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Dynamics Modeling Signaling Pathway Regulating EGF-Induced Cell Adhesion ⋆

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Abstract: A quantitative modeling approach is developed to dissect the signaling pathways involved in the process of the epidermal growth factor (EGF)-induced dynamic change of cell adhesion. The dynamics model will be constructed based on a system identification process, which is regularly employed in control system design to elucidate the unknown structures and parameters of some of the components in the system based on the prior knowledge and the input/output information of the system. The signaling network that is known to regulate the EGF-induced cell adhesion is designated as the controller which controls the physical process of cell adhesion, i.e. the plant. A nanomechanical sensor in quartz crystal microbalance with dissipation monitoring (QCM-D), which is capable of generating real-time, continuous and measurable signals, will be used for evaluating the system output. The interaction of measurement signal with the cell adhesion complex is modeled as plant. From the model, key structures and parameters of the signaling hierarchy were identified and confirmed. The dynamic pathway output agrees well with the measurement result of energy dissipation from the QCM-D sensor. We expect this proposed study will reveal the decisive reactions of the signaling network that are most critical to regulation of EGF-induced changes in cell adhesion at both normal and disease conditions.

Keywords: Dynamics model, micro and nano system, signaling pathway, cell adhesion, nanomechanical senor.

1. INTRODUCTION

Epidermal growth factor (EGF) is a compound that can bind to the cell surface receptor, epidermal growth factor receptor (EGFR) Carpenter and Cohen (1979). The binding will trigger a series of signaling cascades that have complicated effects and implications in the normal function of cells. Specifically, the EGFR pathway could regulate cell migration, growth and proliferation in normal physiological conditions; while in pathological conditions, it is reported to be closely associated with the metastasis of certain cancers Sharma et al. (2007). The EGFR pathway has been shown to be an extremely complicated interconnected network recruiting and involving numerous intermediate molecules Oda et al. (2005). One of the essential consequences of EGFR signaling is the regulation of cell motility through the promotion and inhibition of assembly or disassembly of focal adhesion complexes Lu et al. (2001), or the so called cell-extracellular matrix (ECM) adhesion. Overexpression of EGFR has been associated with cancer metastasis which are featured by compromised cell adhesions. Thus it is of paramount interest to dissect the signing pathways that regulate the cell adhesion after EGFR signaling.

The focal adhesion complex comprises of discrete sites where physical contacts between the cell cytoskeleton and the ECM, extracellular tissue that mainly composed of interlocked fibrous proteins, are established. The foundation of the physical entanglement starts from the binding of integrin molecules of their extracellular domain, a transmembrane receptor, to the RGD sequences in the ECM (Fig. 1A). This binding will recruit a number of proteins in the intracellular domain to tether the integrin molecule to the actin filament, one of the three cytoskeleton elements. Thus, a change in the adhesion site, binding/unbinding of integrin molecules with RGD sequence, assembly/disassembly of focal adhesion complex, adhesion/deadhesion of cell motility, will essentially tip the delicate balance established between the cell cytoskeleton and the ECM Wozniak et al. (2004). The focal adhesion was visualized as discrete sites of contact as shown in Fig. 1B and the cell structure was captured by AFM live cell imaging in Fig. 1C. This biophysical process has been investigated from the ligand-receptor binding dynamics perspective. Most of these models are derivations of the thermodynamic framework pioneered by Bell (1978). The biophysical property of cell adhesion emerges from the delicate equilibrium of integrin-RGD binding, where the stochastic bond formation and dissociation rates were expressed in the form of thermodynamics and the load bore by the integrin-RGD bond. A number of models have extended the reach of the Bell model to more complicated scenarios, such as the cell membrane peeling model when the blood cell in rolling motion through the vein and these extended models have exert influence on the understanding of cell spreading, migration, proliferation and differentiation Dembo et al. (1988).

