be both pathologically and physiologically activated, and because of the availability of quantitative techniques to analyze the signal transduction events of this system [31]. These studies investigated the JNK response to progesterone, a physiological stimulus, and sorbitol, a hyperosmolar pathological stress. Progesterone works through the Mos → MEK → p42 MAPK cascade and can bring about JNK activation, whereas sorbitol activates JNK without going through p42. In pools of oocytes stimulated with progesterone, the JNK activity was graded as a function of progesterone concentration, with a Hill coefficient of 1 [31]. However, a graded response at the population level can have two interpretations regarding the cells behavior at the individual level. The first being that the individual oocytes in the population all produce a graded response to the stimulus, and the second being that the individual oocytes produce bistable responses, but have different thresholds for activation, resulting in a graded response at the population level when all individual responses are averaged out. To uncover which explanation is accurate, the JNK response of individual oocytes was examined. Individual oocytes exhibited a bimodal distribution, with minimal oocytes exhibiting intermediate JNK activity [31]. These findings reiterate the importance of having a method to analyze signal processes at the single cell, quantitative level, as population based results are often not representative of behavior on the single cell level. Furthermore, JNK exhibits an all-or-none response to sorbitol at the individual as well as

population level, indicating that unlike the JNK response to progesterone, the response to sorbitol has minimal variability in the threshold to activation [31]. These results conclude that the all or none activation of JNK in oocytes is not solely limited to the p42 pathway, but is likely intrinsic to the JNK cascade in oocytes.

In general, all-or-none responses are the result of either an ultrasensitive signaling cascade, or a bistable signaling cascade. The latter exhibits hysteresis since the response depends on its history [32]. In the case of hysteresis, once the stimulus surpasses a threshold, the cascade will switch on and be maintained in the on state even when the stimulus falls well below the threshold concentration. In the oocyte study above, the researchers proposed that JNK in oocytes likely exhibited bistability with hysteresis. To determine this feature, the oocytes were stimulated with sorbitol, and then the stimulus was subsequently washed away, while continuing to observe the JNK response of the oocyte. The results illustrated that approximately 70% of the oocytes remained fully active for as long as 16 hours after removal of sorbitol [31]. The persistence of the JNK activity suggests the presence of hysteresis. Overall, these findings suggest that the JNK cascade of oocytes exhibits switch-like behavior, converting the graded input of stimulus concentration into a bistable, all-or-none response.

The major difference between a biological system with a bistable response and one with a graded response lies in the system design of how the system components are connected. For instance, the *lac* operon system can switch between a graded and a bistable state, simply depending on the presence or absence of the physiological inducer lactose [32]. In the presence of the physiological inducer lactose, the internal inducer allolactose is destroyed by the induced product, stopping the positive feedback loop and

converting the switch like output into a graded output. In all, studying the system design of signaling cascades helps explain the information processing mechanism of these pathways, and elucidates how they influence important cellular decision making mechanisms.

Current knowledge of the JNK response has mainly been on the population level, studied in oocytes, or investigated with non-physiological stimuli such as anisomycin in the JNKAR1 study. The development of the novel JNKAR2 probe enables the study of single cell, quantitative data of JNK activity in living cells, induced by physiological stimuli due to the probe's increased dynamic range. Therefore, by investigating the JNK response in mammalian cells, we aim to develop a clearer understanding of the physiological JNK signal transduction mechanism, which may ultimately provide insight into how and where a cell makes the decision to undergo proliferation or apoptosis.

2.2 Materials and Methods

Cell Culture

HEK cells were cultured in DMEM with 10% fetal bovine serum. Cells were transfected in 35mm dishes with Trans-IT 2020 transfection reagent (Mirus Bio) for untargeted JNKAR2 and transfected with Lipofectamine 2000 (Invitrogen) for plasma membrane targeted JNKAR2. All cells were imaged 36-48 hours after transfection.

Imaging

JNKAR2 expressing cells were serum starved in DMEM F12 with 0.1% fetal bovine serum 10-12 hours prior to imaging, and imaged in the same media. These cells were imaged in 8-well chamber slides (Lab-Tek) coated with fibronectin with a Zeiss

Axiovert 200M using a cascade CCD camera (Photometrics) and 40x oil immersed lens at 37C and 5% CO_2, controlled by SlideBook (Intelligent Imaging).

Plasma membrane targeted JNKAR2 expressing cells were serum starved in DMEM F12 with 0.1% fetal bovine serum 10-12 hours prior to imaging, and then washed with Hanks' Balanced Salt Solution (HBSS) buffer and also imaged in HBSS. Plasma membrane targeted JNKAR2 expressing cells were plated onto sterilized glass coverslips that were mounted in 35mm dishes. These cells were imaged with a Zeiss Axiovert microscope with a CCD camera (Roper Scientific), controlled by METAFLUOR software (Universal Imaging).

2.3 Results

2.3.1 JNK Dose Response

The JNKAR2 construct was first characterized in HEK cells transfected with the JNKAR2 probe and stimulated with 5 ng/mL of human TNF. These cells exhibited a 29.1 ± 12.6% (average ± STD, n = 24) increase in FRET to cyan emission ratio (Figure 2-1). This probe exhibits a 45.5% increase in dynamic range compared to the JNKAR1 probe [27], as a result of changing the fluorescent pair used in the reporter. Physiologically, JNK activity generally increases 25-fold within the first ten minutes of TNF stimulation [33, 34]. The probe begins to exhibit a rapid change in FRET to CFP emission ratio 12.25 ± 6.50 minutes (n=24) after 5.0 ng/mL stimulation, suggesting that the probe has a slight delay in showing JNK activity.

