CELL DENSITY AFFECTS ERK SIGNALING HETEROGENEITY

Yen-Der Li

School of Medicine and Department of Physics

National Taiwan University

Outline

Project Summary

Project Summary

Aim:

- To investigate the underlying mechanism leading to cell-to-cell variability of ERK signaling in mammalian cells

Method:

- High-throughput single-cell imaging + computational analysis
- Density-dependent culture experiment
- Conditioned media culture experiment

Result:

- Confirmed higher ERK activation in high density cells
- Found more EGFR degradation (ERK upstream) in high density cells
- Discovered high density conditioned media enhance single cell ERK activation, which suggests a paracrine mechanism

Introduction

Project scope

- Investigate the underlying mechanism leading to cell-to-cell variability of ERK signaling in mammalian cells (use Swiss 3T3 fibroblast cell as a model).

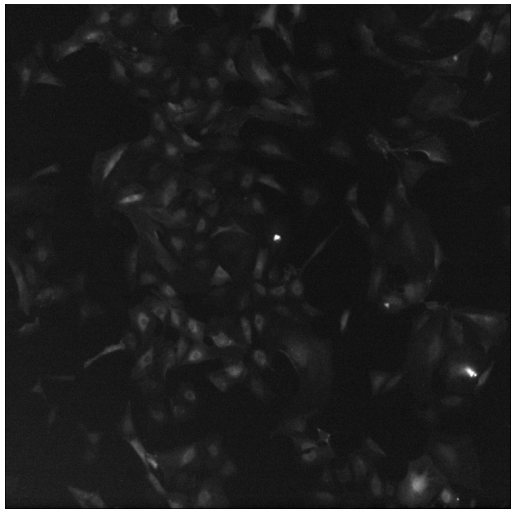
Project background

- Cellular heterogeneity of ERK signaling can lead to entirely different cell fate such as proliferation, differentiation and apoptosis among genetically identical cells.
- However, the mechanism of this heterogeneity cannot be explained by intrinsic stochastic noise alone and sources of extrinsic deterministic noise remain elusive.

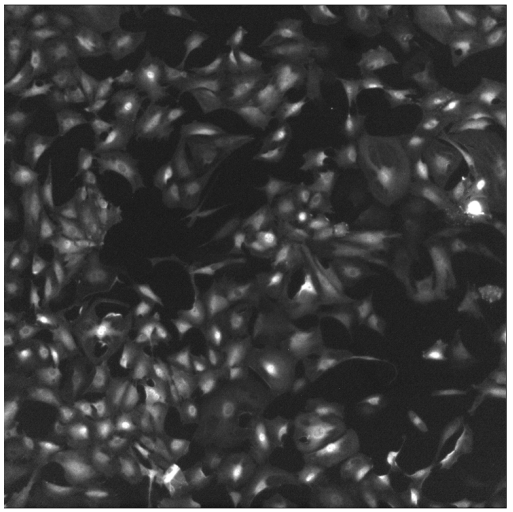
Preliminary data

- In Yangqing's previous study, he found that the activation of MAPK (ERK) in a few mammalian cell types exhibit heterogeneity, sometimes bimodality. However, the source of this signaling heterogeneity remains elusive.

Low single-cell ERK response



High single-cell ERK response



Our hypothesis

- It has been reported that the variability of viral infection is largely determined by micro-environmental context such as cell density and adhesion (see below source).
- Therefore, we hypothesized that cell density affects the heterogeneity of ERK signaling response.
- We focus on cell density rather than cell adhesion first due to its more obvious relationship with ERK signaling observed in preliminary data.

Source: Population context determines cell-to-cell variability in endocytosis and virus infection. *Nature*. (2009)

Our hypothesis

- To deep dive into the molecular mechanism of cell density-dependent ERK signaling heterogeneity, we hypothesized that cell density will affect ERK signaling through paracrine signaling, which can be tested through conditioned media experiment and proteomic screening.

Project aim and experiment design

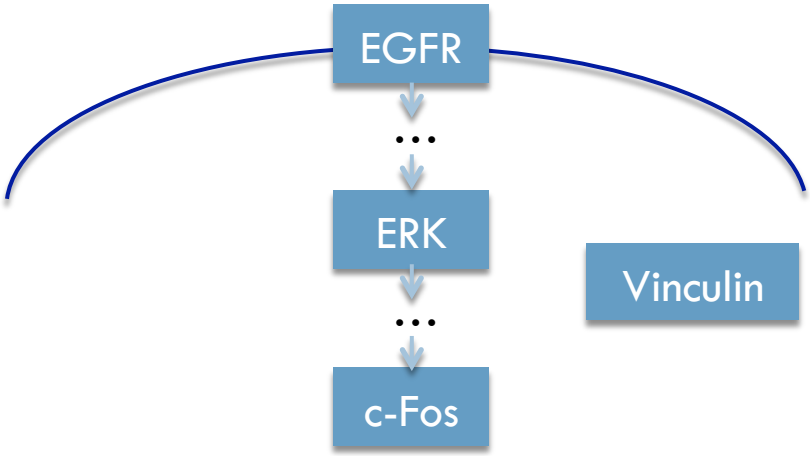
Aim 1: Signaling heterogeneity of ERK:

- Evaluate quantitatively how cell density affects the heterogeneity of ERK signaling response

Project aim and experiment design

Aim 2: Signaling Heterogeneity of ERK upstream and downstream:

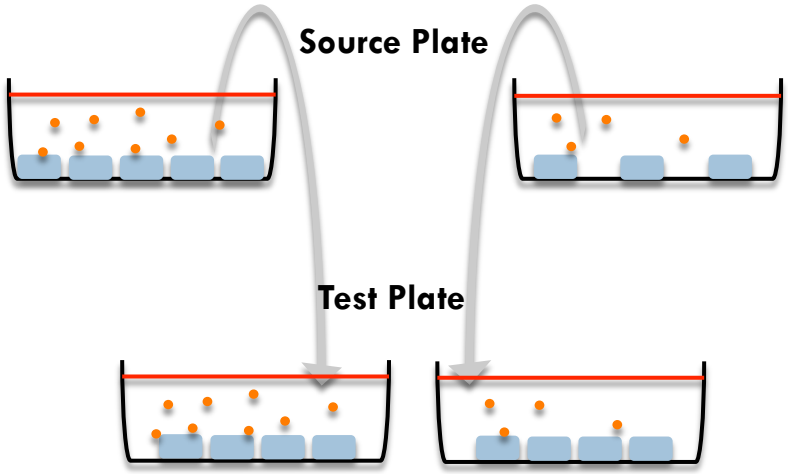
- Evaluate how cell density affects the upstream and downstream of ERK, which may help to elucidate further details of the signaling heterogeneity mechanism



Project aim and experiment design

Aim 3: Molecular mechanism of density-dependent ERK signaling heterogeneity:

- Investigate the molecular mechanism how cell density lead to ERK signaling heterogeneity by using conditioned media and proteomic screening experiments (proteomic screening not done)



Result

Result

- Signaling heterogeneity of ERK
  - Density – ERK exp.
- Signaling heterogeneity of ERK upstream and downstream
  - Density - EGFR endocytosis exp.
  - c-FOS kinetics exp.
- Molecular mechanism of density-dependent ERK signaling heterogeneity
  - Density culture media – ERK exp.
  - Density starvation media – ERK exp.

