**Shedding experimental data**

Although these data were generated in CHO and HMEC cells, the general trends are likely to be the same. What data do we need?

1. Specific **shedding rate** of TGFa +/- induction
2. Specific **internalization rate** of TGFa precursor +/- induction
3. The ligrand in medium vs ligand captured can be used to calculate the diffusion parameters

Data: We had two TCT constructs in CHO cells that lacked EGFR and found that these behaved very similarly to what we observed in HMEC. An advantage of these cells is that they do not have EGFR and thus will not clear the ligand. We looked at both endocytosis of the ligands using a radiolabeled antibody to EGF. Levels of ligands in both total cell lysate and in the medium were done using an EGF ELISA. We looked at internalization and shedding in both control and PMA-stimulated conditions. The problem with the PMA is that it basically induced the release of ALL of the surface ligand into the medium so that you get a sudden release which then stops as the surface ligand is lost. In contrast, the control cells release the ligand continuously and so this can yield a basal rate.

Calculation difficulties: The ELISA measures ligand in the medium and in cell lysate. Unfortunately, the lysate interferes with the measurements and so it is too low. Thus, instead of using the lysate values as a base (substrate) value, I will use the PMA-released value as the original levels. How does this change things?

*Shedding rate constant using the* ***lysate value****:*

B1 = 0.0223 min-1(15 min); 0.0267 min-1(30 min)

B3 = 0.0545 min-1(15 min); 0.0246 min-1(30 min)

*Shedding value using the* ***PMA-release values***

B1 = 0.0075 min-1(15 min); 0.0090 min-1(30 min)

B3 = 0.0127 min-1(15 min); 0.0057 min-1(30 min)

*This can be used as basal level of shedding rate constants*

Interestingly, the PMA-release normalization gives much smaller values

When you look at ligand shedding in control HMEC, the slope is 3.19 & 4.69 per min and the slope of +LPA is 9.03 & 8.40 per min. In other words, LPA causes a 2.2-fold increase in shedding. Adding **TGF-a causes a 2.4-fold increase** after a delay of 15 min.

*LPA is activating Src which phosphorylate iRhome*

Internalization rates were much easier to calculate, and PMA did not cause any discernable changes. This analysis yielded values of **0.058 min-1 for B1** and **0.041 min-1 for B3 (These are basal level rates)**. These are a little bit higher than constitutive internalization rates but are **3-7 fold higher than shedding rates**. Thus, as previously surmised, internalization of the non-released ligand is the usual fate of ligands. Having said this, immunofluorescence suggests that the internalized ligand enters the recycling pathway and so there is likely **a steady-state distribution of ligand precursors between the surface and internal pools**.

*TGF-a secretion rates*: We have a couple of direct experiments. Lee did an experiment on 8-29-07 in which she plated cells in triplicate. For 824K cells, she did an incubation of 3 hrs and collected a total of 1 ml and diluted it to 1.11 mls. Concentration of TGFa was **113 +/- 5 pg/ml**. So, this was **42pg/hr** from 824K cells, or **5.09 x 10-17g per cell per hr**, or **5582 molecules/hr** or **93/min or 1.6 molecule per sec**. So, for a rate of production, it would be **0.00129 nM\_per\_sec**. This is somewhat higher than the values of **15 molecules/min** seen for long term HMEC culture, but those cells were chronically inhibited with 225, so perhaps they had a low release rate.

*This 0.00129 nM/s is a rate describing (ktl \* Vtr / kd), i.e. translation rate constant times transcription rate then divide by degradation rate constant of RNA.*

The important thing is that increasing the shedding rate from **1.6 molecule \* sec-1 to 3.5 molecule \* sec-1** would take occupancy from **1300 receptors to ~3000 receptors** and would induce an almost maximal level of Ras activation, which is consistent with the published data. Still, we need to measure these values again with the MCF10A cells.

If cells are releasing **93 molecules per min**, and the **fractional release rate is 0.01 per min**, then cells are expressing a total of **~10,000 molecules of TGF-a on their cell surface**, which is a reasonable estimate/number. The numbers of **Adam17 are about 15K**, so the numbers are in the same ballpark.

Using the steady states molecule numbers to calculate the activation of iRhom by pERK.