StarCellBio

StarCellBio Exercise 3 – EGFR Function & Signaling

Goal

In this exercise, you will use StarCellBio, a cell and molecular biology experiment simulator, to better understand the function of the epidermal growth factor receptor (EGFR) in signaling and cancer. To do this you will characterize expression levels, subcellular localization, signaling activity, transmembrane orientation and internalization dynamics of two EGFR mutant proteins using western blotting, flow cytometry, and microscopy.

Learning Objectives

After completing this exercise, you will be able to:

1. Use StarCellBio to perform simulated western blot, microscopy, and flow cytometry experiments.
2. Design and implement experiments in StarCellBio using appropriate experimental conditions and relevant positive and/or negative controls.
3. Analyze the results of several different experimental techniques to determine how a protein's characteristics can be altered by a genetic mutation.
4. Hypothesize how genetic mutations alter protein function by assessing specific results gathered from a variety of experimental techniques.

Accessing StarCellBio

To begin:

1. Using **Google Chrome**, navigate to: http://starcellbio.mit.edu.
2. Sign in to your StarCellBio student account. If you need to set up a student account, use the course code **SCB_SampleExercises**. Note: while you can complete these exercises as a guest by clicking on **Try an Experiment** on the right side of the homepage, your work will not be saved.
3. Select "**Exercise 3**" from the **Assignments** window.

Introduction

You are doing an undergraduate summer research project in a lab that studies the role of the epidermal growth factor receptor (EGFR) in cancer. EGFR is the cell surface receptor for the epidermal growth factor (EGF), a small protein ligand that stimulates cell growth, proliferation, and differentiation (Figure 1). Mutations in EGFR or other proteins in the EGFR pathway often result in abnormal cell growth and proliferation and are associated with various cancers, including some types of lung, breast, and brain tumors.

A graduate student in the lab, who is your mentor for this project, previously identified a series of mutations in the human EGFR gene that affect cell growth through two different genetic approaches. She used site directed mutagenesis to separately introduce two of these specific mutations (M1 and M2) into a human EGFR gene construct, and engineered mouse cell lines to stably express the human EGFR mutant variants. These stably expressing cell lines were created using an EGFR-null mouse cell line, which means that they lack endogenous, wild-type EGFR protein and exclusively express one of the mutated EGFR versions.

Your graduate student mentor first confirms that each of the EGFR mutations she identified confers a growth phenotype by comparing the proliferation rate of mutant EGFRs and wild-type EGFR expressing cell

lines in growth media lacking serum, which contains a combination of essential amino acids and salts, but no growth factors. The following are the results of her proliferation analysis for these two cell lines, EGFR-M1 and EGFR-M2, and the wild-type EGFR expressing cell line, WT-EGFR:

Table 1: Doubling time for cell lines expressing wild-type and mutant EGFR proteins.

Cell line	Average doubling time in serum free media ± standard deviation (hours)
WT-EGFR	32.2 ± 1.5
EGFR-M1	60.5 ± 2.3
EGFR-M2	17.8 ± 0.9

Note: a cell line's "doubling time" is the duration of time that it takes for a starting population of cells in the exponential growth phase to double in number.

The goal of your summer research project is to analyze the effects that the M1 and M2 mutations have on EGFR function.