Signaling heterogeneity of ERK

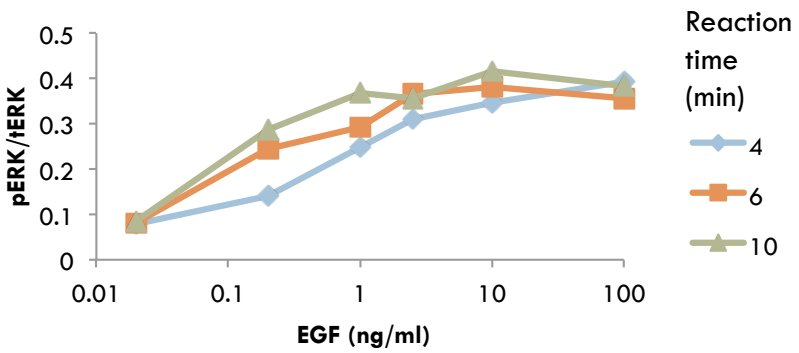
Density – ERK exp.

Result

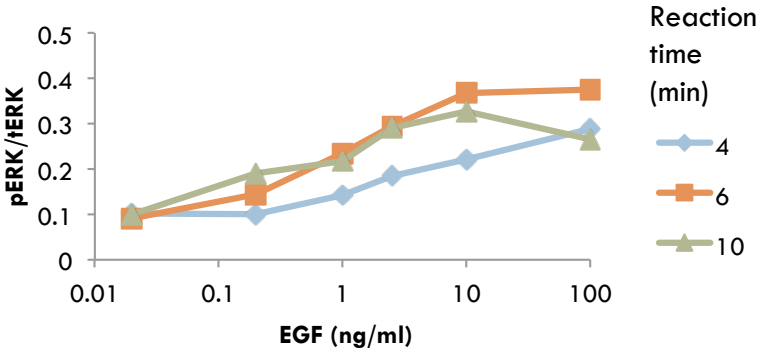
In high density group

- Smaller amount of EGF stimuli can reach the same ERK response
- The maximal ERK response is also higher

High Density ($2*10^4$ cells/ml)



Low Density ($3.3*10^3$ cells/ml)



High density cells have higher single-cell ERK response

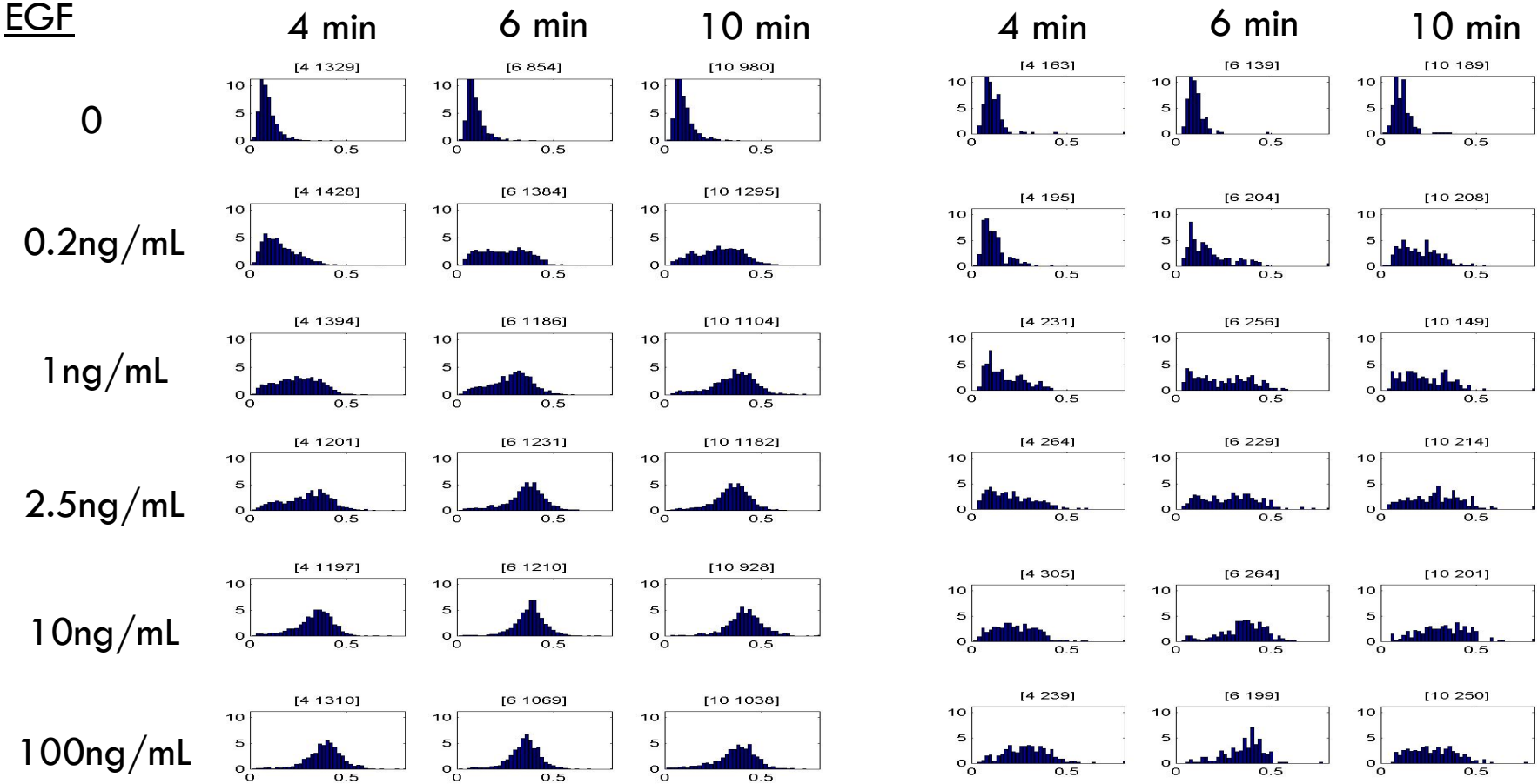
Density – ERK exp.

High Density (2 x 10^4 cells/ml)

Low Density (3.3 x 10^3 cells/ml)

