An Innovative Method for Identification of Tissue Residential Adult Stem Cell by Using GalNAc Carbohydrate Binding Protein Bioprobe

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論 文 内 容 要 旨

1. Introduction

Adult stem cells play a key role in maintaining homeostasis of cell number by repairing damaged or lost cells through regeneration in a variety of tissues. Typical characteristics of stem cells are slow cycle, asymmetric division, DNA immortal chain and resistance to some cytotoxicity drug and so on. Adult stem cells contribute to different cellular turnover and regenerative potential in varies of tissues and organs. Although adult stem cells have been identified in several high cellular turnover tissues, an effective and universal method of precise and

Fig.1 Schematic illustration of innovative method for identification of tissue residential adult stem cell by usingGalNAc carbohydrate binding protein bioprobe

convincing identification for adult stem cells and their locations is still lacking, particularly in low turnover tissues.

In this study, I report the innovative method for identification of tissue residential adult stem cell by using GalNAc carbohydrate binding bioprobe and pulse-chase post injury model in low cellular turnover tissues of mice. Here, 5-fluorouracil (5-Fu) was used to kill the proliferating cells, and then activates the surviving tissue-residential adult stem cells; making the further label possible(KAL method). On one hand, the nucleus of these cells can be labeled by DNA synthesis precursor analogues 5-bromo-2'-deoxyuridine (BrdU) after injury; then stem cells through the divide asymmetrically to retain the "immortal" DNA strands(Fig.1A). Other hand, Dolichos bifows agglutinin (DBA) can specific identify N-acetylgalactosamine (GalNAc) residue (Fig.1B). Further study indicated that specific ligand of DBA-binding has relationship with pluripotent and early differentiation state. For further identifying the heterogeneous and primitive adult

stem cell, by introducing the GalNAc carbohydrate binding bioprobe DBA as a cell-surface indicator of the pluripotency and differentiation state(Fig.1C, D), which finally forms a rapid, reliable and universal identification method(KADL method).

Chapter 2: Using anti-BrdU for identification of functional tissue-resident hepatic stem cell

Here, anti-BrdU was used to tag the possible hepatic stem cells (HSCs) in newborn pups(Fig.2A), followed by a trace period of up to 23 months as a control group for label retaining cells (LRCs). Moreover, HSCs still retained labels and located definitively in the periportal region after a prolonged chase. The number of these LRCs remains nearly constant during the lifespan of the mice (Fig.2B). Furthermore, 5-Fu was used to induce injury, and rapidly kill proliferating cells in adult liver tissue, and activate and label surviving possible hepatic stem cells. The location and kinetics of activated BrdU-tagged HSCs were analyzed at different times after injury (Fig.2C). I observed that the activation of possible hepatic stem cells tagged by the BrdU label
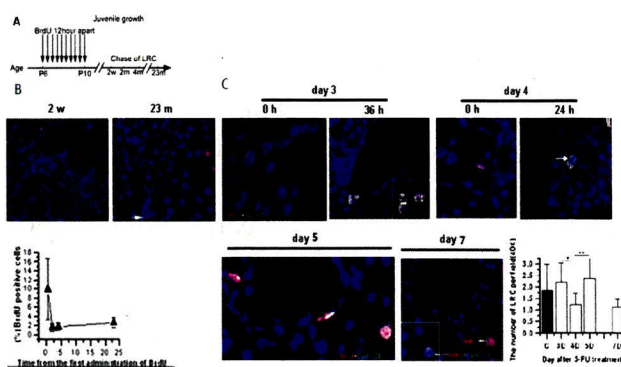

Fig.2 identification and location of hepatic stem cells in liver tissues. A. Schematic outlines of the BrdU pulse chase. B. After a 2-week and 23-month chase; And statistical analysis of BrdU-retaining cells. C. Chasing at day 3 and 4 post-injury; And statistical analysis of BrdU positive cells.

was almost completely inhibited at day 4 post-injury. And the label retaining cells locate primarily in the periportal and pericentral regions. LRCs may represent a part of a heterogeneous population of hepatic stem cells. The label retention combined with 5-Fu induced injury method provided a direct and rapid way to identify hepatic stem cells and their locations within the liver.