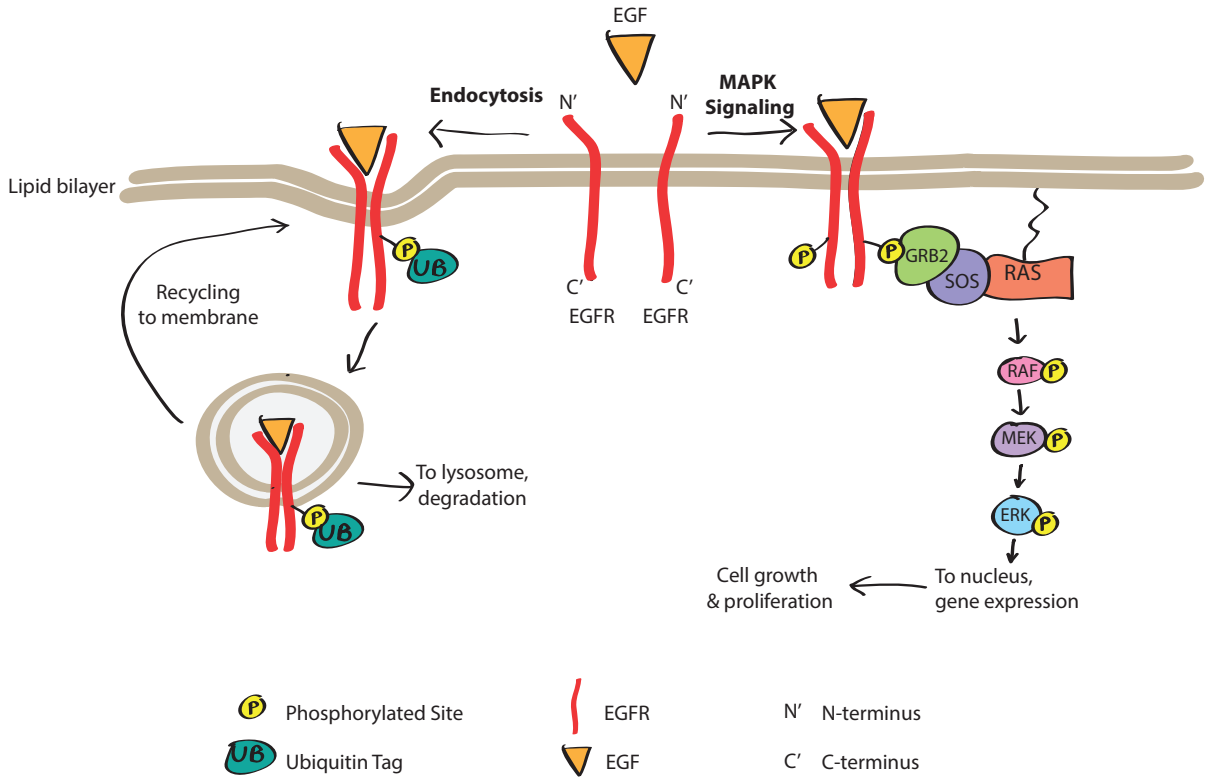

Figure 1. Activation of the EGFR signaling pathway upon EGF binding. Binding of EGF to EGFR results in EGFR dimerization and autophosphorylation (EGFR phosphorylates itself) at various sites on the receptor's intracellular domain. Depending on the specific sites phosphorylated, the downstream effects will be different. Autophosphorylation of specific tyrosine residues results in the activation of the MAPK (MEK/ERK) signaling pathway through the phosphorylation of its downstream components, leading to increased expression of genes that promote cell proliferation and growth. Autophosphorylation of certain tyrosine and serine residues leads to the ubiquitination and internalization of EGFR, followed by either recycling of the receptor back to the cell membrane or lysosome-mediated degradation. EGFR can bind to other ligands besides EGF, and the fate of EGFR after internalization depends on the ligand that induces EGFR signaling, the concentration of ligand present, and the length of time EGFR is stimulated. Internalization and degradation decrease the number of activated receptors on the cell membrane at a given point and attenuate signaling. Several details about EGFR signaling, such as the other pathways, for example the AKT/PKB and STAT5b pathways, which are activated in the presence of EGF, have been omitted in this figure for simplicity.

