Lecture 22: Senescence

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1. Lecture Notes

Why we age?

根據Peter Medawar的理論很可能在我們三十歲時就已經累積了六十歲才會發作的基因突變，然而也有另一種可能性，可能本來就存在這樣的基因缺陷，然而這樣的基因表現在後繁殖期，因此這樣的基因型並不會被天擇淘汰，此外也仍有可能是某種基因型對於年輕個體有益卻對老年個體有害。但這些可能性還是無法解釋老化機制的存在，老化機制之所以在大自然中比較早或晚出現在某些物種很可能和該物種長就以來演化出的生存策略相關，倘若在大自然中有不少生存壓力（比方說狩獵者或是食物來源長期匱乏），這樣的生物在繁殖與修復身體的抉擇時，往往會傾向於選擇繁殖，因為修復身體並沒有辦法順利地將基因型傳遞下去，反之亦然。

How we age?

Telomere在老化當中佔很重要的地位，當telomere過短或是telomerase效能過低，很可能使telomere再也無法保護染色體，細胞也會失去分裂的能力。另一種機制則是來自氧化壓力及自由基的破壞（電子傳遞練、基因體和膜等），然而氧化壓力又是從何而來？現在發現大多是來自於飲食，特別是在熱量控制或節食後對於生物體有著正面的影響。

（題外話，SOD1是清除細胞內自由基的一種酶，在許多SOD1 mutant都有發現漸凍人症ALS，原因正是在於沒被清除的ROS會去攻擊運動神經元的neurofilaments使其開始denature，隨後導致atrophy and impairment of axon，進而影響神經訊息傳遞）

1. Questions and Answers

Question 1:

我很好奇究竟細胞老化是不是一個permanent state？也就是說，細胞老化後會一直維持保持在老化的狀態而無法復原成正常細胞？(senescence可不可逆？) 若是可逆的，則有沒有辦法實現讓人類壽命延長或是「返老還童」？

Answer 1:

我記得老師之前有提過如果讓端粒不會隨著時間而縮短，很可能細胞就不會衰老，但很容易因此產生癌細胞，但若細胞已經進入了老化狀態，有辦法使細胞重新「轉化為」未衰老的細胞嗎？(之前的iPSC是用正常的細胞做，可以使一般體細胞重新變為pluripotent cell，然而若針對已經老化的細胞也不確定是否也可以使用同樣的方法達成目的)

故我認為應先從「加入端粒酶」的方向來看，若在老化細胞中表現端粒酶，是否能讓老化的細胞脫離老化狀態？

而我查到有人曾經做過類似的實驗：

The first candidate we tested for ability to reverse the senescence growth arrest was hTERT, the catalytic subunit and rate-limiting component of telomerase. For these and subsequent experiments, we used two human fibroblast strains: WI-38 (WI) from fetal lung and BJ (from neonatal foreskin). Neither strain expresses the endogenous TERT gene, and both are devoid of detectable telomerase activity, as determined by the telomere repeat amplification protocol (TRAP) assay. [1]

實驗中使用了兩個fibroblast strains，分別為WI (fetal lung) 與BJ (neonatal foreskin)，且兩個strains皆不具有TERT的活性 (TERT為端粒酶中的catalytic subunit，和TERC共同組成端粒酶，故此時strains不具有端粒酶的活性)

We passaged pre-senescent (early passage) cultures (P-WI, P-BJ) until replicative senescence. Unless noted otherwise, senescent cultures (S-WI, S-BJ) contained >99.9% non-dividing cells, as determined by no increase in cell number over >4 wks and <1% [3H]thymidine-labeled nuclei after a 3-day labeling interval (% LN). To express hTERT and other proteins, we used lentiviruses, which efficiently infect and stably express genes in non-dividing cells (Bukrinsky et al., 1993). We verified the infection efficiency by infecting parallel cultures with an equivalent titer of virus expressing green fluorescent protein (GFP), and, where possible, immunostaining for the virally expressed proteins. At the titers employed, the lentiviruses transduced >95% of cells in both pre-senescent and senescent cultures.

實驗中對P-WI, P-BJ(尚未進入senescence的strains)及S-WI, S-BJ(已進入senescence的strains)進行lentiviruses的感染，以將TERT的基因送入細胞中，使細胞表現TERT蛋白，同時使用螢光蛋白(GFP)測定轉染的效率。老化的細胞已喪失細胞分裂的能力。

而實驗結果如(Figure 1A, Figure 1C)

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Description automatically generatedThe hTERT-expressing lentivirus (lenti-hTERT) conferred robust telomerase activity on S-WI cells, whereas S-WI cells infected with lenti–GFP were devoid of telomerase activity. By contrast, pBabe-hTERT, which requires cell proliferation for integration and expression, failed to confer telomerase activity on S-WI cells, although it conferred robust activity on P-WI cells. [1]

(Figure 1A) 測端粒酶活性 (Figure 1C)