To examine the JNK dose response, quantitative single cell analysis was performed by stimulating HEK cells expressing the novel JNKAR2 reporter with varying

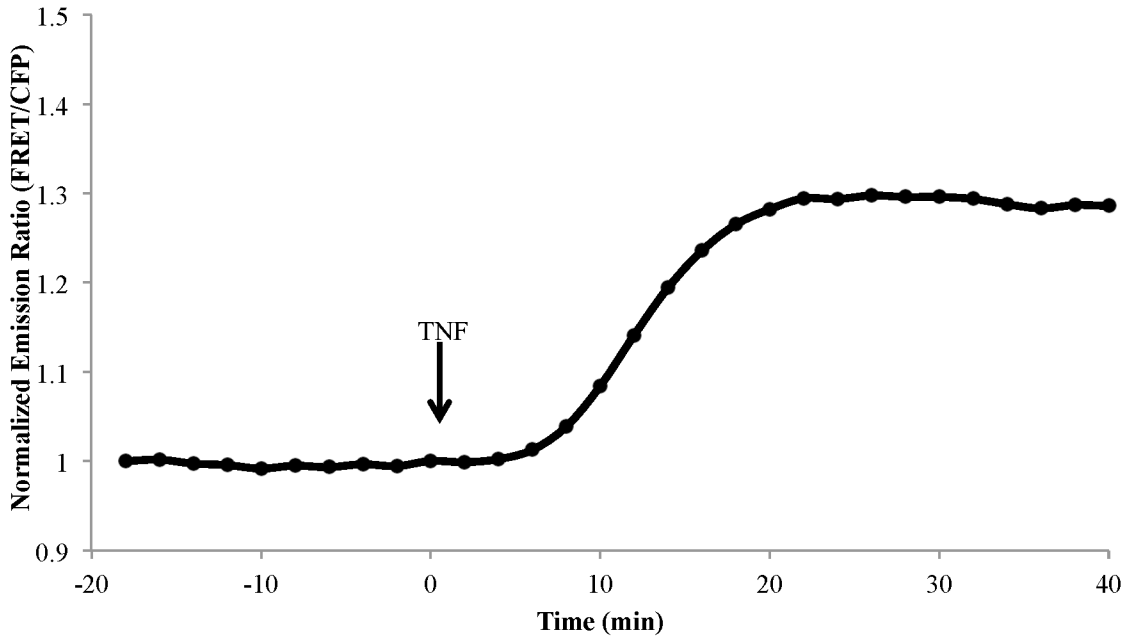

Figure 2-1. JNKAR2 characterization.

Representative normalized emission ratio time course of HEK cells expressing the JNKAR2 probe and stimulated with 5.0 ng/mL TNF.

concentrations of TNF, ranging from 0.005 to 5.0 ng/mL. At the highest dose of 5.0 ng/mL, 100% of cells fully responded to TNF stimulation. At the intermediate doses of 0.5 and 0.05 ng/mL, there was a wide cell-to-cell variation in emission responses, including maximal response, many intermediate responses, and no response. JNK activity in each cell varied and appeared to be highly dependent on the dose of TNF administered. At the lowest dose of 0.005 ng/mL of TNF, the majority of cells did not exhibit an increase JNK activity after exposure to stimulus. Representative curves from the four doses are displayed in Figure 2-2. Two distinct populations of responses were not present, indicating that the JNK response to TNF is not ultrasensitive. Specifically, the average response at the intermediate doses, 13.26% for 0.05 ng/mL TNF and 16.73% for 0.5 ng/mL TNF, cannot be defined as having the same response level as that of the lowest dose at 9.58% or that of the highest dose at 29.13% (Figure 2-2 B, C). The high standard deviation values when calculating the average percent FRET response for the intermediate doses in Figure 2-2C results from cell-to-cell variation in response strength, ranging from maximal response identical to those seen in the 5.0 ng/mL TNF stimulation to no response, similar to the representative curve for the 0.005 ng/mL stimulation.

The responses at each stimulus concentration can be grouped into three categories (Figure 2-3). The distribution of cells in these three categories varies based on the dose of TNF used. As the stimulus concentration increases, the percentage of cells exhibiting maximal response increases from 0 to 100%, whereas the percentage of cells with minimal or no response decreases from 58.8 to 0%.

Furthermore, the stimulus strength also impacts JNK activation kinetics. The JNK activity at each of the four doses exhibits different kinetics, represented by the time to