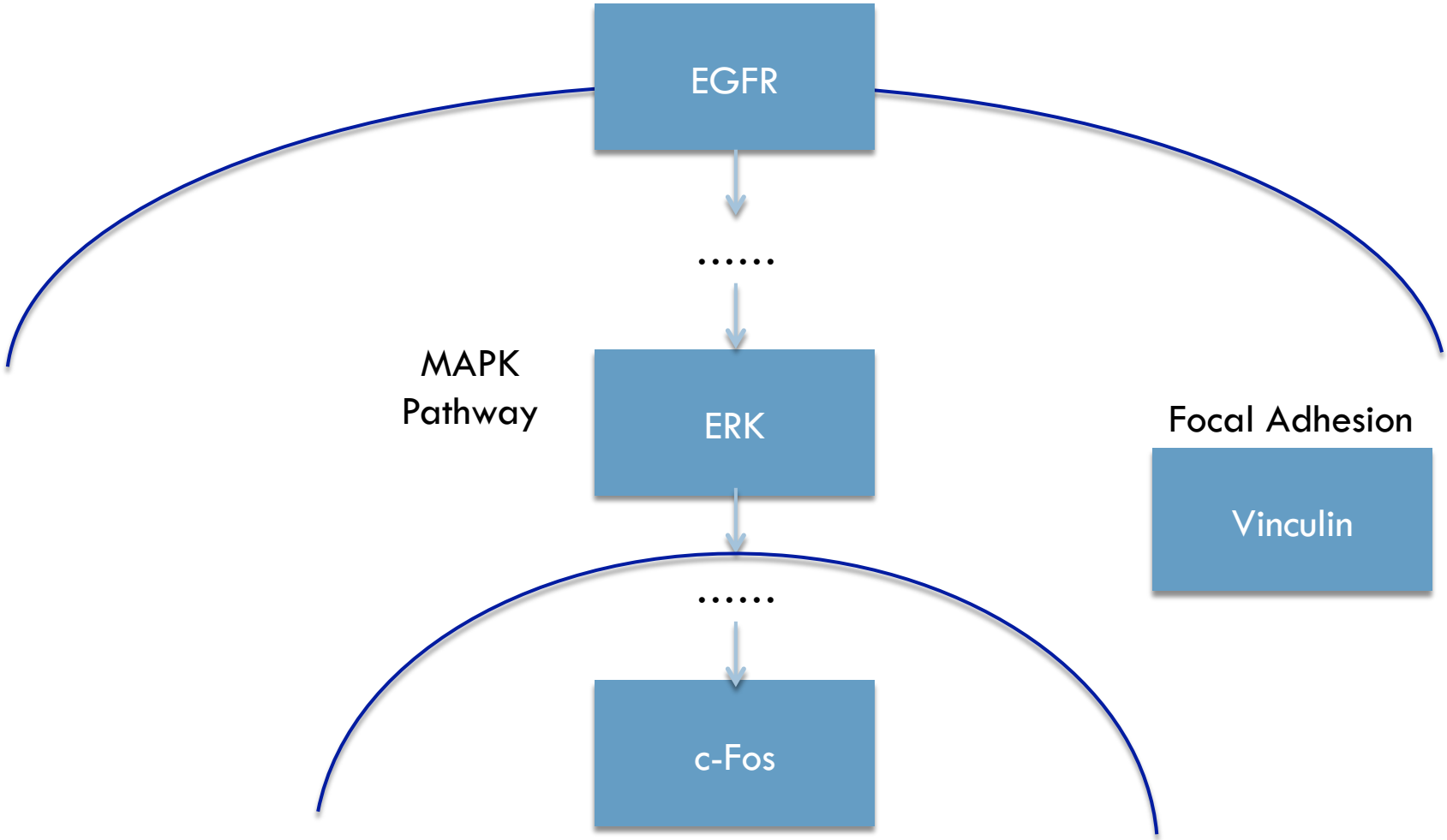
Reaction Time

EGF



Signaling heterogeneity of ERK upstream and downstream

Signaling Heterogeneity of ERK upstream and downstream



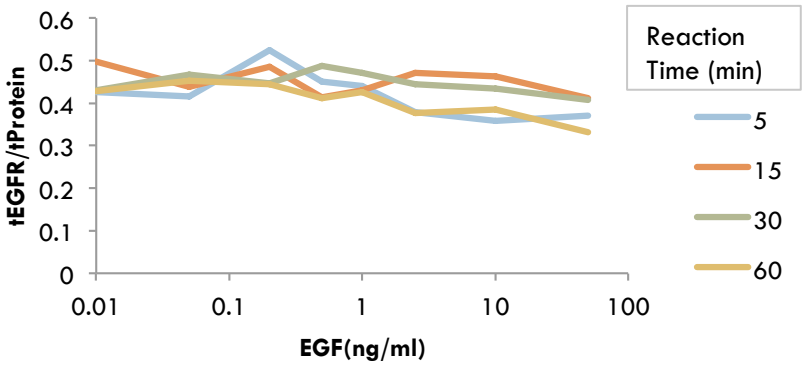
Density - EGFR endocytosis exp.

Result

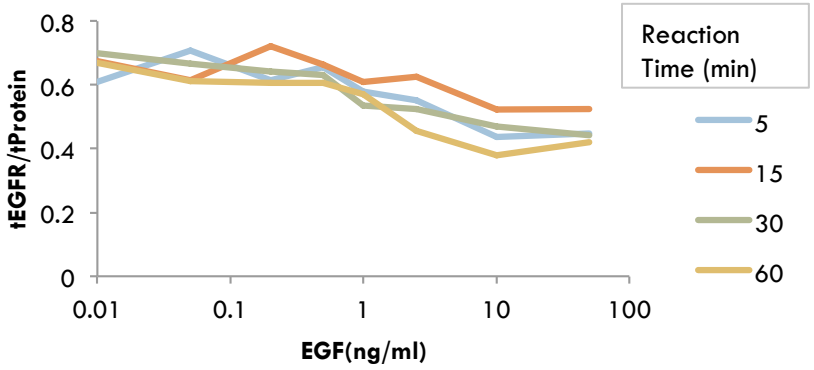
In high density group

- Their EGFR degrades more
- This indicates EGFR may be more activated

Low Density (10^4 cells/ml)



High Density (3 x 10^4 cells/ml)



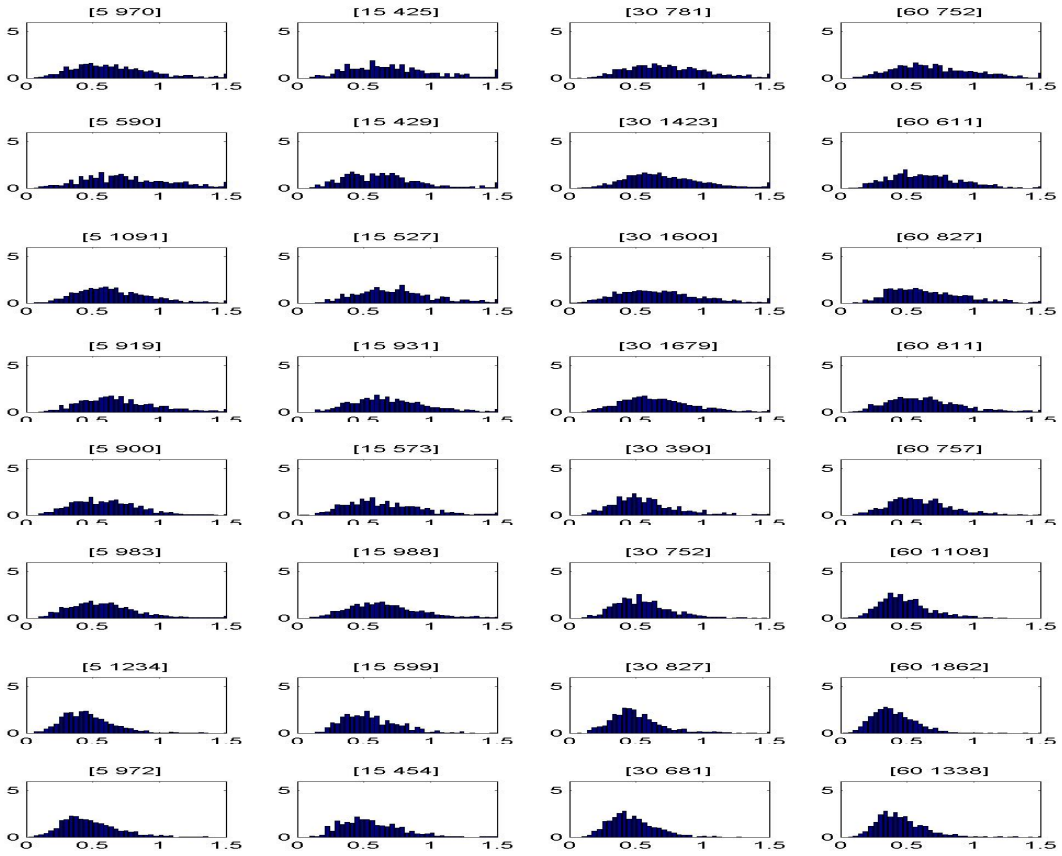
High density cells have higher EGFR degradation

Density - EGFR endocytosis exp.

