ERK activation in murine fibroblast under various growth conditions

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Abstract

Raf/MEK/ERK pathway is targeted as therapeutic pathway for cancer, and the activation is known to lead to many cellular responses such as cell proliferation, differentiation, apoptosis, and transformation. These different cellular responses are known to be effected by the duration of ERK activation and strength and localization of the signal. Therefore, the activation of the ERK pathway can also lead to totally different outcomes such as senescence and growth arrest. It was the aim of this study to see if we can control the outcome of ERK activation by conditional Raf using different activation kinetics. To control the activation of ERKs, western blot and cell counting were done with 3T3BXB-ER. 3T3BXB-ER cells are murine fibroblast cells (NIH3T3) which express Raf-1 kinase domain-estrogen receptor fusion proteins. The ERK pathway is acutely regulated by estrogen and tamoxifen only in 3T3BXB-ER cells. With 3T3BXB-ER cells, it is known that high concentrations of tamoxifen/estrogen make high ERK signals and lead cells to senescence. On the other hand, it is known that growth factor, FCS, makes low ERK signal and leads cell to proliferate with all fibroblast cells. To control the outcomes, parameters were adjusted by looking at ERK activation signals. First, the ERK activation was observed with time course and different levels of growth factor, FCS, and tamoxifen. The concentration of tamoxifen was lowered to lower ERK activation to make the cell proliferate. After, the ERK activation signal was regulated by MEK inhibitor called PD184352. Even though the cells grow to optimal levels with activation of Raf- constructs, the cells did not reach to accelerated proliferation level with the conditions tested.

Introduction

Raf/MEK/ERK pathway

Raf/MEK/ERK pathway is known to regulate numerous cellular events such as cellular proliferation, differentiation, apoptosis, and transformation (Lovric *et al.*, 1998). To activate this pathway, substances such as hormones, growth factors, differentiation, and tumor-promoting substances are used (Kolch, 2000). As shown in the figure 1, receptor tyrosine kinases (RTKs) gets activated by the substances, and then recruit SOS which exchange GDP for GTP to make Ras activated. Ras is common upstream activator of Raf which activate both MEK1 and MEK2. After activation of MEK, MEK activate ERK1 and ERK2 via phosphorylation of Thr-Glu-Thy motif in an activation loop (Kolch, 2000) ERK then phosporylates many transcription factors such as Ets-1. (SRF). This pathway is guided by scaffolds proteins, and it is said that the substrates which ERK feature are around 150 substrates in cytoplasm and in nucleus (Wellbrock *et al.,* 2004).

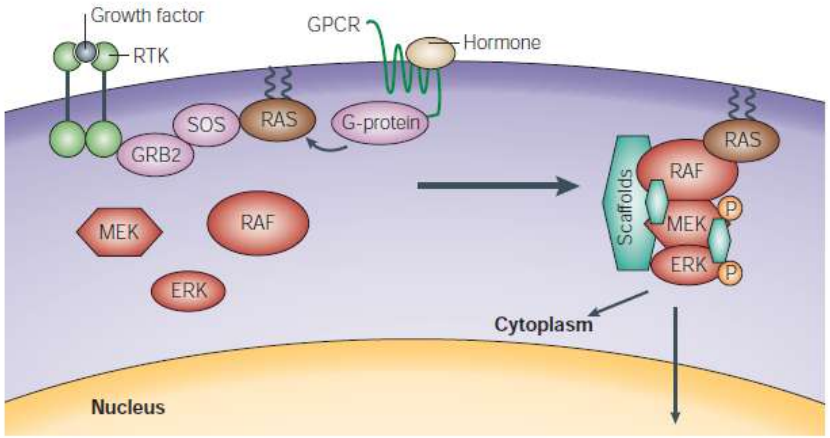


Figure 1: Raf/MEK/ERK Pathway (Wellbrock *et al.,* 2004)

Raf exists in three isoforms in mammalian cells, namely Raf-1/C-Raf, B-Raf, and A-Raf. C-Raf relates of process of mediating the cellular effects of growth factor signal (Matallanas *et al.,* 2011) and has general role in tissue formation (Kolch, 2000). B-Raf is known to have mutations in 66% of melanomas and in many other cancers such as colon cancer. (O'Neill *et al.,* 2003). Compared to these two Raf isoforms, A-Raf remains under mystery of its function (Matallanas *et al.,* 2011).

All these Raf isoforms have three conserved regions which are CR1, CR2, and CR3. CR1 is Ras-binding domain which is necessary for interactions with Ras. CR2 is cysterine-rich domain for secondary Ras-binding site with Raf auto-inhibition, and CR3 features kinase domain, which includes activation segment (Matallanas *et al.,* 2011; Baccarini *et al.,*2005).

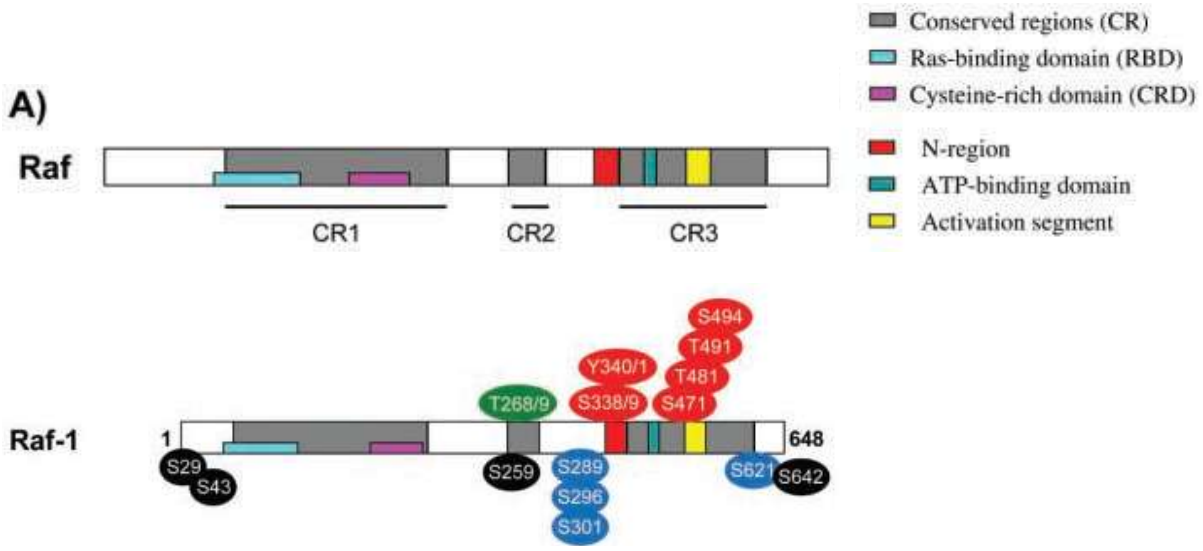


Figure 2: Raf construct (Matallanas *et al.,* 2011).

The top Raf represents the common Raf domains. All Raf isoforms have different phosporylation sites. The red circles represent activating phosporylation sites in Raf isoforms. Black ones show the inhibitory sites, and blue ones are both activating and inhibitory sites. Raf is inactive when 14-3-3 binding sites (S259, S621) are phosphorylated. When Ras is active, pS259 gets dephosporylated, and Raf gets recruited to the membrane and bind to Ras-GTP. After, the N-region and activation segment get phosphorylated and Raf goes to either heterodimerization with B-Raf or homodimerization. This causes the full activation of Raf-1. Then the N-region and activation segment of Raf-1 gets dephosporylated, and other inhibitory sites get phosphorylated. This leads to deactivation of Raf-1 (Matallanas *et al.,* 2011).