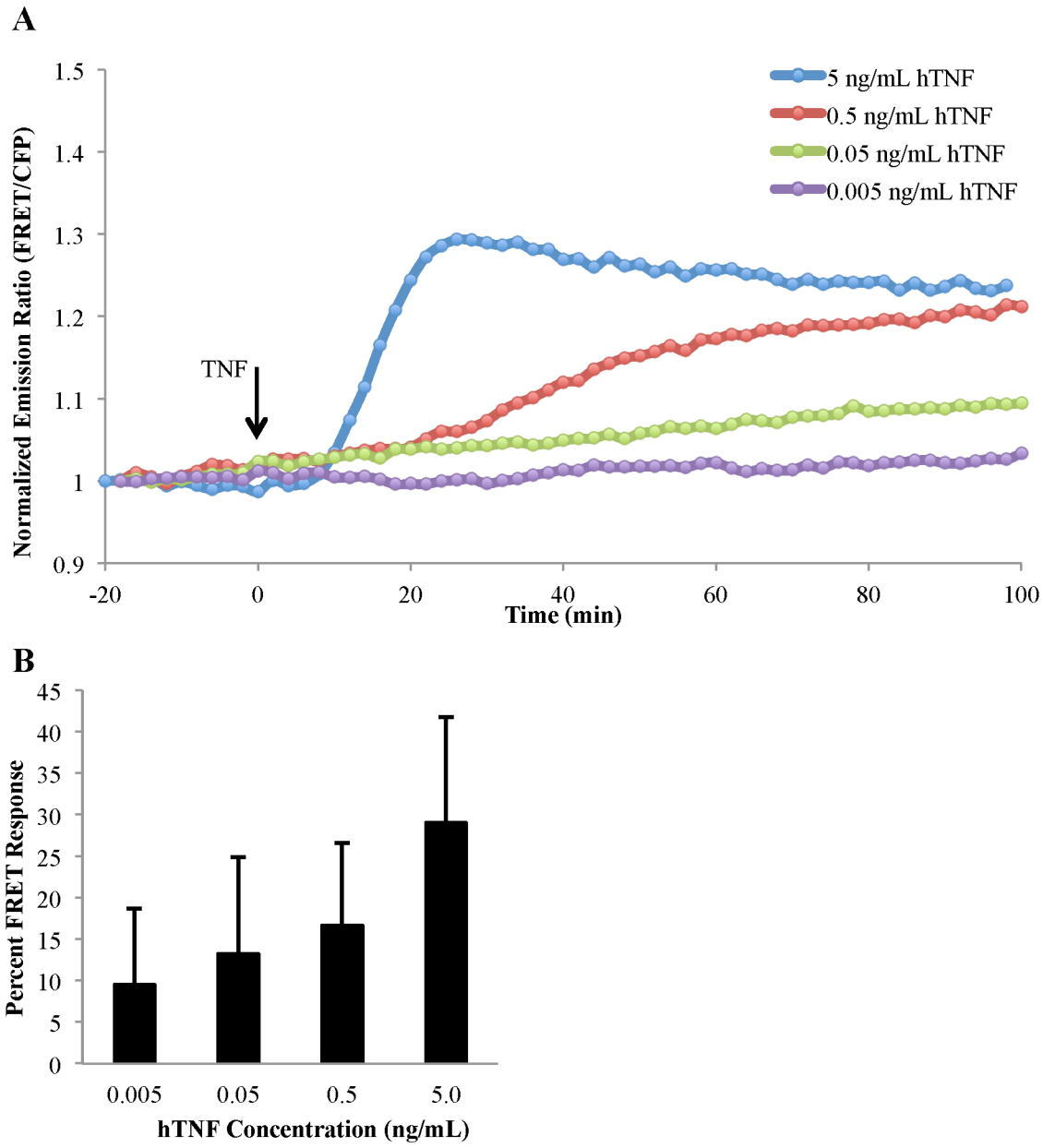

Figure 2-2. Graded JNK response to TNF stimulation

(A) Normalized emission ratio time courses from representative experiments of HEK cells treated with different TNF doses (5 ng/mL, 0.5 ng/mL, 0.05 ng/mL, and 0.005 ng/mL). **(B)** Average percentage change of yellow FRET to cyan emission ratio in response to four different doses of TNF.

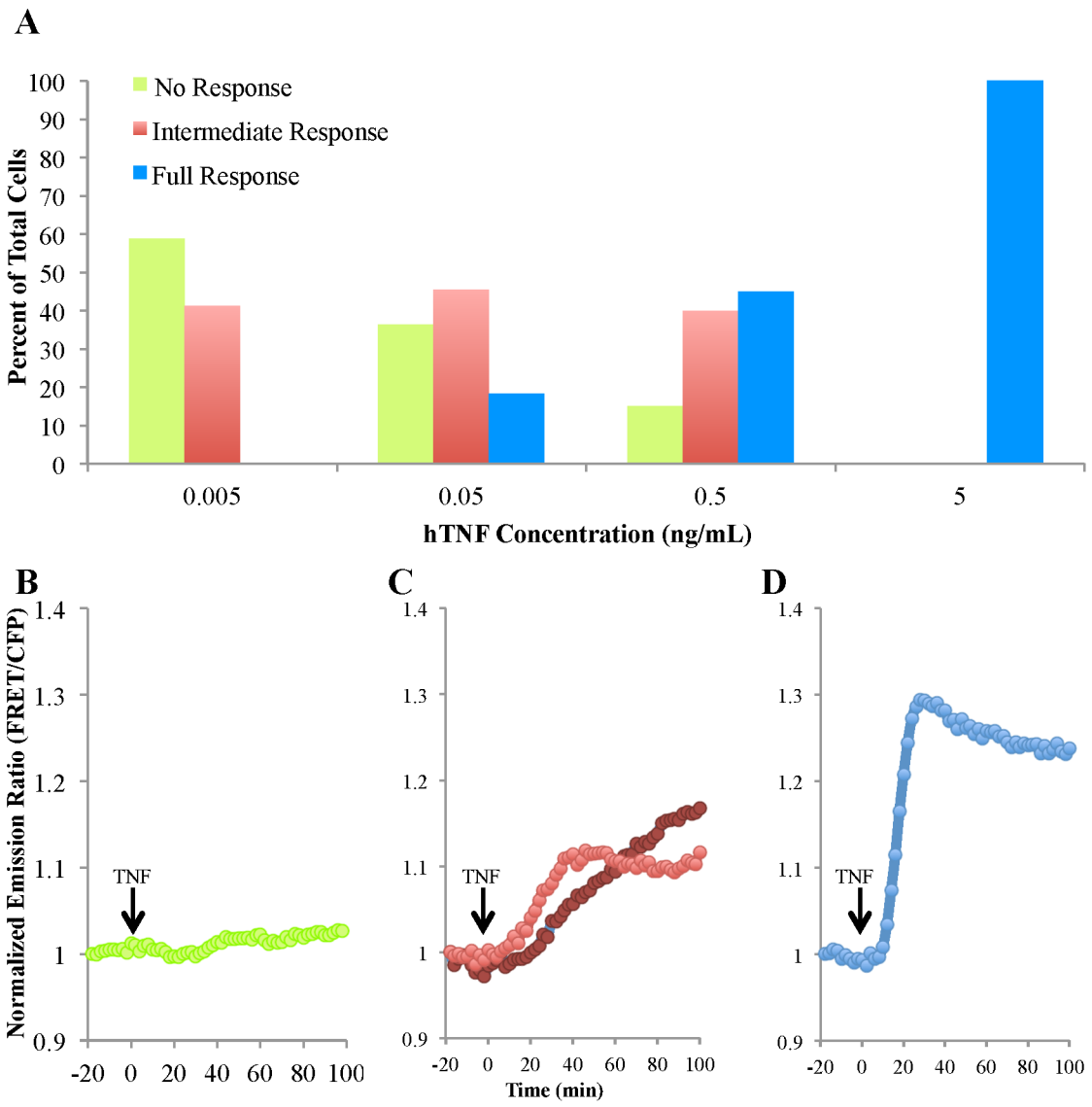

Figure 2-3. Categorization of JNK Responses

(A) Percentage of total cells in each of the three response categories based on stimulation concentration. **(B)** Representative curve for the "No Response" category, defined as a change in emission ratio that does not exceed 5%. **(C)** Representative curves for the "Intermediate Response" category, defined as either a slow and gradual rise in emission ratio or a slow rise followed by plateau. **(D)** Representative curve for the "Full Response" category, characterized by a rapid increase in FRET emission ratio upon stimulation followed by a plateau of greater than 10% increase in emission ratio.

reach half the maximal response ($t_{1/2}$). The $t_{1/2}$ was 60.5 ± 17.87 minutes (n=8) for 0.005 ng/mL TNF, 42.0 ± 21.5 minutes (n=13) for 0.05 ng/mL TNF, 33.64 ± 10.68 minutes (n=14) for 0.5 ng/mL TNF, and 14.17 ± 5.90 minutes (n=24) for 5.0 ng/mL TNF. The $t_{1/2}$ decreases as the stimulus concentration increases, indicating faster JNK activation kinetics when cells are subjected to higher concentrations of TNF (Figure 2-4).

