【Title】

Analyzing the expression pattern of TERT in planarian toward elucidating the mechanism for acquiring a life span.

【Background】

Why do we have a life span? There are various theories about what influences life span, for example senescence by oxidative stress. The most representative is due to telomere shortening (1). Telomeres are repetitive sequences in the end of chromosomes. If DNA is replicated many times, replication problems take place in the end regions. If cells exceed a limit of divisions called the Hayflick limit, cells acquire a life span. For example, it is reported that human cells divide about 60 times and die if we culture them in dishes because telomeres shorten every time they divide (2). But the telomere shortening is not observed in stem cells and cancer cells. This is because they have high telomerase activity (3). Telomerase is composed of protein complex, TERT is the most important protein because it has reverse transcriptional activity. Recent research reported positive correlation between expression of TERT and telomerase activity (4).

Planarians have high regeneration ability in order to form individuals from one cleaved piece (reference1). Planarians which multiply by fission (innate asexual: AS) distribute totipotent stem cells throughout the body. These cells are capable of regeneration by proliferation and differentiation (5). AS's totipotent stem cells keep maintenance of telomere length in the whole body because they express TERT.

On the other hand, another planarians form testis and ovary for sexual reproduction due to environmental change including low temperature or starvation. Planarians which were born from eggs (innate sexual: InS) express TERT only in germ cells, not somatic cells. As a result, telomere shortening and a life span is acquired in that individual (6). Recent research about relationship between reproductive patterns and life span reports that telomere shortening occurs only InS, not sexualized planarians (Acquired sexual: AqS) (7). As above, the relationship between reproductive pattern and life span has observed, the details have not been clear.

At present, the relationship needs elucidation concerning mechanisms at the molecular level. Further research in this area is required.

【Purpose of the research】

We hypothesize that telomere shortening in InS is caused by suppressing expression of TERT. We therefore address expression pattern of TERT analysis in the developmental process. Determining TERT suppression stage and area is equal

to acquire onset of life span system. Elucidating mechanism for acquiring life span in planarian can cause development of life span because planarian is the first triploblastic animal in the process of evolution.

【Result of the study】

1. In developmental process of planarian (innate Sexual: InS), we produced anti-planarian TERT antibody to detect planarian TERT in order to reveal the presence of the protein.

2. We conducted Western blotting to detect planarian expression of TERT *in vivo* by use of TERT antibody. We also confirmed availability of the antibody in this process. Especially due to the fact that planarians are assumed to have different base sequences of *tert* in planarian of related species, so we confirmed detection of TERT in *Schmidtea mediterranea*, *Dugesia japonica*, *Dugesia ryukyuensis*.

3. We conducted immunostaining with AS, InS and AqS to compare and examine the different localization of TERT in adult planarian by the difference in reproductive patterns and development. We also examined the optimal condition for the concentration of antibodies in this process.

4. In order to specify phase and area of TERT suppression in developmental process of InS, we collect samples at various developmental stages from fertilized egg to hatching and detect expression and localization of TERT by immunostaining. We also determine the telomere length in each developmental stage and clarified the relationship between expression of TERT and telomere length.

5. Through examining the relationship between silence of TERT and individual life span, we breed AS, AqS and InS long-term, to measure individual life span, analyze expression pattern of TERT and changes in the telomere length.

【Future study plan】

1. It has been reported already that someone has detected planarian TERT, so we decided to produce polyclonal antibody. The base sequence of *tert* is been cloned recently, so we predicted the amino acid sequence by the base sequence of *tert* and selected the region of the antigen. We cooperated with K.Masutomi at National Cancer Center in this process. We identify high antigenic region in N-terminal

region of planarian TERT as being homologous to human TERT K.Masutomi has. These sites (CSEIKKRSPIYKSSNSYS) are as antigen, we had him produce synthetic peptide, immunized with rabbits and produce polyclonal antibodies.

2. First of all, we conducted Western blotting with the use of individual asexual of *S.mediterranea* to confirm utility of planarian TERT antibody. The result from experiment using ×500 antibody, we detected band as size of planarian TERT being speculate from base sequence. Thereby, we confirmed availability of TERT antibody in *S.mediterranea* and succeeded in detecting planarian TERT for the first in the world. Also, we used samples not cut (not inducing regeneration), so it reveals that asexual worms of *S.mediterranea* express TERT in the case of not regenerating. We have been able to detect TERT in other species (*D.japonica*,*D.ryukyuensis*).

3. We conducted immunostaining using asexual worms of *S.mediterranea* and *D.japonica*, and changed combination that primary antibody diluted to 100times and 500 times, secondary antibody diluted 2000 times and 5000 times, cut planarian and uncut planarian. The result of that, we reveal that TERT localized in almost whole body without pharynx in *S.mediterranea* under conditions of primary antibody diluted to 500 times and secondary antibody diluted to 2000 times. But signal intensity is unbalance, so we can't confirm localization in detail. Now, we have conducted experiments many times, for example conducting immunostaining with the use of sliced sample, for establishment of optional conditions.

4. We don't have experiments from here, we assume that expression of TERT can be seen by blastula stage and TERT is inhibited onward from the blastula stage in InS. This is because TERT expressed in humans is not seen in somatic cells by the blastocyst stage (inner cell mass) correspond to blastula stage from fertilized egg.

5. We think that individual life span of AqS is as long as AS and telomere shortening does not occur because TERT is expressed over whole body. Individual life span of InS is shorter than AS and AqS, we speculate that expression of TERT is observed not in somatic cells but only in the ovary and testis, so the telomere length will be shorter.

【Reference】

We will examine difference of interspecific TERT localization using TERT antibody in planarians. We hope to establish an experimental method to acquire sexual worms from asexual worms for investigation. After that, we will use immunostaining to determine expression of TERT stage and area in developmental process. We will reveal TERT localization suppressing expression of TERT in developing AqS cells. After that, we can identify the suppression factor collecting these cells. Next, we will produce knockdown planarians in these factor using RNAi, and observe the difference of life span to elucidate molecular mechanisms. In the future, we would like to approach and elucidate the essence of life span systems in all organism including human being based on this investigation.