Your graduate student mentor suggests that you perform experiments to characterize the following properties and functions of the EGFR-M1 and EGFR-M2 mutant proteins:

1. Protein size and relative expression levels
2. Subcellular localization
3. Activation of downstream signaling molecules
4. Orientation within the membrane
5. Internalization/degradation of the receptor upon EGF stimulation

Background Information

Cell Lines

You are provided with the following cell lines:

Name	Description
WT-EGFR	A cell line that expresses the wild-type EGFR protein.
EGFR-Null	A cell line that does not express EGFR protein (neither wild-type or

	mutant).
EGFR-M1	A cell line stably expressing a mutant version of EGFR, EGFR-M1, without endogenous expression of the wild-type protein.
EGFR-M2	A cell line stably expressing a mutant version of EGFR, EGFR-M2, without endogenous expression of the wild-type protein.
NoUB	A cell line stably expressing a mutant version of EGFR where four serine residues that mediate EGFR degradation have been replaced by alanine residues to inhibit degradation upon EGF ligand binding. This cell line does not express the wild-type EGFR protein.
ConstActive	A cell line stably expressing a mutant version of EGFR that is constitutively active and does not require EGF binding to initiate MAPK signaling. In this cell line, the kinase domain of EGFR is always in its 'active' state. However, the mutant protein is internalized and degraded normally upon EGF binding. This cell line does not express wild-type EGFR protein.
His-EGFR-FLAG	A cell line stably expressing an epitope-tagged version of the wild-type EGFR with a 6xHis tag on the N-terminus and a FLAG tag on the C-terminus, without endogenous expression of the wild-type protein.
His-EGFR-M1-FLAG	A cell line stably expressing an epitope-tagged version of the EGFR-M1 protein with a 6xHis tag on the N-terminus and a FLAG tag on the C-terminus, without endogenous expression of the wild-type protein.
His-EGFR-M2-FLAG	A cell line stably expressing an epitope-tagged version of the EGFR-M2 protein with a 6xHis tag on the N-terminus and a FLAG tag on the C-terminus, without endogenous expression of the wild-type protein.

Treatments

You are provided with the following treatment options:

Treatment	Treatment Duration	Description[1]
Growth media only	30 sec	Cells are cultured in serum-free growth media[1] for various periods of time.
	1 min	
	6 hrs	
Growth media + EGF	30 sec	Cells cultured in serum-free growth media[1] are incubated with a high concentration of EGF for 30 seconds, washed to remove EGF, and collected.
	1 min	Cells cultured in serum-free growth media[1] are incubated with a high concentration EGF for 1 minute, washed to remove EGF, and collected.
	6 hrs	Cells cultured in serum-free growth media[1] are incubated with a high concentration EGF for 6 hours, washed to remove EGF, and collected.
Growth media + buffer	30 min	Cells cultured in serum-free growth media[1] are collected. Intact cells are then incubated with Proteinase K buffer only for 30 min.
Growth media + ProK	30 min	Cells cultured in serum-free growth media[1] are collected. Intact cells are then incubated with the Proteinase K (ProK) enzyme for 30 min to digest any extracellular peptides[2].

Western Blotting

You are provided with the following antibodies for western blotting experiments:

Antibody	Description	Expected molecular weight of detected band(s) (kDa)
Mouse anti-EGFR	Primary antibody recognizing both the	120 – 150 → depending on the