High Density (3 x 10^4 cells/ml)

Reaction Time

EGF	5 min	15 min	30 min	60 min
0	[5 970]	[15 425]	[30 781]	[60 752]
0.05ng/mL	[5 590]	[15 429]	[30 1423]	[60 611]
0.2ng/mL	[5 1091]	[15 527]	[30 1600]	[60 827]
0.5ng/mL	[5 919]	[15 931]	[30 1679]	[60 811]
1ng/mL	[5 900]	[15 573]	[30 390]	[60 757]
2.5ng/mL	[5 983]	[15 988]	[30 752]	[60 1108]
10ng/mL	[5 1234]	[15 599]	[30 827]	[60 1862]
50ng/mL	[5 972]	[15 454]	[30 681]	[60 1338]



c-FOS kinetics exp.

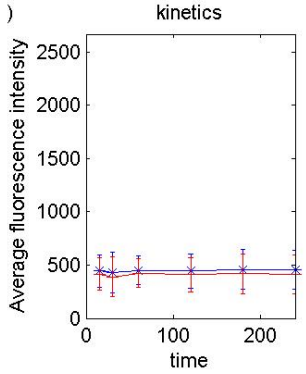
Result

In different EGF concentration stimuli

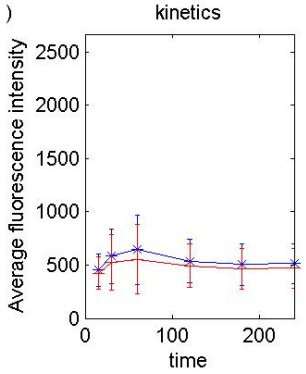
- c-FOS increases with stimulation time, and peaks at 1hr
- c-FOS is then turned off after 1hr

EGF

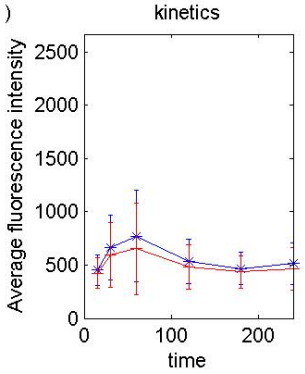
0



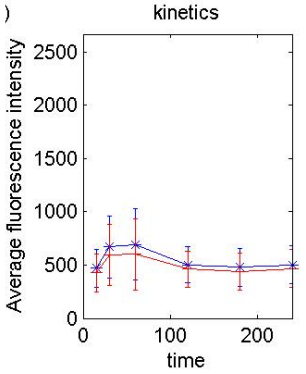
1ng/mL



5ng/mL



50ng/mL



Time Point : 5min, 30min, 1hr, 2hr, 3hr, 4hr

Density (2 x 10^4 cells/ml)

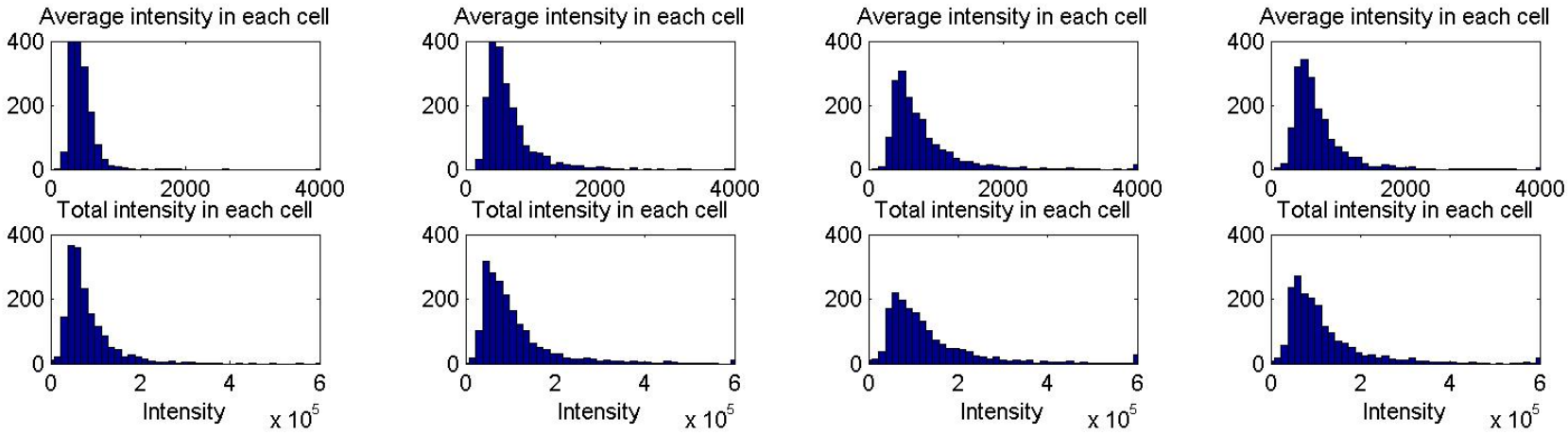
c-FOS expression peaks at 1hr after EGF stimulation

c-FOS kinetics exp.

Time Point : 1hr

Density (2 x 10^4 cells/ml)