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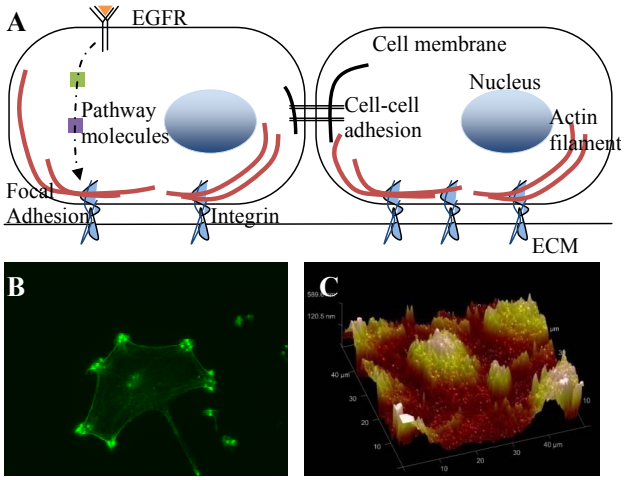

Fig. 1. Illustration of EGF induced cell adhesion change

The regulation of cell adhesion by EGFR signaling pathways has been reported to work through three branches of cascades and their inter-branch crosstalks: the phosphoinositide 3-kinase (PI3K) pathway, the mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) pathway, and the phospholipase C (PLC) pathway as shown in Fig. 2 Pece and Gutkind (2000). Significant progress has been made since the discovery of EGF and EGFR, resulting in the identification of key molecules in these pathways. Nevertheless, the complexity of these pathways and their crosstalks prevents the full understanding of impacts of each pathway to the adhesion/deadehsion process, and ultimately cell motility and migration in a bigger picture Joslin et al. (2010). From the intricacy of the pathway dynamics it also raises the nonlinearity issues that are inherent to the various feedback and feedforward loops prevalent in these networks of molecules, issues of paramount interest to the modeling and control community Morris et al. (2011).

To understand these exquisite balances of molecules in the process of cell motility regulation, system biologists often refer to the computational modeling techniques at the system level to elucidate the steps and molecules, and try to assess quantitatively the rise and fall of these molecules in terms of expression level or concentrations at each step, as well as break down the contributions from each component during the dynamic interactions Joslin et al. (2010). The resulting product is computational models written in the form of ordinary differential equations (ODE) derived from the reactions of chemical species based on the kinetics of reaction with knowledge of sets of rate constants. This approach has produced a number of well-defined pathway structures for various aspects of EGFR signaling Bagheri et al. (2011). To analyze the modeling results and verify them by experiments, however, requires significant amount of biochemical assays, which are often time consuming themselves.

To circumvent the problem, researchers have proposed a paradigm-shifting methodology to integrate the biophysical model of cell structure and its dynamic remodeling and reconfiguration process with the control and regulation from the signaling cascade upon stimulation by various extracellular cues. The only concrete model so far that has been proposed for mechanistic regulation of cell adhesion by signaling pathways was developed by Besser and Schwarz (2007). The Rho pathway was considered in the model as the main regulator of cell

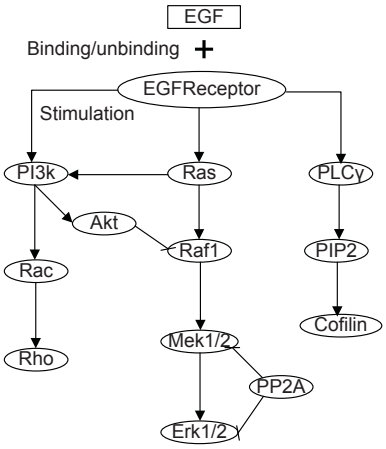

Fig. 2. Three entangled pathways that are believed to regulate cell adhesion

adhesion and the level of enzymatic activities were converted to applied load similar as the Bell model. The lack of components from other key molecules poses a challenge for the general acceptance of the model. The linkage between the expression level of Rho and the applied load to the molecular bond is debatable.

The physical properties of the focal adhesion and its impact on the cell mechanical property as a whole provide unique peek into the end product of the signaling pathway controlled cell adhesion. The mechanical characterization of the strength of focal adhesion by an oscillating quartz crystal microbalance with energy dissipation (QCM-D) and its implication to cell mechanical state by atomic force microscopy (AFM) probing has been reported previously Yang et al. (2012). The relationship between the size of focal adhesion and the readout from the QCM-D sensor has been elaborated Chen et al. (2012). Thus to build an integrated EGFR signaling/cell adhesion model, we tried to shed the light from the system control perspective, in which the cellular physical state and its interaction with the nanomechanical sensor are deemed as the micro and nano mechanical system under the control of the signaling pathway cascade. Leverage on the unique capability of QCM-D which can provide real-time continues mechanical measurement, a mechanical model can be built as the plant, and then various signaling pathway structures and molecular interactions of different rate constants and parameters can be put on trial against the real-time data as to verify their legitimacy and accuracy.