	phosphorylated and unphosphorylated forms of EGFR. Note: This antibody recognizes an epitope on the N-terminal end of the protein, and can recognize this epitope in wild-type and mutant forms of EGFR in all cell lines.	extent of phosphorylation and whether the protein is tagged or untagged.
Mouse anti-pEGFR	Primary antibody recognizing EGFR phosphorylated on tyrosine 1088, an indicator that EGFR is 'active'. Note: Tyrosine 1088 is on the C- terminal end of the protein. This antibody can recognize this epitope in wild-type and mutant forms of EGFR in all cell lines.	140 – 150 → depending on the extent of phosphorylation and whether the protein is tagged or untagged.
Mouse anti-pMEK	Primary antibody recognizing the phosphorylated (active) form of MEK.	45
Mouse anti-pRAF	Primary antibody recognizing the phosphorylated (active) form of RAF.	74
Mouse anti-PGK1	Primary antibody recognizing PGK1, a housekeeping protein expressed in all cell types at relatively equal levels.	44
Rabbit anti-6xHis	Primary antibody recognizing the 6xHis epitope tag.	Varies depending on the molecular weight of the 6xHis tagged protein. The 6xHis tag adds about 1 kDa to the molecular weight of the tagged protein.
Rabbit anti-FLAG	Primary antibody recognizing the FLAG epitope tag.	Varies depending on the molecular weight of the FLAG tagged protein. The FLAG tag adds about 1 kDa to the molecular weight of the tagged protein.
Rabbit anti-mouse HRP	Secondary antibody recognizing mouse primary antibodies, conjugated to horseradish peroxidase (HRP)[3].	Varies, depending on primary antibody used.
Goat anti-rabbit HRP	Secondary antibody recognizing rabbit primary antibodies, conjugated to horseradish peroxidase (HRP)[3].	Varies, depending on primary antibody used.

Flow Cytometry

You are provided with the following conditions for flow cytometry experiments:

Antibody conditions	Description
EGFR A488	Incubation with mouse anti-EGFR primary antibody recognizing the EGFR protein, followed by incubation with secondary antibody conjugated to Alexa Fluor 488 fluorophore (green)[4]. Note: The anti-EGFR primary antibody recognizes an epitope on the N-terminal end of the protein, and can recognize the wild-type and mutant forms of EGFR in all cell lines.

Microscopy

You are provided with the following conditions for immunofluorescence microscopy experiments:

Antibody	Description

conditions	
EGFR A488	Incubation with mouse anti-EGFR primary antibody recognizing the EGFR protein, followed by incubation with secondary antibody conjugated to Alexa Fluor 488 fluorophore (green)[4,5]. Note: The anti-EGFR primary antibody recognizes an epitope on the N-terminal end of the protein, and can recognize the wild-type and mutant forms of EGFR in all cell lines.
PM A488	Incubation with primary antibody recognizing LCK, a protein that localizes to the **plasma membrane** (PM), followed by incubation with secondary antibody conjugated to Alexa Fluor 488 fluorophore (green)[4,5].
Cyto A488	Incubation with primary antibody recognizing RPS20, a protein that localizes to the **cytoplasm** (Cyto), followed by incubation with secondary antibody conjugated to Alexa Fluor 488 fluorophore (green)[4,5].
Nuc A488	Incubation with primary antibody recognizing histone H2B, a protein that localizes to the **nucleus** (Nuc), followed by incubation with secondary antibody conjugated to Alexa Fluor 488 fluorophore (green)[4,5].
ER A488	Incubation with primary antibody recognizing calnexin, a protein that localizes to the **endoplasmic reticulum** (ER), followed by incubation with secondary antibody conjugated to Alexa Fluor 488 fluorophore (green)[4,5].
NM A488	Incubation with primary antibody recognizing lamin B1, a protein that localizes to the **nuclear membrane** (NM), followed by incubation with secondary antibody conjugated to Alexa Fluor 488 fluorophore (green)[4,5].

Notes:

[1] Serum-free growth media does not contain growth factors, including EGF.

[2] Proteinase K is an enzyme that digests proteins. It cannot penetrate the plasma membrane when the plasma membrane is intact, which means that the cell membrane has not been disrupted or permeabilized. As a result, incubating intact cells with Proteinase K results in the digestion of extracellular peptides only.

[3] These secondary antibodies are conjugated to horseradish peroxidase (HRP). Horseradish peroxidase catalyzes a reaction that produces light as a by-product, which is detected using photographic film

[4] The secondary antibodies used for these experiments are conjugated to fluorescent molecules, or fluorophores. In this case, the fluorophore is Alexa Fluor 488 (A488), which fluoresces or emits green light when excited by light with a wavelength of 488 nm.

