Making new stable cell line and measuring mitochondrial activity in many conditions for making clear the Raf-mitochondrion interaction.

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Abstract

Mitochondrion activity is important. If something is happened in the cell, for example apoptosis, the cytochrome C is released from mitochondrial membrane and it will be dead. It is because mitochondria can receive some signals via some proteins as second messenger. Recently, it is discovered that Raf which is one protein of the MAP K cascade interacts with mitochondrion. (H. G. Wang, Rapp, & Reed, 1996). However, there are many unclear things about mitochondrion, for example the pH gradient change between OMM (outer mitochondrial membrane) and IMM (inner mitochondrial membrane). I was interested in this kind of interaction and want to make clear this with Dr. Josip Lovric (University of Manchester). Lovric has made BXB-ER cell from NIH 3T3 cells and its activity of Raf is probably controlled by estrogen because of some Raf coded area is inserted the estrogen receptor protein. Hence, we can see the cell and mitochondria viability with or without estrogen. For these reason, I try to measure the mitochondrial viability in many conditions by MTT assay with BXB-ER cell line and make new stable cell line for more deep analysis of Raf-mitochondria interaction. Finally I succeeded to measure that estrogen activates mitochondrial activity, the mitochondria activity of BXB-ER cell line is smaller than NIH 3T3 cell line and the MEK inhibitor suppress the mitochondrial activity. Also, I established base of the new stable cell lines from NIH 3T3 cell lines. For these points, the research of Raf-mitochondria interaction will be improved.

Introduction

1) Ras/Raf/MAP/MEK

Ras is the head protein of the pathway to activate ERK1/2, it is one of the MAPK (Mitogen-activated protein kinase) cascade. When the Receptor Tyrosine Kinase (RTK) recognize the growth factor, RTK activate its self and it will be recognized by Grb2. Grb2 recruit Sos it is the Guanine exchange factor and exchange the GDP

which is binding Ras to GTP. Ras binding GTP is active and phosphorylates Raf. Phosphorylated Raf will phosphorylate MEK and the phosphorylated MEK phosphorylates the ERK. ERK1 and ERK2 will make dimer and it goes into nuclear and control the translation, growth, mitogen. From previous research, the Raf interact with mitochondria also apoptosis of the cell. 出典

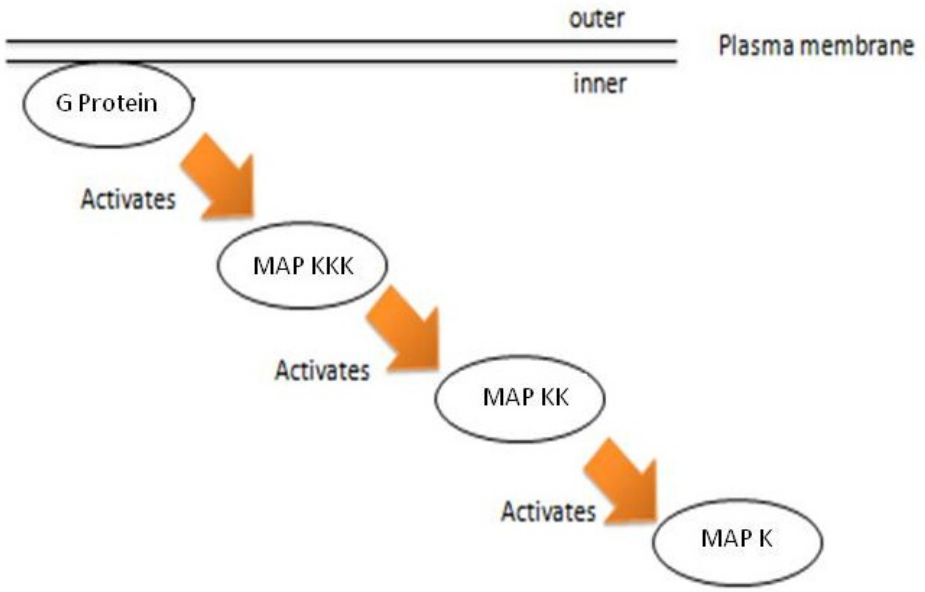


Figure 1 The model of the MAP K cascade. G protein is activated by the replaced GTP when the receptor receives the messenger. (Here, it is not showed) Then, G protein activates (phosphorylates) MAP KKK protein. Similar to this pathway, MAP KK is activated by MAP KKK and MAPK is activated by MAPKK. Finally, MAP K activates the transcription factors.

2) BXB-ER cell line

In the previous work in Dr. Lovric Lab, BXB-ER cell line is established. It is made from NIH 3T3 cell line. There are three types of Raf isoforms in mammals: Raf-1(C-Raf), A-Raf and B-Raf In these three types of isoforms have three conserved regions; CR1, CR2, CR3. Function of CR1 is Ras-Raf interaction, CR2 phosphorylates and protein-protein interactions can influence localization and activation of Raf. CR3 is the kinase domain of Raf (Chong, 2003). I used BXB-ER cell line its CR1 and CR2 of Raf-1 is transformed by estrogen receptor fusion protein. Hence, activity of CR1 and CR2 of Ras-1 in the BXB-ER cell line is regulated by estrogen. Adding the estrogen will activate Raf. Raf interacts mitochondria, not only the mitogen.

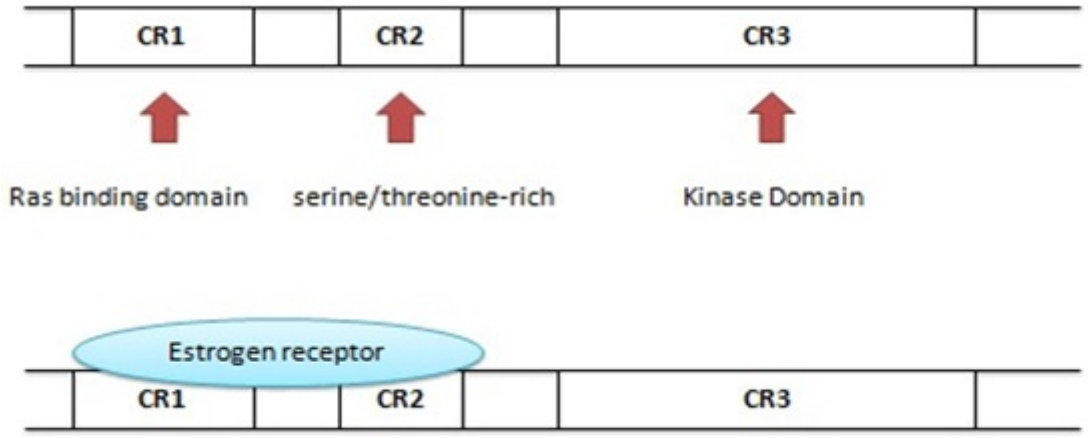


Figure 2: **The model of the conserved genome region of the Raf; CR1, CR2, CR3.CR1 is Ras binding domain and CR2 is serine/threonine richdomain. Lovric have done the transformation from NIH3T3 cell s. It is called BXB-ER cells which CR1 and CR2 domain inserted the estrogen receptor.**