Biological Functions

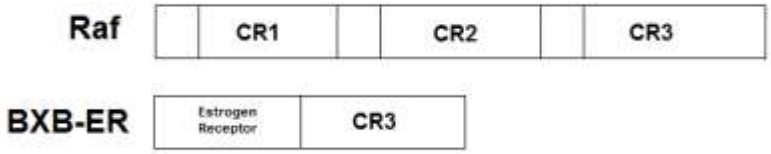
As stated in the beginning, the activation of this pathway would lead to many different biological functions. It is known that accurate regulation of ERK is a key to balance these responses (Wellbrock *et al.,* 2004), but it remains unclear how the activation of one signaling molecule ERK leads to specific cellular responses (Ebisuya *et al.,* 2005). It is known that different duration and intensity of ERK activation signals effect these decisions of different biological responses in fibroblast cells (Wellbrock *et al.,* 2004). For duration, sustained ERK activation is needed for quiescent cells to proliferate (Cook *et al.,*1996). Phosporylation of ERKs (p42 and p44) is required for proliferation of fibroblast cells (Page *et al.,* 1993). Disruption of sustained ERK1 with MEK inhibitor, PD98059, resulted in G1 growth arrest (Weber *et al.,* 1997). Also, sustained ERK level induce cyclin D1 protein (Balmanno and Cook, 1999; Weber *et al.,* 1997) and mRNA levels but disrupted ERK level with PD98059 resulted in decrease of cyclin D1 protein and mRNA levels (Weber *et al.,* 1997). These cyclin D1 and mRNA level which are important for S phase entries that are increased by sustained ERK level (Weber *et al.,* 1997). For intensity, strong prolonged activation of ERK would lead the cells to go in senescence (Martin *et al.,* 2010). With strong intensity of ERK level, the CDK inhibitor p21 is elevated (Coleman *et al.,* 2003) which decreases CDK activity (Ebisuya *et al.,* 2005) and inhibits DNA synthesis and mitosis in proliferating cells (Kerkhoff *et al*., 1998). Also, prolonged activation leads to changes in the level and distribution of heterochromatic histone mark (Martin *et al.,* 2010).

Also, ERK is constitutively activated in many cancers (Chambard *et al.,* 2006 and Torii *et al.,* 2006). Since ERK pathway is related to cancer, this pathway was highlighted as therapeutic

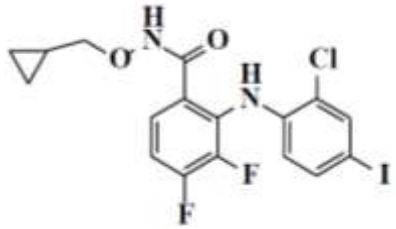
target for cancer treatment. To study more about this pathway, the parameter which the cells induce accelerated cell growth is needed. By regulating the duration and intensity of ERK activation, parameters where cells induce accelerated growth are tested.

3T3BXB-ER

In the laboratory of Dr. Lovric, mouse fibroblast cells, NIH3T3 and 3T3BXB-ER (Lovric *et al.*, 1998), were used for experiments. 3T3BXB-ER cells are NIH3T3 cells which express Raf-1 kinase domain-estrogen receptor fusion which can be regulated by OHT (Lovric *et al.*, 1998). This protein does not have regulatory domain which are CR1 and CR2. With addition of estrogen, these 3T3BXB-ER cells showed robust induction of BXB-ER kinase activity which was stabled for several hours and declined after 9 hours (Josip *et al.,*1998). In previous studies, the author was unabled to induce accelerated growth by activating Raf constructs.



MEK inhibitor: PD184352



(Sebolt-Leopold *et al,* 1999)

There are no substrates that are identified which MEK phosporylates other than ERK1/2 (Pearson *et al.,* 2001). Therefore, ERK is thought to be only substrates that are downstream of MEK. If MEK inhibitor is used, it is thought that it inhibits only ERK activity and its downstream substrates. PD184352 is 2-(2-chloro-4-iodo-phenylamino)-N-cyclopropylmethoxy-3,4-difluoro-benzamide (Sebolt-Leopold *et al,* 1999). . MEK inhibitor would inhibit ERK activity, and it has been reported that inhibition of ERK activity with MEK inhibitor will lead to cell growth arrest (Weber *et al.,* 1997).

Materials and Methods

Cell Culture

Two types of mouse fibroblast cells, NIH3T3 and 3T3BXB-ER (Lovric *et al*., 1998), were used for experiments. 3T3BXB-ER cells are NIH3T3 cells which express Raf-1 kinase domain-estrogen receptor fusion which can be regulated by estrogen (Lovric *et al*., 1998). These cells were maintained in Dulbecco's Modified Eagle's medium, DMEM (PAA), with 10% Fetal Bovine Serum (SIGMA), 1% 200mM L-Glutamine (SIGMA), and 1% Penicillin Streptomycin. This medium was

kept in 4°C, and was warmed to 36.7°C every time before using. Cells were subcultured into fresh 100x20mm cell culture treated dishes (SARSTEDT) every 2-3 days at dilution of 1:3 to 1:6 to keep them below 100% density. The cells were washed twice with Trypsin-EDTA (SIGMA), detached from plates by vigorous shaking, and separated by repeated pipetting. The cells were kept in a humidified incubator at 37°C with 5.0% carbon dioxide.

Proliferation Rate/Cell Counting

To examine how the cells proliferate under different conditions such as with tamoxifen, estrogen, and different levels of fetal bovine serum, the cell numbers were counted for each condition. For these experiments, $3.3x10^4$-$6.6x10^4$ cells were plated in each well of 6-well plates (Costar multiwall with Corning cell BIND surface) using full medium with 10% FCS. For various experiments the medium was changed after allowing the cells to attach for 15-18 hours. The cells were kept in the incubator with 37°C with 5.0% carbon dioxide, and were counted on the 4^{th} to 5^{th} day of the experiments with a haematocytometer. Images were taken with Canon IXY 14.1 mega pixels with an inverted light microscope.

Western Blot

For generation of Western blot lysates, $1.5x10^5$ cells were plated in each well of 6 well dishes. Around 24-27 hours later, the cells were put in starved medium which is DMEM (PAA), with 0% Fetal Bovine Serum (SIGMA), and 1% Penicillin Streptomycin. After 15-16 hours starvation, cells were induced with substances such as tamoxifen and FCS for the indicated durations. The cells were put on ice, washed with 2ml PBS twice, and lysed with 100µl of lysis buffer per well. (Lysis Buffer: 50mM Tris pH8, 150mM NaCl, 1% Triton X-100, and 1% protease inhibitor) For the harvest, the cells were incubated on ice for 2 minutes with the lysis buffer. The cells were centrifuged at 13000 rpm for 5 minutes. The supernatant was mixed with 4X SDS sample loading buffer. (200mM Tris pH 6.8, 40% glycerol, 16% β-mercaptoethanol, 6% SDS, and bromophenol blue) These samples were heated at 95°C for 3 minutes.

The samples were loaded in 12% acrylamide gel, using 250mM Tris Base, 1.9 M glycine, and 1% SDS as a running buffer. After running, gels were blotted to PVDF membrane which was put in methanol. Blotting was with transfer buffer which is made up of 10% 10X Towbin Buffer and 20% methanol. (10X Towbin Buffer : 250mM Tris Base & 1.9 M Glycine) These membranes were put in methanol and then blocked with 5% Albumin, Bovine Serum, Fraction V, Fatty Acid-Free (BSA) (Cal Biochem) with PBS. After blocking, primary antibody such as P-p44/42 MAPK (T202/Y204) (Cell Signaling) or Anti-MAP Kinase (ERK-1, ERK-2) (SIGMA) were applied with 1% BSA in PBS. These antibodies were kept in -20°C. At -20°C, antibody p-ERK was in liquid and antibody ERK was in frozen in aliquots. They were applied in specific dilutions (anti-p-ERK 1:1,000/anti-ERK 1:20,000) for overnight, and secondary antibody (Stabilized Peroxidase Conjugated Goat Rabbit (Thermo)) with 1% BSA in PBS (anti-Rabbit 1:100- 500) was applied for 2 hours. Luminata Crescendo/Classico Western HRP Substrate (Milipore) and Kodak Films were used for protein detection.