[5] When the green channel of the microscope is chosen, the fluorescence emitted by a sample is captured as an image taken with a black and white camera.

Question 1

As a first step, your graduate student mentor advises you to look at whether the M1 and M2 mutations in the EGFR gene affect the expression levels and/or size of the corresponding EGFR-M1 and EGFR-M2 proteins.

To do this, you perform a western blotting experiment to analyze the protein expression levels and molecular weight of the mutant EGFR-M1 and -M2 proteins in the **absence** of EGF (Growth media only treatment), making sure to include any relevant controls and using the appropriate primary and secondary antibody combinations. To arrive at your answer, you do not need to use all available cell lines, treatments, and antibodies, only those relevant to this experiment.

a. How does the molecular weight of the EGFR-M1 and EGFR-M2 proteins compare with the wild-type EGFR protein? Explain how you arrived at your answer using your experimental results.

b. How does the expression of the EGFR-M1 and EGFR-M2 proteins compare to that of wild-type EGFR? Explain how you arrived at your answer using your experimental results.

c. Can the growth phenotypes observed in EGFR-M1 and EGFR-M2 expressing cells (Table 1) be explained by increased or decreased levels of mutant EGFR transcription or translation rates? Explain your answer using your experimental results.

Question 2

To further characterize the mutant cell lines, your mentor suggests you determine whether the mutant EGFR proteins localize correctly to the plasma membrane since the correct localization of the receptor is essential for EGF binding and proper EGFR signaling.

To determine if the EGFR-M1 and EGFR-M2 proteins localize to the plasma membrane, you perform an immunofluorescence (IF) microscopy experiment, making sure to include any relevant controls. First, you fix and permeabilize cells mounted on microscope slides to allow antibodies to cross the plasma membrane and have access to intracellular components. Then, you incubate them with the appropriate primary and secondary antibody combinations. To arrive at your answer, you do not need to use all available cell lines, treatments, and antibodies, only those relevant to this experiment.

a. What are the subcellular localizations of the EGFR-M1 and EGFR-M2 proteins? Justify your answer using your experimental results.

b. Does the localization of either mutant EGFR protein differ from that of wild-type protein?

Question 3

Next you would like to examine MAPK signaling in EGFR-M1 and EGFR-M2 expressing cells upon EGF stimulation.

To do this, you perform a western blotting experiment to examine the expression and activation of proteins in the MAPK signaling pathway following EGF stimulation (see Figure 1), making sure to include any relevant controls. Your laboratory has antibodies against several MAPK signaling components, including: 1) phosphorylated (active) EGFR, 2) phosphorylated (active) RAF, and 3) phosphorylated (active) MEK. To arrive at your answer, you do not need to use all available cell lines, treatments, and antibodies, only those relevant to this experiment.

a. How does EGF-induced MAPK signaling in the EGFR-M1 cell line compare with the WT-EGFR cell line? Explain using your experimental results. If there are differences, include which signaling components are different and how.

b. How does EGF-induced MAPK signaling in the EGFR-M2 cell line compare with the WT-EGFR cell line? Explain using your experimental results. If there are differences, include which signaling components are different and how.

**** Answer Questions 4 and 5 for any mutant EGFR proteins that localize to the plasma membrane. For proteins that are not localized to the plasma membrane, skip to Question 6. ****

Question 4

The orientation of EGFR is important for its proper functioning and activation of downstream signaling pathways. EGFR is a single-pass transmembrane protein with the N-terminus, responsible for ligand binding (EGF), outside the cell and the C-terminus, responsible for initiating signaling, inside the cell. Your graduate student mentor advises you to perform a western blot experiment to determine the orientation of the mutant EGFR proteins that localize to the plasma membrane.