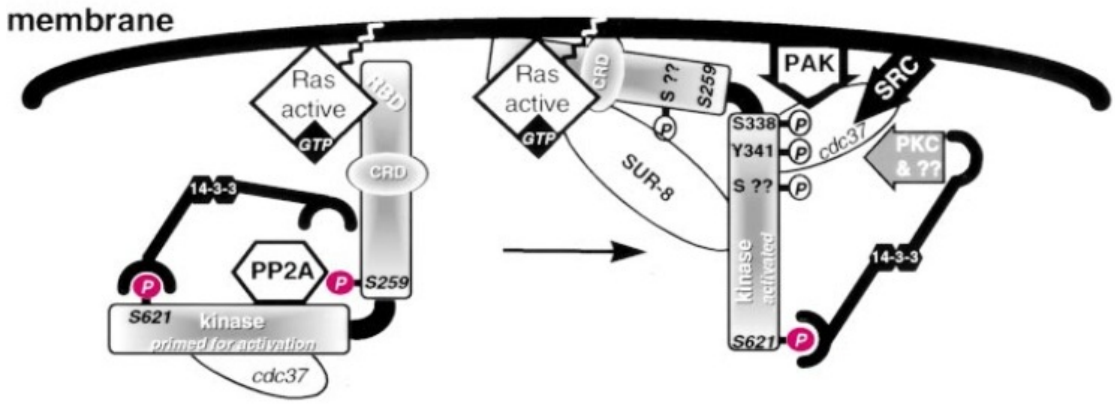


Figure 3: Meaningful relationships: the regulation of the Ras/Raf/MEK/ERK pathway by protein interactions, Walter KOLCH, Biochem. J. (2000) 351, 289–305 (Printed in Great Britain) 289.

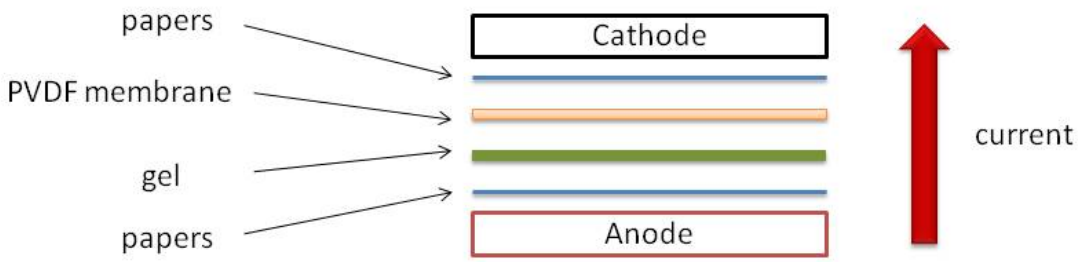
This figure shows the RBD (Ras binding domain) and the kinase domain of Raf. Both domain has quite different function because of structure problems. Hence, comparing two cell lines is very important for understanding the function of Raf. Two cell lines are; one is BXB-ER cells and the other is the new stable cell line which all CR domains are transformed to estrogen dependence.

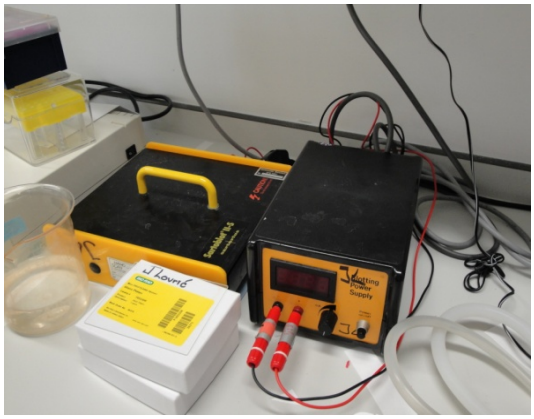
3) MTT assasy

Active mitochondria have reduction power because of its high density of hydrogen. 3-(4,5- Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide compound (MTT) has yellow colour but once it is reduced, the colour will change to purple and become crystallize as formazan dye. In 570nm length wave visible light can measure the density of this frmazan crystal. Hence, adding MTT solution into the reductive solution, we can measure the reduction power of the solution. (Nomura et al., 1996) Crude mitochondrion has reduction power because of its high density of hydrogen. So, we can measure the mitochondria activity indirectly.

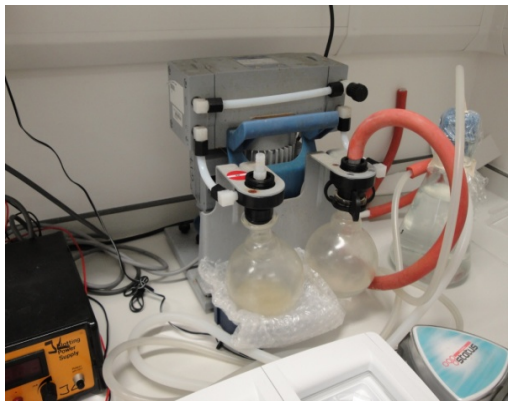
5) Western Blot

Western Blot is one of the traditional ways to detect the proteins which we want to see what happen in the cell (ex. phosphorylation) and the size of it. Before running the Western Blot, it needs running SDS-PAGE. SDS-PAGE (Sodium Dodecyl Sulfate polyacrylamide gel electrophoresis) is the powerful tool to separate the proteins by the molecular weight accurately for cutting the disulfide bonds by the ß-mercapto ethanol. After running the SDS-PAGE, transfer the proteins to membrane (ex. PVDF membrane) and treat with antibodies. After that, soak the membrane into the luminescence solution and the protein will be detected by emission the light because of the chemiluminescence reaction.