Stripping membrane

After the p-ERK Western blot, the membranes were stripped with the solution of 2% SDS, 0.6% Tris, and 1%, and B-meracaptonol. This was warmed with microwave with 1 minute. The membranes were put in this solution for 30 minutes, and were washed with miliQ twice. The membranes were put in methanol and were blocked with 5% Albumin, Bovine Serum, Fraction V,

Fatty Acid-Free (BSA) (Cal Biochem) with PBS. After that, primary antibody was put, and the same procedure as western blot was repeated.

Results

Time course of ERK activation: Difference between activation by FCS and tamoxifen

NIH3T3 cells and 3T3BXB-ER cells were used for the experiments. 3T3BXB-ER cells are NIH3T3 cells which express a fusion protein that contain parts of the human estrogen receptor. Therefore, the activation of MEK and ERK of 3T3BXB-ER could be regulated with estrogen and tamoxifen. It is known that Fetal Bovine Serum, FCS, is necessary for cell proliferation, and strong ERK phosporylation levels by tamoxifen leads to cell senescence. These different outputs of cells are thought to be dependent of ERK activated duration and strength (Yamamoto and Nishida, 2007). Also, the activation depends on various anchor, adaptor, and scaffold proteins. By changing duration/strength and finding right levels for activation of ERK by conditional Raf, the control of outcomes may be possible. Also, the study of ERK pathway would be easier to research if the parameters, which cells give outcome of proliferating by activation of conditional Raf, are found.

To examine the difference of ERK activation by induction of FCS and tamoxifen, time course experiments were done on NIH3T3 and 3T3BXB-ER cells. Time course was to see how ERK phosphorylation levels change for both cells, and to see if there are any similarities. With activation by FCS, ERK got phosphorylated within 2-5 minutes with both types of cells. After 10 minutes, the ERK phosporylation levels went down. On the other hand, ERK phosporylation increased as the time with 100nM tamoxifen with 3T3BXB-ER cells (Figure 1). 100nM tamoxifen is showed to induce a near maximal ERK phosphorylation with previous laboratory work. Compared to activation by FCS, 100nM tamoxifen took longer time to induce ERK in 3T3BXBER but signals were stronger than FCS. ERK phosphorylation was not seen with NIH3T3 cells with 100nM tamoxifen which was expected.

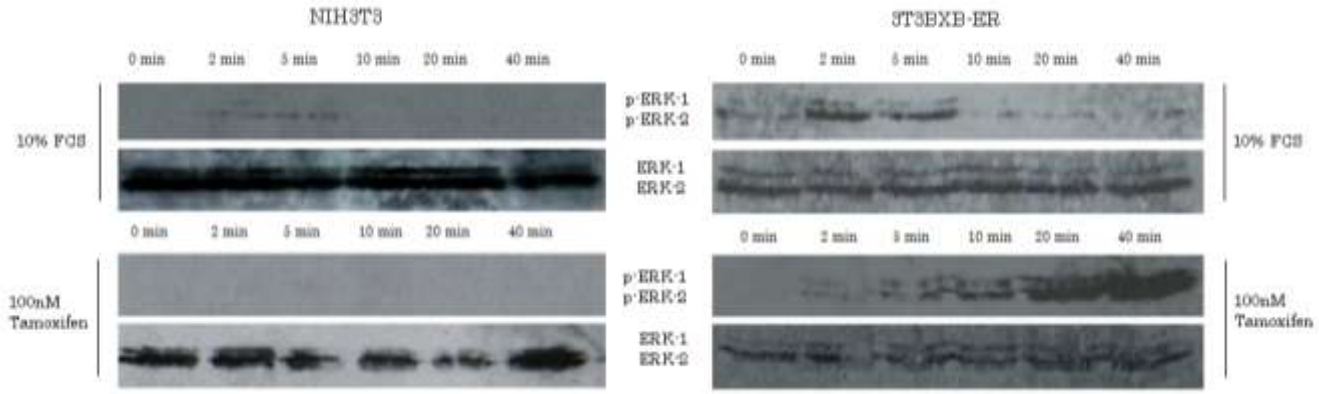


Figure 1: $1.5x10^5$ cells were plated in each well of 6-well plates. It was starved with 0% serum the next day. The cells were starved for 17 hours, and 10% FCS/ 100nM tamoxifen were induced. The membranes were stripped after antibody p-ERK, and were put in antibody ERK. The amounts of protein and exposure time of western blots of were the same. ERK levels of NIH3T3 were higher than that of 3T3BXB-ER cells. However, the ERK phosporylation levels were higher with 3T3BXB-ER cells with induction of 10% FCS and 100nM tamoxifen. Similar results were seen when repeated.

Cell Growth: FCS concentration and ERK activation

Fetal Bovine Serum (FCS)

To find out if steady state level of ERK phosporylation could be correlated with speed of growth, the cells were treated with different concentrations of FCS. Different concentration of FCS giving different p-ERK levels could affect proliferating rates. These cells were counted for growth and phosporylation ERK levels were measured.

As shown from the graphs, the cells were proliferated the fastest with 10% FCS. As the FCS percentage that is included in the media went down, the cells decreased their proliferating rates. Also, different concentrations of FCS gave different strength of p-ERK signals. Unlike with tamoxifen, ERK got phosphorylated with both NIH3T3 and 3T3BXB-ER cells with FCS as expected. As shown in Figure 2, 10% had the strongest level with NIH3T3 and 5%-10% with 3T3BXB-ER cells. Starved cells were treated with 0% FCS.

Also, both NIH3T3 cells and 3T3BXB-ER cells had similar trend with proliferating rates and signal strength, but the NIH3T3 cells grew faster than 3T3BXB-ER cells.

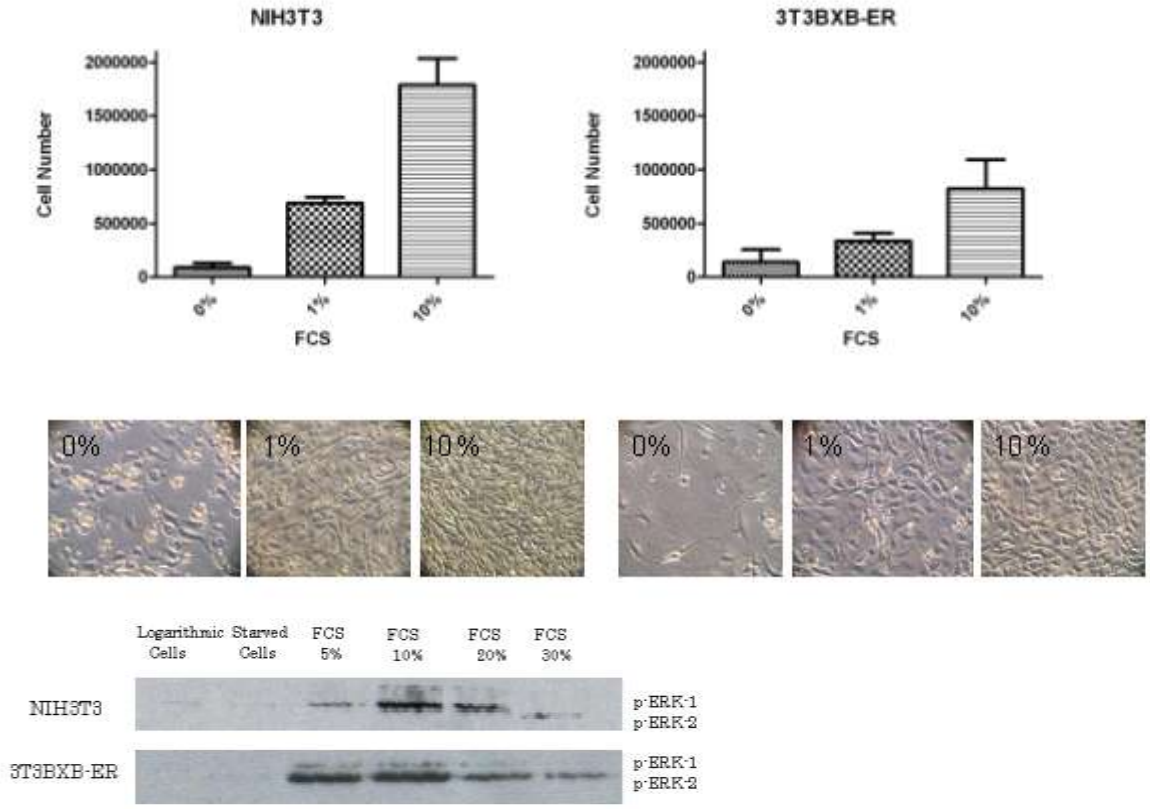


Figure 2: NIH3T3 and 3T3BXB-ER cells were used for experiments. In order to compare how proliferation rates differ and how concentration of FCS affect the cells, $6.6x10^4$ cells were plated for each conditions and were counted after 4 days. NIH3T3 cells grew faster than 3T3BXB-ER cells with all the concentrations of FCS. Even though the 1% FCS was not tested with western blot, cells increased its numbers as the concentration of FCS increased. NIH3T3 cells have tendency to grow on top of each other, so the confluence is more than 120%. The western blots

show the phosphorylation levels after 5 minutes inductions. Comparing with results with it, the level of phosphorylation was strongest in 10% for NIH3T3 and 5-10% for 3T3BXB-ER cells.

