Lecture: 13—Cell Apoptosis and Senescence

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1. Brief Intro

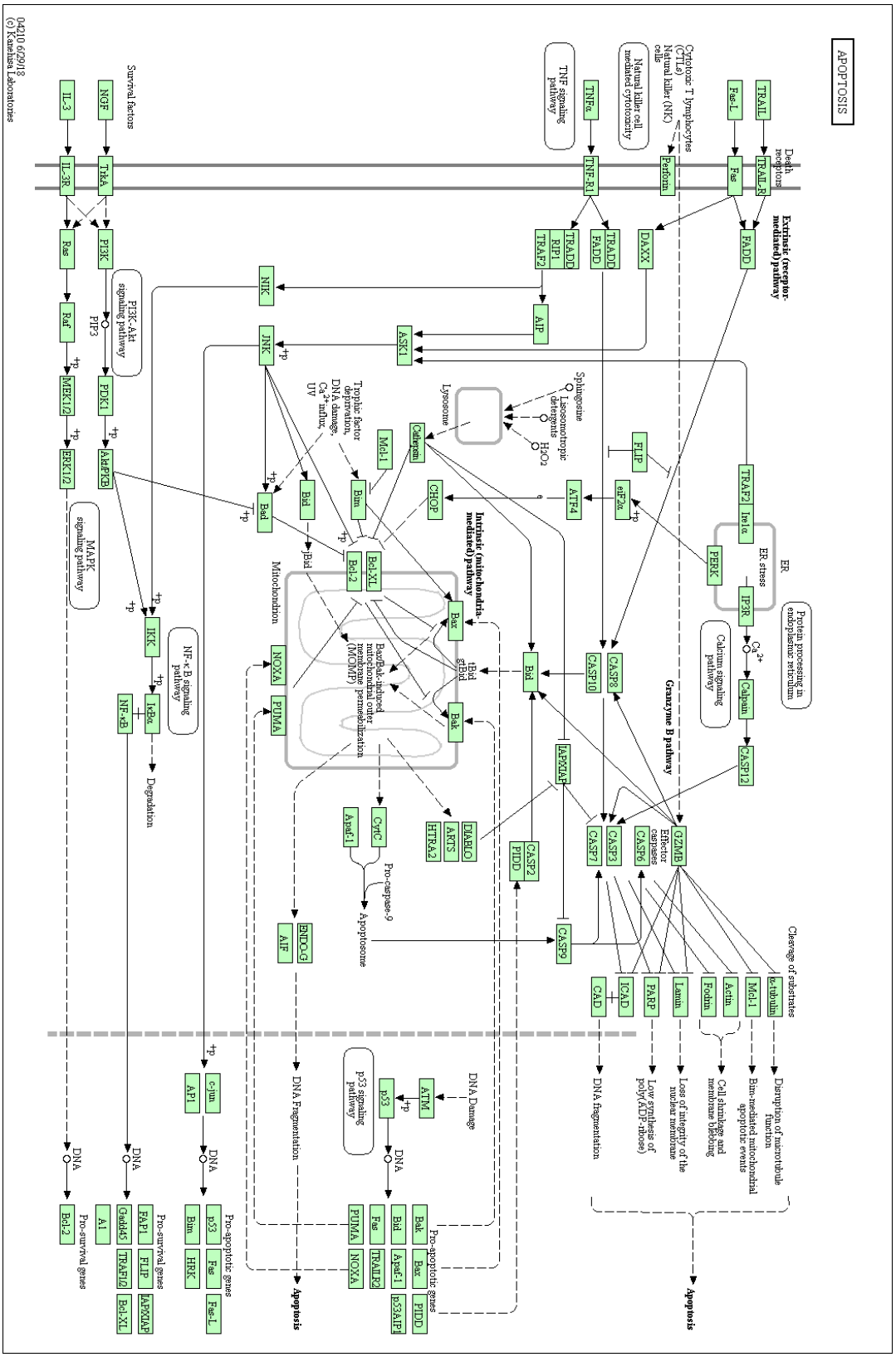

Figure 1. Signaling pathway of apoptosis in human body[1][2][3]

TNF signaling pathway: [4][5][6][7][8][9]