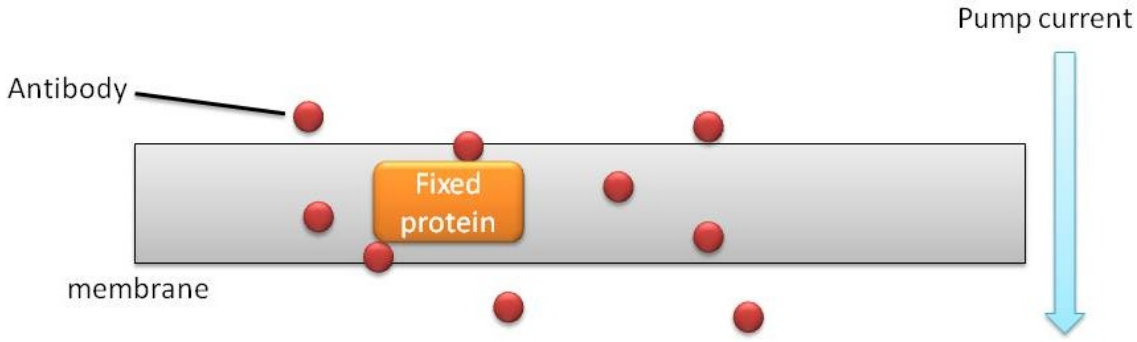




left figure is the transferring machine. (see above figure.) It make electrical current between the gel and PVDF membrane. Finally, the proteins which separated by SDS-PAGE move on to PVDF membrane.



Both two figures are the equipment for detection the protein. Left one is blotting machine and right one is vacuum pump. These two are connected and work very powerful. See below. Transferred protein is tightly fixed in PVDF membrane. The best effort of the detection depends on how many antibodies meet protein. Hence, pump the air and vacuum the antibody solution helps good detection because the antibodies goes in membrane easily than without the pump current.



Materials & Methods

1) Western Blot-reagents

Western Blot Lysis Buffer: 150mM NaCl, 1% Triton X-100, 50mM Tris pH8.0. 4xSDS sample loading buffer: 200mM Tris pH6.8, 40% Glycerol, 16% ß-mercaptoethanol, 6% SDS, Bromophenol. 10x Western Blot Transfer Buffer: 250mM Tris pH 9.5, 1.11M Glycine. 1xWestern Blot Transfer Buffer: 25ml 10xWB Transfer Buffer, 25ml MtOH, 225ml Water.

Blocking solution: 1% BSA, Primary antibodies: (1.5ml PBST + 1µl antibody)/1 membrane. Normal dilution is 1:5000, so I calculated like this… Traditional method needs 10ml antibody solution with designed dilution. So, in traditional method we need 10ml PBST and 2 µl antibody. However, in SNAP id Kit, we have to make it more concentrated. The recipe is 3ml PBST + 2µl antibody (same amount). Secondary antibody:

(1.5ml PBST + 1.25 µl antibody)/1 membrane

I diluted it in same way as primary antibody assuming that the normal dilution is 1:4000.

2) Western Blot-methodos-

2-1) Lyse the cells(10cm dish)

Wash the plates with Ice cold PBS (5ml) twice. Add 600µl Western Blot Lysis Buffer (with PROTEASE INHIBITOR) and scrape them off. Put the solution into epis. After that, epis were vortexed twice for 5seconds each. Centrifuge them in 13000g, 10min, at 4˚C. Take out SN and put into new epis. (throw pellet away) Add the 4XSDS loading buffer. Volume= (Volume of SN)/3. Boil them in 3min at 95˚C . Load 20µl of them to gel.

***Store the samples in -20 ˚C. *Every time re-boil before using sample**

***If the plate was small ones (e.g. 24wells plate)…**
Wash them with PBS 2times on the plate. Add 50 µl Western blot lysis buffer (on ice with PROTEASE INHIBITOR) leave it on for 2 min. Resuspend it and transfer it in an Epi for centrifugation, trying to avoid foaming. Then go back to the process 3 of 10cm dish.

2-2) Electrophoresis

Protein marker should be warmed up in 42˚C 1min. (Protein marker 7µl + loading buffer 12 µl + water 12 µl) Load the 20µl sample and Protein marker. Run the power supply in 180mA 1.5h
***All of the wells should be filled with the sample or loading buffer.**

2-3) Transfer the protein to the membrane

Use the PVDF membrane. Before using it, treat it with Methanol well. Soak the PVDF membrane, paper filters, gels in transfer buffer. Run the power supply of electrical blotting 500mA, 1hour. Take out the membrane from the blotting equipment and soak it in Methanol again.

2-4) Blocking the protein

Block the protein on the membrane by treating with 5% BSA in 10ml PBST 30min.

2-5) Detection

Take out the membrane from BSA and washing it with PBST 3min 3~5 times. Throw away the PBST and treat it with the primary antibody (high-HA affinity) 1 hour. Throw away or keep the antibody in fridge and washing the membrane with PBST 3min 3~5 times. Throw away the PBST and treat it with the secondary antibody 1 hour. After treating with secondary antibody, wash it with PBST 3min 3~5 times. Treat the membrane with chemiluminescent working solution 5min. (mix the Luminol and Peroxide 5ml each). Expose the X-ray sheet to the light of chemiluminescent. (Exposure time depends on how the bands looks like).