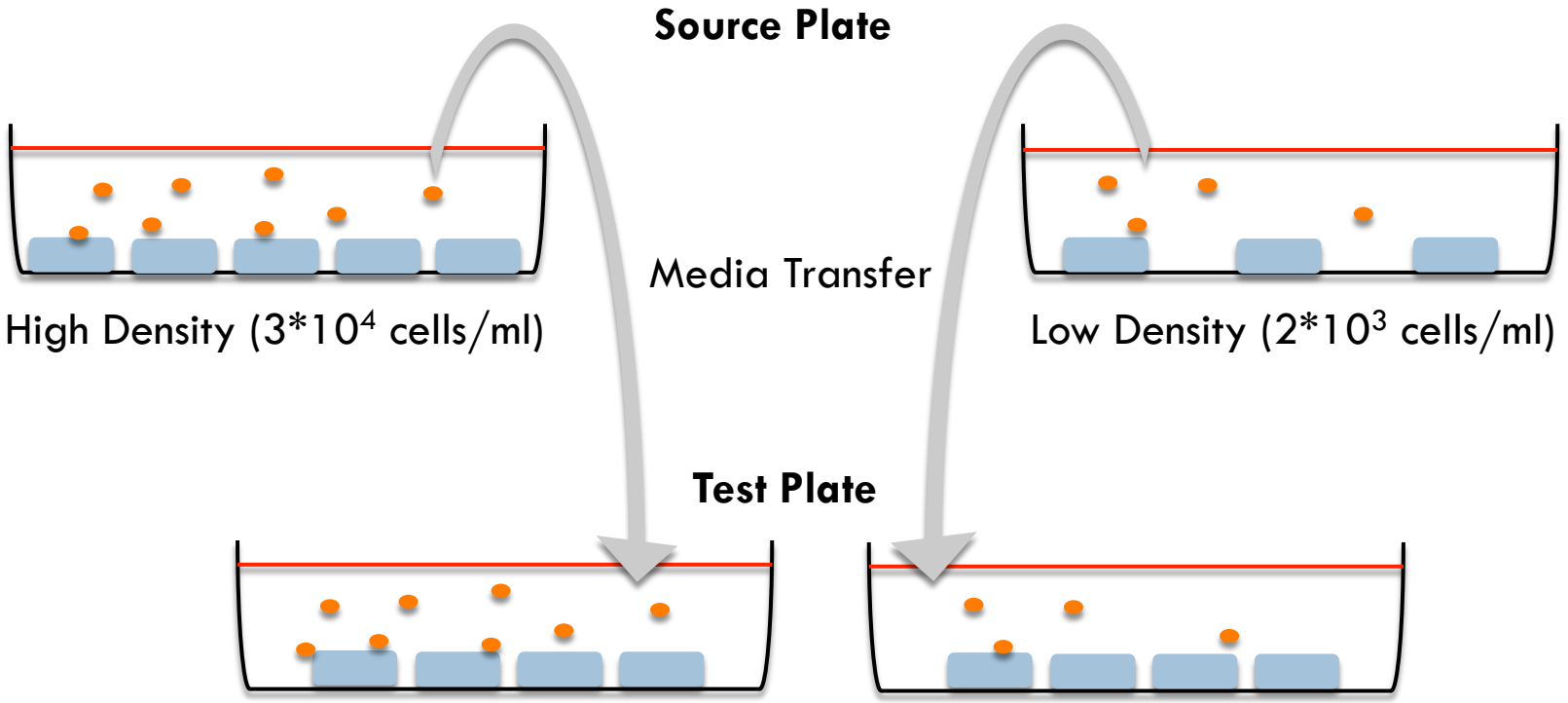
EGF



Molecular mechanism of density-dependent ERK signaling heterogeneity

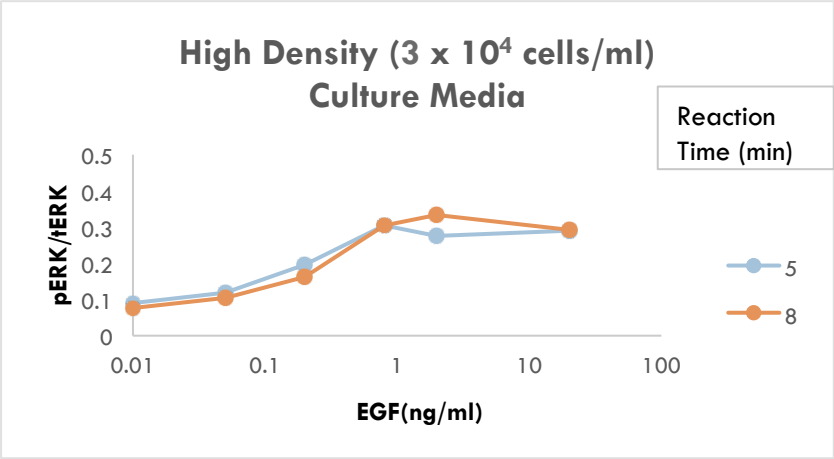
Density conditioned media

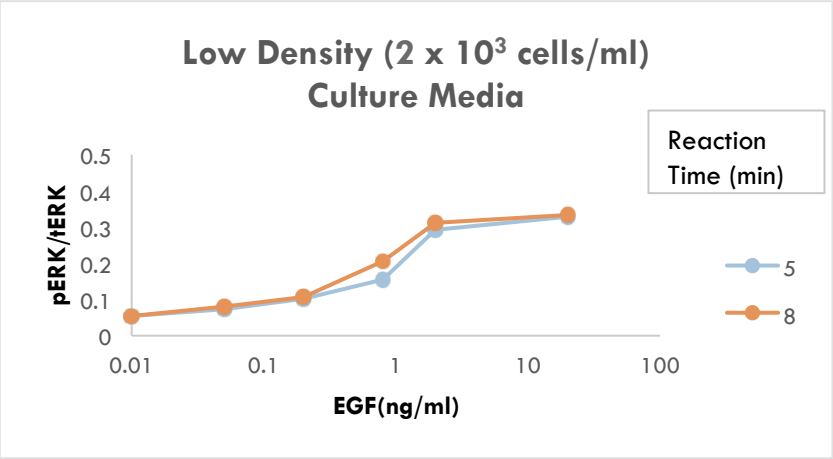
Growth factors in the media?



Density conditioned culture media – ERK exp.

The ERK response difference between different density conditioned **culture media**



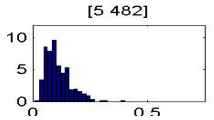
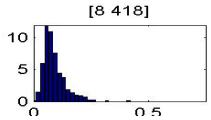
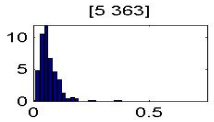
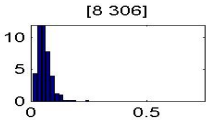
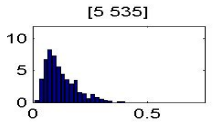
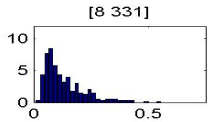
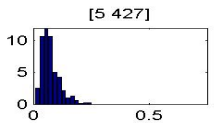
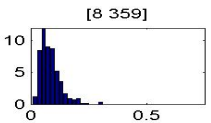
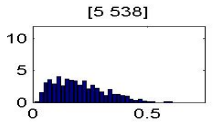
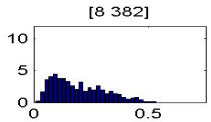
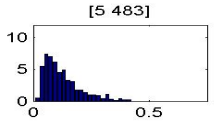
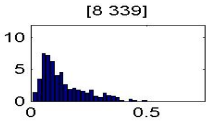
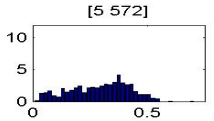
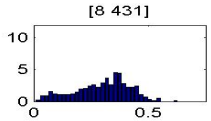
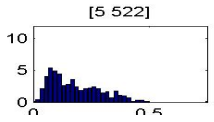
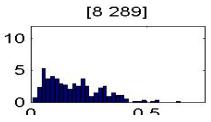
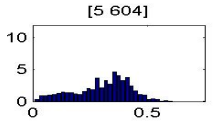
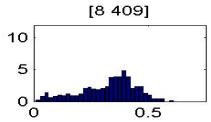
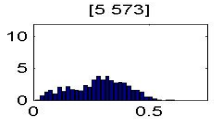
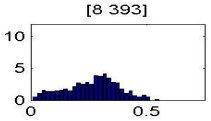
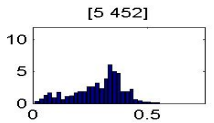
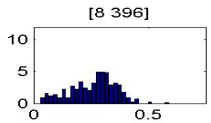
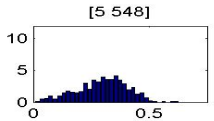
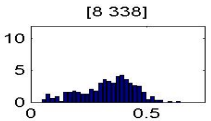


High density conditioned culture media group have higher single-cell ERK response after EGF stimulation

Density conditioned culture media – ERK exp.

