Lecture 20: Stem Cell and Regenerative Medicine

10801040 莊博恩、10801128 陳俊鴻

1. Lecture Notes

Stem cell會有兩種特性，首先是他能self-renewal，再者是他能分化成各式各樣的細胞。而Stem cell在生物體中的功用可以分成兩種階段，在embryo development主要目標便是增加身體細胞的個數和分化成各式各樣的細胞，在成人體內則是不斷提供新的細胞來補充死亡或老化的細胞。

說到stem cell如何形成，就得先回到受精卵，受精卵本身就是totipotent stem cell，具有形成完整個體的能力，接著會分化成pluripotent stem cell，他們能夠形成一個器官中各式各樣的組織但不能形成一個完整個體，pluripotent stem cell再分化成multipotent stem cell，multipotent stem cell能增加特定細胞的數目。然而究竟是什麼使stem cell具備self-renewal和differentiation的特性？這必須要看到stem cell在身體內所處的位置和環境。基本上stem cell在分裂後會有兩種路可以選擇，正式self-renewal和differentiation，然而只有在分裂後和niche cell連結的細胞才會走向self-renewal並繼續作為stem cell，沒有連結的細胞則是會開始受到其他環境因子的誘導走向differentiation，因此這就關乎stem cell分裂時的方向了，倘若分裂方向和連結的方向平行，則離niche cell較遠的細胞變會走向differentiation，倘若分裂方向和連結方向垂直，則兩細胞最終都會維持stem cell。然而在這兩個特性間決定性的關鍵則是和以下三個transcription factors有關，Oct4, Sox2, Nanog。當這三個transcription factors表現，便會促進細胞self-renewal基因的表現，另一方面也會抑制細胞走向differentiation的基因表現。

1. Question and Answer

Question 1:

這週的主題其實令我最困惑的是Shinya Yamanaka的研究為何可以如此篤定，因此我拜讀了他的Induction of Pluripotent Stem Cells from Mouse Embryonic and Adult Fibroblast Cultures by Defined Factors，也確實對於他的研究有不少疑惑但這些疑惑可能要作者本人才能解答。

Answer 1: [1]

首先是Yamanaka從24個基因選出10個更有潛力的基因時，為何在十六天以來colony number 明顯較低的1號factor沒被選擇，反而是前十天沒有colony但到第十六天的colony number特別多的第11和21號被選擇？如果選擇標準是第十天的colony number，那做到第十六天的意義和目的又何在？而且為何是第十天？莫非是和他們實驗室MEF的doubling time有關？又或是他們有測試相當多天後發現第十天有明顯差異？但也沒看見他們有重複實驗（畢竟沒看到p value），這個部分我不確定是不是我才疏學淺還是他們確實不夠嚴謹，四處查資料也沒查到合適的解釋（甚至有稍稍請教北榮的老師怎麼看，但似乎這篇文章內確實很難看到原因）。不過最終的結果確實是有做出來，相形之下這雖然是一個枝微末節的小問題，但我仍舊很好奇他是基於什麼原因。

Question and Answer 2:

我想到一個方向：reprogram所使用的somatic cell的端粒長度應是不完整的，這樣一來當somatic cell reprogram成Totipotent或Pluripotent後再分裂出來的細胞不就繼承了原本不完整的端粒長度？這樣一來reprogram後分裂出來的細胞是不是就會較短？

想到這裡，我也很快想到了桃莉羊的壽命通常不長，會不會剛好就是這個原因造成的？若桃莉羊細胞真的繼承了原本somatic cell用剩下的端粒長度，那的確會造成桃莉羊很快老化，甚至提早死亡。

1. 首先我想問問看，是否真的如我假設的，reprogram後的細胞端粒會變短，也因此壽命減少？另外，這個原因是否為造成桃莉羊壽命不長的原因？

若細胞有辦法解決這個問題，我認為應該有兩種應對方式：

(a)細胞有辦法可以把端粒延長

(b)reprogram後的細胞有特殊功能，能夠使端粒不隨細胞分裂而變短，或是細胞能減緩端粒縮短的速度？

經查詢，發現reprogram後的細胞端粒長度會恢復到人類受精卵中的端粒長度，且reprogram後的細胞端粒長度不受somatic cell原始端粒長度長短的影響(也就是說，老年人和年輕人的somatic cell reprogram後的產生的細胞端粒是一樣長的)，另外在不同的年齡、性別、reprogramming factors或medium種類下，也不影響reprogram後的細胞端粒延長。這些Totipotent或Pluripoten細胞分化後端粒又會再次縮短，和受精卵中端粒的機制相同。