Before performing the western blotting procedure, you collect His-EGFR-M1-FLAG and/or His-EGFR-M2-FLAG cells and incubate intact cells with Proteinase K (ProK), an enzyme that digests proteins. Because Proteinase K cannot penetrate the membrane of intact, non-permeablized cells, only intracellular proteins and peptides will remain after incubation with Proteinase K. After Proteinase K digestion, you lyse the cells and perform a western blot analysis to determine the presence or absence of each epitope tag using the appropriate primary and secondary antibody combinations, while ensuring to include any relevant controls. To arrive at your answer, you do not need to use all available cell lines, treatments, and antibodies, only those relevant to this experiment.

a. What is the orientation of the mutant EGFR protein(s) in the plasma membrane with respect to the N- and C-terminus? Justify your answer using your experimental results.

b. Does the orientation of the mutant EGFR protein(s) differ from that of the wild-type protein? If so, how? Explain.

Question 5

Internalization and degradation of EGFR initiated by EGF binding is an important mode of regulation and is essential for proper signaling (Figure 1). The fate of EGFR after internalization (whether it is recycled back to the membrane or degraded) depends on the ligand that induces EGFR signaling, the concentration of ligand present, and the length of time EGFR is stimulated. For example, the presence of high concentrations of EGF for prolonged periods of time (i.e. > 60 min) leads to ubiquitination of EGFR's intracellular domain, which in turn results in EGFR internalization and lysosome-mediated degradation.

To examine the internalization dynamics of EGFR-M1 and/or EGFR-M2, you perform flow cytometry experiments to measure the amount of mutant EGFR protein on the cell surface following stimulation with EGF, making sure to include any relevant controls. Prior to flow cytometry, you incubate live cells with EGF for different time periods. After EGF stimulation you collect and fix intact cells for flow cytometry analysis, followed by incubation with anti-EGFR primary antibody and the appropriate fluorescently-labeled secondary antibody. The primary antibody used in this experiment (mouse anti-EGFR) recognizes an epitope on the N-terminal end of the EGFR protein (note that the N-terminal end of WT-EGFR is extracellular). Since antibodies cannot cross the plasma membrane of intact, non-permeabilized cells, binding to the N-terminal EGFR epitope by the anti-EGFR antibody will only occur for receptors with an extracellular N-terminal domain that have not been internalized. To arrive at your answer, you do not need to use all available cell lines, treatments, and antibodies, only those relevant to this experiment.

Note: Assume that the conditions of the experiment, including EGF concentration and incubation time, result in most of the internalized EGFR being trafficked to the lysosome-mediated degradation pathway rather than being recycled to the membrane. Therefore, the rate of internalization will give you a good estimate of the rate of degradation in these mutants.

a. Do the internalization dynamics of the mutant EGFR protein(s) differ from that of the wild-type protein? Justify your answer using your experimental results.

b. After incubating cells with high concentrations of EGF for 6 hours, would you expect the EGFR-M1 and/or EGFR-2 cell lines to show higher, lower, or similar levels of EGFR degradation compared to the WT-EGFR cell line? What experiment(s) could you perform with the provided reagents to test your hypotheses? Explain.

Question 6

a. Using all of your experimental results and conclusions from Questions 1-5, propose a hypothesis that explains how the EGFR-M1 and EGFR-M2 mutations result in the abnormal growth phenotypes observed in the EGFR-M1 and EGFR-M2 expressing cell lines, respectively (Table 1). Make sure to include the results that allowed you to arrive at your conclusion.

i. EGFR-M1

ii. EGFR-M2

b. A member of your lab studying the same mutant cell lines discovers that when EGF is added to the EGFR-M2 cell line at high concentrations for a prolonged period of time, the levels of ubiquitinated EGFR-M2 protein are surprisingly low.

Does this observation lend support to your hypothesis regarding EGFR-M2 in Question 6a.ii? Explain why or why not. If necessary, use Figure 1 to help you answer this question.