Source Plate Density | High (3 x 10^4 cells/ml) | Low (2 x 10^3 cells/ml)

Reaction Time

EGF	High: 5 min	High: 8 min	Low: 5 min	Low: 8 min
0				
0.05ng/mL				
0.2ng/mL				
0.8ng/mL				
2ng/mL				
20ng/mL				

Culture Media

In source plate: 24hr

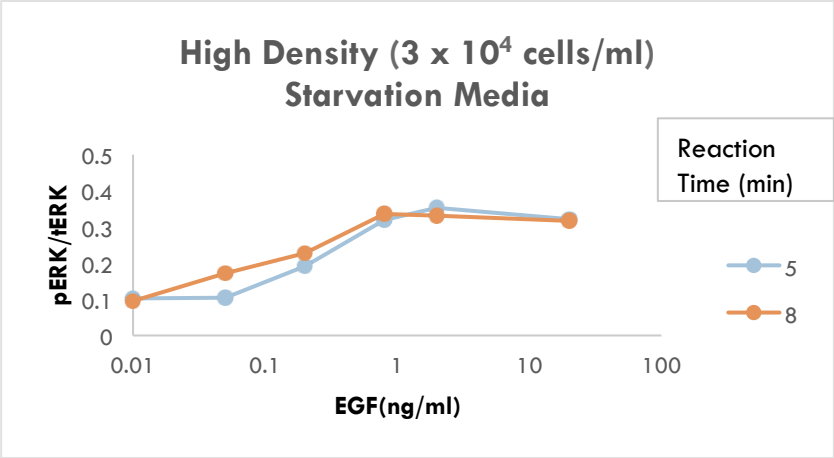
In test plate: 24hr

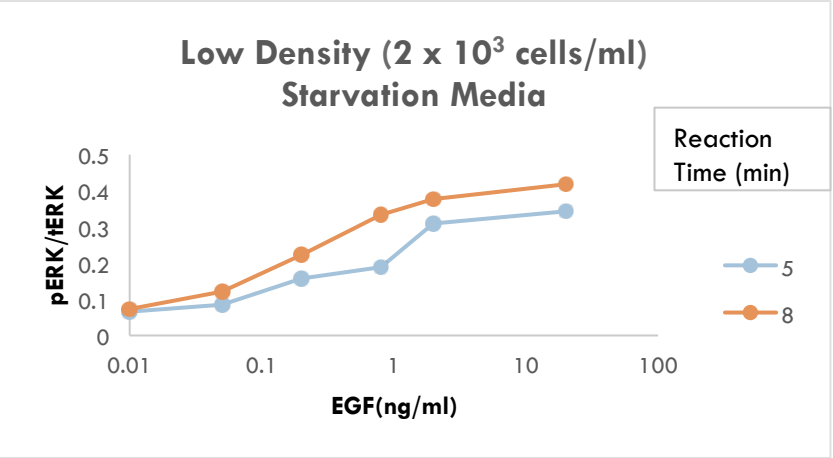
Test Plate Density

10^4/well

Density conditioned starvation media – ERK exp.

The ERK response difference between different density conditioned **starvation media**



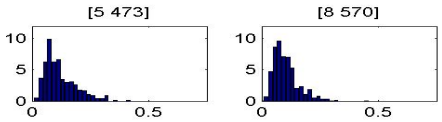
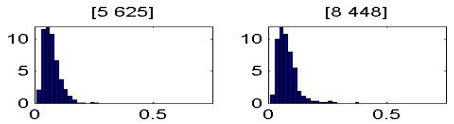
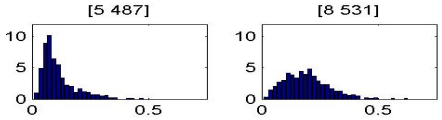
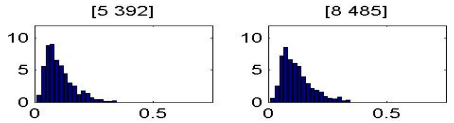
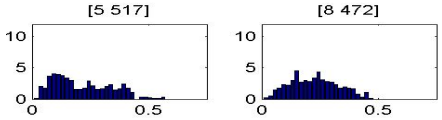
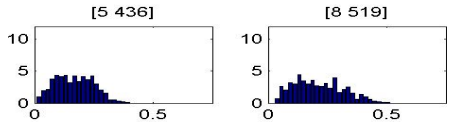
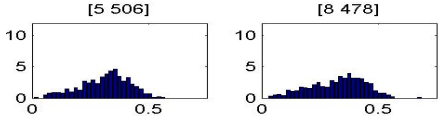
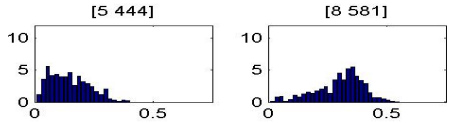
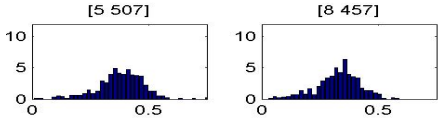
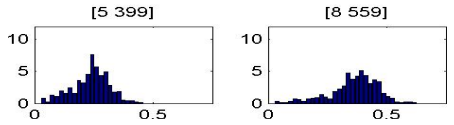
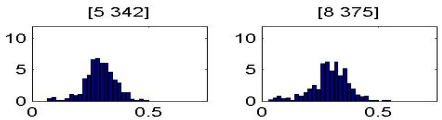
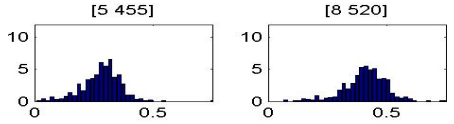


High density conditioned starvation media group have higher single-cell ERK response after EGF stimulation

Density conditioned starvation media – ERK exp.

Source Plate Density | High (3 x 10^4 cells/ml) | Low (2 x 10^3 cells/ml)

Reaction Time

EGF	5 min	8 min	5 min	8 min
0				
0.05ng/mL				
0.2ng/mL				
0.8ng/mL				
2ng/mL				
20ng/mL				

Starvation Media

In source plate: 24hr

In test plate: 2hr

Test Plate Density

10^4/well

Discussion

Aim 1: Signaling heterogeneity of ERK

- Our result validates Yangqing's preliminary data that the high density Swiss 3T3 cells have higher ERK signaling response compared with the low density group.

Aim 2: Signaling Heterogeneity of ERK upstream and downstream

- We investigate the upstream (EGFR) and downstream (c-Fos) of ERK signaling pathway.
- We see high density group also shows higher EGFR degradation, which is consistent with the more activated ERK signaling.
- In c-Fos experiment, however, we didn't observe expression difference between high density and low density group. But we have characterized c-Fos kinetics under our experiment condition, which is also important if we want to further optimize our c-Fos experiments.

Aim 3: Molecular mechanism of density-dependent ERK signaling heterogeneity

- The high density conditioned media groups show higher ERK response in both conditioned culture media and conditioned starvation media experiments. This implies that the high density cells are modifying its populational ERK response through conditioned media.

Aim 3: Molecular mechanism of density-dependent ERK signaling heterogeneity

- In addition, the conditioned culture media experiment shows more obvious difference between the high density conditioned group and low density conditioned group compared with its counterpart in the conditioned starvation media. The reasons may result from the difference in incubation period of conditioned media (conditioned culture: 24hr, conditioned starvation: 2hr).
- This result also aligns with our previous interpretation that high density cells are modifying its populational ERK response through conditioned media, and also implies that the ERK signaling response is related to the exposure level to conditioned media.