Though the IPSC lines in this report were isolated from only two subjects of very disparate ages, these results suggest that like animal cells reprogrammed to pluripotency, reprogrammed human somatic cells will generally restore telomeres to lengths characteristic of human ESCs. It follows that IPSC-derived cells will not reach replicative senescence prematurely, regardless of donor subject age.[1]

Similar to what was observed in mouse IPSCs(四基因法), our results also suggest that variables in the IPSC conversion process including the age and sex of input cells, reprogramming factors used (Oct4 and Sox2 coupled with either Nanog and Lin28 or KLF4 and c-Myc), and media conditions for early establishment (HES medium with either 100 ng/zFGF or 4 ng hFGF) do not preclude telomere elongation in human IPSCs nor shortening following redifferentiation. [1]

然而，仍不知道為什麼telomere可以變長，是什麼機制或是什麼特殊基因被turn on影響造成的嗎？

至於機制的方面，我查詢到了和telomerase有關，telomerase可以作用在端粒的末端並延長，而機制如下：

Interestingly, the resulting iPS cells showed a decreased density of H3K9m3 and H4K20m3 heterochromatic marks at telomeres compared to the parental MEF, reaching similarly low levels to those of ES cell telomeres. These results are in line with a higher plasticity in the chromatin of pluripotent ES cells compared to that of differentiated cells. Also in agreement with an opening of the telomeric chromatin associated with nuclear reprogramming, we observed similarly elevated telomere recombination frequencies both in ES cells and in iPS cells when compared to parental MEF, which showed much lower frequencies of recombination. Finally and in agreement with their longer telomeres, TERRA levels are efficiently increased in iPS compared to the MEF. This accumulation of TERRA in turn may serve as a mechanism to negatively regulate telomere elongation by telomerase once the iPS cells reach the ES cell telomere length." Together, these observations demonstrate that generation of iPS cells involves a change in the epigenetic status of telomeres towards a more open chromatin conformation with a lower density of heterochromatic histone marks, which is coincidental with increased TERRA transcription, increased telomere recombination and continuous telomere elongation until reaching ES cell telomere length. Since TERRA has been proposed to negatively regulate telomerase activity, increased expression of TERRA in iPS may serve as a counting mechanism of telomere length that would inhibit telomerase activity once the iPS cells reach the ES cell telomere length. These results prove that telomeric chromatin is dynamic and reprogrammable depending of the differentiation stage of cells.[2]

Telomerase-mediated telomere elongation and maintenance depend on telomere structure, which is regulated by epigenetic modifications at telomeres and by telomere binding proteins. Nuclear reprogramming requires the removal of the tissue-specific epigenetic pattern imposed on the chromatin during cellular differentiation and division, which is accomplished through large scale reorganization of chromatin structure and functions, as global changes of DNA methylation or histone modifications that lead to a more open state of the chromatin.2 These epigenetic alterations have been shown to also alter telomere chromatin during reprogramming. The reprogramming of telomeric chromatin into a more open conformation observed in iPS cells may be required to allow telomerase access to the end of the telomere and posterior telomere lengthening (Fig. 2) On the other hand, epigenetic changes occurring during reprogramming could also have a direct impact in telomerase expression.[2]

Finally, telomere binding proteins are also mediators of telomere length that may inhibitor facilitate the binding of telomerase to telomeric DNA. A possibility exist that the expression or function of these proteins is regulated during reprogramming to contribute to telomere rejuvenation, although data supporting this hypothesis is not available. Thus, future studies defining the role of chromatin modifying activities and telomere binding proteins on telomere reprogramming would be of great interest.

[2]

實驗發現，iPS cells具有相似於ES cell的以下特性：

(a) decreased density of H3K9m3 and H4K20m3 heterochromatic marks at telomeres

(b) elevated telomere recombination frequencies

(c) High TERRA levels

相較於parental MEF(原始的somatic cell)來說，iPS cells皆展現出和ES cell telomeres較相似的特質，另外由(a)與(b)可以得知，iPS cells telomeres的epigenetic status改變成more open chromatin conformation，具有higher plasticity，使telomerase比較容易進入telomere的末端，進而延長telomere

另外，因為nuclear reprogramming requires the removal of the tissue-specific epigenetic pattern imposed on the chromatin(亦即，epigenetic status改變不只發生在telomere)，會改變了部分染色體的結構，很可能使原本不會表現的染色體開始轉錄，進而加強了telomerase的expression

簡單來說：telomerase延長telomere的機制可能如下，都和epigenetic status改變有關：

(1) telomeric chromatin變比較鬆(open)，使telomerase可以進入反轉錄並延長

(2) telomerase的expression加強，telomerase activity提高，使細胞獲得能延長telomere的能力。

(3)(僅為猜測，尚未證明)因為telomere binding proteins也會影響telomere structure，很可能reprogramming時也能改變這些proteins的表現，進而改變其(蛋白)在telomere structure的作用，使telomere更容易被telomerase延長