Tamoxifen/Estrogen: Strong Activation of ERK leads to cell cycle arrest

To see how tamoxifen inductions affect the cells, tamoxifen/estrogen 10nM, 30nM, and 100nM were used. With NIH3T3, the cell number increased slightly after the treatment. With 3T3BXB-ER cells, the cells decreased after the same inductions. As shown on Figure 3, ERK did not get phosphorylated with 100nM tamoxifen with NIH3T3 cells while 3T3 BXB-ER cells had strong phosphorylation levels. With 3T3BXB-ER cells, the ERK phosporylation reached maximal with 100nM tamoxifen. As the phosporylation levels went up, the cell proliferation decreased. The strong ERK phosporylation levels led to cell senescence.

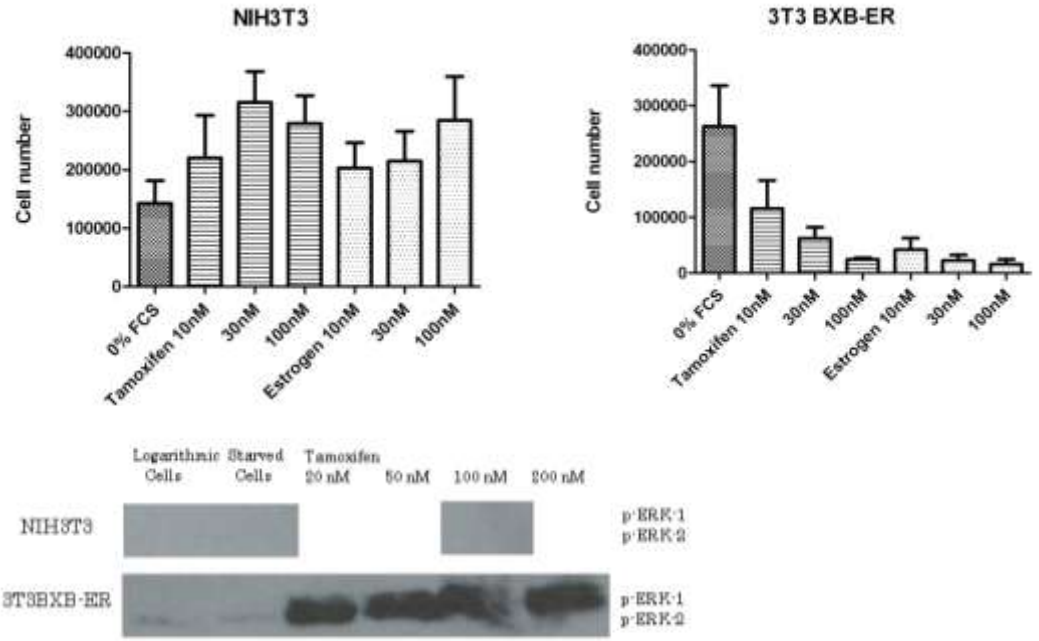


Figure 3: Cell growth experiment: $6.6x10^4$ cells were plated, and they were starved with media including 0% FCS, tamoxifen (10nM, 30nM, 100nM) and estrogen (10nM, 30nM, 100nM). After 4 days, the cell numbers were counted. Western blot: $2.4x10^5$ cells were plated in each well of 6-well plates. They were starved with 0% serum for 17 hours, and were induced with tamoxifen 10% FCS, 0% FCS, and tamoxifen (20nM, 50nM, 100nM, 200nM) for 40 minutes.

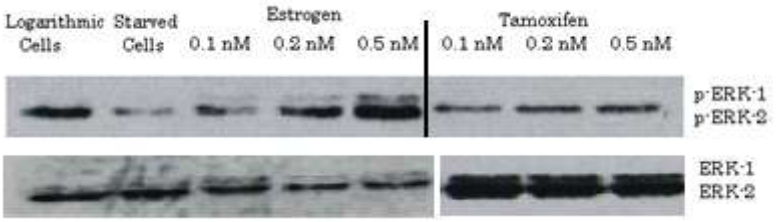
Lower phosphorylation of ERK and Cell Growth

Even though both FCS and Tamoxifen/Estrogen phosphorylate ERK with 3T3BXB-ER cells, FCS made cells to proliferate with 10% but Tamoxifen/Estrogen made cells not to proliferate with concentrations of 10nM, 30nM, and 100nM. The difference considered is the levels of phosphorylations. As shown in Figure 3, the phosphorylation levels are a lot stronger than the

levels activated by FCS. Since it is known that strong level of ERK phosphorylation leads to senescence, lowering phosporylation ERK levels were considered.

It is also known that MAPK pathway is important in proliferation mechanism, and it may be possible to make cells proliferate with lower levels of ERK phosphorylation. The conditions for cells to grow with activation of MAPK pathway is needed to see if it is really the level of phosporylation ERK which could regulates the proliferation. Also, if the conditions for cells to grow are found, it is possible to study more about this pathway.

Lower amount of concentrations of estrogen/tamoxifen were tested to see if the phosphorylation of ERK levels could get close to logarithmic cells which are put in 10% serum. 0.1nM, 0.2nM, and 0.5nM were tested for both estrogen and tamoxifen. As shown in Figure 5, the phosporylation ERK levels with these concentrations are similar to 10% FCS (logarithmic growth). The cells with these conditions were counted. Figure 5 shows that cells did not grow a lot more than the control. The cell numbers were similar to the control, not similar to the logarithmic cells.



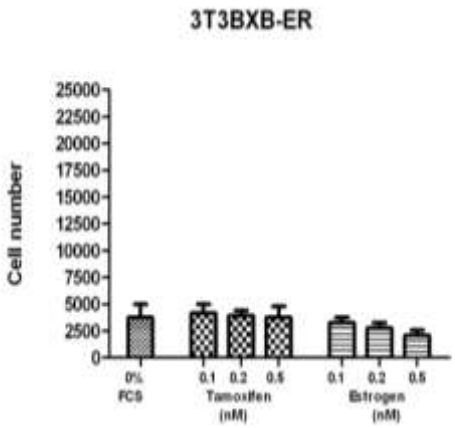


Figure 5: (Western Blot) $1.5x10^5$ cells were plated in each well. The cells were starved for 17 hours and were induced with 0.1nM, 0.2nM, 0.5nM of estrange/tamoxifen for 40 minutes. (Cell Count) $6.6x10^4$ cells were plated for each conditions, 0% FCS, 0.1nM, 0.2nM, and 0.5nM of tamoxifen, and were counted after 4 days.

Cells were put under even lower concentrations from 0.001nM to 0.1nM of tamoxifen/estrogen. The cells grew to the optimal levels with figure 6-B and C of 3T3BXBER, but did not go over the number dramatically. If the ERK levels regulated the cell growth, it could be said that with same level ERK phosporylation with 10% FCS would lead to accelerated cell growth. The cells should grow as much as cells with 10% FCS would. Also, cell counts were not stable for all the experiments with low concentration of tamoxifen/estrogen.