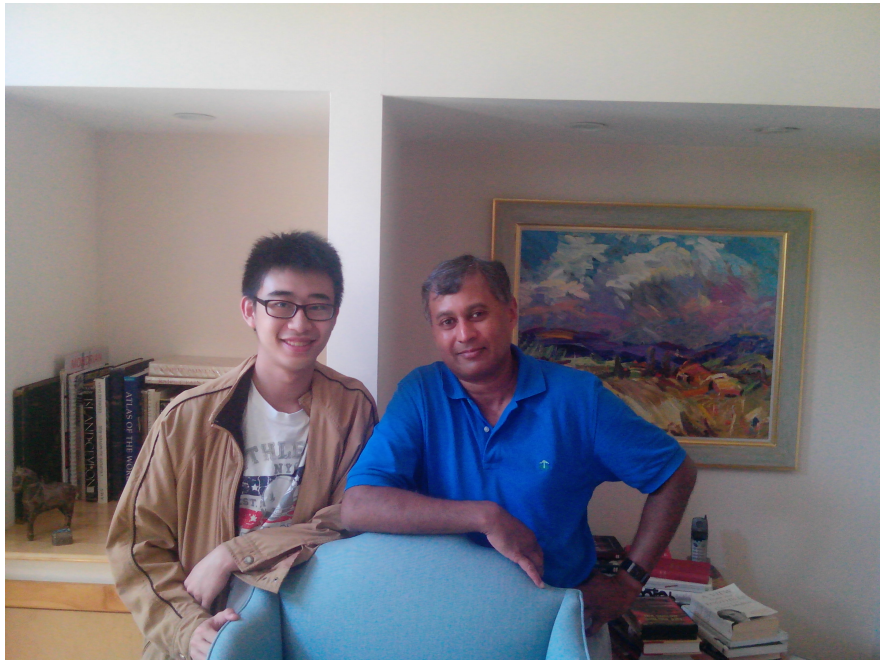
Aim 3: Molecular mechanism of density-dependent ERK signaling heterogeneity

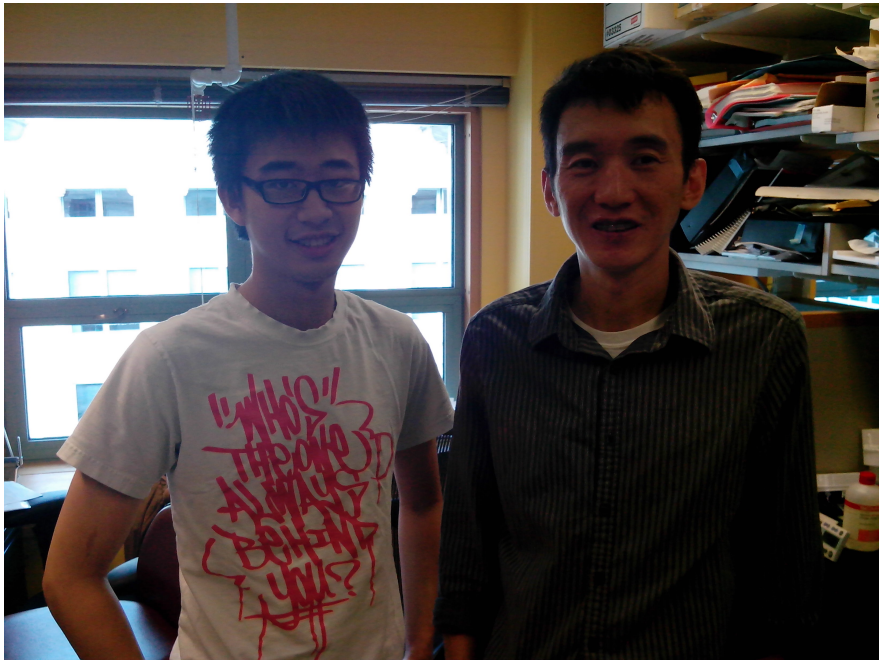
- Hence, the conditioned media experiment result strengthens our hypothesis that cell density are affecting ERK signaling heterogeneity through paracrine signaling. In order to determine what is the paracrine mechanism at molecular level, we need to identify which molecule induce the modification of single-cell ERK signaling. One method to achieve this goal is through proteomic screening, but we don't have time to proceed the study to this stage.

Acknowledgement

Acknowledgement

Much appreciation to Dr. Jeremy Gunawardena for giving me the opportunity to be here and to Dr. Yangqing Xu for the excellent mentorship on the project





Backup

Method

Day 1

- Plate Cells

Day 2

- Starvation

Day 3

- Stimulation
- Fixation

- Immunofluorescence
- Image Analysis

Method

Plating and Stimulating Protocol

- Day 1
  - Plate cells
  - 2 x 10^4 cells/ml x 150 ul per well in 96-well plate unless otherwise indicated
- Day 2
  - Starve with 300 ul of starvation medium (0.25% Bovine Calf Serum)
- Day 3
  - Stimulate cells with serial dilutions of EGF for several time points.
  - Fix the cells with 3.7% Formaldehyde. Wash with PBS and store the sample in 100 ul of PBS.

Method

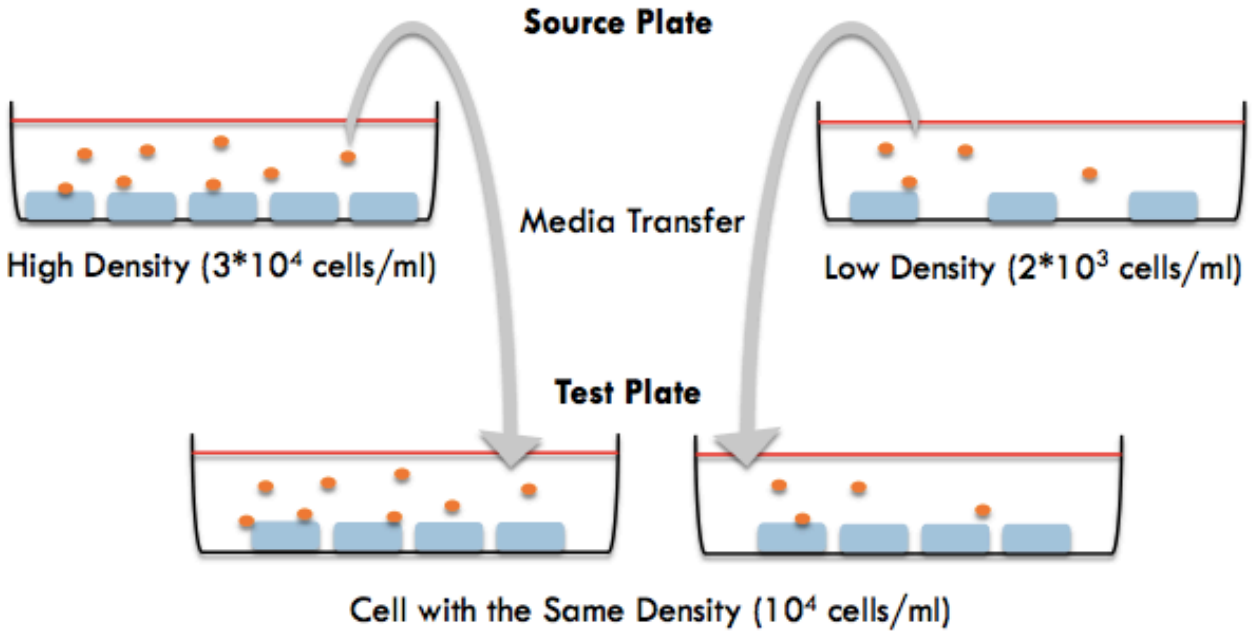
Immunofluorescence Protocol

- Permeabilize the cells with 0.2% Triton for 5 min
- Block the cells with 3% BSA for 30 min
- Primarily staining with desired antibodies in typically 60 ul of reaction buffer (PBS + 0.3% Triton + 1.5% BSA). 2-4 hours at RT
- Wash with 125 ul of 0.5% Triton 3 x 5min.
- Secondary staining in reaction buffer (normally 60 ul of 1 ug/ml of each antibody) x 1-2 hr.
- Wash with 125 ul of 0.5% Triton 3 x 5min.
- DNA-counter-staining by Hoechst 1:6000 x 5 min.
- Wash with 200 ul of PBS twice. Store in 100 ul of PBS.