2. CHARACTERIZATION OF CELL ADHESION

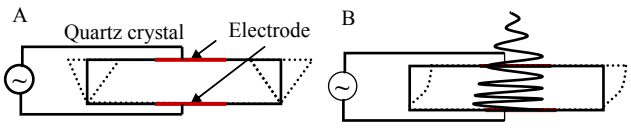

Fig. 3. The probing principle of QCM-D

QCM-D is an acoustic sensor that probes the physical interaction of the sensors with the material, normally in the form of thin film, through high frequency oscillation of quartz crystals. An alternating voltage will cause relative lateral movement of the two surfaces and thus apply a shear force to the surface material (Fig. 3A). Traditional applications of quartz crystal

oscillator have been mainly in the field of physical and materials sciences to inquire the mechanical property of the film deposited on top of the sensor or as a metrology instrument to determine the thickness of the deposition Rodahl and Kasemo (1996). Once extended to the biomedical field, the QCM-D sensor took on the insurmountable task of cell mechanics Lord et al. (2006). A highly sensitive device, small perturbations of the fluid on the top or reorganizations of the cell structure could be captured with high frequency measurement pulses as shown in Fig. 3B. The measurement results of QCM-D are normally in the form of frequency shifts and energy dissipation variations which are mainly caused by the oscillation decay of the acoustic wave over the course of observation. The frequency shifts often indicates the mass of deposit layer of films; while under liquid environment, the energy dissipation variations were analyzed to generate the viscoelastic properties of the film Voinova et al. (1999).

The QCM-D sensor disk is an AT-cut quartz crystal operated at an oscillation frequency of 5 MHz. The disk has a diameter of 14 mm and is deposited on top a layer of gold or silicon dioxide of 50 nm in thickness. The penetration depth of the shear wave propagating in perpendicular direction to the sensor surface can penetrate a depth of $\delta = \sqrt{\frac{\eta}{\pi\rho f}}$, in which η is the viscosity of the bulk liquid, ρ is the density of the liquid and f is the operating frequency of the sensor. A third overtone is used and the estimated depth of penetration is around 100- 200 nm Guillou-Buffello et al. (2011).

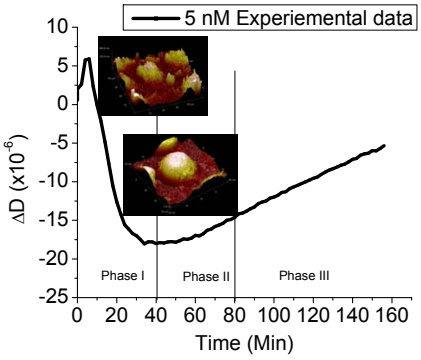

Fig. 4. Real time measurement of energy dissipation upon EGF treatment of A431 cells

The EGF treatment of A431 cells has long been reported to provoke discernable physical alterations, such as rounding and stiffening of the cell body Chinkers et al. (1981), as also verified by AFM live cell imaging shown in the insect of Fig. 4 before and after stimulation. The result of the EGF-EGFR signaling pathway regulation displays its capability in control the cell fate in a full spectrum. At the basal area, subtle reorganization and remodeling of the focal adhesion complex is the direct result of the expression of certain pathway molecules which disrupt the normal probability of ligand-receptor binding and dissociation. The alterations in the focal adhesion complex have been correlated with the change of energy dissipation factor from the QCM-D measurement. From Fig. 4, the energy dissipation factor change ΔD firstly dropped to a minimum level at around 40 minutes after treatment (Phase I), then it maintained at that level for about 30-40 minutes (Phase II) before climbing back up (Phase III). It was also reported that the change of ΔD is almost linearly correlated with the size of the focal adhesion visualized by fluorescence labeling Chen et al. (2012). The small kink at the onset of first few minutes of stimulation is caused by the perturbation of adding the EGF solution. They will be omitted in later simulations.