而關於(c)，因為TERRA會抑制telomerase activity，且在telomeres 變長時，TERRA 的濃度會隨之提高(cells with long telomeres exhibit greater TERRA expression while short telomeres have relatively lower TERRA expression)，故可以將TERRA的堆積視為telomere length的倒數機制，若telomere length長度夠長了即抑制telomerase，使telomerase不再延長telomere，控制telomere的長度，另外，TERRA的增加也可以當作telomere確實有被延長的證據。

那控制此機制的基因究竟為何？

在實驗中使用的方式為：根據iPS cells的研究，4個factor的gene作用下可以使somatic cell轉換成ES，故應科學家假設其中一個GENE應有控制telomere length功能的效果，故作了以下的實驗：

把只有三個factor加入(c-myc沒有加)，去觀察細胞telomere的長度是否變長

實驗發現若c-myc沒有加，Telomeres仍然會延長，但無法達到把四種factor都加進去的telomere長度

Next, we addressed whether telomeres were elongated during the reprogramming of 3F and 4F iPS cells (all at passage 8) compared to control ES cells by measuring telomere length in individual clones of these cells using two independent techniques, Southern telomere restriction analysis (TRF) and quantitative telomere FISH (Q-FISH) on metaphase spreads. Telomeres were elongated in wild-type 4F iPS cells compared to the corresponding parental wild-type MEF, both as determined by TRF and Q-FISH analyses. Telomeres were also elongated in 3F iPS cells but did not reach the length of 4F iPS cells, although both had similar levels of telomerase activity. [1]

Next, we addressed whether telomere elongation in iPS cells was mediated by telomerase activity and/or by telomere-lengthening mechanisms alternative to telomerase, which are based on recombination between telomeric sequences and are described to mediate telomere elongation in early cleavage embryos

). To this end, we measured telomere length in iPS cells (3F) derived from G1 Terc−/− MEF. As shown both by TRF and Q-FISH techniques, telomeres were further shortened in iPS cells derived from G1 Terc−/− MEF compared to the parental wild-type MEF, indicating that telomerase is the primary activity responsible for telomere elongation in telomerase-proficient iPS cells. Signal-free ends (chromosome ends with undetectable telomere signals or critically short telomeres) were decreased in telomerase-proficient 3F and 4F iPS cells compared to parental MEF, reaching similar levels to those of control ES cells , in agreement with re-elongation of short telomeres by telomerase during iPS cell generation. In contrast, this was not observed in Terc−/− iPS cells, which showed a further increase in signal-free ends compared to the parental G1 Terc−/− MEF. Finally, the fact that G1 Terc−/− iPS cell telomeres shortened compared to the parental MEF argues against telomere recombination mechanisms operating to elongate telomeres during iPS cell nuclear reprogramming, at least in early-generation G1 Terc−/− cells. These results are in agreement with telomere recombination operating from zygote to blastocyst and switching to telomerase at the blastocyst stage. [1]

接下來實驗中把分別由G1 Terc−/− MEF(3F)和parental wild-type MEF cells reprogram((3F))出來的iPS cell做比較，發現缺少Terc(telomerase的一個subnit)的MEF reprogram出來的iPS cell telomeres的短非常多，說明在iPS cell中telomerase activity確實擔任了重要角色(註1)

另外，telomerase-proficient 3F 和4F iPS cells都能有效減少Signal-free ends的數量，說明缺乏c-myc造成的short telomere很可能是因為telomerase activity不夠所致，因此c-myc很可能就是負責調控telomerase，使其activity增加的基因。

註1：這個地方我想了很久，我認為從這個發現可以得出的c-myc在此的功能應該不是調控telomerase activity，因為由G1 Terc−/− MEF和parental wild-type MEF cells reprogram出來的iPS cell都缺少了c-myc，若c-myc功能為增加telomerase activity，那這兩種iPS cell應該telomerase activity都偏低，不會因為Terc−/−的差異再次影響造成telomere長短差異，這個推論也符合之前查到的敘述，(Telomeres were also elongated in 3F iPS cells but did not reach the length of 4F iPS cells, although both had similar levels of telomerase activity.)，說明telomerase activity是不變的，證明c-myc在此的功能並非調控telomerase activity。

然而，這個推論在「telomerase-proficient 3F和4F iPS cells都能同樣有效減少Signal-free ends的數量」卻產生了衝突，我也因此去查了其他資料。

The proto-oncogenec-Myc, one of the four factors involved in the reprogramming by defined factors, transcriptionally regulates Tert, 100 suggesting that could be responsible for the telomerase activation observed in iPS cells. However, iPS cells generated with or without c-Myc showed similar levels of telomerase activity.[2]