【a list of references】

(1) Omer T. Njajou, Wen-Chi Hsueh, E. H. Blackburn et al. Association between telomere length, specific causes of death, and years of healthy life in health, aging, and body composition, a population-based cohort study. J Gerontol A Biol Sci Med Sci. Aug;64(8) 860-864, 2009

(2) Hayflick L, Moorhead P The serial cultivation of human diploid strains. Exp Cell, 25:585-621, 1961

(3) Kim NW, Piatyszek MA, Prowse KR et al. Specific association of human telomerase activity with immortal cells and cancer. Science 266: 2011–5, 1994

(4) K.L. Kirkpatrick, G. Clark, M. Ghilchick, et al. hTERT mRNA expression correlates with telomerase activity in human breast cancer. European Journal of Surgical Oncology Volume 29, Issue 4, 321-326, 2003

(5) D. E. Wagner, I. E. Wang, P. W. Reddien et al. Clonogenic neoblasts are pluripotent adult stem cells that underlie planarian regeneration. Science 332, 811-816, 2011

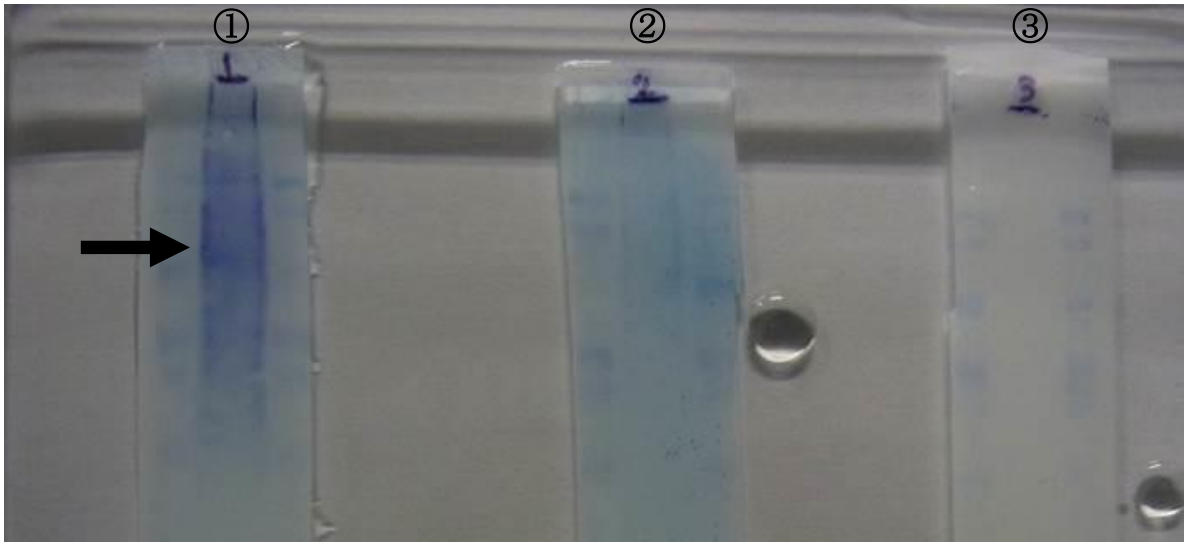
(6) T . C. J. Tan, Ruman Rahman, Farah Jaber-Hijazi et al. Telomere maintenance and telomerase activity are differentially regulated in asexual and sexual worms. PNAS, vol.109 no.11, 4209–4214, 2012

(7) K. Tasaka, N. Yokoyama, H. Nodono, et al. Innate sexuality determines the mechanisms of telomere maintenance. Int. J. Dev. Biol. 57: 69-72, 2013

(reference1) RIKEN development and regeneration - Behavior of cell we can come to see.-

Figure1：Cleaved planarian(left) and regenerating planarian(right).

Figure2



The result of Western blots using TERT antibody is different to antigen.

①TERT antibody (No.1)(left)

②TERT antibody (No.2)(middle)

③not using TERT antibody (right)

※We confirm TERT expression in No.1 antibody(arrow). But No.2 antibody weren't observed any bands.

Figure3：Immunostaining of S.mediterranea (inate asexual:AS) with TERT(×500) and Alexa Fluor® 488(×2000, green). TERT expression was observed whole-body.

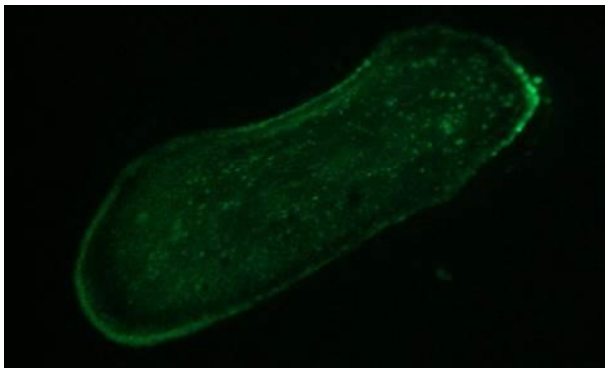


Figure4：Dilation of Figure 3. Spotty TERT expression was observed.