3. DYNAMICS MODEL

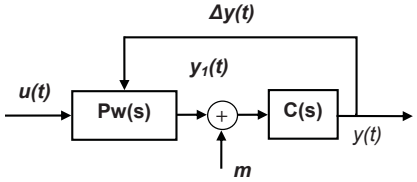

Fig. 5. The signaling pathway control model architecture

We propose to use quantitative modeling to dissect the signaling pathways involved in the process of the EGF-induced dynamic change of cell adhesion. The model will be constructed based on a system identification process, which is regularly employed in control system design to elucidate the unknown structures and parameters of some of the components in the system based on the prior knowledge and the input/output information of the system. A nanomechanical sensor in QCM-D, which is capable of generating real-time, continuous and measurable signals, will be used for evaluating the system output. The proposed system structure is illustrated in Fig. 5. The signaling network that is known to regulate cell adhesion is designed as the controller ($Pw(s)$) of the system. The controller can convert the input signal $u(t)$ (e.g., EGF stimulation) to the execution signal $y_1(t)$, which is responsible for controlling the cell adhesion complex, the plant $C(s)$ of the system. Feeding the plant $C(s)$ with a measurement signal m from the QCM-D will result in the dynamic output $y(t)$, a quantity that correlates with the time-dependent ΔD signal of the QCM-D measurement. Meanwhile, the cells can also actively adjust to the stimulation through a self-imposed adaption by sending the feedback signal $y(t)$ to the controller.

3.1 Pw(s) the controller

We define the controller as the EGF-mediated signaling network that regulates cell adhesion (i.e., restructuring of focal adhesions). This controller includes the PLC pathway (Pw_a), the PI3K pathway (Pw_b), and the MAPK/ERK pathway (Pw_c) (Fig. 2). Some of the signaling molecules that are involved in EGFR activation and its downstream cascades will not be included in the initial model, but will be considered during the model refinement Schoeberl et al. (2002). The state variable of Pw is designated as the concentration of a specific downstream effector molecule that is directly involved in regulation of restructuring of focal adhesions (e.g., calpain in the MAPK/ERK pathway). The change in concentration of a species involved in a signaling reaction is usually described with an ODE. For example, when EGF binds to the EGF receptor on the membrane of the cell: [EGF] + [FreeEGF-receptor] $\rightleftharpoons$ [Bound-EGF-Receptor] (BEGFR), and the reaction is regarded as a mass reaction, thus the time-dependent change in concentration of BEGFR can be described mathematically by an ODE:

$$\frac{d[B_{EGFR}]}{dt} = k_{rbEGF}[EGFR][EGF] - k_{ruEGF}[B_{EGFR}] \qquad (1)$$

where [EGFR] is the concentration of EGF receptors on the cell membrane, [EGF] is the concentration of EGF and is the overall input of the system u, k_{rbEGF} and k_{ruEGF} are the forward and

reverse reaction rate constants (binding/unbinding of EGF to the free EGF receptor). The concentration of [EGFR] can then be determined based on these parameters, which are available from previous biochemical, biophysical, and modeling studies Brown et al. (2004)]. For a Michaelis-Menten reaction, like the Rac molecule activates Rho, the process can be defined by:

$$\frac{d[ActRho]}{dt} = +k_{Rho}[ActRac]\frac{[InActRho]}{[InactRho]+k_mRho} \quad (2)$$

where [ActRho] and [InActRho] are the concentrations of active and inactive Rho, and [ActRac] is the concentration of active Rac; k_{Rho} is the rate constant for the reaction and k_{kmRho} is the Michaelis constant for the Michaelis-Menten reaction.

The cross-talk between these pathways occurs when proteins from different pathways interact. For example, PI3K in Pw_b could regulate the hydrolysis of PIP2 in Pw_a by catalyzing the phosphorylation of PIP2 to form PIP3. Thus the crosstalk will be introduced to the description of the output by combining state variables from Pw_a and Pw_b in one expression. Furthermore, for the different type of molecular reactions such as ligand-receptor binding, phosphorylation, or hydrolysis, differential equation terms will be defined and standardized to establish a database. A combination of equations can easily be built for a more specific and complicated signaling network based on the registered items in the database. The overall signal output from the entire signaling network will be represented as a weighted linear combination of three pathways:

$$y_1 = \alpha Pw_a + \beta Pw_b + \gamma Pw_c \quad (3)$$

where α, β and γ are mapping parameters.