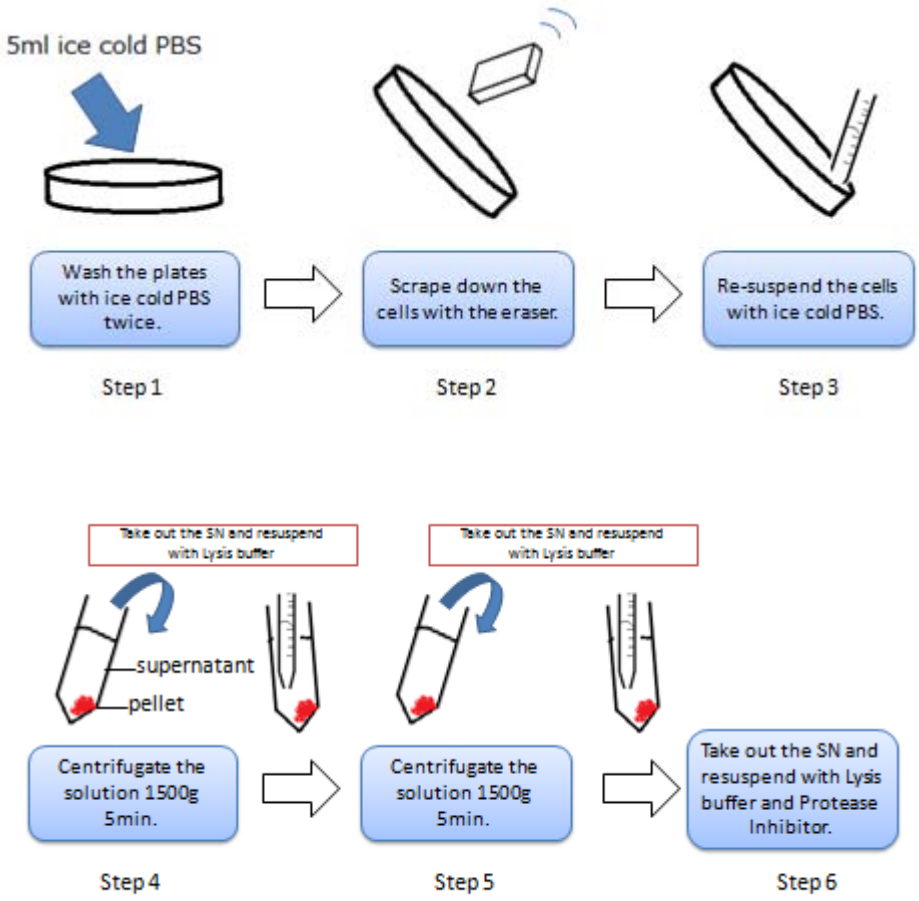
3) Reagents for MTT assay

MTT stock solution; 1ml MTT solution(5mg/ml) + 9ml Complete Medium(10% FCS, 1%L-Glutamin, 1% Pen-strep), MTT working solution; 1.5ml MTT stock solution + 500mM MgCl 30μl + 30mg BSA,

Lysis buffer; 1M HEPES (pH7.2) 2.5ml + 2M Sucrose 5ml + 500mM EDTA 250μl + 500mM EGTA 250μl + MilliQ (fill up to 50ml), PBS, protease inhibitor, estrogen, FCS, UO 126 (MEK inhibitor).

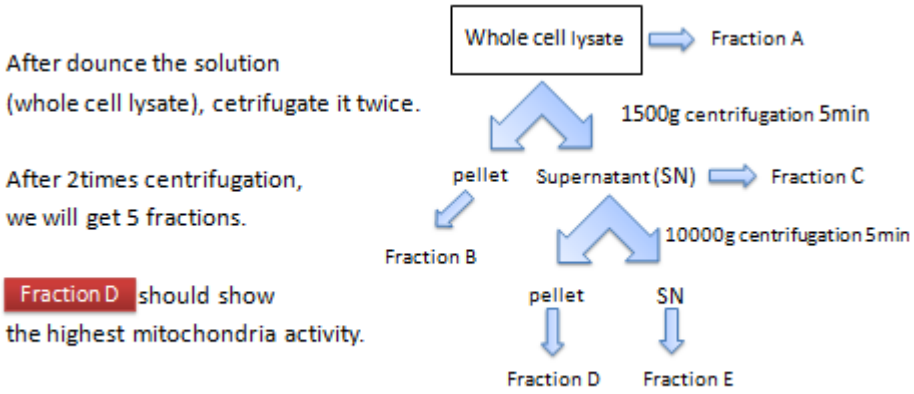
4) Washing the cells and lysing them

Wash cells twice in ice cold PBS and scrape them off the plates with ice-cold ethanol sterile eraser. Re-suspend in 5ml/plate ice cold PBS. Centrifuge cells at 1500 g for 5 min. Discard supernatant. Carefully re-suspend cells pellet in 5ml Lysis buffer and centrifuge it at 1500 g for 5 min. Re-suspended pellet in 1.5ml Lysis buffer (with protease inhibitor), put it into the dounce homogenizer and dounced it in 35 strokes.



4) Method of isolating mitochondria

Wash cells twice in ice cold PBS and scrape them off the plates with ice-cold ethanol sterile eraser. Re-suspend in 5ml/plate ice cold PBS. Centrifuge cells at 1500 g for 5 min. Discard supernatant. Carefully re-suspend cells pellet in 5ml Lysis buffer and centrifuge it at 1500 g for 5 min. Re-suspended pellet in 1.5ml Lysis buffer (with protease inhibitor) and dounced it 35 strokes with the small douncer. Take 100µl from this (as fraction A) and rest of it is centrifuged at 1500g for 15min. Nuclei/cells accumulated as the pellet. Re-suspend this pellet with 100µl Lysis buffer with 0.1% protease inhibitor (B fraction).Collect SN containing mitochondria (C fraction(take 100 µl)) and centrifuge at 10000g for 5min. Pellet (D fraction; re-suspend with 100 µl with protease inhibitor) should contain crude mitochondrial fraction. And the SN is fraction E.



5) Adding MTT solutions and incubation

Add 20µl of each sample fraction and 20µl MTT working solution in each well. Then, incubate them at 37˚C, 7% v/v CO2 during 60mins. At the same time, add 20 µl Lysis buffer and 20 µl MTT working solution into the same plate. After incubating, add 20µl 10%SDS in each well and wait 2min until all proteins are dissolved. After then, add 50µl iso-propanol with HCl to each well and dissolve the all formazan crystals.