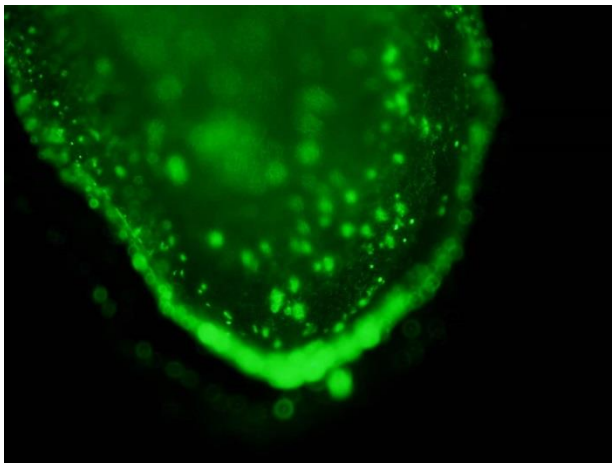


Figure5：Immunostaining of D.japonica (inate asexual:AS) with TERT(×500) and Alexa Fluor® 488(×1000, green). We was not able to observe localization of spotty TERT, whole staining is background.

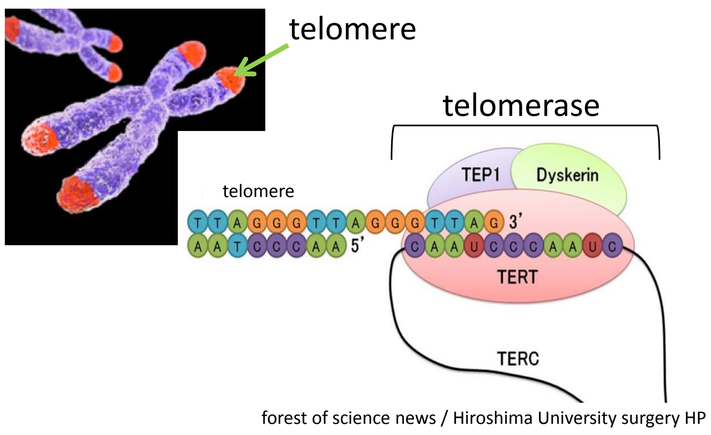
Analyzing the Expression Pattern of TERT in Planarian toward Elucidating the Mechanism for Acquiring a Life Span

[redacted]

Abstract

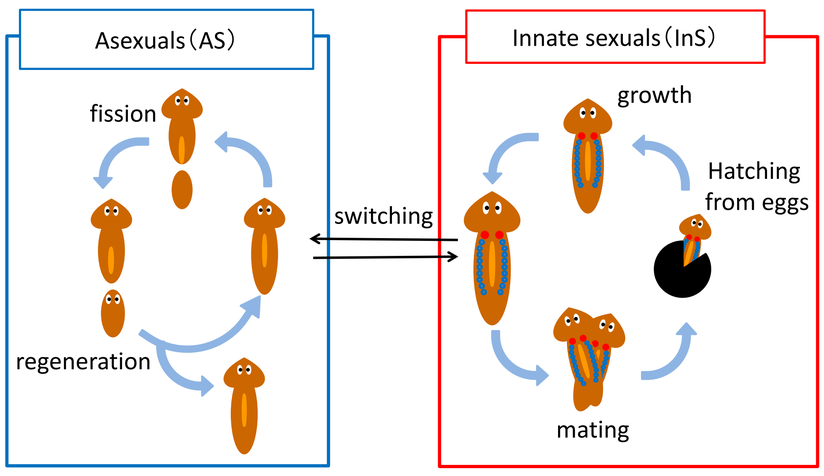
The representative factor deciding whether to acquire a life span is shortening of telomere. Telomeres are elongated by telomerase. In telomerase, TERT is the most important. Recently, *tert* gene was isolated in planarians and it reported that asexually reproducing planarians (AS) do not have telomere shortening and do not acquire a life span because they distribute totipotent stem cells throughout the body, while planarians which were born from eggs (sexually reproducing planarian:InS) have telomere shortening and acquire a life span. Additionally, InS scarcely express TERT. Thus we hypothesized that telomere shortening is caused by suppressing TERT expression in developmental process from fertilized egg to hatching. We have studied in order to determine TERT suppression stage and area in developmental process and grasp moment of acquiring a life span. To date, we revealed TERT expression in whole body of planarian by western blotting and immunostaining in AS. We will reveal expression pattern of TERT in InS's development process and analyze suppression factor of TERT after collecting the cells suppressed TERT expression. We want to elucidate the mechanism for acquiring a life span and approach the origin of a life span in organism including human being.

Background

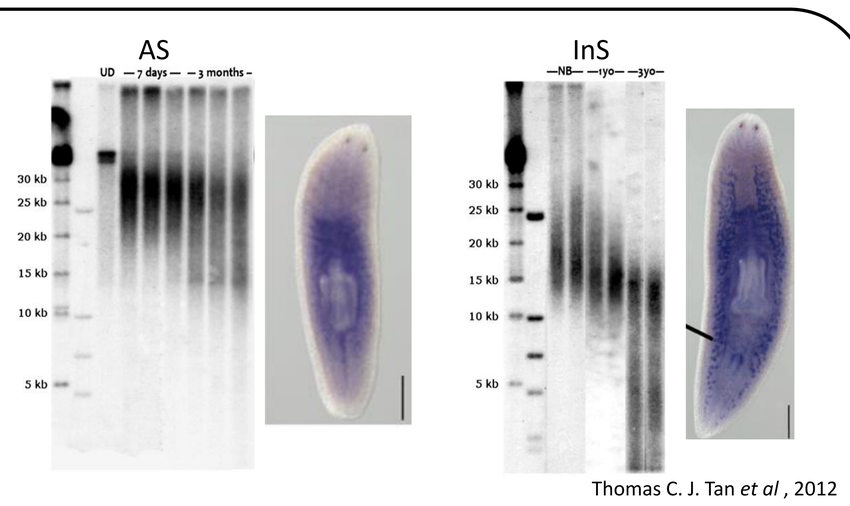


forest of science news / Hiroshima University surgery HP

Telomeres are repetitive sequences in the end of chromosomes. If cells exceed a limit of telomere shortening, cells acquire a life span. But telomere shortening is not observed in stem cells and cancer cells. This is because telomeres are elongated by telomerase. Telomerase is composed of RNA and protein complex, TERT (telomerase reverse transcriptase) is the most important protein.