Chapter 3: Under conditions of the homeostasis and regeneration stress model, pulse chasing the cardiac stem cell on low regenerative potential/low cellular turnover tissue through anti-BrdU label

BrdU was used here to tag the possible cardiac stem cells (CSCs) in newborn pups and young mice post-5-Fu treatment (Fig.3A). In the pulse-chase model, a trace period of up to 24 months was performed. The result from the immunohistostaining with anti-BrdU label suggested that label retaining cells(LRCs) definitively exist in the heart tissues of adult mice (Fig.3B, C), and some LRCs express the stem cell marker, Sca-1 or c-Kit, and are located primarily in the myocardium and vascular endothelial regions. Moreover, the number of LRCs remains nearly constant during the lifespan of the mouse (Figure 3D). After injury induced by 5-Fu, the proliferating cells were almost completely cleared on day 3(Fig.
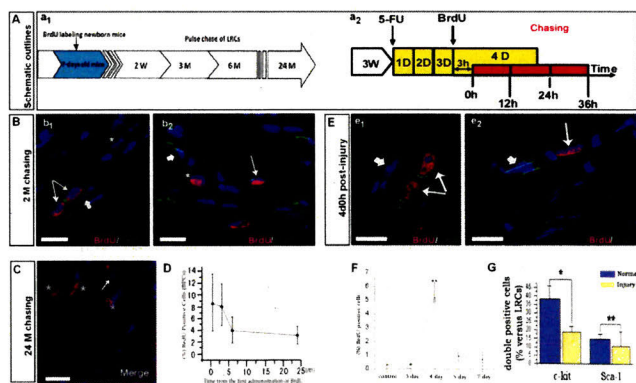

Fig.3 Identification and location of cardiac stem cells. A. Shows schematic outlines of pulse chase and post-injury. B. Shows the molecular characteristics of cardiac stem cells after 2 months chasing. C. Shows 24 months chasing. D. Shows statistical analysis of BrdU label-retaining cells. E. Shows the molecular characteristics of cardiac stem cells at 0 hour on day 4 post-injury. F. Shows statistical analysis of BrdU label-retaining cells at day 3 to day 7. G. Shows statistical analysis of double positive cells (BrdU and Sca-1 or c-Kit)

3F), while a great number of activated-CSCs were observed on day 4 (Fig.3F) and these cells were in charge of regeneration of heart tissues post-injury. A small percentage of the CSCs express Sca-1 or c-Kit post-injury

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(Fig.3E). The percentage of double positive cells (BrdU+/c-Kit+ or BrdU+/Sca-1+) was approximately 38% and 15% respectively at 2 months of chasing, 18% and 11% on day 4 post-injury (Figure 3G), which suggested that several type of cardiac stem cells possess the ability of retaining BrdU label and take part in the regeneration of injured heart.

Chapter 4: The *Dolichos biflorus* Agglutinin as protein bioprobe for further monitoring asymmetric division of hepatic stem cell during homeostasis and repair status

Based on our previously reported method, I further introduce the dolichos biflorus agglutinin (DBA) as a suitable pluripotency associated cellular surface bioprobe for identification of primitive hepatic stem cell. DBA/BrdU-double staining for liver tissue sections was performed at different time point during the process of liver regeneration. The results further analyzed verify that more primitive stem cells are existed in the liver tissues. Immunohistochemistry of liver tissue sections was performed with anti-BrdU and DBA bioprobe for mice of chasing 2 weeks (Fig.4A). There are two types of cell subpopulations (hepatic stem cells) with different characteristics in liver tissue (Fig.4a$_1$), that is BrdU label retaining cells (LRCs), which can be classified BrdU high and BrdU low. Another is DBA positive subpopulation cells (DBA high and DBA low). I further
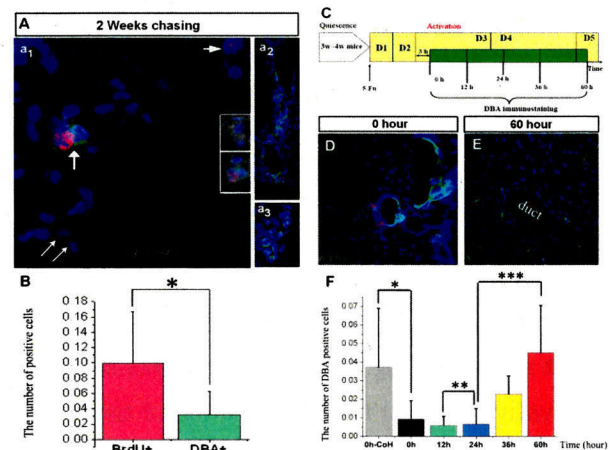