S-WI cells infected with lenti-hTERT did not proliferate, despite expressing high levels of telomerase. Moreover, they did not lose the senescent morphology (Figure1C) or senescence-associated β-galactosidase (SA-Bgal) expression. Identical results were obtained when S-BJ cells were infected with lenti-hTERT. Lenti-hTERT did not alter average telomere length in S-WI cells, even when tested up to 6 weeks after infection; however, the same virus elongated telomeres in P-WI cells , indicating that the virus expressed a functional hTERT protein. [1]

結果為：

1. lenti-hTERT, S-WI cells：有telomerase activity(轉染成功)
2. lenti–GFP, S-WI cells：無telomerase activity(無加入hTERT，為合理結果)
3. pBabe-hTERT, S-WI cells：無telomerase activity
4. pBabe-hTERT, P-WI cells：有telomerase activity

(3)(4)說明pBABE-puro載體需在能進行細胞分裂的細胞中才可以表現目標基因，但這不是主要實驗目標，僅略提。

同時，lenti-hTERT, S-WI cells雖有telomerase activity，但無法進行細胞分裂，且仍具有senescent morphology (在Figure 1C中，lenti–GFP, S-WI cells(不具telomerase activity)和lenti-hTERT, S-WI cells的morphology是差不多的)，也會表現senescence-associated β-galactosidase，可以看出lenti-hTERT, S-WI cells仍沒有脫離老化的狀態，且觀測到的lenti-hTERT, S-WI cells的端粒長度不會變長，lenti-hTERT, P-WI cells的端粒卻會變長。

故可以推得以下結論

1. 端粒酶須在細胞能進行細胞分裂時才能將端粒延長。
2. 端粒酶無法反轉senescent cells回正常細胞，不論是使細胞進行proliferate或脫離senescent morphology皆無法成功。

故得知重新表現telomerase的方法是不可行的，不過這篇論文發現有另一個方法能讓老化細胞離開senescent stage：

Consistent with results from SV-40 infection and plasmid microinjection experiments (Gorman and Cristofalo, 1985; Sakamoto et al., 1993; Hara et al., 1996b) (Figure 2A), LgT stimulated a substantial fraction (60–70%) of S-WI cells to synthesize DNA. CDK4m also stimulated DNA synthesis in S-WI, albeit to a lesser extent (35–40%). By contrast, GSE-22 and LgT-K1 were essentially inactive (<5%). These data suggest that S-WI cells can re-enter the cell cycle upon inactivation of the pRB pathway (by LgT or CDK4m), but inactivation of the p53 pathway alone (by LgT-K1 or GSE) has no effect in these cells. However, regardless of ability to stimulate DNA synthesis, none of the lentiviruses, alone or in combination, efficiently stimulated S-WI cells to proliferate (Figure 2A). We conclude that although S-WI cells enter S-phase upon inactivation of the pRB pathway, they cannot complete the cell cycle and proliferate. [1]

In contrast, a substantial fraction of S-BJ cells initiated DNA synthesis in response to each of the four lenti-expressed proteins (LgT, LgT-K1, GSE-22 and CDK4m) (Figure 2B). Moreover, GSE and LgT-K1 were as effective as LgT, each stimulating 70–90% of the cells (Figure 2B). CDK4m was less effective (25–30%) (Figure 2B). Most striking, all four lentiviruses each stimulated S-BJ cells to complete the cell cycle and proliferate. The extent of proliferation was approximately equal to the extent of DNA synthesis. Proliferation was assessed by the formation of colonies (>50 cells) (Figure 2B) and loss of senescent morphology (Figure 2C). Thus, in contrast to S-WI cells, the growth arrest of replicatively senescent BJ fibroblasts was completely reversible, and p53 inactivation was sufficient to induce both DNA synthesis and proliferation. [1]

方法是透過對p53與pRB的抑制，因為p53和pRB的活化都會使細胞進入senescence(見FIG.1與FIG.2)，故p53與pRB的活性可能也是細胞維持senescence狀態的必須條件，因此實驗透過抑制p53與pRB的活性並觀察。

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(FIG.1) (FIG.2)

而實驗發現若將S-WI的p53與pRB都抑制，有60-70％的細胞會開始合成DNA，而若只抑制pRB，有20-30％的細胞會合成DNA，若只抑制p53，則幾乎沒有細胞會合成DNA，然而，在這三種情形下細胞皆不會分裂(就算細胞能進行DNA合成也一樣)，故細胞皆無脫離senescent stage

而S-BJ不論只抑制p53或pRB，或兩者皆抑制，都能進行DNA合成並開始分裂(兩者都抑制或只抑制p53，結果都是有70-90％的細胞進行DNA合成與分裂，而只抑制pRB，則只有25-30％的細胞進行DNA合成與分裂)，細胞開始分裂時會失去senescent morphology並形成colonies，故應可推知應是離開了senescent stage。

而依照S-BJ的結果，因為抑制p53可以讓大部分的細胞脫離senescent stage，可以推測反轉老化細胞主要應是因為p53的作用，然而，為何在S-WI中使用相同方法，沒有細胞的senescent stage能被反轉？(在後續會討論)