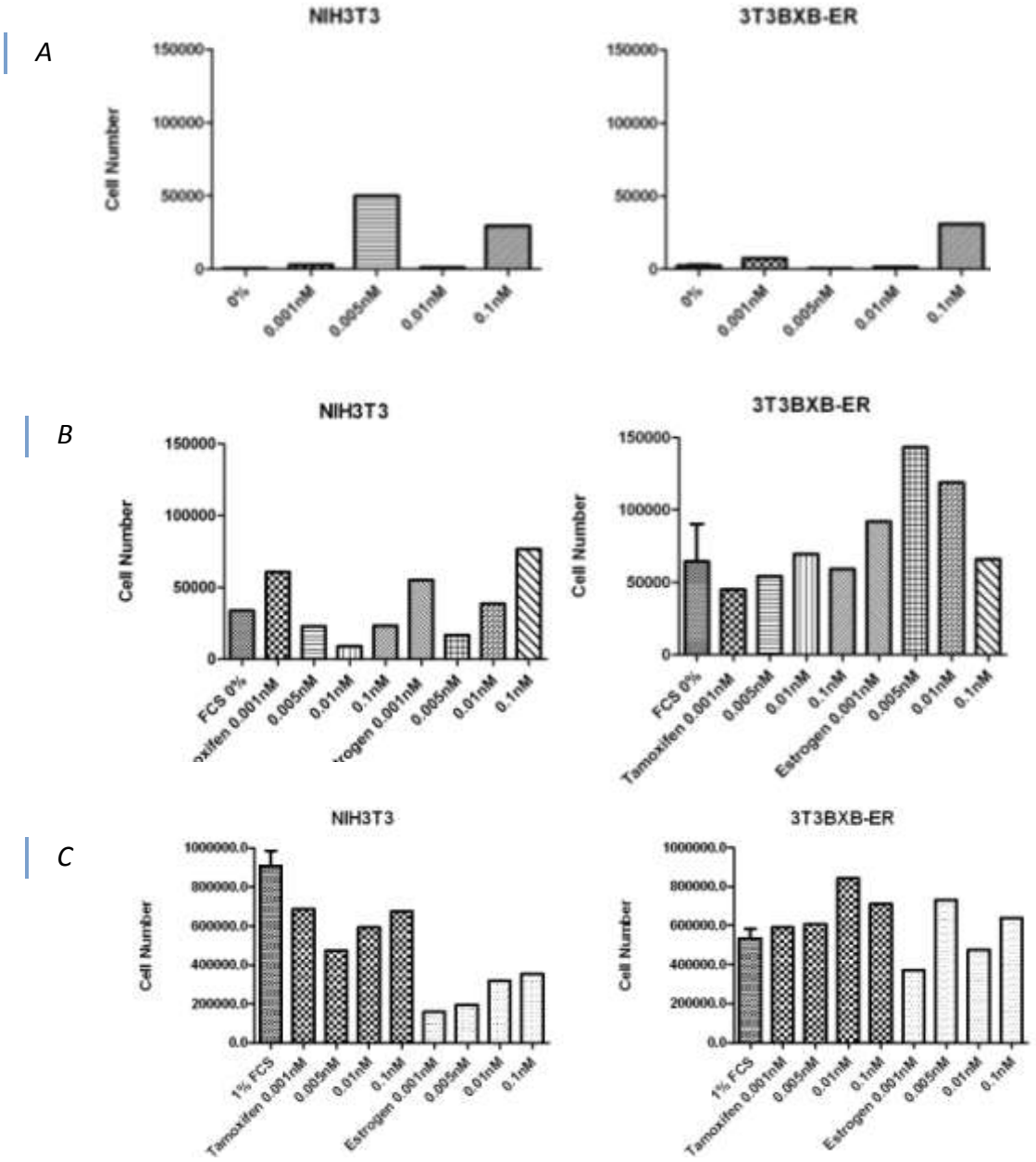


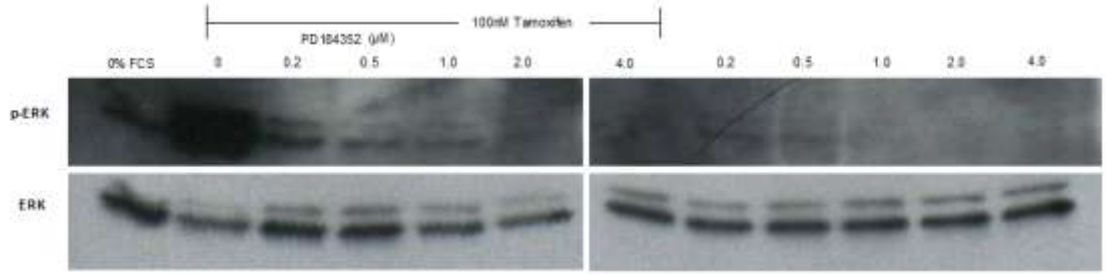
Figure 6: 6.6x10^{4}cells were plated. The cells were put in conditions with various concentrations from 0.001nM to 0.1nM. In figure 6-A and B, cells were put under 0% serum. With figure 6-C, the cells were put in 1% serum. The experiments were done three times (A, B, and C), and were done on different days.

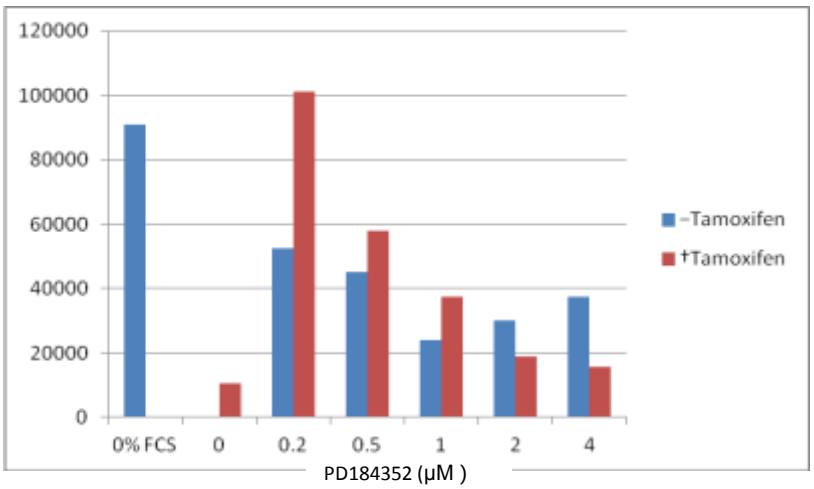
Tamoxifen Induction with MEK inhibitor PD184352

To control the outcomes of cellular responses and to find conditions for cells to have accelerated proliferation, MEK inhibitor PD184352 was used. PD184352 inhibits phosporylation of ERK. Different concentrations of PD184352 were tested with 100nM tamoxifen and without 100nM tamoxifen. Various concentrations of PD184352 should give different levels of ERK phosporylation. The experiments were done with 0%, 1%, and 10% serum.

0% FCS

In 0% serum, 0μM, 0.2μM, 0.5μM, 1.0μM, 2.0μM, and 4.0μM of PD184352 were tested on cells with and without 100nM tamoxifen. Figure 7 shows the condition with 0.2μM PD184352 with 100nM tamoxifen gave the highest cell number. As the concentration of inhibitor increases, the cell number decreased. Also, 0.2μM, 0.5μM, and 1.0μM tamoxifen gave higher cell number with 100nM tamoxifen.