3.2 C(s) the plant

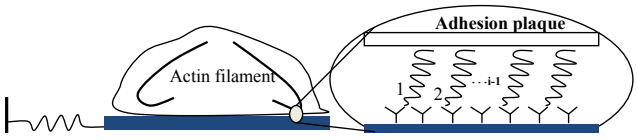

Fig. 6. Mechanical system of the cell adhesion and its interaction with QCM-D sensor disk

As the plant C(s), the adhesion complex of adherent cells can receive the input signal u_2 from the EGF-activated signaling pathways. In response, the adhesion complex will undergo restructuring through dynamic assembly or disassembly of focal adhesions, and will result in the dynamic change in cell adhesion, which will be assessed based on the output of the system. The correlation between the level of cell adhesion and the ΔD response have been established in our previous study Chen et al. (2009). The cell layer attached to a QCM-D sensor can be model as a sensor disk anchored to a spring with an initial momentum (Fig. 6). The dynamic equation for the movement of this sensor disk is defined as:

$$M\ddot{Y} + \eta\dot{Y} + fu_2 + KY = 0 \quad (4)$$

where M is the mass of the disk and η is the damping coefficient, Y is the index of the disk horizontal motion, K is the spring constant and f is the mapping coefficient from the focal adhesion to the sum of bond forces between the sensor disk and the cell adhesion complex. Therefore, $\eta\dot{Y}$ represents damping by the trapping fluid and the interactive bond force is defined by the mapping fu_2. KY is the force in the spring and $M\ddot{Y}$ is the force resulting from the acceleration of the sensor disk. This equation is derived based on the assumption that the friction between the cell and the sensor disk and the viscous damping between the cell body and the liquid trapped underneath the cell body are the primary causes of the energy loss of the sensor disk. This assumption can be verified by comparing the sum of the friction and the viscous damping to the quantity of energy dissipation measured with the QCM-D.

The system dynamics can be transformed to a state space model as:

$$\begin{bmatrix}\dot{Y}_1\\ \dot{Y}_2\end{bmatrix} = \begin{bmatrix}0 & 1\\ -\frac{K}{M} & -\frac{\eta}{M}\end{bmatrix}\begin{bmatrix}Y_1\\ Y_2\end{bmatrix} + \begin{bmatrix}0\\ -\frac{f}{M}\end{bmatrix}u \quad (5)$$

with the two state variables Y_1 and Y_2 denoting the position and velocity of the oscillating sensor disk. These two states specify the input of the QCM-D measurement signal $m = [Y_1 Y_2]^T$. The output from the mechanical model is defined as the rate of dissipation of kinetic energy:

$$Y = \frac{d}{dt}(\frac{1}{2}MY_2{}^2) \quad (6)$$

which is quantitatively related to the ΔD response measured with the QCM-D. A transfer function can be obtained:

$$c(s) = \frac{1}{625s(3.5\times 10^9 s+1)} \quad (7)$$

The mechanical oscillation by the sensor disk would be damped by the interaction of the molecular bonds between the cell and the disk. The process of bond friction relies on breaking of bonds to dissipate the kinetic energy of the disk, and this process is a stochastic process defined by Bell's model Bell (1978), which in the overall control architecture determines the bond number conversion to the friction force or $f(u_2)$. Plugging the friction force to the second order ODE will result in the relationship between the number of bonds u_2 and the output Y which is the energy dissipation factor, the quantity that measured by QCM-D in the real-time experiment.