2.3.2 JNK Plasma Membrane Localization

Investigating the cellular localization and spatial regulation of JNK within the cell is vital to fully understanding the JNK mechanism. Several JNK responses have been shown to either originate or be mainly prevalent in a specific region of the cell. For instance, cardiomyocyte cell death can be induced by JNK mitochondrial signaling [35]. Overall, JNK can phosphorylate physiological substrates in different subcellular locations and the activation kinetics and differences between TNF-induced JNK activities in these different areas of the cell have not been systematically analyzed.

To analyze JNK activity dynamics at the plasma membrane, we performed quantitative single cell analysis by stimulating HEK cells expressing the plasma membrane targeted JNKAR2 probe with 5.0 ng/mL hTNF. The average response was a 16.10 ± 8.62% (n=12) increase in upon stimulation (Figure 2-5). All cells responded to the stimulation, but with highly varying amplitudes. These results indicate that TNF can facilitate JNK activity at the plasma membrane.

Furthermore, the kinetics of JNK activation at the plasma membrane, calculated as $t_{1/2}$, was 34.92 ± 9.01 minutes (n=24). This 146.61% increase in $t_{1/2}$ compared to whole cell JNKAR2 with the same stimulus concentration clearly indicates that JNK at subcellular locations exhibit different activation dynamics. The increase in $t_{1/2}$ is due to

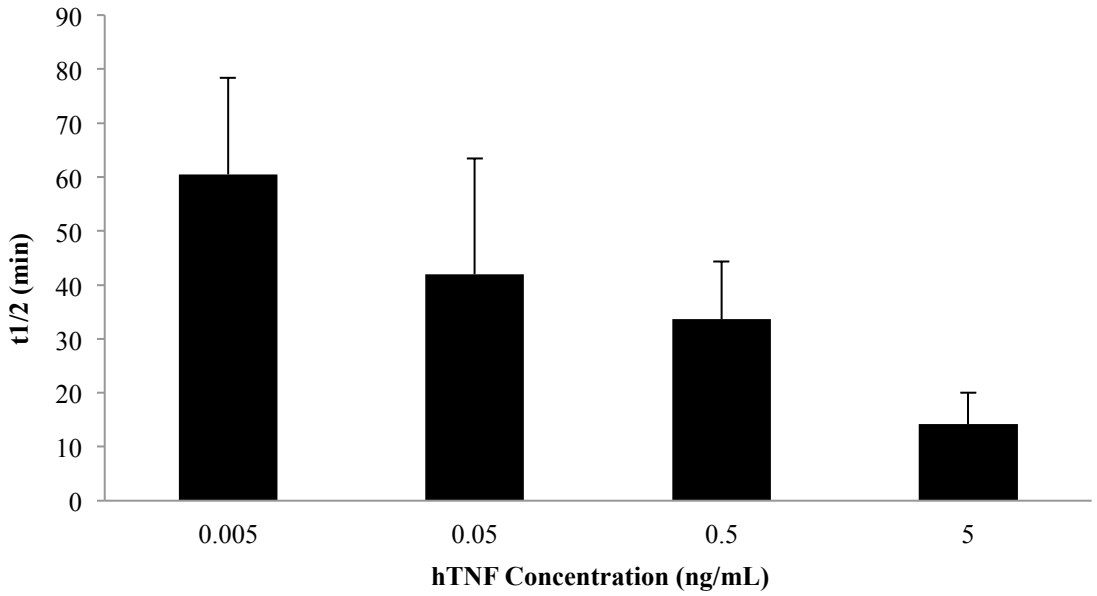

Figure 2-4. Faster JNK response at higher TNF concentration

The JNK response of HEK cells when subjected to increasing doses of TNF subsequently decreases the time to reach half maximal emission ratio ($t_{1/2}$).

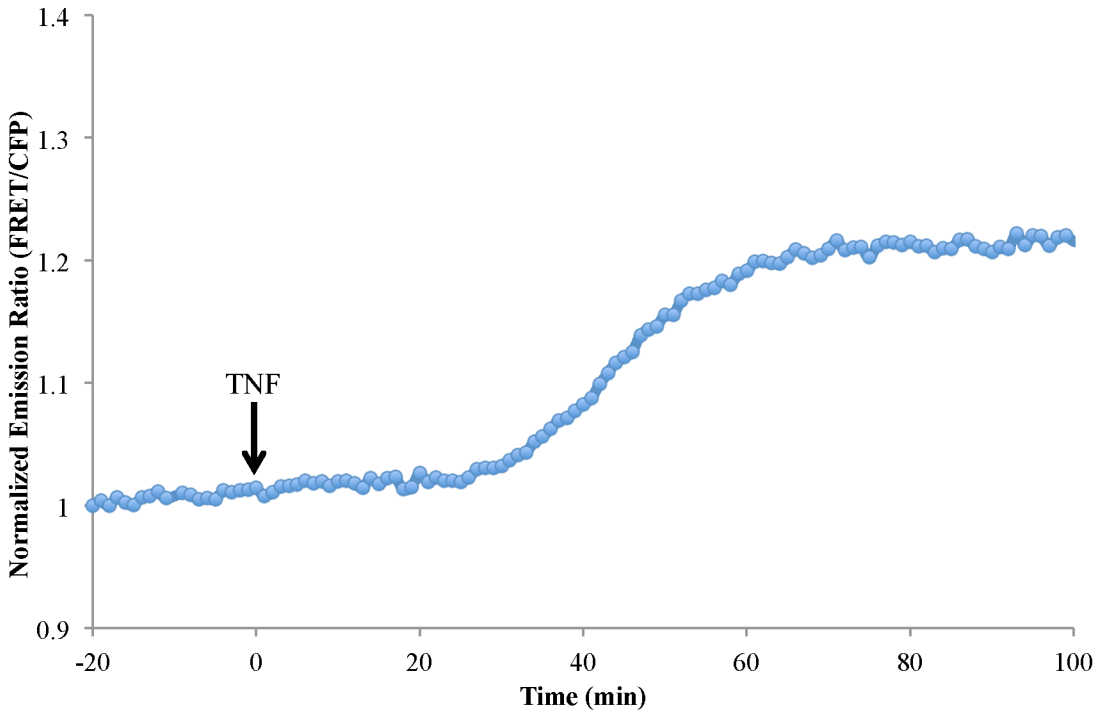

Figure 2-5. Plasma membrane targeted JNKAR2 response.