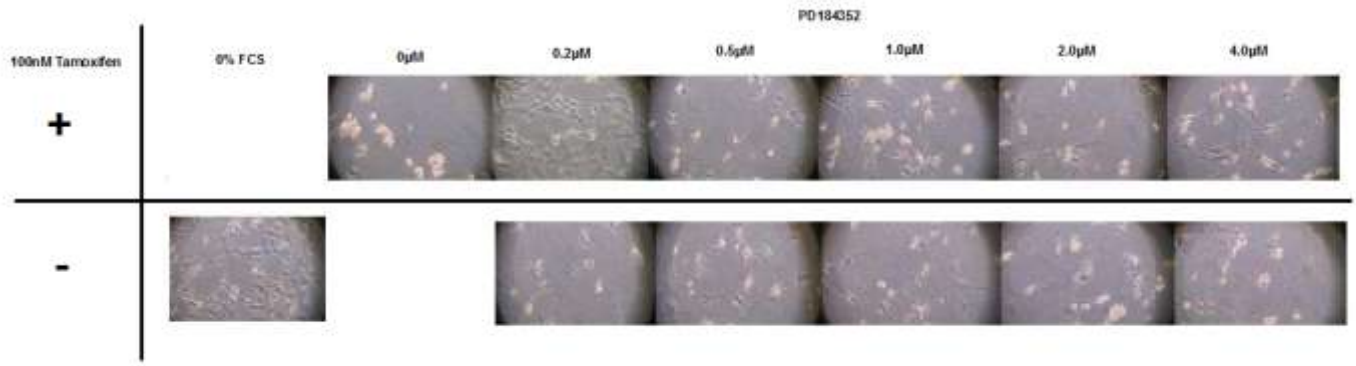
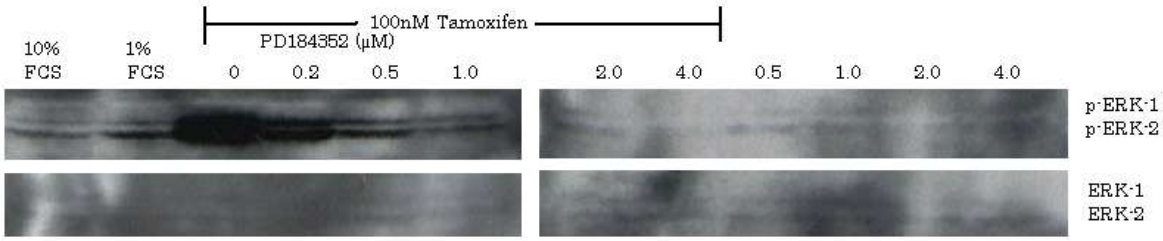


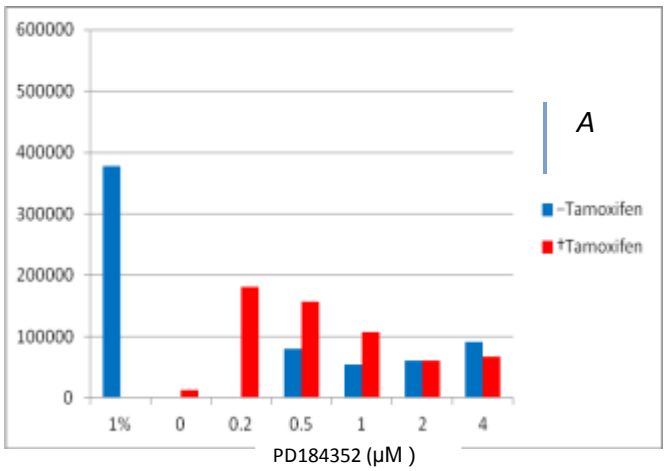
Figure 7: (Western Blot) $1.5x10^5$cells were plated and were put in conditions shown in the figure for 24 hours. The cells were lysed and the samples were run in the 12% acrylamide gel (Cell Count) $6.6x10^4$ cells were plated on each well. It was stared conditions were 0% FCS, 0μM, 0.2μM, 0.5μM, 1.0μM, 2.0μM, and 4.0μM of PD184352 with and without 100nM of tamoxifen. The cells were put in these conditions for 4 days. The pictures were taken before counting.

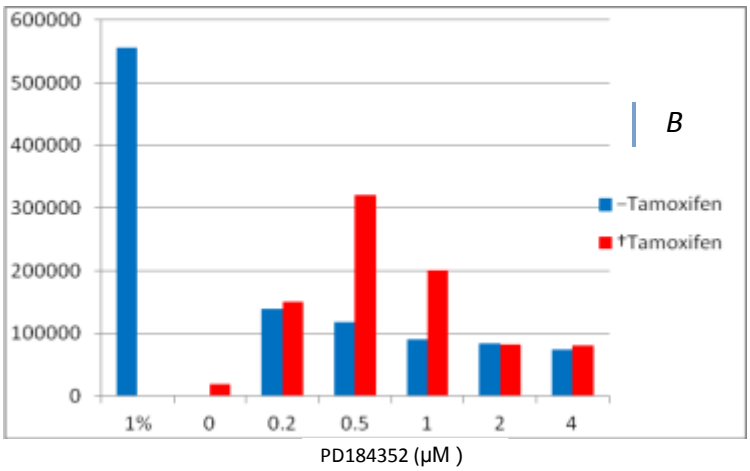
By adding serum into the conditions, the proliferation rate may be accelerated. To test this, experiments with 1% and 10% serum were done.

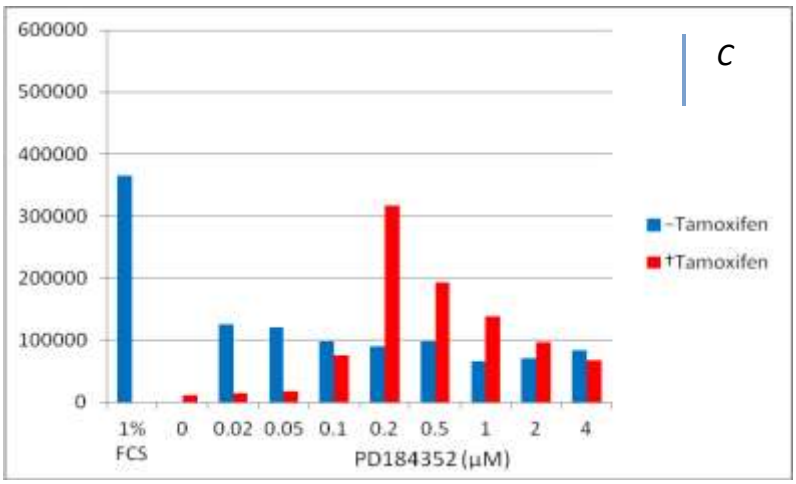
1% FCS

In 1% serum, 0μM, 0.2μM, 0.5μM, 1.0μM, 2.0μM, and 4.0μM of PD184352 were tested on cells with and without 100nM tamoxifen. With western blot, the highest signal is at 100nM tamoxifen with 0μM PD184352. As the concentrations of the MEK inhibitor, PD184352 increases, the phosporylation ERK levels decreased. With cell counting, the cells were proliferated the most under induction of 100nM tamoxifen with 0.2μM-0.5μM of PD184352.