Together, the results indicate that p53 maintains the senescence growth arrest of S-BJ cells, and that inactivation of p53 alone is sufficient to reset their replicative lifespan. A third human fibroblast strain (82-6, from skin) displayed an intermediate response to p53 inactivation: 20–25% of senescent 82-6 cells initiated DNA synthesis in response to GSE-22 (not shown). Thus, some human fibroblast strains have phenotypes intermediate between WI-38 and BJ with respect to reversibility of the senescence growth arrest by p53 inactivation. [1]

p53的inactivation可以讓S-BJ重新變成正常細胞(有70-90％的細胞進行DNA合成與分裂)，但p53的inactivation對S-WI卻幾乎沒有作用(只有不到5％的細胞開始合成DNA)。而使用第三種人類成纖維細胞株(82-6,皮膚)進行p53的inactivation則有20-25％的細胞進行了DNA合成。因此，某些人類成纖維細胞株對p53的inactivation的反應是介於WI-38和BJ之間，說明並不是所有細胞對p53的inactivation的反應皆相同。

Why do S-BJ cells resume growth upon p53 inactivation, without pRB inactivation, whereas S-WI cells fail to proliferate (despite undergoing DNA synthesis) even when both pRB and p53 are inactivated? One possibility might be intrinsic differences in the ability to induce p16 at senescence. WI-38, like several human epithelial cells, appear to undergo replicative senescence prior to critical telomere shortening owing to induction of p16 by as yet unidentified factors. Thus, p16 may impose a proliferative block that cannot be overcome by p53 inactivation. Consistent with this idea, WI-38 cells consistently expressed higher levels of p16 than BJ cells, whether pre-senescent or senescent (Figure [3](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC175806/figure/cdg417f3/)A and B). Moreover, S-BJ cells that were rescued from senescence by GSE-22 ceased proliferation (after >20 additional PDs; Figure [2](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC175806/figure/cdg417f2/)D) with low but significant p16 expression (Figure [3](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC175806/figure/cdg417f3/)C), and could not be rescued from this second growth arrest by LgT (not shown). Finally, senescent 82-6 fibroblasts expressed p16 at levels intermediate between S-WI and S-BJ cells; immediately after rescue by GSE-22, the cells had substantially less p16 than the starting population (not shown), suggesting that GSE rescued only those cells that expressed little or no p16. Thus, p16 may prevent reversal of the senescence arrest by p53 inactivation. [1]

(上述提出的問題)然而，為什麼S-BJ cells只要p53 inactivation就能分裂並脫離老化狀態，而S-WI經過了p53 與pRB inactivation卻仍沒辦法分裂？經推論有可能是S-BJ與S-WI的p16的表現量不同影響，由FIG.1可以知道，p16會促使pRB表現，並使細胞進入senescence，故p16表現量若夠大，造成的proliferative block可能會使p53 inactivation仍無法讓細胞脫離senescence的狀態，而結果和推論也符合，S-BJ cells的p16的確比S-WI cells還要少很多，且剛才提到的senescent 82-6 fibroblasts的p16表現量也介於S-BJ cells與S-WI cells間，故可以推論p16表現應可以阻止p53 inactivation。

CDK4m may also act indirectly on the p53 block by sequestering p21.

Although p21 binding by CDK4m may partially relieve the p53 block, it is not equivalent to p53 inactivation. [1]

補充：為何pRB inactivation也可以造成20-30％的細胞開始合成DNA？不是沒有進行p53 inactivation嗎？因為實驗中使用CDK4m來進行pRB inactivation時，CDK4m可以和p21 binding，產生了部分p53 inactivation的效果，但是效果沒有直接進行p53 inactivation來的顯著。

結論：故最後結果和我當初假設的相當不同，加入telomerase沒辦法讓在senescence stage的細胞脫離老化狀態(在這篇論文中，要能進行細胞分裂，並失去senescent morphology才算離開senescence stage)，而是必須藉由p53 inactivation才能達成，然而p53 inactivation並不是對所有細胞都有效，而需要看該細胞p16的表現量，若p16表現量不夠小，很可能儘管進行了p53 inactivation，細胞仍無法離開senescence stage。

回到一開始「延長壽命」或「返老還童」的論點，我覺得應是不可行的，首先

p53 inactivation是把一個人體很重要的抑癌基因無效化，如此會讓人體暴露在易得到癌症的風險中，另外，也不是所有細胞都能成功反轉senescence stage回到正常細胞的狀態，若只讓人體部分的細胞脫離senescence stage，另一部分的細胞卻仍維持在senescence stage，也無法保證會不會有其他問題增生。

**Reference**

[1] Beauséjour, C. M., Krtolica, A., Galimi, F., Narita, M., Lowe, S. W., Yaswen, P., & Campisi, J. (2003). Reversal of human cellular senescence: roles of the p53 and p16 pathways. *The EMBO journal*, *22*(16), 4212–4222.

(FIG.1)：Campisi J. Cellular senescence as a tumor-suppressor mechanism. *Trends Cell Biol*. 2001;11(11):S27-S31. doi:10.1016/s0962-8924(01)02151-1

(FIG.2)：Hickman ES, Moroni MC, Helin K. The role of p53 and pRB in apoptosis and cancer. *Curr Opin Genet Dev*. 2002;12(1):60-66. doi:10.1016/s0959-437x(01)00265-9