Normalized emission ratio time courses from representative experiments of HEK cells transfected with the plasma membrane targeted JNKAR2 probe, treated with 5.0 ng/mL TNF.

both a slower rise in FRET emission ratio as well as a delayed start to activation, as shown from representative curves in Figure 2-6. The delayed activation at the plasma membrane indicates that JNK activity upon activation is not uniform throughout the cell. JNK may be sequestered within the cytoplasm, leading to the faster response in the cytosol and postponing the activation in the plasma membrane. Alternatively, JNK may need to interact with other proteins prior to reaching the plasma membrane, resulting in the delay. For instance, in fibroblast growth factor-2 (FGF2) activation of JNK, JNK has been found to translocate to the plasma membrane after being recruited by the scaffold protein, JNK/ stress-activated protein kinase-associated protein 1 (JSAP1) [36].

2.4 Discussion

JNK signaling is widely involved in a plethora of cellular processes. Up until recently, its mechanism of action during the cell's decision making process has been ambiguously defined. However, the recently developed JNKAR2 probe allows us to analyze JNK activity at the single cell level, within the cell's native environment at high signal-to-noise ratios. An analysis of the FRET emission using this probe indicates that the JNK response to TNF in HEK cells likely does not exhibit switch-like behavior in response to TNF stimulation, but rather relays the graded signal from the input to JNK's downstream effectors to interpret and convert into a digital signal for subsequent execution of important decisions.

Moreover, studying the subcellular localization of JNK provides insight into the spatiotemporal regulation of JNK throughout the cell. Evidently, JNK activation at the plasma membrane is both slower and delayed compared to that of the whole cell. These findings demonstrate that once JNK is activated, it does not immediately distribute

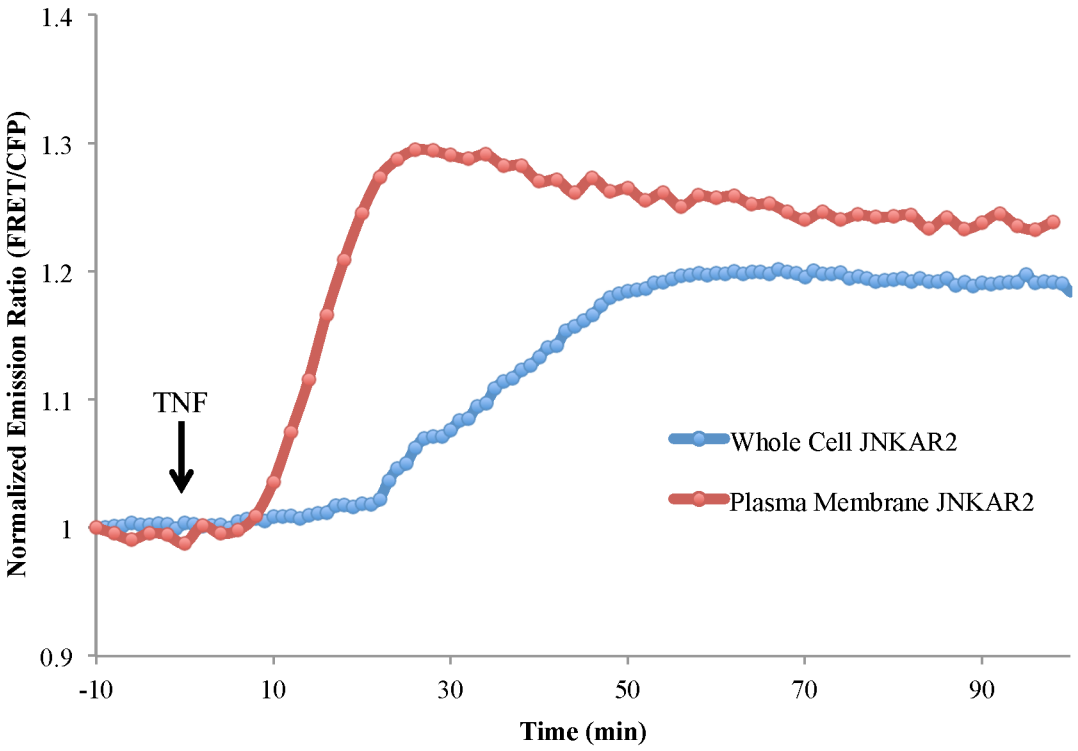

Figure 2-6. Different JNK activation kinetics at the plasma membrane.

Representative normalized emission ratio time courses of HEK cells expressing either whole cell JNKAR2 or plasma membrane targeted JNKAR2, illustrating the difference in activation kinetics.

uniformly throughout the cell, but rather reaches subcellular locations at varying time points. Further analysis of the JNK response at other subcellular locations, including the mitochondria and the nucleus may elucidate more into the subcellular localization of JNK within the cell.

Additionally, important characteristics such as JNK's sensitivity to repeated stimuli have yet to be determined. Bistable signaling has frequently been described as a characteristic of signaling pathways [37], but these findings oppose this hypothesis, previous findings in the oocyte maturation process [31], as well as HeLa cells subjected to anisomycin stimulation [27]. Thus, the JNK pathway response is likely dependent on a variety of factors including the cell line and the stimulus, emphasizing that the mechanism by which JNK processes graded input signals is still extremely ambiguous. The JNKAR2 probe provides a unique opportunity to further study the JNK mechanism in quantitative detail.

CHAPTER 3: JNK SENSITIVITY

3.1 Introduction

Although TNF is a pivotal cytokine that regulates the cell's immune response, its tolerance and sensitivity in mammalian cells to repeated TNF stimulation is unknown. Tolerance to the toxic effects induced by TNF has been found to develop after repeated administration of low doses of TNF in rats, as selective TNF effects are reduced following repetitive stimulus administration [38]. However, the mechanism by which this tolerance develops is unclear and has also not been investigated in mammalian cells.

Furthermore, cells must make critical decisions to determine a specific cell fate in the presence of noisy signals. A true signal is the intended change, for example in the stimulus concentration, whereas noise is stochastic fluctuations. The mechanism by which cells are able to reliably detect and respond to the true stimuli lies in the sensitivity of the cell to concentration changes of the stimuli input signal. Because random fluctuations in the signal concentration are often present, the cell must utilize its signal processing mechanism to delineate between noise and the intentional signal. Therefore, understanding the sensitivity and tolerance of a signaling cascade to input signals such as TNF, can provide vast knowledge into the cell's immune response, the mechanism of the JNK pathway, and how the cascade will react and execute information given by the stimulus.