6) Method of Inducing estrogen and starvation

Estrogen is reserved in ethanol. When we use this for inducing cells, we have to dilute this solution 1:10000. First, we mix the 10µl stock estrogen and ethanol solution with 990µl PBS. It is 1:100 dilution. Then, put 100 µl estrogen solution into 10ml medium (into plates). This is 1:100 dilution too. Thus, finally we can dilute estrogen solution as 1:10000. Starvation medium has the same components as Complete medium without FCS. Starvation medium includes 2.5ml FCS (as 0.5%). The day before evening inducing the estrogen, remove all medium from cell culture and replace it with starvation medium (10ml each). It should be warmed

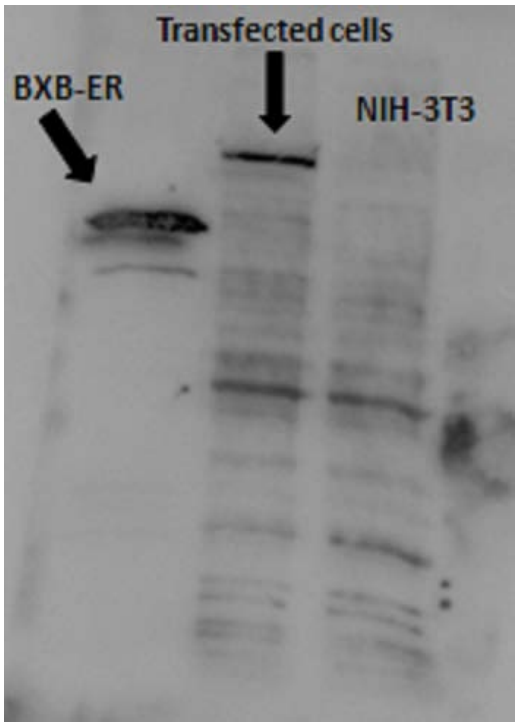
before put it in. Next morning, induce pure ethanol to half of dishes and estrogen to the others (100μl each). Then, incubate 20minutes. After incubating, take out dishes from incubator and put these on the ice →use for experiment. Replace the complete medium to the starvation medium. After 17 hours, induce some reagents and wait 20min.

*When UO126 is induced, 6ml medium will be replaced from all plates before adding it.

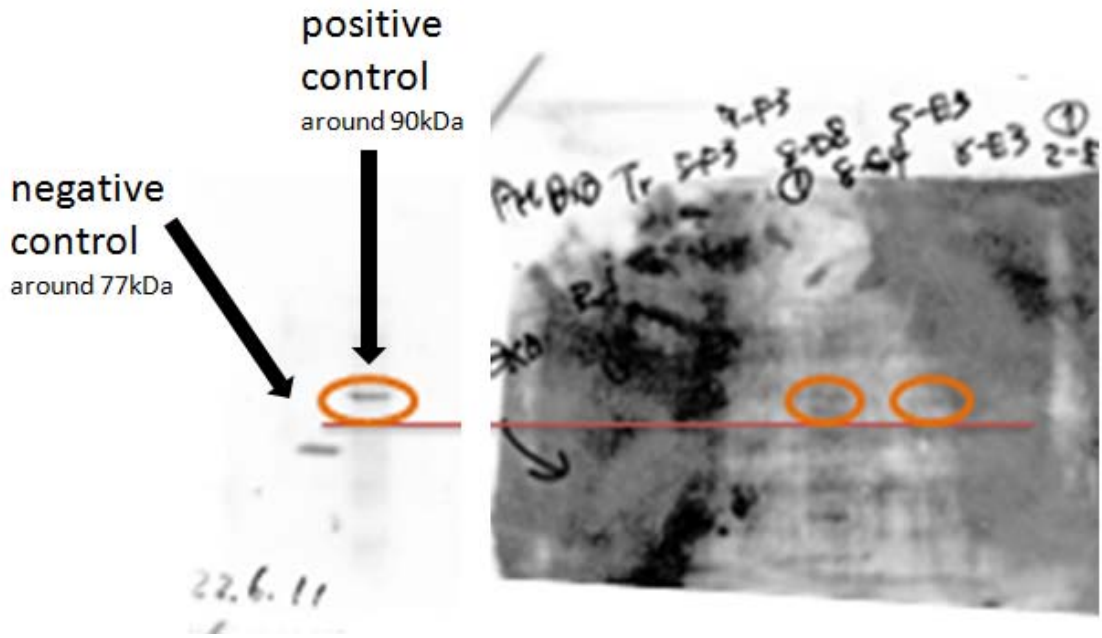
*The final volume includes...30% of FCS and 0.2% of UO126

Starved cells treated with FCS...add 4.3ml FCS (10ml*3/7=4.3ml). Starved cells treated with UO126...add 8μl UO126 (4ml*0.2/99.8=8μl). Starved cells treated with UO126 and FCS...add 1.73ml FCS and 11μl UO126 (4ml*30/69.8=1.73ml, 4ml*0.2/69.8=11μl)

Results (Western Blot)



This figure shows the result of Western Blot using BXB-ER cells, transfected cells (transient transfection) and NIH-3T3 cells. BXB-ER cells can be a negative control and Transient transfected cells can be positive control. Transient transfection is not stable, because this is just 24hours later from transfection. Thus, some of the induced DNA has not insert into chromosomes. So, there is full length of Raf CR domain band, but it cannot be suggested that is stable new cell line from this band. After the selection by G418, isolate them as one single cell. After cloning the one single cells in G418 medium one to two weeks, the clone have grew and become stable cell lines. Then the cells are checked by Western Blot again. The result is next.



From this Western Blot, some cell lines show the full length Estrogen receptor CR domain bands (see the orange circles). Other cell lines which doesn't show the bands probably the resistance gene for G418 is inserted but the Estrogen receptor domain wasn't inserted.

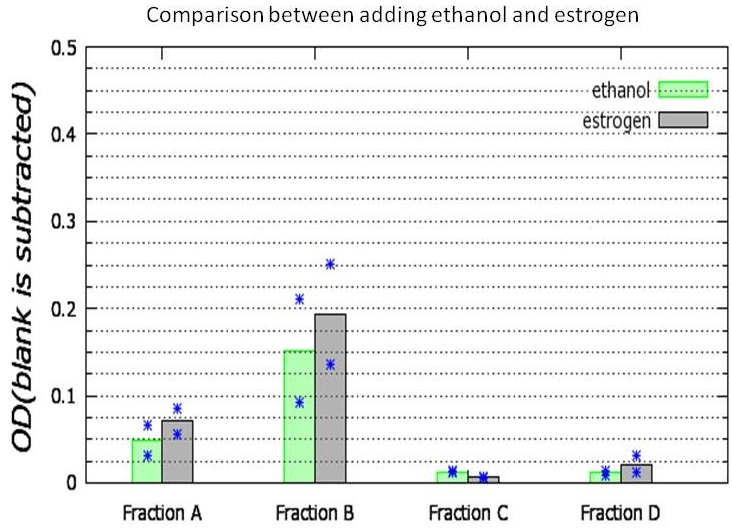
Results (MTT assay)