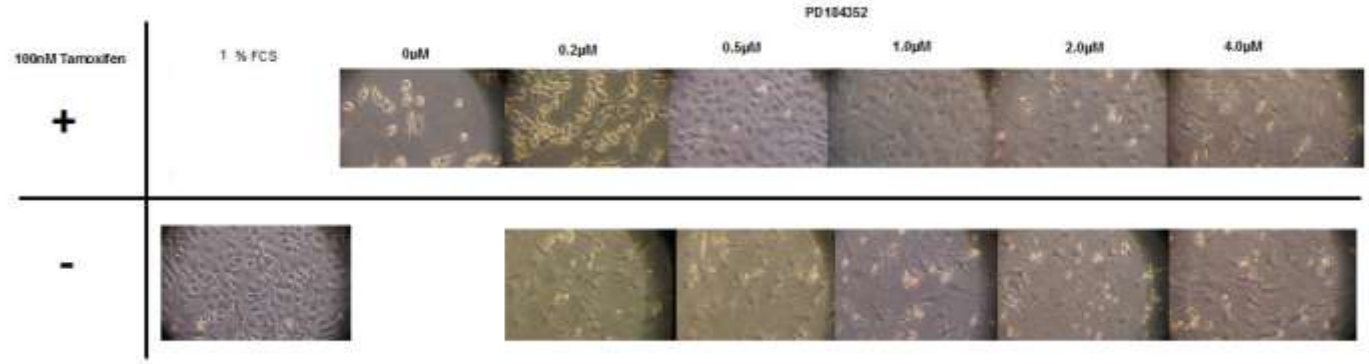
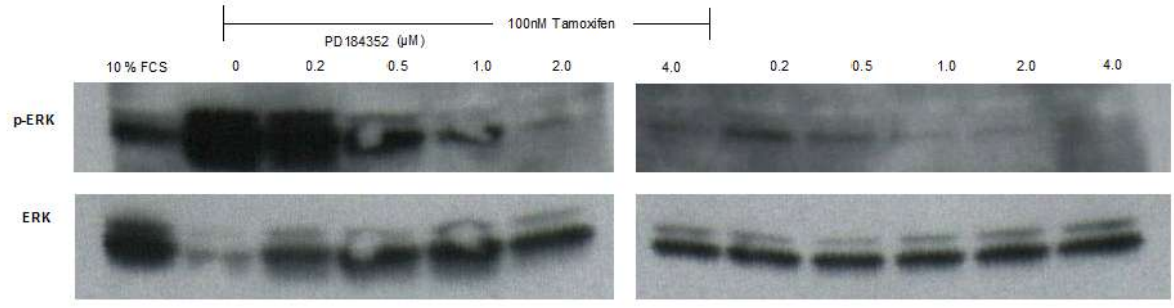
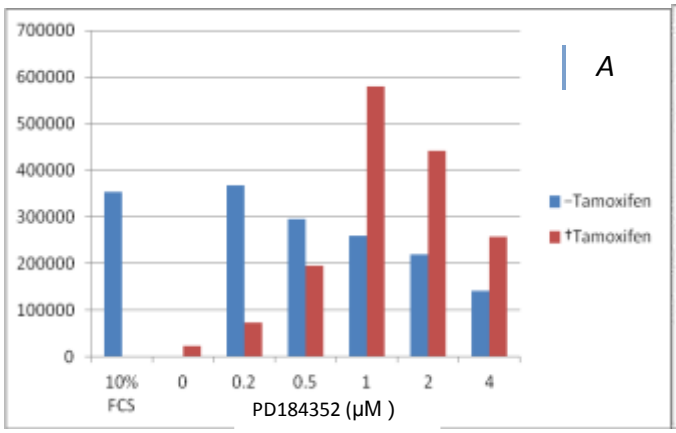


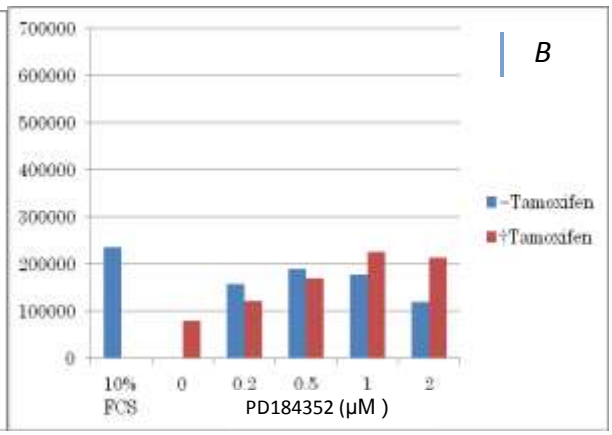
Figure8: (Western Blot) $1.5x10^5$cells were plated and were put in conditions shown in the figure for 24 hours. The cells were lysed and the samples were run in the 12% acrylamide gel. (Cell Count) $5.5x10^4$cells were plated in each well. The concentrations of different PD184352 were tested from 0 to 4.0μM. The pictures were taken from cells of figure B.

10% FCS

In 10% serum, 0μM, 0.2μM, 0.5μM, 1.0μM, 2.0μM, and 4.0μM of PD184352 were tested on cells with and without 100nM tamoxifen. Under 100nM tamoxifen, the cells grew the fastest with 1.0μM of PD184352.







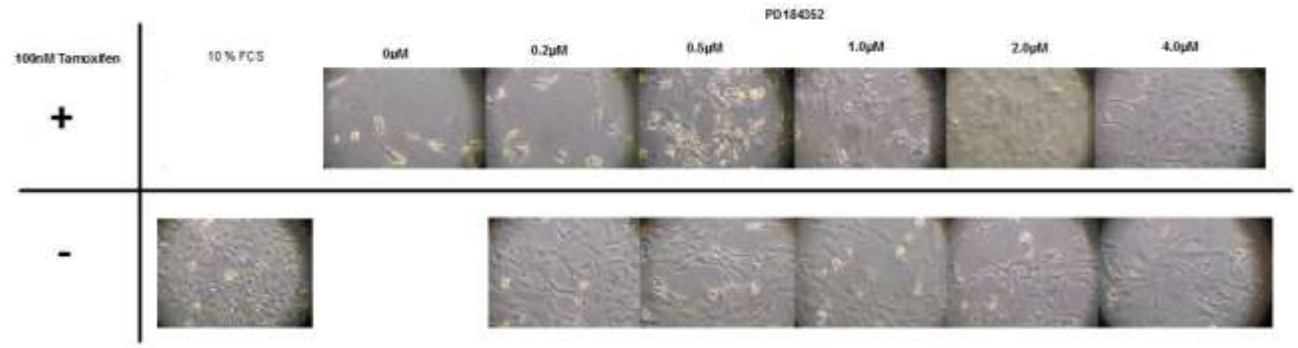


Figure9: (Western Blot) $1.5x10^5$cells were plated and were put in conditions shown in the figure for 24 hours. The cells were lysed and the samples were run in the 12% acrylamide gel. (Cell Count) $5.5x10^4$cells were plated in each well. The concentrations of different PD184352 were tested from 0 to 4.0μM. The cells were in these conditions for 3-4 days. The cell number was the highest with 1.0μM PD184352 under 100nM tamoxifen induction. The pictures of cells were taken before counting from experiment A.

Discussion

Differences in ERK expression level and phosphorylation in NIH3T3 cells and 3T3 BXB-ER cells after stimulation with 10% FCS

NIH3T3 cells have higher ERK signal than 3T3BXB-ER cells, but NIH3T3 cells have lower phosporylation signals when induced with 10% FCS (Figure 1). It could be said that phosporylation ERK signal is higher in 3T3BXB-ER cells. This may be due to “leakiness” (Kerkhoff *et al,* 1998; Martin *et al.,* 2010; Joseph *et al.,* 2002) of the induction system of 3T3BXB-ER. Leakiness allows 3T3BXB-ER to activate conditional Raf when endogenous Raf is not activated by mitogenic stimuli. With previous studies, it has been seen that there is consistent and slight “leakiness” of the conditional Raf with 3T3BXB-ER cells (Martin *et al.,* 2010). Therefore, the signal of phosporylation of ERK may be higher with 3T3BXB-ER than NIH3T3 due to the addition of ERK by two different activations: activation of “leakiness” BXB-ER and activation of endogenous Raf-1 by FCS.

Proliferation rate of NIH3T3 and 3T3BXB-ER cells are different when activated by 10% FCS (Figure 2). 3T3BXB-ER has lower cell number after 4 day growth. The difference of the rate of cell growth may be due to ERK phosporylation signal levels. Usually, phosporylated ERK gives negative feedback towards Raf-1 by phosporylation on T292 (Eblen *et al.,* 2004) which would lead to lower interactions of Raf-1 and MEK (Frost *et al.,* 1997 and Eblen *et al.,* 2002). This feedback would reduce activation of ERK pathway. With 3T3BXB-ER induced with FCS 10%, this negative feedback may be higher than NIH3T3 because of “addition “of two different activations that are discussed above. This may lead to lower phosphorylated ERK after the activations which may interfere with the proliferation rate. That may cause prolonged checkpoint period for 3T3BXB-ER cells.

Different kinetic between activation of ERK by BXB-ER and by serum

The activation of ERK by BXB-ER follows a different kinetic than the activation of ERKs by Serum. Activation of ERK by FCS is highly regulated by cells, but activation by BXB-ER is not regulated by the cell. Therefore, ERKs could get activated in the wrong cell compartment in the wrong time thus phosphorylated wrong substrate for growth.