3.1.1 Receptor Sensitization and Desensitization

Continued or repeated stimulation of a signaling receptor by its agonist can lead to changes in the receptor responsiveness. These changes can be categorized as either sensitization or desensitization. Receptor sensitization is the concept that repeated stimulus administration will result in amplification of response, as for example more

receptors to that stimulus will move to the surface in anticipation of an incoming stimulation. In contrast, receptor desensitization occurs when a receptor's response to a stimulus is decreased after repeated stimulation for example due to a decrease in the number of receptors at the cell surface. These two phenomena have been identified in many signaling processes. For instance, in the repeated stimulation of cyclic AMP (cAMP) by its agonist, cAMP levels plateau or even return to basal levels within minutes of continued exposure [39].

In this section we investigate the tolerance and sensitivity of HEK cells in response to TNF by quantifying the JNK response to a single dose of TNF stimulation compared to that of two repeated doses of TNF stimulation using the JNKAR2 probe to track and provide quantitative single cell data. Specifically, we would like to determine whether the JNK pathway in response to TNF displays receptor sensitization or desensitization by looking at the kinetic differences in the responses.

3.2 Materials and Methods

Cell Culture

HEK cells were cultured in DMEM with 10% fetal bovine serum. Cells were transfected in 35mm dishes with Trans-IT 2020 transfection reagent (Mirus Bio) and imaged 36-48 hours later.

Imaging

Cells were serum starved in DMEM F12 with 0.1% fetal bovine serum 10-12 hours prior to imaging, and imaged in the same media. Cells were imaged in 8-well chamber slides (Lab-Tek) coated with fibronectin with a Zeiss Axiovert 200M using a

cascade CCD camera (Photometrics) and 40x oil immersed lens at 37°C and 5% CO_2, controlled by SlideBook (Intelligent Imaging).

3.3 Results

HEK cells transfected with the JNKAR2 probe were first stimulated with 0.05 ng/mL TNF, one of the intermediate concentrations used in the dose response results of Chapter 2, for 42 minutes, and then immediately stimulated with additional TNF to reach a final dose of 5.0 ng/mL TNF. To emphasize the presence of three separate responses to three different stimulation doses (no TNF, 0.05 ng/mL TNF and 5.0 ng/mL TNF), other types of responses, such as cells that displayed no change in JNK activity upon initial stimulation, were excluded from the analysis. A representative time course emission ratio illustrating JNK activity throughout the experiment is shown in Figure 3-1. The cell responds with a slow and gradual rise in JNK activity after the initial stimulation, with a 9.00 ± 4.68% (n=10) increase in FRET emission ratio, similar to the results of the 0.05 ng/mL TNF stimulation in Chapter 2 as expected. This response was followed by a rapid rise and subsequent plateau in JNK activity upon stimulation with a total dose of 5.0 ng/mL TNF, which resulted in a 26.14 ± 10.95% (n=10) increase in emission ratio, when normalized to the timepoint immediately preceding 5.0 ng/mL stimulation.

The results further validate that HEK cells stimulated with TNF illustrate a graded response. The initial stimulation cannot be categorized as a bistable, all or none JNK response, but instead elicits an intermediate level of slowly rising JNK activity. The representative time course clearly portrays three distinct JNK responses—no activity when no stimulus is present, intermediate and slowly increasing JNK activity with 0.05 ng/mL stimulation, and high activity level with 5.0 ng/mL stimulation.

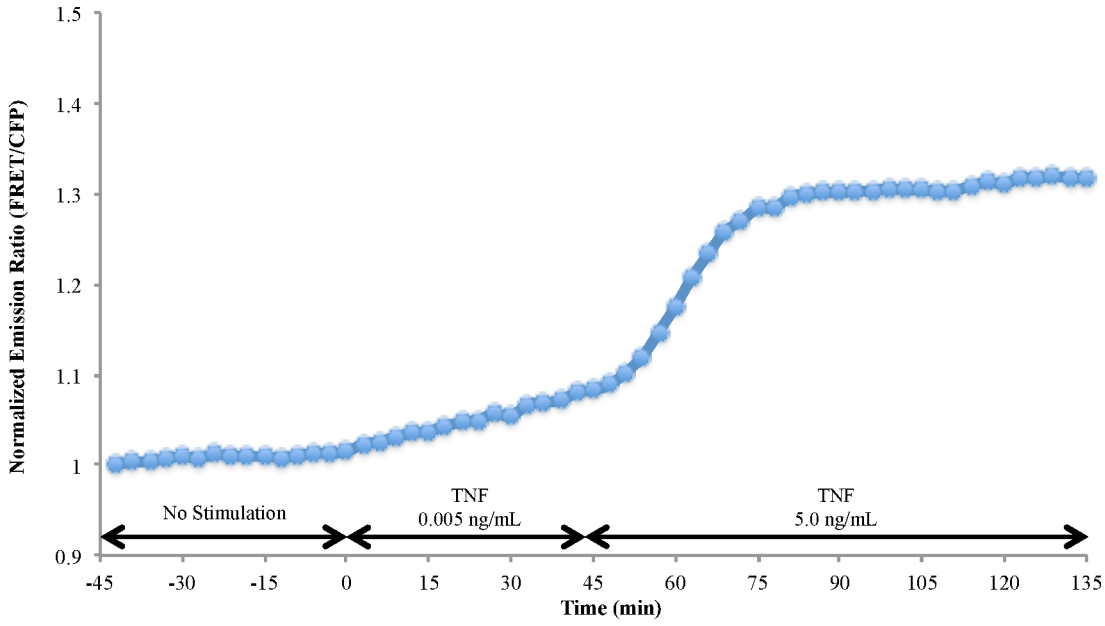

Figure 3-1. Repeated stimulation of JNKAR2.

Representative emission ratio time course of HEK cells transfected with JNKAR2, stimulated with an initial dose of 0.05 ng/mL TNF for 42 minutes and then subsequently stimulated with a total dose of 5.0 ng/mL TNF.