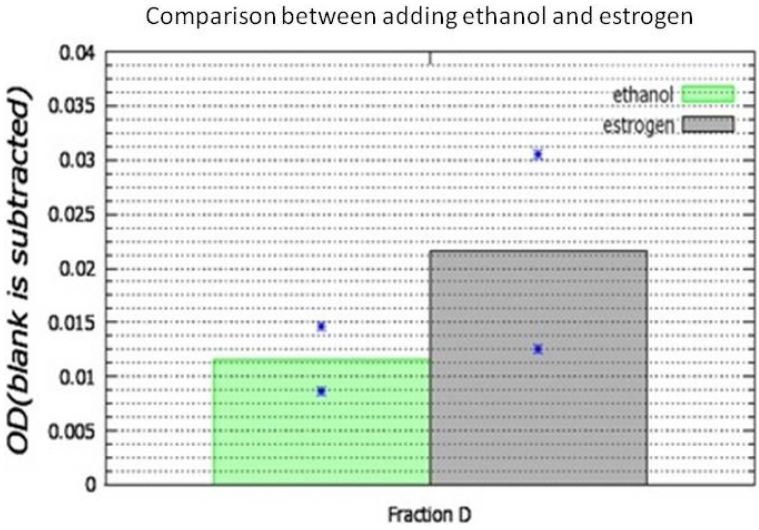
Table 1

KH4	Raw Data (570)				
	A	B	C	D	E
Raw treated with ethanol	0.214	0.276	0.196	0.192	0.171
Raw treated with estrogen	0.239	0.32	0.189	0.196	0.175
Lysis buffer + MTT + isopropanol	0.194	0.187	0.191	0.176	0.169
(treated with ethanol) - (control)	0.0306	0.0926	0.0126	0.0086	-0.0124
(treated with estrogen) - (control)	0.0556	0.1366	0.0056	0.0126	-0.0084
treated with ethanol calculated	2.295	0.7408	0.819	0.0688	-0.682
treated with estrogen calculated	4.17	1.0928	0.364	0.1008	-0.462

Table 2

KH5	Raw Data				
	A	B	C	D	E
Raw treated with ethanol	0.243	0.389	0.191	0.192	0.164
Raw treated with estrogen	0.263	0.429	0.185	0.208	0.171
Lysis buffer + MTT + isopropanol	0.196	0.177	0.182	0.163	0.169
(treated with ethanol) - (control)	0.0656	0.2116	0.0136	0.0146	-0.0134
(treated with estrogen) - (control)	0.0856	0.2516	0.0076	0.0306	-0.0064
treated with ethanol calculated	4.92	1.6928	0.884	0.1168	-0.737
treated with estrogen calculated	6.42	2.0128	0.494	0.2448	-0.352





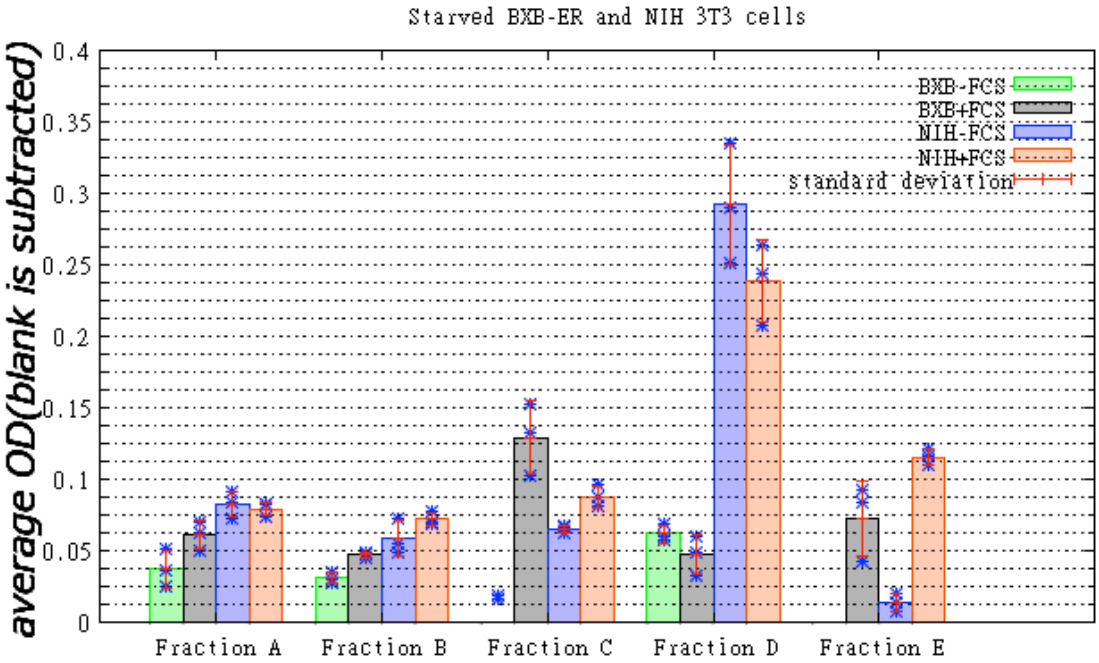
From this result, we can see the difference between treated with ethanol and estrogen in fraction D. Fraction D is crude mitochondria and we can know the mitochondrial activity from fraction D. activity which are treated with estrogen are higher than ethanol.

Table 3

starved -FCS					
Raw data	A	B	C	D	E
BXB	0.206	0.211	0.197	0.238	0.166
	0.217	0.209	0.036	0.25	0.172
	0.232	0.216	0.2	0.241	0.169
NIH	0.265	0.23	0.247	0.516	0.201
	0.254	0.254	0.244	0.432	0.195
	0.272	0.236	0.249	0.471	0.189
blank	0.186	0.177	0.187	0.176	0.178
k is subtra	A	B	C	D	E
BXB	0.206	0.211	0.197	0.238	0.166
	0.217	0.209	0.036	0.25	0.172
	0.232	0.216	0.2	0.241	0.169
NIH	0.265	0.23	0.247	0.516	0.201
	0.254	0.254	0.244	0.432	0.195
	0.272	0.236	0.249	0.471	0.189
blank	0.1808				
average	A	B	C	D	E
BXB	0.218333	0.212	0.144333	0.243	0.169
NIH	0.263667	0.24	0.246667	0.473	0.195