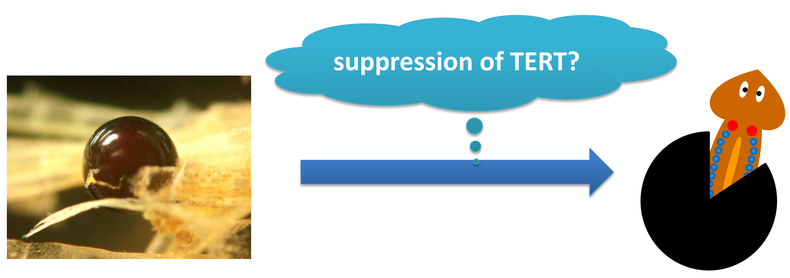
Planarians have high regeneration ability in order to form individuals from one cleaved piece. In the same species, there are asexual planarians (AS) and innate sexual planarians (InS). AS multiply by fission. InS multiply by laying eggs. Also, some species can switch reproductive pattern from each other due to environmental change including low temperature or starvation.



Thomas C. J. Tan *et al* , 2012

AS distribute totipotent stem cells throughout the body. AS's totipotent stem cells keep maintenance of telomere length in the whole body because they express TERT. InS express TERT only in germ cells, not somatic cells. As a result, AS has telomere shortening and a life span in that individual.

Purpose

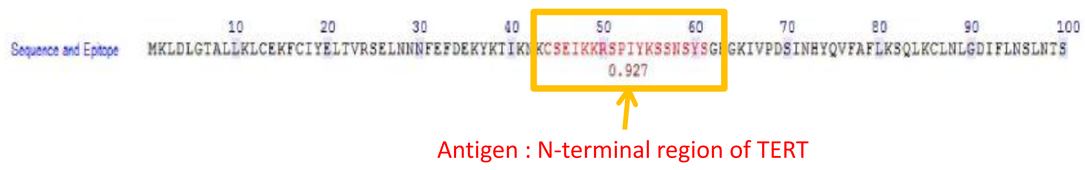


We hypothesize that telomere shortening in InS is caused by suppressing TERT expression. TERT suppression stage is equal a moment of acquiring a life span. We therefore analyze the expression pattern of TERT from egg to hatching in developmental process. We can approach the origin of a life span in organism including human by these findings because planarian is the first triploblastic animal in the process of evolution.

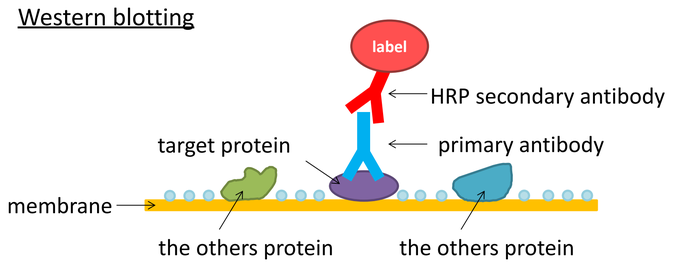
Method

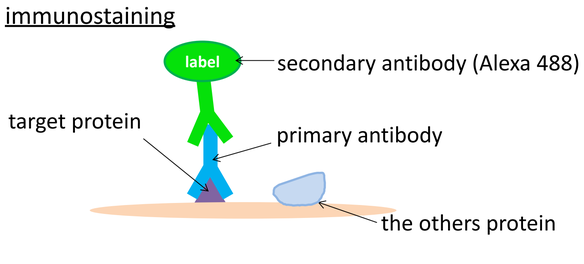
①Change asexual planarian into sexual planarian and get eggs for experiment (in progress)

②Produce polyclonal anti-TERT antibody in order to detect TERT in Planarian



③Confirm availability of anti-TERT antibody by western blotting and immunostaining





Result

Fig. 1

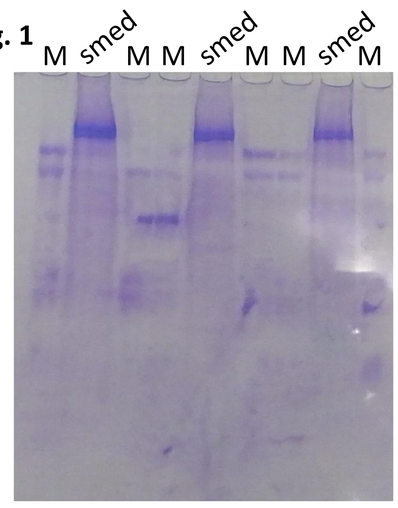
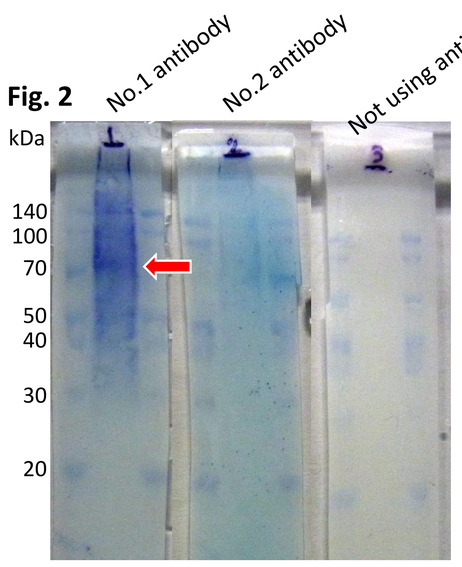
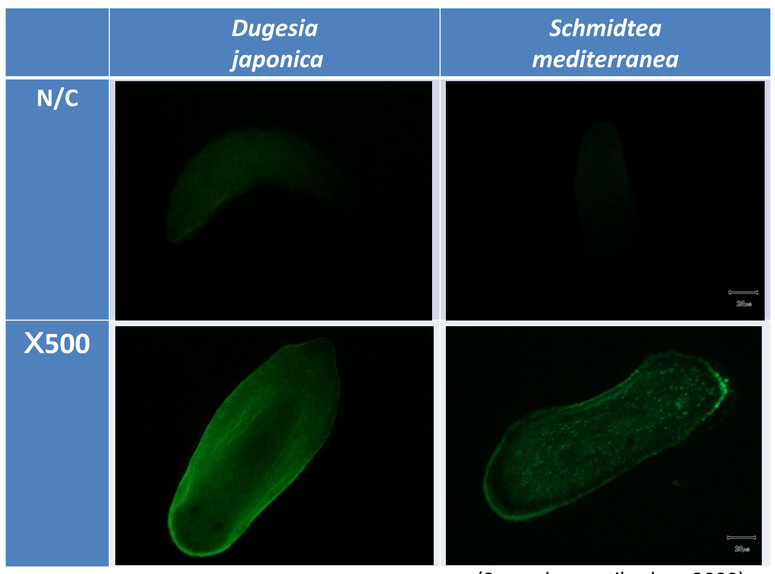