TNF-α是一種主要由macrophage分泌的cytokine，當他與細胞上的TNFR1(一種transmembrane protein)結合，會出現以下三種pathway。

a. TNFR1會與TRAF2和RIP結合，TRAF2又再與kinase IKK結合，此時旁邊的RIP活化IKK，活化的IKK會去磷酸化IκBα，IκBα平常與NF-κB結合並抑制其translocation，被磷酸化的IκBα則會降解並釋放NF-κB。此時，NF-κB會translocate to nucleus並促使一些有關cell survival, proliferation, inflammatory response, anti-apoptotic factors的蛋白質transcription(c-FLIP, Bcl-2, cIAP1/cIAP2等等)。

b. 接在TNFR1上的TRAF2/Rac會活化JNK-inducing kinase，其中活化的MLK2/MLK3磷酸化MKK7，被磷酸化的MKK7再活化JNK，使JNK translocate to nucleus並活化transcription factor including c-Jun and AP1，這些transcription factor則會促使一些pro-apoptotic gene表現，比方說p53, Fas, FasL, Bim, HRK。

c. TNF-α的結合會引發trimerization of TNFR1，這會導致dissociation of SODD(the inhibitory protein) from death domain，此時adaptor protein TNF-receptor-associated death domain(TRADD)會與death domain結合，另一個adaptor protein Fas-associated death domain(FADD)會再與TRADD結合，此時procaspase 8與FADD結合並被活化為Caspase 8，Caspase 8會引發execution of apoptosis和activation of downstream caspase，其中這些被活化的caspase很多會去cleave components of NF-κB，比方說RIP, IKK, subunits of NF-κB。

Fas signaling pathway(Extrinsic pathway): [4][5][10]

要引發Fas signaling pathway有兩種可能，一種是DNA damaging agents活化了p53-dependent pathway，進而造成expression of Fas(First-apoptosis signal, a transmembrane protein of the TNF family)；另一種則是cellular stress或mitochondrial dysfunction，這也會導致expression of Fas。當細胞膜上有Fas表現，同時附近的細胞的膜上也有表現FasL(Fas ligand, 本身是一種trimer)，此時Fas與FasL會結合並引發trimerization and aggregation of Fas，FADD則會在trimerization of Fas之後與Fas trimer結合並再與procaspase 8結合與活化。與此同時會形成由Fas, FADD, Caspase 8組成的death-inducing signaling complex(DISC)，他會transduce造成apoptosis的downstream signal cascade，比方說activation of downstream caspase。

Intrinsic pathway: [4][5][11]

在經歷不論是TNF signaling pathway或是Fas signaling pathway後，與Bcl-2(B-cell lymphoma 2, homologous to ced-9 in C. elegans) family息息相關的調控機制正準備開始。Bcl-2 family中同時有著pro-apoptotic proteins和anti-apoptotic proteins，比方說Bid(BH3 interacting domain death agonist), Bax(Bcl-2 associated X protein), Bak(Bcl-2 homologous antagonist/killer) and Bok(Bcl-2 family apoptosis regulator)就是pro-apoptotic proteins，而Bcl-2, Bcl-XL, and Mcl-1則是anti-apoptotic proteins。當activated Caspase 8一出現，Bid便會被它切斷，剩餘的tBid(C-terminal part of Bid)便會translocate to mitochondria，並促使mitochondrial dysfunction，進而導致release of cytochrome c and Diablo/Smac into cytosol透過permeabilization of the outer mitochondrial membrane。可是Bcl-2 homodimer會阻止這件事的發生，因此這時需要Bax(其實是alternatively spliced Bcl-2)產生進入mitochondria並與

Bcl-2結合行程沒有抑制作用的Bcl-2/Bax heterodimer。在cytochrome c與Diablo/Smac釋放到cytosol後，cytochrome會先與Apaf-1(homologous to ced-4 in C. elegans)結合並使Apaf-1可以與ATP連結同時讓整個complex可以進行oligomerization。Oligomerization會促使Caspase 9 precusor與complex結合，並自我活化形成apoptosome。但此時cytosol中會存在IAP(inhibitor of apoptosis)透過將Caspase precursor與activated Caspase結合來抑制其活性，方才與cytochrome一同被released的Diablo/Smac便派上用場了，他會抑制IAP的活性使caspase主導的apoptosis得以繼續進行。Caspase 9首先會活化Caspase 3(homologous to ced-3 in C. elegans)和Caspase 7，這兩個都是進行apoptosis中抑制DNA repair和起始DNA degradation最主要的protein，其中Caspase 3會剪DFF(DNA fragmentation factor) dimer的其中一個subunit，另一個被活化的subunit便會去降解nuclease。接著各種caspase都會被活化進行apoptosis，當中Caspase 2, 8, 9, 10是負責initiation of apoptosis，Caspase 3, 6, 7則是主要進行apoptosis。(Caspase 6是負責disintegration of the lamina and cytoskeleton)

2. Quiz
   a. What is the biochemical assay Dr. Wang designed to isolate and reconstitute cellular components which can activate Ced3 protein in vitro?