4. EXPERIMENTAL AND SIMULATION RESULTS

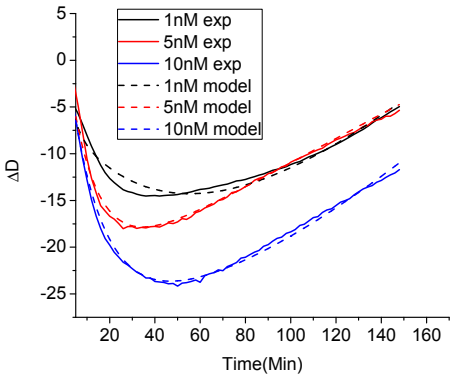

Fig. 7. Comparison of simulation results with experimental data

To determine the concentration of a downstream effector may require solving a series of ODEs derived from sequential signaling reactions upstream in the pathway. The detailed system consists of 14 molecules that primarily determine the output of the effector molecule for cell adhesion regulation. From the 14 molecules, we employed 26 state variables based on their active and inactive status (the list of the first 7 variables is shown in

Table 1. State variables and ODEs

Variables	Physical Meaning	ODEs	Reactions
X_1	[EGF]	$\frac{dX_1}{dt} = -k_{rbEGF}X_1X_2 + k_{ruEGF}X_3$	EGF+ FreeEGFRec ⇔ BoundEGFRec
X_2	[Free EGF Receptor]	$\frac{dX_2}{dt} = -k_{rbEGF}X_1X_2 + k_{ruEGF}X_3$	EGF+ FreeEGFRec ⇔ BoundEGFRec
X_3	[Bound EGF Receptor]	$\frac{dX_3}{dt} = +k_{rbEGF}X_1X_2 - k_{ruEGF}X_3$	EGF+ FreeEGFRec ⇔ BoundEGFRec
X_4	[Active Ras]	$\frac{dX_4}{dt} = +k_{Ras}X_3\frac{X_5}{X_5+k_{mRas}} - k_{ERKRas}X_{18}\frac{X_4}{X_4+k_{mERKRas}}$	RasInactive → RasActive
X_5	[Inactive Ras]	$\frac{dX_5}{dt} = -k_{Ras}X_3\frac{X_5}{X_5+k_{mRas}} + k_{ERKRas}X_{18}\frac{X_4}{X_4+k_{mERKRas}}$	RasActive → RasInactive
X_6	[Active Raf]	$\frac{dX_6}{dt} = +k_{Raf}X_4\frac{X_7}{X_7+k_{mRaf}} - k_{AktRaf}X_{10}\frac{X_6}{X_6+mAktRaf}$	RafInactive → RafActive
X_7	[Inactive Raf]	$\frac{dX_7}{dt} = -k_{Raf}X_4\frac{X_7}{X_7+k_{mRaf}} + k_{AktRaf}X_{10}\frac{X_6}{X_6+mAktRaf}$	RafActive →RafInactive

Tab. 1). The structure is shown in Fig. 2 on which the group of ODEs was based.

If the concentration or the rate constant of a particular species is not readily available, an estimate based on the information on a related species may be used for the calibration of the initial model. Such estimated parameters can be determined eventually through model fitting of the experimental data. The rate constants for the first 7 state variables ODEs are listed in Tab. 2. The parameter for the control model $Pw(s)$ was obtained mostly from the paper by Brown et al. (2004). For the unknown parameters, we used a fitting algorithm to fit the experimental data with the model and obtain an optimal parameter set. For the EGF treatment of the A431 cells, the real time energy dissipation factor data were then fitted. The data would then be tested when one or several of the pathways were to be blocked.

The real-time measurement of energy dissipation variations is compared against the simulation data from the model in Fig. 7. Based on the parameters from literature, the model fits the experimental data with precision for concentrations of 1, 5 and 10 nM. The dynamics model shows discrepancies with the experimental data only at the first phase of the cell adhesion transition (Phase I) when the cell deadhesion dominates the process with drastic restructuring. Features in Phase II and III with stable configuration and gradual recovery of cell adhesion are well captured.

Of equal importance, the expression of these key molecules in the pathway can be analyzed. It has been reported Chen et al. (2012) that the three branches of pathway cascade induce different outcomes of cell adhesion. Specifically, the PLC pathway strengthens the focal adhesion complex and promote the assembly of focal adhesion while inhibit the disassembly of ell adhesion. On the contrary, both the MAPK pathway and the PI3K pathway destabilize the focal adhesion structure and compel the disassembly of the focal adhesion. As the cell experience the rise and fall of the cell adhesion both in size and strength as indicated by the energy dissipation and fluorescence labeling, the expression of these key molecules also fluctuates.