Fig. 2



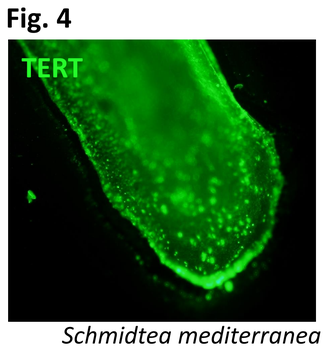
We extracted protein from *Schimidtea mediterranea*, separated by molecular weight using electrophoresis (fig. 1). The blue-violet staining are proteins. Gel was transcribed into membrane, after that membrane was reacted with two kinds of antibodies which differ on antigen of TERT (No. 1 and No. 2). We predicted amino acid sequences from base sequences of planarian *tert* gene. Predicted TERT molecular weight was 38kDa～79kDa, so it is speculated that blue band is TERT (Fig. 2 arrow). This result revealed that No.1 antibody can detect TERT specifically. But No.2 antibody can not be detect TERT.

Fig. 3



(Secondary antibody : x2000)

Fig. 4



Schmidtea mediterranea

Whole-mount immunostaining by No.1 primary anti-TERT antibody. *Dugesia japonica* and *Schimidtea mediterranea* are AS (Fig. 3). Spotty TERT localization were detected in whole body only *Schimidtea mediterranea* (Fig. 4). It revealed that TERT express in cells throughout the body at *Schimidtea mediterranea* AS.

As a result, we confirmed availability of planarian anti-TERT antibody.

Future plan

In this research, we get anti-TERT antibody which is available for western blotting and immunostaining in planarian. Next, we have some following strategies. First, we will make AS into InS by feeding *Bdellocephala brunnea*. Second, we get eggs from InS, observe and delimit developmental process from fertilized egg to hatching. Third, we will reveal the expression pattern of TERT in each developmental stage and decide a moment of suppressing TERT expression by western blotting and immunostaining. In parallel, we determine the telomere length in each developmental stage by quantitative PCR. Finally, we would like to reveal mechanism for suppression of TERT and approach the origin of a life span in organism including human.

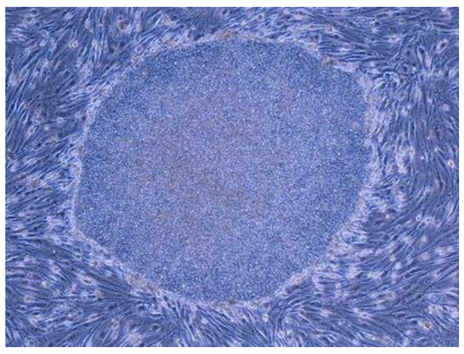
老化の進んだ細胞からのiPS細胞作製効率亢進へのアプローチ

Abstract

iPS細胞は様々な細胞に分化できる人工の幹細胞であり、再生医療や創薬分野への応用が期待されている。高齢化が進む現在の日本では老化の進んだ細胞からiPS細胞を作製することが求められているが、その効率は非常に低く、今後の研究に注目が集まっている。ヒトの体細胞ではテロメア配列が細胞分裂を繰り返すごとに短縮し、これが細胞老化につながるとされている。短縮したテロメア配列はテロメラーゼにより伸長され、テロメラーゼの活性はTERTの発現により調節されている。また近年、マウスES細胞においてTERTの発現がWntシグナルにより調節されていると報告された。以上のことからわたしは、老化の進んだ細胞にWnt刺激を与え、予めテロメア長を回復させることで、老化の進んだ細胞からでも効率良くiPS細胞を作製できるのではないかと考え研究を進めている。現在までに、マウス胎児の線維芽細胞に山中４因子を導入し、iPS細胞のような形態をした細胞をクローニングすることに成功している。今後は、老化の進んだ細胞をWnt処理することでそのテロメア長がどのように変化するのか、iPS作製効率はどの程度亢進するのかを明らかにしていきたい。