Differences in kinetics are shown with time, durations, and intensity of phosporylation ERK signals. First, activation time and duration are different with the two activations. When 3T3BXB-ER is induced with serum, the ERK gets phosphorylated after two minutes, and gets inhibited after five minutes after the induction. However, ERK start to get phosphorylated 2-5 minutes and stay phosphorylated after 40 minutes by activation of BXB-ER. Second, intensity of phosphorylation of ERK is different with activations by BXB-ER and by serum. When 3T3BXB-ER cells are induced with 100nM tamoxifen, it gives higher intensities than the cells induced with serum. Therefore, the kinetics of these two activations are shown to be different.

Cells proliferate the most after stimulation with FCS 10%- ERK signal difference after stimulation of 0%, 1% and 10% FCS

Proliferation rate differs with concentrations of FCS. Serum plays an important role for inducing cells to go on to S phase. Another author states that serum concentrations above 2.5% are able to increase the rate of cell cycling by inhibiting density dependent negative regulation of NIH 3T3 (DiSalvo *et al.,* 1995).Increase in G1 phase time is inhibited by increasing serum concentration (DiSalvo *et al.,* 1995). As an experiment, this was shown that high concentration of FCS (10%) gives shorter time for G1 phase by giving faster proliferation rate than others (1% and 5% FCS).

Possible effect of Ethanol on mouse fibroblasts

Tamoxifen and estrogen are stored in ethanol. When inducing with OHT, it is likely some of ethanol (0.34, 1.0, 3.4μ l) is put in cell culture as well. With previous study, it has been seen that ethanol inhibits FGF-mediated MAPK activation (Ghiselli et al., 2003). With NIH3T3 cells, OHT is thought to not have effect. However, addition of tamoxifen and estrogen with ethanol induced slight proliferation on NIH3T3 cells (Figure 3). To test whether this slight growth was due to OHT or ethanol, ethanol induction was tested (Figure not shown). Ethanol amount is just 0.017%, 0.05%, 0.17% of media. With these experiments the results varied with each experiment. Some experiments induced growth with only ethanol. On the other hand, other experiments did not induce growth. It does not show if ethanol induced this slight growth on NIH3T3 cells, but there may be possible affect on fibroblast cells (NIH3T3) with ethanol induction.

ERK activation is optimal in growing cells, further activation by BXB-ER leads to slower growth under all serum concentrations tested (OHT>10nM →cell arrest with strong pERK signal)

Both cell types (NIH3T3 and 3T3BXB-ER) grew fast with10% FCS (Figure 2). With starved cells (0% FCS), the cells cycles were put in arrest. This way, ERK activation is necessary for optimal growth cells. Also, 3T3BXB-ER was induced with tamoxifen and estrogen with concentrations of 10nM, 30nM, and 100nM (Figure 3). As the concentration got higher, the level of pERK increased with 3T3BXB-ER cells. For earlier studies, it has been known that high-levels of Raf signal lead to cellular proliferation arrest (Sewing *et al.,* 1997; Woods *et al.,* 1997). With 3T3BXB-ER cells, the higher the phosporylation ERK, the cell number decreased. ERK activation is needed with growth factor such as FCS, but further activation with OHT on 3T3BXB-ER cells lead cells to senescence.

Activation of ERK during sub-optional induction of BXB-ER (tamoxifen 0.1 nM, 0.2 nM, and 0.5 nM) results in similar proliferation rate as the control(0% serum)

Phosporylation of ERK by minimum of 10nM tamoxifen prevents cell growth. However, lower concentrations of tamoxifen were tested to see if they give different results. The 3T3BXB-ER cells were activated by low concentrations of tamoxifen (0.1, 0.2, and 0.5nM), and it was shown that they do not give cell arrest but slower cell growths. The growth rates were similar to the control cell growth rates which were in 0% serum. The intensities of phosporylation ERK signals were close to logarithmic growth cells (10% serum) cells. However, it did not reach to logarithmic cell growth rates when the cells are induced. Since the kinetics of activations by tamoxifen and by serum is different (discussed above), it may be impossible to compare just signals of phosporylation ERK with accelerated growth rates.

If ERK activation is inhibited by PD184352, the cells grow slower. Under these conditions, additional ERK activation enhances the growth of cells.

3T3BXB-ER cells were put in different concentrations of PD184352 (Figure 7,8,9). PD184352 is MEK inhibitor which disables phosporylation of ERK by MEK. This is shown by western blots with lower phosporylation levels of ERK when induced with PD184352. With different concentrations of PD184352 are put inside the media, the cell mostly decreased its numbers (all concentrations (0%, 1%, 10% FCS) tested). Under these conditions, the additional ERK phosporylation induced by tamoxifen (100nM) enhanced growth. With 0% serum, cells under 0.2μ M with 100nM tamoxifen condition grew the most. With 1% serum, 0.2-0.5μ M with 100nM tamoxifen, and with 10% 1.0μ M. As the percentage of FCS increases, the higher the concentration of PD184352 it is for the highest proliferation rates. These may be due to the balance of ERK activation by serum and tamoxifen. Even though it has different kinetics for activations by FCS and tamoxifen, these two activations may influence each other with proliferation.

To simplify, in order to have high proliferation rates
0% Serum (Lower p-ERK) and 100nM tamoxifen&0.2μ M (Higher p-ERK)
10% Serum (Higher p-ERK) and 100nM tamoxifen & 1.0μ M (Lower p-ERK)

These combinations may have high proliferation rates because of the right phosporylation- ERK intensities for proliferation.

Pure p-ERK does not induce accelerate growth

Even though the cells grew to optimal levels by controlling phosporylation of ERK signals, the cells did not induce accelerated growth. Phosporylation of ERKs (p42 and p44) is required for proliferation of fibroblast cells (Page *et al.,* 1993). However, there must be more than pure p-ERK levels responsible for growth. Proteins such as scaffold proteins and inhibitor protein of this pathway provide variations of duration and magnitude of ERK pathway (Marshall *et al.,* 1995 and Ebisuya *et al.,* 2005). Scaffold protein acts as docking platforms and anchors of the signaling components, bringing together the different modules of the cascade (Matallanas *et al.,* 2011). Protein Sprouty, Sped, Sef , KSR1, CNK, and many other proteins are known to be scaffold of ERK pathways (Matallanas *et al.,* 2011). Unfortunately, it is quite difficult to study scaffold proteins because they are highly dependent on concentrations and stoichiometric ratios with respect to client protein (Matallanas *et al.,* 2011). Also, P13K pathway is known to be related to proliferation. Controlling this pathway along with ERK pathway may be necessary to induce accelerated cell growth.

Experimental Error

For western blot, the loading control was cell numbers. Same amount of cells were plated with all the conditions done on the same day. Since the same amount of cells should have been plated on each well, each well should contain same amount of proteins which are loaded on western blot. However, as the experimental error, the cells may have been slightly different with handling error. If cells were not pipetted up and down so that cells distribute equally in media, different amount of cells may be loaded on certain well. Other possible ways of loading control are protein (Bradford assay), SDS PAGE gel stained with CBB or silver, or strip the blots and probe with antibody tubulin or ERK. For most of the experiments, the western blots membranes were stripped and were put in antibody ERK to see there were equal amount of protein loaded in each well.

Also, depending on the day of the experiments, the cell growth numbers and western blot intensities were inconsistent. This may be due to the incubation conditions. Even though the temperature and percentage of carbon dioxide should stay constant inside the incubator, there are some days when doors are opened many times which makes temperature and percentage of carbon dioxide change. This may affect the cell numbers that are proliferating.

Conclusion

In fibroblast at various concentrations of serum, the induction of ERK seems to be optimal. In any case, BXB-ER activation cannot induce additional growth. However, if MEK activity is inhibited by PD184352, additional BXB-ER activation can restore ERK phosporylation levels, and also growth rates to the levels they were before MEK inhibition, but not more. Therefore, the cells seem to control the level of ERK activity very tightly, to be optimal for growth.

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