      Wang研究的其實並非Ced3，而是其homolog—CPP32。Wang先將apoptosis分為兩個小主題，activation of CPP32和DNA fragmentation。

      在確認activation of CPP32是dATP-dependent後，Wang又將樣本通過“phosphocellulose column”，並發現吸附在column上的物質和流出column的物質都是proteins required for dATP-dependent activation of CPP32，因此命名流出column的為apoptotic protease activating factor-1 (Apaf-1)，而留在column的為apoptotic protease activating factor-2 (Apaf-2)。

為了解析何為Apaf-2，Wang在樣本中加入 “ammonium sulfate” 讓大多數protein precipitated，上清液中Apaf-1和Apaf-2的功能卻還尚在，因此取上清液通過 “phenyl sepharose column” ，再將eluate通過一個 “gel filtration column”。此時，Apaf-2會流過Mono Q column並停在Mono S column，Wang再將Mono S column上的物質以100–300 mM NaCl linear salt gradient搜集並拿去跑 “SDS PAGE” 以silver staining染色，可以發現用這個方法會搜集到非常純的Apaf-2而不會有其他protein。由於purified Apaf-2有著明顯的粉色，因此Wang嘗試將它拿去測他的spectrophotometric absorbance，沒想到spectrum和cytochrome c的一模一樣。為了確認cytochrome c是不是就是Apaf-2，Wang之後還特地買了cytochrome c確認是否能initiate the dATP-dependent activation of CPP32並和Apaf-2有一樣的效率，當然結果確實是對的。[12]

b. What is the original question the author intends to answer in this study?
在introduction中，其實不難看出作者本來想要找出*c-myc*在G_0/G_1 transition中詳細的調控機制，因為除了introduction最後一段外，其他段全都在談論*c-myc*和growth arrest, quiescent, mitogen, proliferation等等的關係，甚至多次提到這之中有著unknown mechanism存在，卻只在最後一段倒數兩句才說本篇在討論的其實是apoptosis。[13]

c. How to estimate % of cells in S phase when Rat-1/myc cells grow in 0.1% of serum and what is the result?
“Cells were labeled for 1hr with 2mM “BudR”, trypsinized, fixed in ethanol, and stained with propidium iodide and appropriately conjugated anti-BudR antibody (i.e., traversing S phase) as described. “Flow cytometric analysis” was carried out on a Beckton-Dickson FACSstar plus.”

"The results of this analysis are shown in Figure 2 and, paradoxically, appear to show that there is a complete block to growth arrest in cells constitutively expressing *c-myc*. To accommodate these two apparently conflicting results, we examined the possibility that Rat-1/*myc* cells were dying in low serum. Microscopic inspection of such cultures revealed this to be the case."

他是利用BudR只會被DNA合成中的細胞(S phase) 攝入的特性還有flow cytometric analysis可以仔細數每一個細胞，才設計出這樣的實驗，因為他寫得相當詳細而且切中老師的問題，我便不再贅述。[13]

d. Is Rat-1 a primary cells?

是。[14]

e. In Table one, cotransformating and apoptosis inducing activities of Myc mutants were compared. What is the cotransforming activity?

Cotransforming activity是指兩個或多個基因的同時進行transformation的機率。只有在同一染色體附近的基因才有機會一起被transformed，所以當兩個基因越靠近，cotransforming activity就越高。至於此篇文章，作者為了找出Myc protein是哪個特定位置進行cotransformation，因此將*c-myc*各個片段突變的個體拿來測試其cotransforming activity。

f. Is myc-induced apoptosis a cell cycle dependent event? What experiment results support your answer?

是。由引用的文獻可以看到只有在G2 phase時myc才是必要的，在G1 phase時*c-myc*$^{-/-}$細胞的apoptosis根本不受影響。[15]

g. What is the question you are most interested after reading this paper and how to answer your question by experiment?

文章中提到 "Successful proliferation would then presumably occur only if apoptosis were actively inhibited, perhaps by activation of complementary

signal transduction pathways.”也已經知道Myc protein進行proliferation和apoptosis是使用同一個region，因此我有個疑問「Inhibition of apoptosis是在Myc的上游、下游、還是Myc本身？」要回答這個問題，我認為可以從Myc是如何induce apoptosis著手，因為apoptosis的pathways其實相當多種，但失去任何一條apoptosis還是可以進行，所以我想先把每一條pathway比較下游的gene mutated，先確認Myc是induce哪一條apoptosis pathway。在知道主要是哪一條pathway後，則開始從該條pathway已知的部分開始測試，每一個protein和Myc的上下游關係。當確認好Myc是從哪一步開始涉入apoptosis，便能開始驗low/high serum時各個protein的表現量，不論是在control還是c-myc mutant，最後就能明確知道inhibition of apoptosis是在Myc的上游、下游、還是Myc本身。

Reference

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