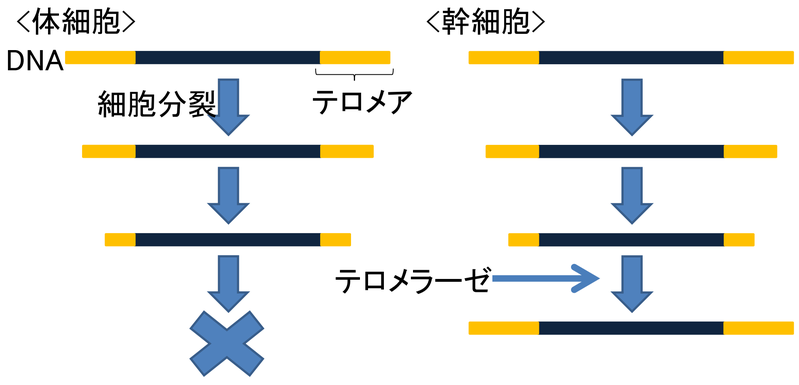
Background

iPS細胞



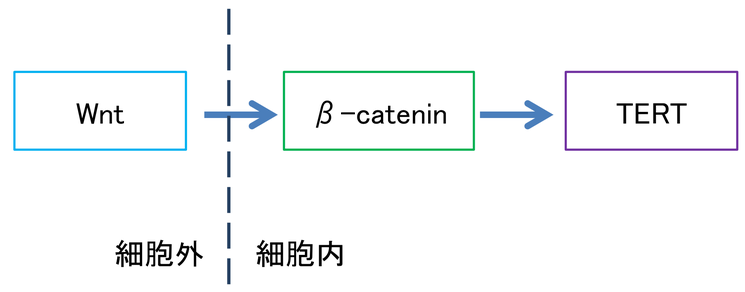
高い増殖能力と多能性を持ち、再生医療や創薬分野への応用が期待されている。上図は2006年に京都大学山中教授がマウス胚繊維芽細胞から作製したもの。2007年にはヒトiPS細胞の作製も報告されている。

テロメア・テロメラーゼ・TERT



テロメアは染色体の末端にある構造で細胞分裂のたびに短縮していき、やがて細胞は分裂を止めてしまう。それを避けるためにはテロメア長を伸長しなければならない。これを担うのがテロメラーゼという酵素で、その中で重要なサブユニットがTERTタンパク質である。

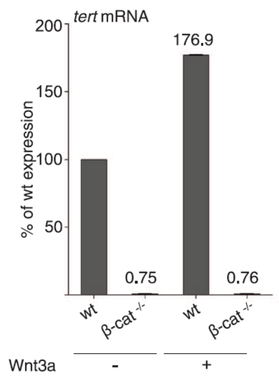
Wnt/β−cateninシグナル伝達経路



Wntは分泌性タンパク質で、それが細胞に作用することによりβ−cateninが活性化する。 活性化したβ−cateninはいくつかのタンパク質と複合体をつくり、DNA上のTERT転写開始領域に結合することでTERTの転写が調節される。

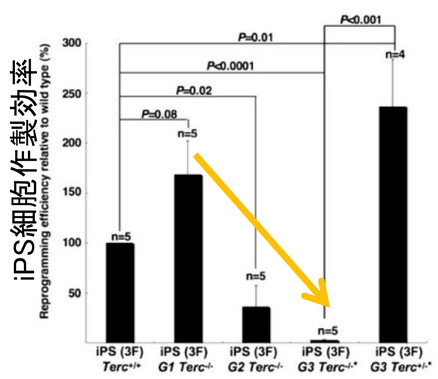
Wnt刺激がTERTの発現に与える影響

ES細胞



Katrin Hoffmeyer ,et al. Science.2012

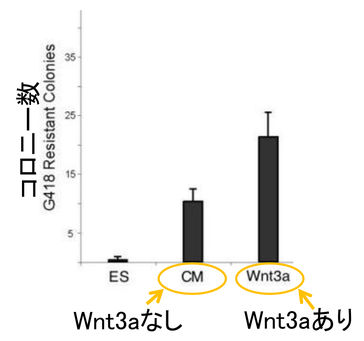
テロメア長とiPS細胞作製効率との関係



Marion MR,et al. Cell stem cell. 2009

Wnt刺激によるMEFからのiPS細胞作製効率の亢進

MEF

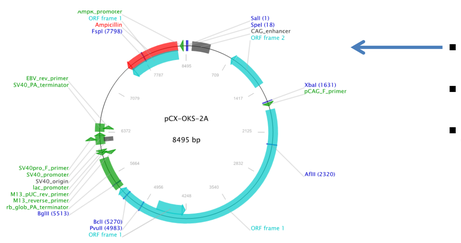


Marson A,et al.Cell Stem Cell.2008

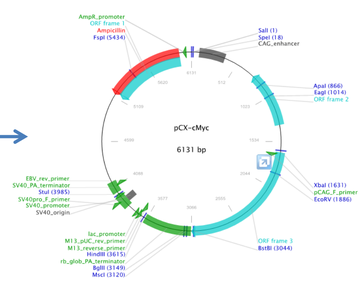
Purpose

現在日本では社会的に高齢化が進んでいるため、老化の進んだ細胞からiPS細胞を作製することが求められているが、その効率は若い細胞からiPS細胞を作製した場合に比べ、非常に低いことが報告されている。本研究の目的は、iPS化の前段階であらかじめWnt刺激を与えることによって、若い細胞から作製した際と同程度までにiPS細胞作製効率を亢進させることである。