Moreover, the second stimulation with the 100-fold greater dose of TNF changes the percent FRET change as well as produces altered JNK activity kinetics compared to the same stimulation dose alone. A comparison of the average percent change in yellow FRET to cyan emission ratio between the 5.0 ng/mL TNF stimulation with prestimulation of 0.05 ng/mL TNF and the 5.0ng/mL TNF stimulation alone indicates that the presence of prestimulation produces a greater change in emission ratio, and thus a heightened level of JNK activity (Figure 3-2 A,B). Furthermore, an analysis of the time to half maximal JNK activity for the two sets of experimental conditions portrays important differences in the activation kinetics (Figure 3-2 A,B). Overall, the 20.53% increase in percent FRET change and the 20.96% decrease in $t_{1/2}$ when compared to no prestimulation, clearly demonstrate that by initially pretreating the HEK cells with a low dose of TNF, the response to a subsequent dose elicits a greater and quicker response, possibility due to increased sensitization of the TNF receptors at the cell surface.

3.4 Discussion

Evidently, pretreatment of HEK cells with a low dose of TNF elicits an altered JNK response in both the level of JNK activity as well as the activation kinetics. These findings elucidate information about the JNK pathway's tolerance and sensitivity in HEK cells in response to TNF. In HEK cells, initial stimulation with low dose of TNF followed by stimulation with a higher dose does not result in tolerance, but rather displays increased sensitivity to the stimulus, as demonstrated by the faster activation kinetics and increased level of JNK activity. Tolerance may develop following additional repeated stimulation at a low dose of TNF, but two TNF doses in close temporal proximity does

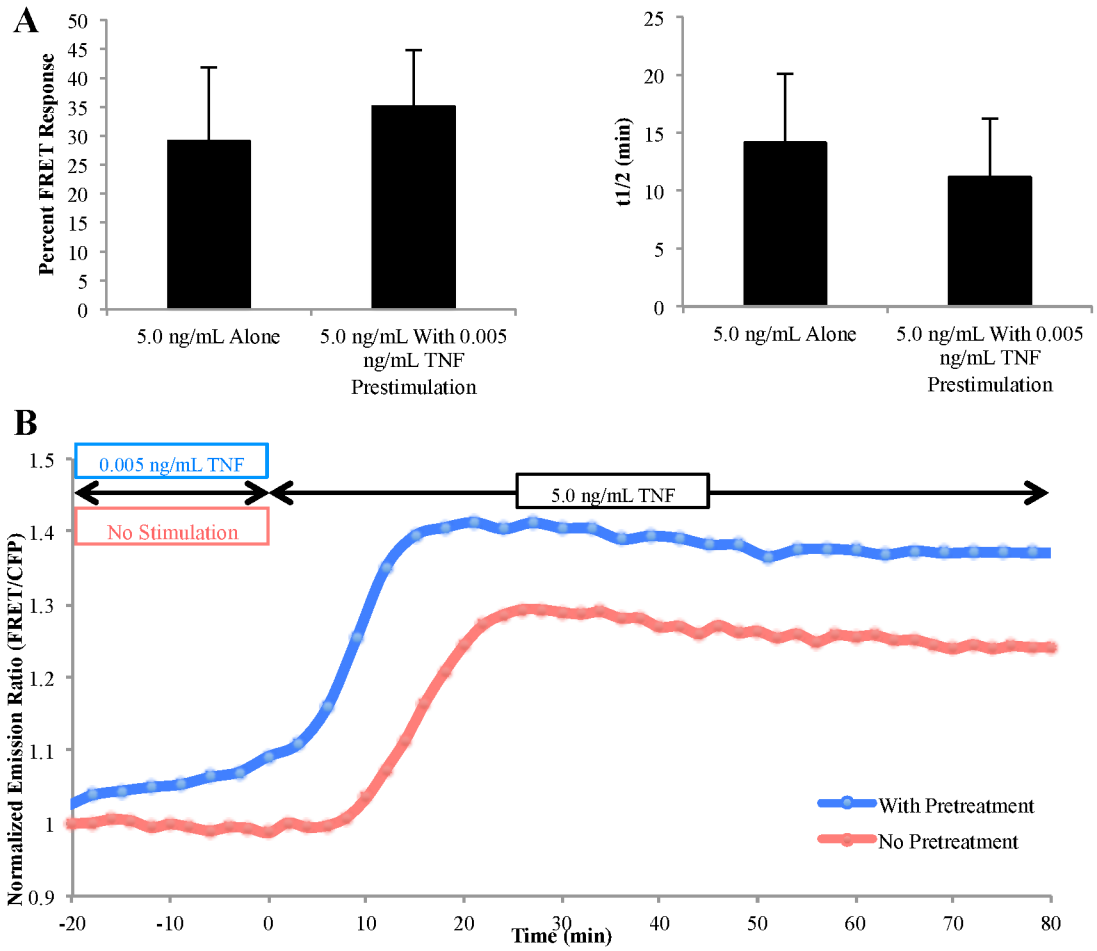

Figure 3-2. Different JNK kinetics in the presence of prestimulation

(A) HEK cells expressing the JNKAR2 probe were pretreated with 0.005 ng/mL TNF and subsequently stimulated with 5.0 ng/mL TNF. The percent increase in FRET signal for the 5.0 ng/mL stimulus without pretreatment was 29.13 ± 12.67% (n=24), and with pretreatment was increased to 31.55 ± 9.75% (n=10). The $t_{1/2}$ for the 5.0 ng/mL stimulation alone was 14.17 ± 5.90 (n=24), whereas for the 5.0 ng/mL after "priming" with an initial dose was 11.20 ± 5.00 minutes (n=10). **(B)** Normalized emission ratio time courses from representative experiments of HEK cells with and without pretreatment, portraying the differences in maximum percent FRET change and kinetics.

not result in tolerance to TNF's effects on the JNK pathway of HEK cells. These findings suggest receptor sensitization of TNFR1 to repeated TNF stimulation.

The mechanisms underlying such sensitization are currently unknown. It is possible that the receptor levels of biochemical states change in response to the first dose. The initial dose of TNF may cause an excess amount of TNFR1's to move to the cell surface in anticipation for incoming TNF stimulus. Other possible mechanisms may involve crosstalk between the JNK signaling pathway and other pathways. Previous findings have demonstrated that the TNFR1 induced apoptotic signaling pathway results in the formation of two distinct signaling complexes. The initially formed complex I at the plasma membrane rapidly triggers an NF-κB cell survival response, whereas the subsequently formed complex II involves FADD and procaspases, but not TNFR1, and develops in the cytoplasm [40]. Activation of complex II results in programmed cell death when the NF-κB cell survival signal from complex I fails to induce expression of anti-apoptotic proteins. Studies have also investigated the crosstalk between TNF-induced NF-κB and JNK pathways and its regulation on cell fate determination. The level of JNK activation from TNF stimulation appears to be regulated by NF-κB activation. Future research can examine whether the first dose of TNF induces any changes to the crosstalk, thereby influencing the sensitivity of the system to subsequent doses.