Table 4

starved +FCS					
Raw data	A	B	C	D	E
BXB	0.244	0.228	0.313	0.214	0.224
	0.251	0.226	0.334	0.241	0.273
	0.231	0.23	0.283	0.23	0.265
NIH	0.255	0.251	0.262	0.388	0.291
	0.263	0.258	0.277	0.425	0.302
	0.263	0.25	0.268	0.445	0.297
blank	0.198	0.202	0.216	0.268	0.254
k is subtra	A	B	C	D	E
BXB	0.244	0.228	0.313	0.214	0.224
	0.251	0.226	0.334	0.241	0.273
	0.231	0.23	0.283	0.23	0.265
NIH	0.255	0.251	0.262	0.388	0.291
	0.263	0.258	0.277	0.425	0.302
	0.263	0.25	0.268	0.445	0.297
blank	0.2276				
average	A	B	C	D	E
BXB	0.242	0.228	0.31	0.228333	0.254
NIH	0.260333	0.253	0.269	0.419333	0.296667



BXB+FCS in fraction C and Fraction E (BXB and NIH) were precipitated, so these bar are higher than the real OD. From this graph, I can suggest Raf-ER domain is not so activated by FCS. Also, FCS decreases the mitochondrial activity little bit. See the gray bar and red bar. Red bar is almost 5times higher than gray bar. It mentions that Raf-ER domain is not activated by FCS. If it is activated by FCS radically, the OD of BXB-ER cells will be same as NIH one. Next, we can see the difference between NIH 3T3 cells +/- FCS (BXB as well). Except fraction D, in all fractions which are treated with FCS cells show higher OD than that are not treated. However, in fraction D, blue bar is higher than red one. It means mitochondrial activity will be decreased little bit by FCS.

Table 5

27.1.11 nothing and FCS					
Raw data	A	B	C	D	E
	0.234	0.235	0.225	0.242	0.193
nothing	0.247	0.244	0.23	0.265	0.211
	0.242	0.242	0.238	0.24	0.209
	0.237	0.255	0.24	0.242	0.199
ated with l	0.249	0.247	0.246	0.25	0.199
	0.244	0.251	0.243	0.256	0.192
blank	0.222	0.224	0.225	0.213	0.194
blank is subtracted					
	A	B	C	D	E
	0.234	0.235	0.225	0.242	0.193
nothing	0.247	0.244	0.23	0.265	0.211
	0.242	0.242	0.238	0.24	0.209
	0.237	0.255	0.24	0.242	0.199
ated with l	0.249	0.247	0.246	0.25	0.199
	0.244	0.251	0.243	0.256	0.192
blank	0.2156				

Table 6

27.1.11 UO and UO+FCS					
Raw data	A	B	C	D	E
	0.222	0.224	0.211	0.216	0.189
UO	0.233	0.237	0.209	0.231	0.193
	0.235	0.23	0.221	0.224	0.195
	0.216	0.23	0.218	0.211	0.194
UO+FCS	0.239	0.22	0.215	0.228	0.189
	0.231	0.245	0.225	0.231	0.198
blank	0.212	0.207	0.198	0.201	0.195
blank is subtracted					
	A	B	C	D	E
	0.222	0.224	0.211	0.216	0.189
UO	0.233	0.237	0.209	0.231	0.193
	0.235	0.23	0.221	0.224	0.195
	0.216	0.23	0.218	0.211	0.194
UO+FCS	0.239	0.22	0.215	0.228	0.189
	0.231	0.245	0.225	0.231	0.198
blank	0.2026				

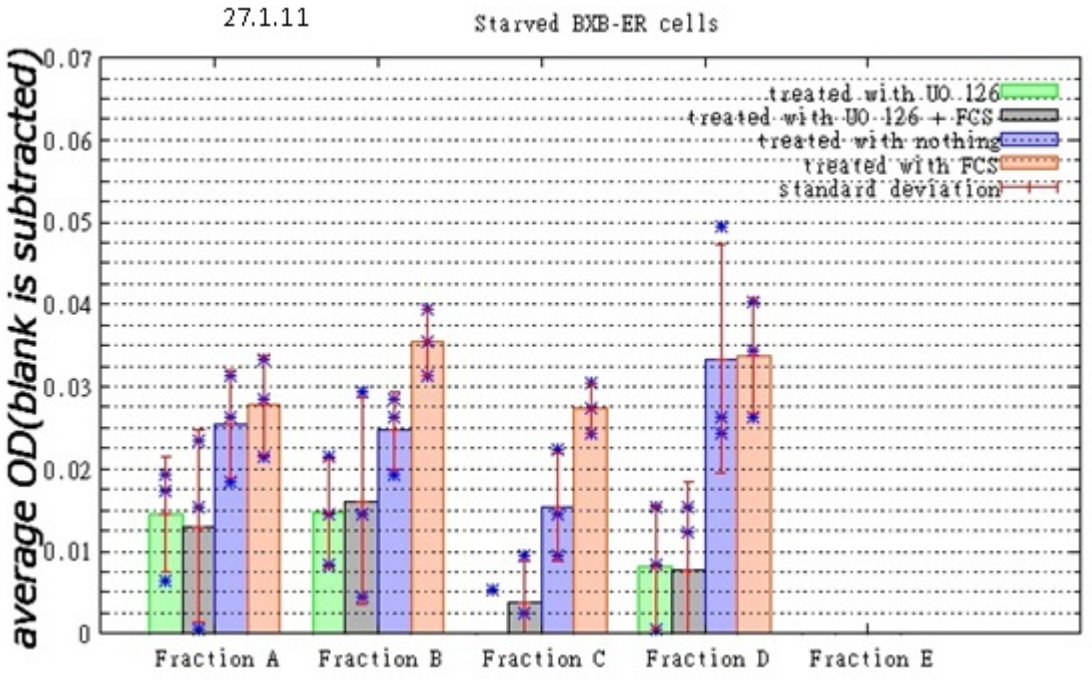


Figure 9

From this result, I can suggest the activity of MEK is relation to mitochondria activity. See the difference between "treated with FCS after treating with UO 126 (gray bar)" and "treated with FCS (red bar)". Red bar is quite higher than gray bar. When we treat the BXB-ER cells, Raf will be activated and MEK/ERK will be activated too. On the other hand, when I added the UO 126 (MEK inhibitor) before adding FCS, Raf will be activated. However, MEK is inhibited by UO 116, so MEK and ERK will not be activated. From this comparison, MEK/ERK may activate the mitochondria activity.