Method

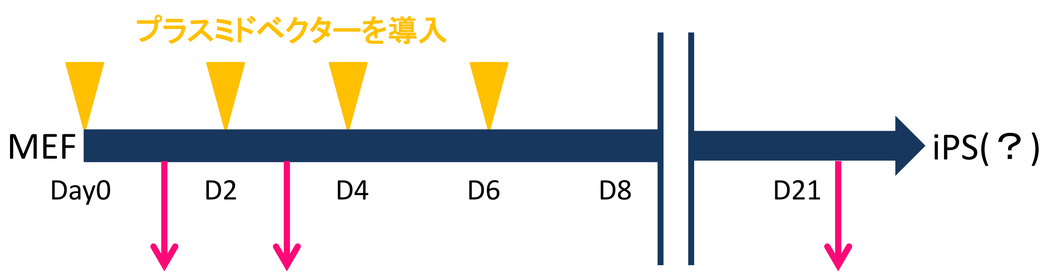


・Oct3/4
・Klf4
・Sox2

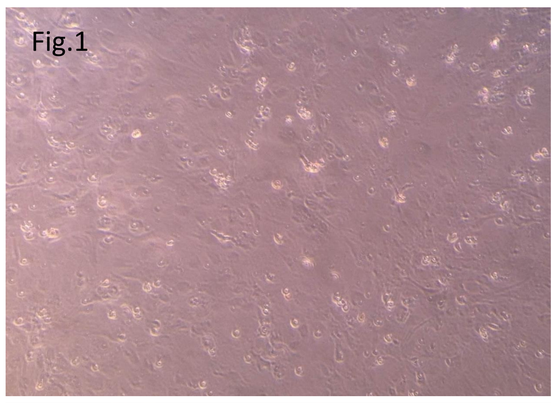


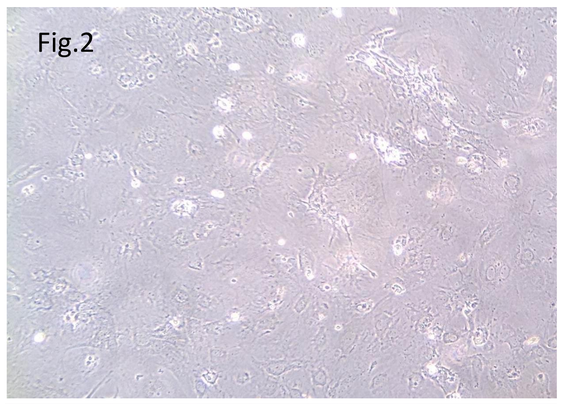
・c-Myc

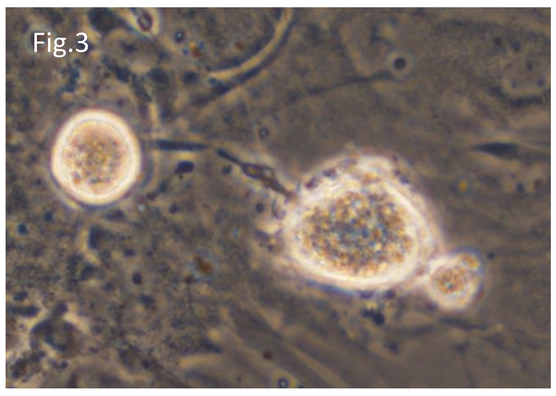
山中４因子(Oct3/4,Klf4,Sox2,c-Myc)が組み込んだプラスミドベクター(pCX-OKS,pCX-cMyc)をそれぞれ大腸菌にトランスフェクションし、増殖させた。そしてリポフェクション法によりMEF(マウス胚線維芽細胞)に2種類のプラスミドベクターを1日置きに計4回導入し、遺伝子組み換えを行った。その後数日培養していき、iPS細胞を作製した。また導入後の細胞の変化を観察していく。プラスミドベクターをMEFに導入した翌日には必ずメディウムを交換した(MC)。その際、2回目にプラスミドベクターをMEFに導入した翌日まではフィーダーメディウムを、それ以降はESメディウムを用いた。D30にはiPS細胞のコロニーをピックアップし、クローニングした。
左図は実際に今回導入したプラスミドベクターの模式図で、下図は実験計画を大まかに模式図にしたものである。



Result







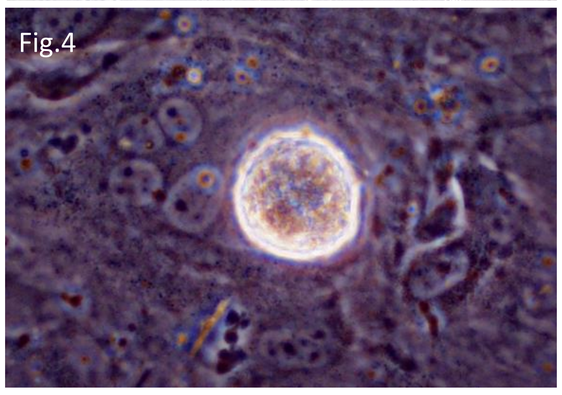


Fig.1) 2種類のプラスミドベクターをMEFに1回導入した細胞。 Fig.2) 2種類のプラスミドベクターをMEFに2回導入した細胞。Fig.1に比べてFig.2は少しコロニーが大きくなっている。
Fig.3, 4) 2種類のプラスミドベクターをMEFに4回導入し、約2週間後の細胞のうちコロニー様の細胞を拡大したもの。Fig.1やFig.2の細胞と異なり、丸みを帯びた大きなコロニーを形成しており、形態的にiPS様の細胞をクローニングできた。

Future plan

継代を重ねていないMEF（若い細胞）からiPS細胞を作製するとともに、継代を重ねたMEF（老化が進んだ細胞）からiPS細胞を作製しその作製効率を比較する。その後、①継代を重ねていないMEF （若い細胞）にWnt刺激を与えた上でiPS細胞を作製し、その効率が何も刺激を与えない場合よりも上がっていることを確認する。そのうえで、②継代の重ねたMEF （老化が進んだ細胞）にWnt刺激を与えた上でiPS細胞を作製し、その効率が①の場合と同程度まで亢進するかどうかを明らかにする。