說明c-Myc是負責調控Tert (telomerase的subunit)，和註1的論點也不符，我認為可能是因為其中的影響因素很多或機制複雜(例如：c-Myc也許能同時調控telomerase activity與telomeric chromatin)，不易用如此限定的方式區分。

1. 另外，上述論文提到了儘管4個factor都有被引入，仍可能出現telomere沒有被延長的情況，這讓我很好奇，在只有telomere出狀況的情形下，reprogram後的細胞有沒有辦法偵測到telomere太短，因此乾脆使細胞無法進行分裂(就像cell cycle的checkpoint在DNA有損壞時不會進行cycle)，既然細胞端粒短就代表著這個細胞分裂分化的子代細胞都無法活得很久，那是否也有機制在調控，若偵測到端粒太短就會阻止細胞分裂？

TELOMERASE ACTIVATION IS ESSENTIAL FOR THE "GOOD QUALITY OF THE RESULTING iPS CELLS

Reprogramming efficiency of cells derived from increasing generations of telomerase-deficient mice, which present a higher frequency of critically short telomeres and chromosome end-to-end fusions, was dramatically reduced, indicating that a minimum telomere length is required for efficient reprogramming. Crucially, reintroduction of telomerase reduced the frequency of short telomeres and largely restored iPS cell generation efficiency. These results suggested that damaged/uncapped telomeres are responsible for their failure to reprogram and highlighted the existence of reprogramming barriers that abort the reprogramming of cells with uncapped telomeres. Since p53 has a key involvement in preventing the propagation of DNA-damaged cells, including those containing short telomeres, its possible involvement as a reprogramming barrier was readily tested." Abrogation of p53 allowed efficient reprogramming of cells with critically short telomeres and other types of DNA damage," demonstrating that p53 is critical in preventing the generation of human and mouse pluripotent cells from suboptimal parental cells, including those with critically short telomeres. In line with these results, other studies have also shown that p53 limits the production of iPS cells.%99 Overall, these findings demonstrate that telomere length and telomeric chromatin are rejuvenated during in vitro reprogramming and highlight the important role of telomere biology and dynamics in iPS cell generation and functionality. [2]

經查詢是有的，而此基因就是p53基因，under short telomeres的情形下，reprogram後的細胞難以進行分裂，但若把p53移除，會發現under short telomeres時那些reprogram的細胞仍分裂了，說明p53基因就調控此機制的基因。

最後回到一開始提出的桃莉羊，究竟短壽的桃莉羊是否為short telomeres造成？

It is well accepted that the telomeres of the first cloned mammal Dolly were 20% shorter when compared with those of age-matched controls. [3]

關於短壽桃莉羊的原因眾說紛紜，但的確有short telomeres的可能性，大部分的資訊都有提到其實有壽命正常的桃莉羊(並不是每一隻桃莉羊都短壽)，這也和目前的理論符合，我認為桃莉羊應該是「under short telomeres」又同時「缺乏p53」共同導致的結果，才會使具有較短telomeres的細胞仍能分裂分化形成一隻壽命較短的羊。

**Reference**

[1] [Kazutoshi Takahashi](https://www.cell.com/fulltext/S0092-8674(07)01471-7), [Koji Tanabe](https://www.cell.com/fulltext/S0092-8674(07)01471-7), [Mari Ohnuki](https://www.cell.com/fulltext/S0092-8674(07)01471-7), [Tomoko Ichisaka](https://www.cell.com/fulltext/S0092-8674(07)01471-7), [Kiichiro Tomoda](https://www.cell.com/fulltext/S0092-8674(07)01471-7), and [Shinya Yamanaka](https://www.cell.com/fulltext/S0092-8674(07)01471-7). (2007). Induction of Pluripotent Stem Cells from Adult Human Fibroblasts by Defined Factors. *Cell*. *131*(5). 861-872.

[2] Suhr, S. T., Chang, E. A., Rodriguez, R. M., Wang, K., Ross, P. J., Beyhan, Z., Murthy, S., & Cibelli, J. B. (2009). Telomere dynamics in human cells reprogrammed to pluripotency. *PloS one*, *4*(12), e8124. <https://doi.org/10.1371/journal.pone.0008124>

[3] The Cell Biology of Stem Cells , Eran Meshorer, Kathrin Plath,p.125-p.127

[4] Lei Yang, Xuefei Liu, Lishuang Song, Guanghua Su, Anqi Di, Chunling Bai, Zhuying Wei, Guangpeng Li.(2020). Melatonin restores the pluripotency of long‐term‐cultured embryonic stem cells through melatonin receptor‐dependent m6A RNA regulation. *Journal of Pineal Research*, 10.1111/jpi.12669, **0**, 0.