Table 7

raw data 26.1.11	A	B	C	D	E
starvedc cells treated with UO 126 and FCS	0.096	0.111	0.062	0.221	0.031
starved cells treated with UO 126	0.106667	0.121667	0.065334	0.226334	0.031667
starved cells	0.144667	0.239667	0.084667	0.251667	0.154
starvedc cells treated with FCS	0.166667	0.28	0.089334	0.250667	0.251
relative data 26.1.11	A	B	C	D	E
starvedc cells treated with UO 126 and FCS	1	1.156249	0.645835	2.302079	0.322919
starved cells treated with UO 126	1.111111	1.26736	0.680557	2.357634	0.329863
starved cells	1.506943	2.496523	0.881945	2.621522	1.604165
starvedc cells treated with FCS	1.736109	2.91666	0.930556	2.611106	2.614578

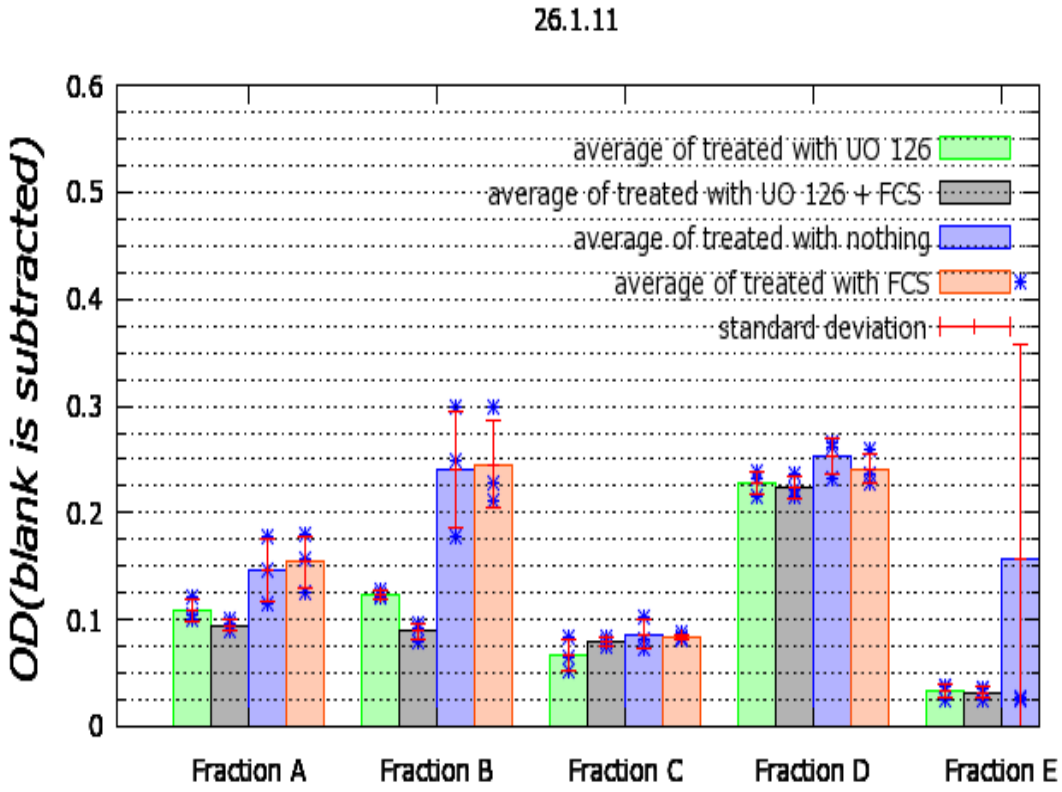


Figure 10

NIH cells

Compare these three graphs. We can't see any activity of UO 126 treated lines in 28.1.11. Also, the I repeated the same experiments. After one night starvation, treat with UO 126 20min, UO 126 + FCS, treat with nothing, treat with FCS. From this result, in NIH cells, there is not so radical difference between each line (maximum is 9%)

Discussion

See the figure 7, the graph shows estrogen activates mitochondrial activity. The samples of this experiment are only two in each condition, so to improve this result, we need more samples to calculate standard deviation. Next, figure8. There are very big difference between NIH 3T3 cells and BXB-ER cells in fraction D. In fact, in all fractions, NIH show bigger activity than BXB, however, fraction D is quite bigger than the other fractions. Fraction D is crude mitochondria, so I can suggest that BXB-ER's ER domain has very important function for mitochondrial activity.

See the difference figure 9 and 10. Fraction D is crude mitochondria, and we can see the obviously difference between NIH 3T3 cells and BXB-ER cells in fraction D. NIH 3T3 cells have the mitochondrial activity even though it is treated with UO 126 (MEK inhibitor). On the other hand, BXB-ER cells show lower activity than control. It suggest that, Raf can activate mitochondria without MEK, but the cells have inactivated Raf cannot activate the mitochondrial activity. So, it supports the assumption that Raf activates mitochondria by itself. Also I can assume Raf has other pathway does not thorough the MEK to activate the mitochondria.

Future works

The new cell line is established, but still unstable. It means the new cell line is different from original one, especially become weaken because of suppressing the MAP K cascade. After detecting the best culturing way, it will be used for experiment. The CR1,2,3 domain of new cell line has estrogen receptor, so it can be use for more detail analysis of Raf function. MAP K cascade and mitochondria is very important to analyse the cancer mechanism, aging mechanism. Hence, to analyse the function of Raf also mitochondria in the cell is very useful for human.

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References

Chong, H., Vikis, H. G., & Guan, K.-L. (2003). Mechanisms of regulating the Raf kinase family. *Cellular Signalling*, *15*(5), 463-469. doi:10.1016/S0898-6568(02)00139-0

Lovrić, J., Dammeier, S., Kieser, a, Mischak, H., & Kolch, W. (1998). Activated raf induces the hyperphosphorylation of stathmin and the reorganization of the microtubule network. *The Journal of biological chemistry*, *273*(35), 22848-55. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9712920

Nomura, N., Saijo, K., Kato, M., Wang, P.-chao, Ohno, T., & Matsumura, M. (1996). bromide) ASSAY FOR THE MEASUREMENT OF VIABLE ANIMAL CELL NUMBER IN POROUS CELLULOSE CARRIERS. *Biotechnology Techniques*, *10*(I), 883-888.

Wang, H. G., Rapp, U. R., & Reed, J. C. (1996). Bcl-2 targets the protein kinase Raf-1 to mitochondria. *Cell*, *87*(4), 629-38. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8929532

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