シロイヌナズナにおけるカルシウムの吸収に関する遺伝子の解析

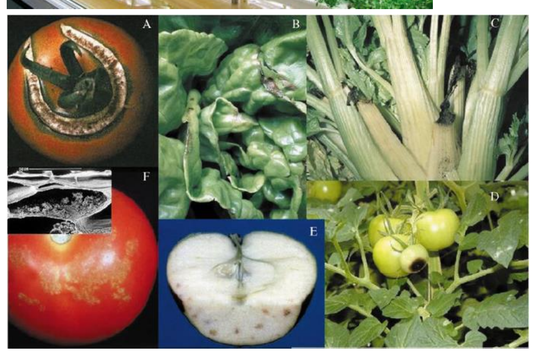
要旨

私たちは、限られた栄養源でも育つおいしい野菜を作ることを目標に研究を行っている。植物にとってCaは必須栄養素の一つである。例えば、Caは細胞壁などの中にも多く含まれ、それらの合成に必要であるため、植物においてCaは重要な役割を果たす。しかしCaは土壌中に大量に存在しながら欠乏症になりやすい栄養素の一つでもある。Caは土壌中で難溶性の形で存在すること、植物体内で移動しにくいことが欠乏症に陥りやすい原因と言われている。植物の生長速度にCaの移動が追いつけず、生長点でCaが欠乏することが多い。それによって味や見た目が悪くなるチップバーンという症状などを引き起こす。遺伝子レベルでCa欠乏の原因を解明することで、Ca欠乏に強い植物を作ることを目的とする。シロイヌナズナのCaD428変異体は、Ca欠乏条件においてCa充分条件と比べて、地上部が小さくなる傾向が認められた。変異の原因遺伝子が染色体のどこに存在するのかを調べるため、雑種第２代を用いてマップベースクローニング法を行っていく。CaD428変異体の原因遺伝子を特定して植物の栄養吸収における機能を明らかにし、日本の新たな農業に貢献したい。

背景と目的

(a)

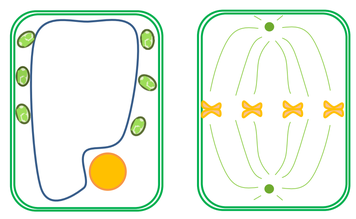
(b)



(PHILIP J. WHITE & MARTIN R. BROADLEY , 2003)

現在、放射性物質などによる土壌汚染問題が話題になっている。それらの問題を解決する技術として植物工場が注目を浴びている。植物工場は無菌状態で水耕栽培をするクリーンな農法である(a)。しかし、問題点の一つとして必須元素のCaの欠乏が報告されている。細胞レベルでは、Caは細胞壁の形成や細胞分裂時に現れる紡錘体の合成に必須である。 Caが欠乏することで味や見た目に悪影響を及ぼす(b)。これを解決するためにCaの栄養吸収の理解が求められている。

(c)



モデル植物
（シロイヌナズナ）

- 染色体5本

- ゲノムの塩基配列が全てわかっている
- 二か月で次世代へ

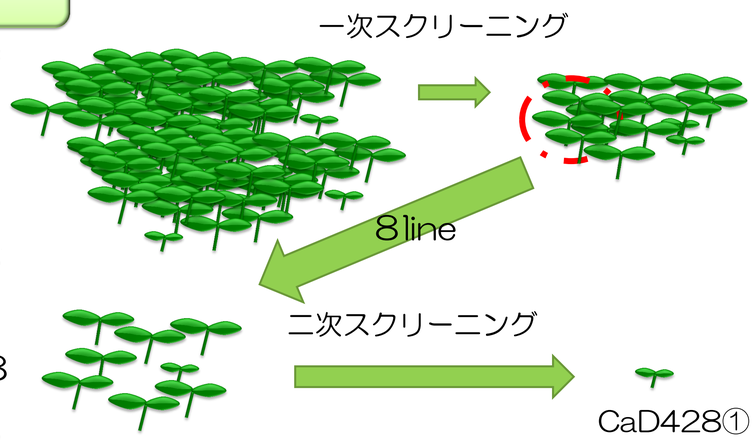
私たちは、遺伝子解析がしやすいシロイヌナズナを用いて、Ca吸収の機構を解明することを目標としている。これらの研究を下記の手順ですすめていく。そのうち変異体の生育条件の検討と雑種第二代を得ることを並行して行っている。

変異体の作出
↓
変異体の生育条件の検討
↓
雑種第二世代を得る
↓
変異の場所を見つける

変異体の選抜

数万系統の変異体を一定の栄養条件で1次スクリーニングが行われ、数百系統が選抜されている。私たちはそのうち8系統をさまざまな栄養条件で生育させ、表現型を観察した（2次スクリーニング）。その結果有意な差を示す変異体を1系統得ることができた。

インキュベーター内で16時間明期・8時間暗期・22度と温度管理等し、シロイヌナズナを生育させスクリーニングを行った(a)。



(a)

スクリーニングの結果

カルシウム欠乏条件(Ca0.15mM)

428①　Col　Ler

カルシウム通常条件(Ca2.0mM)

428①　Col　Ler

Ca通常条件では野生型Col、Lerと428①の成長の具合が一緒だが、Ca 欠乏条件では428①の葉がCol、Lerと比べて極端に小さくなった。Ca吸収に関する遺伝子に変異が起こっていると考えられた。

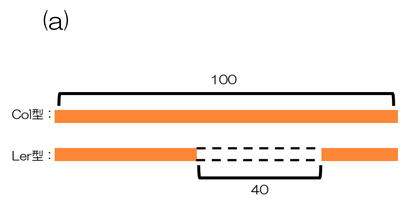
結論と今後の予定

2次スクリーニングの結果、CaD428がCa欠乏感受性の変異体として選抜された。今後は、原因遺伝子をマップベースクローニング法を用いて同定し、機能を解析していく。

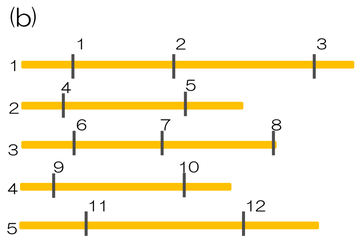
変異場所の特定

マーカーの場所

(a)



(b)



染色体上のどのあたりに変異が起きているかを絞り込むために、今回SSLPマーカーを用いた。SSLPは繰り返し塩基配列の長さによってCol系統かLer系統かを区別するものである(a)。そのマーカー設計し、シロイヌナズナの5本の染色体につけた(b)。

PCR結果

PCR法を用いてマーカーの領域を増幅させ、電気泳動した。その結果全てのマーカーでLerとColのバンドの差が見られ、ヘテロでLerとCol両方のバンドが見られた。これらのマーカーを用いて変異の場所を同定していく。

C… Columbia 、L… Landsberg erecta 、H…ヘテロ