CHAPTER 4: CONCLUSIONS

Using a genetically encodable FRET-based biosensor for JNK, we were able to characterize the JNK cascade in response to TNF stimulation. Our results indicate that HEK cells stimulated with TNF elicit a graded JNK response. These findings strongly suggest that the characteristics of the JNK response vary depending on cell line and stimulus, as HeLa cells subjected to anisomycin and oocytes stimulated with sorbitol and progesterone both exhibit a bistable response to graded input signals [37]. Moreover, the JNKAR2 probe also allows study of JNK's subcellular localization in areas such as the plasma membrane. Comparing the kinetics of JNK activation of whole cell JNKAR2 and plasma membrane targeted JNKAR2, we found that JNK activation is delayed at the plasma membrane. Further investigation of JNK subcellular localization can reveal more about the spatiotemporal regulation of JNK upon activation with stress stimuli. Lastly, by pretreating HEK cells with a lower dose of TNF, a subsequent TNF stimulation exhibits a faster and increased JNK response, suggesting that JNK signaling can be sensitized. Overall, the JNKAR2 probe has allowed us to characterize JNK activity under tight, complex spatiotemporal regulation and further investigation of JNK activation dynamics can be conducted using the novel FRET-based probe to investigate how the JNK response varies across different cell lines and subjected to various physiologically relevant stimulation. Due to JNK's vast involvement in vital cellular decision making such as cellular apoptosis and differentiation, investigating its response to immune system stimulus, such as TNF as seen here, can provide significant insight into the development of novel therapeutics towards cancers and various diseases.

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Curriculum Vitae

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- B.S. in Biomedical Engineering with minor in Entrepreneurship and Management (May 2013)
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Research and Professional Experience

Johns Hopkins University Signal Transduction and Cell-Cell Communication Lab, Baltimore, MD

Research Assistant (January 2011—Present)

- Characterize how the c-Jun N-terminal (JNK) signaling cascade responds to tumor necrosis factor alpha (TNFα) at the single cell level using a novel JNK Fluorescence Resonance Energy Transfer probe
- Investigate the possible mechanisms behind bistability in the JNK signaling cascade
- Determine if there are any additional feedbacks in the JNK pathway and characterize them in mammalian cells

Accenture LLP, Arlington, VA

Systems Integration Functional Analyst (June—August 2012)

- Designed and tested a unified approach to reporting standards by streamlining multiple reporting units on a Federal Civilian client project
- Wrote test conditions and test scripts for Systems Integration Testing and Customer Acceptance Testing
- Ran queries in SQL to identify discrepancies in report calculation and analyzed client's business logic to locate source of discrepancies
- Developed a new data breakdown approach to deliver enhanced clarity into report calculations
- Wrote simplified instructions that summarized step-by-step how to identify discrepancies in report calculation in future reports

Johns Hopkins Center for Bioengineering Innovation & Design, Baltimore, MD
Team Member (May 2011—May 2012)

- Designed and engineered a prototype for a custom wheelchair cushion that supports patients with limited mobility and rapidly progressing bone deformities
- Analyzed target market to determine best means of market entry, and wrote project proposals for grants and business plan competitions

American College of Obstetricians and Gynecologists, Washington, DC
Research Intern (January—April 2012)

- Analyzed over 1,000 neonatal encephalopathy and cerebral palsy references to update the 2012 edition of the *Neonatal Encephalopathy and Cerebral Palsy* Report
- Developed a simplified system for organizing and storing references to allow users to more easily locate relevant references

National Institutes of Health, National Eye Institute (Lab of Immunology), Bethesda, MD
Student Intern (June—August 2010, October 2008—August 2009)

- Analyzed the effects of overexpressing proteins involved in hypoxia on ARPE-19 cells
- Conducted tail genotyping of transgenic and knock-out mice to conduct mouse modeling to understand cellular mechanisms that regulate immune homeostasis

Leadership Experience

Hopkins Medical Device Network (HMDN), Baltimore, MD
Graduate Advisor (August 2013—Present)

- Oversee HMDN activities and events and provide guidance to the current board, based on prior experience as Events Coordinator

Events Coordinator (August 2011—May 2013)

- Organized HMDN Speaker Series events and coordinated with various other student groups to gather funding for collaboration events

Bringing Books Back, Baltimore, MD
Assistant Vice President (August 2012-May 2013)

- Established a focused direction for the newly created student group aimed at contributing back to the neighborhood by organizing book donations and a tutoring program for local Baltimore students

Biomedical Engineering Society (BMES), Baltimore, MD
Student Mentor (August 2011—May 2013)

- Provided freshmen biomedical engineering students at Johns Hopkins University with an opportunity to connect with upperclassmen and seek advice on topics such as career development for both industry and graduate studies

Teaching Experience

Johns Hopkins University Whiting School of Engineering, Baltimore, MD
Graduate Teaching Assistant for Accounting and Finance: EN.662.611 (August 2013—December 2013)
Graduate Teaching Assistant for M.S. in Engineering Management Seminar: EN.660.811 (August 2013—December 2013)

Graduate Teaching Assistant for Marketing Communication and Strategy: EN.662.651 (January 2014—Present)

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Course Assistant for Financial Accounting: EN.660.203 (January 2012—May 2012, August 2012—May 2013, August 2013—Present)

Awards

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Johns Hopkins University Elevator Pitch Competition—3rd Place Winner (2012)
Tau Beta Pi Engineering Honors Society Active Member (2011—Present)
Golden Key International Honour Society Nominee (2011, 2012)
National Society of Collegiate Scholars (2011)
Johns Hopkins University Dean's List (2010—Present)

Skills

Computer skills: MATLAB, SQL, Benthic, Microsoft Office Suite
Language skills: Mandarin (Fluent), Spanish (Basic)
Wetlab skills: Mammalian cell culture, fluorescence microscopy, image processing and analysis, DNA plasmid purification, RNA isolation, PCR, gel electrophoresis, western blotting, lentiviral transduction, transfection, bacterial transformation, surgical mice dissection, DNA extraction