As shown in Fig. 8, the expression levels of Rho, MEK and PLCγ over the course of preservation are plotted. Both the levels of Rho and MEK went up in Phase I, though the temporal dynamics of these two molecules did not overlap. The upregulation of MEK experienced a more drastic boost during the first 20 minutes of EGF stimulation and reached plateau 15 minutes into the observation; while the deregulation had a similar temporal dynamics. The expression level of Rho increased at a pace shared with the overall deadhesion. It plateaued at around the end of Phase I and the inception of Phase II, and subsequently dropped at a slower rate than MEK. Since both pathways are responsible for the disassembly of focal adhesion, the increase of both would shadow or at least neutralize the promoting effect of the PLC pathway. Additionally, the downregulation of PLCγ begins slowly after receiving EGF signal, which exacerbate the disassembly of the focal adhesion. After Phase II, however, the combined effect of both decreased levels of MEK and Rho, plus the upswing of the PLCγ would essentially bring back the focal adhesions that were previously disrupted. A concerted effort of these key molecules in the pathway dynamics accomplished the delicate task of cell adhesion regulation.

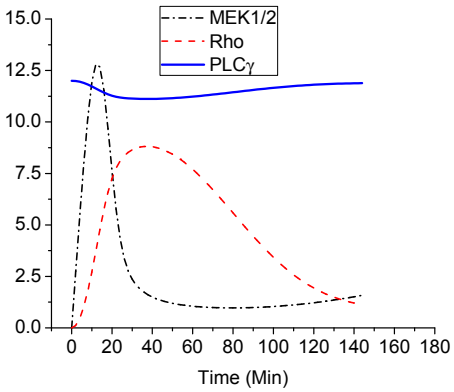

Fig. 8. Expression levels of MEK1/2, Rho and PLCγ

The overall effect of the molecule dynamics after EGF stimulation is manifested through the biophysical properties of the focal adhesions as well as the cytoskeleton mechanics, as the real-time measurement data from QCM-D tracks the change of energy dissipation that are directly related to the size and strength of focal adhesion. Leveraging on this functional study, we were able to provide a multifaceted characterization of the EGF induced cell adhesion change. The analysis from the signaling pathway control model yields a unique peek into the biochemical dynamics of the regulation as well as the biomechanical dynamics of the adhesion. The simulation result compares well with the realtime measurement data. More importantly, the simulation provides the molecule dynamics in the process of regulation which can be verified by a biochemical assays, such as Western blot. At the minimum, from the biochemical assay, we can build a calibration standard for each key molecule over the time course and we'll prevent the messy experiments from repeating. If the prediction from the model proves correct, we will find similar levels of protein expression during the EGF stimulation process.

5. CONCLUSION

The study proposed a signaling pathway control model that aims to decipher intricate signaling pathways that regulate cell adhesion during EGF stimulation. The model leverages on the capabilities of nanomechanical senor in QCM-D which pro-

Table 2. Rate constants

Reaction name	Parameter	Value
EGF binding	k_{rbEGF}	2.185E-5
	k_{ubEGF}	0.0121008
Ras activation by bound EGFR	k_{Ras}	694.731
	k_{mRas}	6086070
Ras deactivation by ERK1/2	k_{ERKRas}	1611.97
	$k_{mERKRas}$	896896
Raf activation by Ras	k_{Raf}	1509.36
	k_{mRaf}	1432410
Raf deactivation by Akt	k_{AktRaf}	15.1212
	$k_{mAktRaf}$	119355

vides real time measurement of cell adhesion strength. A mechanical model was built to quantitatively relate the measurement value, energy dissipation (ΔD) with the dynamic bond formation/dissociation. This mechanical model was considered the plant controlled by the signaling pathway model. Upon obtaining the measurement output in ΔD and with prior knowledge of EGF concentration, we identified the key steps and branches of signaling pathways in the regulation process, or more specifically the dynamics of key molecules. The study initiates a unique methodology in delineating pathway dynamics that essentially eliminates the necessity of the messy, time-consuming biochemical assays, at least significantly reduces the amount of them, which will shed insight on the system biologists' approaches, as well as be of importance to the the modeling and control community. The result of the study will advance the understanding of the EGF regulated cell adhesion and benefit the development of therapeutics in cancer treatment with overexpression of EGFR.

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