Method

Protocol for conditioned media exp. (From Yangqing)

- First of all, prepare starvation media for 1.5 plate. (0.25% serum and 250 ul media each well, for about 150 wells, so about 40 ml of media)



Method

- Source plate
  - Pipette out 150 ul of media from all rows of the source plate. Note, media from the left and the right side of the plate, i.e., low-density and high-density cell-conditioned culture media, must be handled separately and put in different container. (Do NOT mix them!) You should get about 7.5 ml of conditioned media from each density of cells. Try not to touch the bottom of plate to avoid lifting cells directly.
  - Dump the rest of the media on paper towel.
  - Replenish with 250 ul of starvation media in each well. You should still have about 15 ml of starvation media left at this point.
  - Centrifudge down the conditioned culture media at 300 g x 5 min. The two tubes should be placed symmetrically to balance the rotor.

Method

- Test plate
  - Pipette starvation media and the two conditioned culture media in separate reservoirs. So now you have three reservoirs of media ready, with 15 ml of starvation media or ~ 7 ml of conditioned media.
  - Dump the media on paper towel.
  - Replenish with 250 ul of starvation media for the top half of the plate (Row 1-4). You would need about 12 ml.
  - Replenish with 250 ul of conditioned culture media for the bottom half of the plate (Row 5-8). You would need about 6 ml for each side of the plate.
- Note

  Always have media ready in reservoir and tips ready on the multichannel pipette before you dump the plate empty. Minimize the time that the plate is dry.

Experiment Timeline

- ☑ Density – ERK exp. (07/08-07/12)
- ☐ pEGFR antibody characterization (07/08-07/12)
- ☐ Density media – ERK exp. (07/15-07/22)
- ☑ pERK antibody characterization (07/22-07/25)
- ☐ Density – c-FOS exp. (07/24-07/30)
- ☑ Density – EGFR endocytosis exp. (07/24-08/06)
- ☐ Density – Vinculin, c-FOS exp. (07/29-08/07)
- ☑ Density media – ERK exp. 2 (08/07-08/12)
- ☑ c-FOS kinetics (08/12-08/15)
- ☐ c-FOS antibody characterization (08/12-08/15)
- ☐ Density – EGFR endocytosis exp. 2 (08/14-08/21)
- ☐ Density – c-FOS exp. 2 (08/19-08/22)

Antibody Characterization

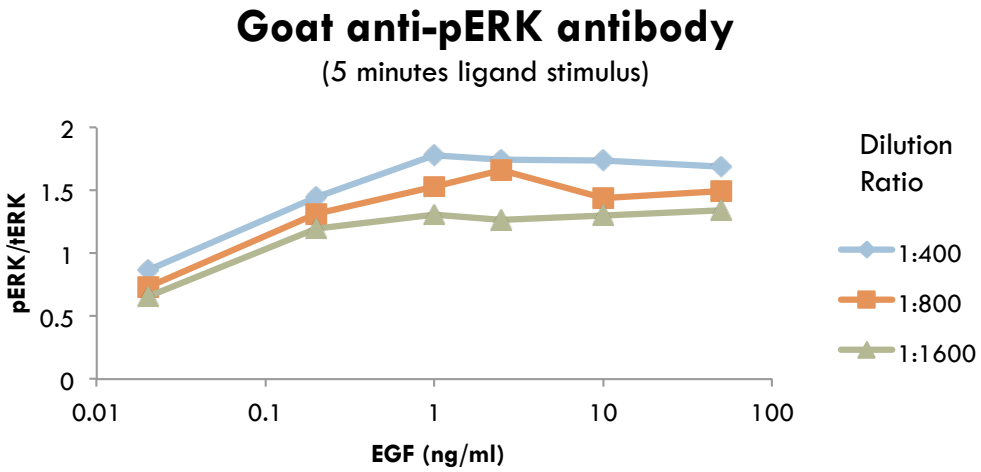
- pEGFR antibody
  - Characterization failed, so we used EGFR endocytosis to measure activation
- New pERK antibody
  - Characterization succeeded, so we got new pERK antibody which allowed us to do more concurrent measurment
- New c-FOS antibody characterization
  - Test failed, but we already had one worked

Antibody Characterization

- We want to get as more characterized antibody as possible. Many of the experiments require concurrent measurements of several marker proteins. For instance, in the same cell, we may need to measure the expression/activation of EGF-receptor, MEK, ERK as well as the focal adhesion pattern by Vinculin. In ensure high-quality multiplexed measurements, some of the antibodies need to be tested and titrated.

pERK antibody characterization

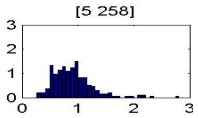
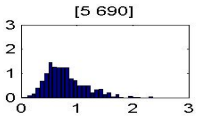
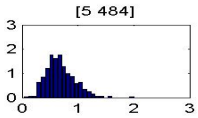
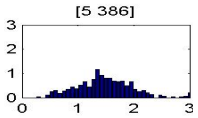
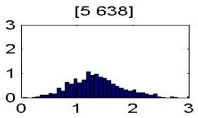
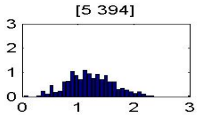
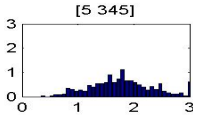
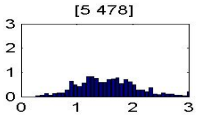
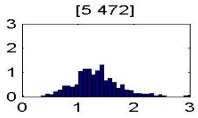
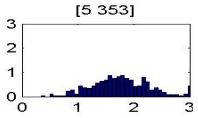
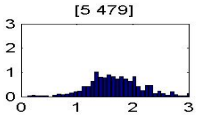
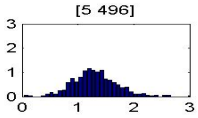
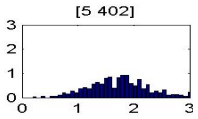
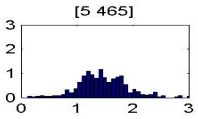
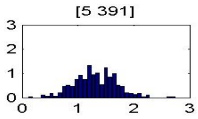
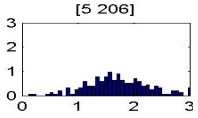
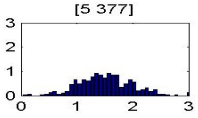
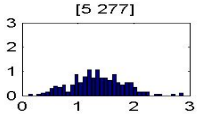
Titrate Goat anti-pERK antibody with 1:400, 1:800, 1:1600 dilutions



We used the antibody at 1:1250 dilution ratio for later experiments

pERK antibody characterization

Dilution Ratio

EGF	1:400	1:800	1:1600
0			
0.05ng/mL			
0.2ng/mL			
0.8ng/mL			
2ng/mL			
20ng/mL			

Stimulation Time

5 min