Fig.4 Immunohistochemistry analysis with anti-BrdU label and DBA bioprobe. A. Chasing for 2 weeks. B. Statistical analysis result. C. Scheme of experiment post-injury. D. Shows 0 hour chasing at day3 post-injury. E. Shows 60 hour chasing at day3 post-injury. F. Statistical analysis result.

found that identified liver cells are potential more primitive stem cells, which can perform asymmetric cell division. It produces two offspring with affinity of DBA lectin; one of the offspring can keep the label retaining. Further observation showed that these DBA positive cells have a high nucleo-cytoplasmic-ratio, which indicated that these cells probably are immature cells. Moreover, this further indicated that stem cells pool is heterogeneous in the liver tissue. Additionally, most of DBA positive cells distributes in groups and located mainly in the vicinity of duct-like structure, containing Cannal of Hering of billiary tree (Fig. 4a$_2$-a$_3$). The number of BrdU positive cells is more than three times as many as that in DBA positive cells (Fig.4B), suggested that the DBA positive cells are most likely more primitive hepatic stem cells. With the 5-Fu induced injury model plus DBA & BrdU-label (Fig.4C), I still can observe the DBA-affinity cells (Fig.4D, E). According with statistical analysis, kinetics of BrdU-positive and DBA-positive cells showed similar tendency post-injury (Fig.4F). That is, DBA as a reliable protein bioprobe can be used to identify more primitive hepatic stem cell during homeostasis and repair status.

Chapter 5: Identification of more primitive tissue-residential cardiac stem cell by introduction of GalNAc carbohydrate-binding protein bioprobe

A subpopulation of DBA positive cells can be reappeared in heart tissues post 5-Fu induced-injury. Immunohistochemistry analysis of heart tissue sections was performed with FITC labeled DBA lectin bioprobe and anti-BrdU antibody (Fig. 5A). After treatment with 5-Fu for 3days, a population of DBA+ cells can be found at that time (Fig.5B KADL). However, almost none of BrdU positive cells appeared in the cardiac tissues (Fig.5B KAL), which indicated that the DBA+ cells are possible more primitive cardiac stem

cells, which firstly be activated. Cardiac stem cells were activated and their differentiation was initiated on day 4, however, DBA+ cells rarely are detected (Fig.5C). BrdU+ cells and DBA+ cells show opposite trend in change of cell number. According with the statistical analysis, it is significant that reduction of DBA+/BrdU- cells (~1.15%) is almost equal to the increase of BrdU+/DBA- cells at the same time (~1.24%) (Fig.5E). On day 5, I detected some DBA+/BrdU+ cells (Fig. 5D), which showed that DBA+ cells along with differentiation pathway, still capable of resistant to toxicity of 5-Fu. The results suggested that the cardiac stem cell pool is heterogeneous, and the pool is composed of primitive stem cell (PASC), secondary primitive stem cell (secondary-PASC), and adult stem cell (ASC), committed adult stem cell (Fig.5F).It further demonstrated that the innovative method for identification of tissue residential adult stem cell in low cellular turnover tissues of mice is reliable.
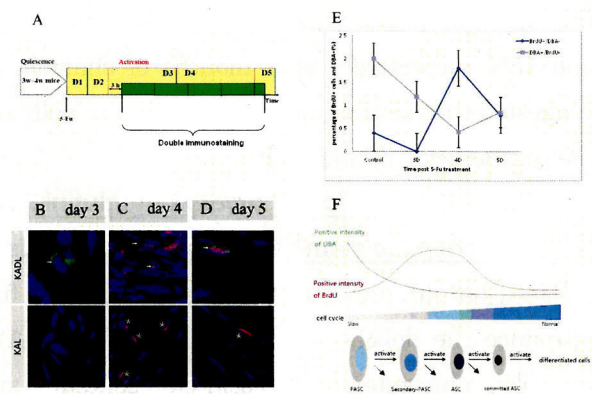

Fig.5 Immunostaining of DBA-bioprobe for heart tissue sections post induced injury. A. Scheme of liver injury and timing of double immunostaining with DBA bioprobe and anti-BrdU antibody. B-D. Immunohistochemistry of heart sections with KAL and KADL methods. E. Statistical analysis of BrdU+ cells and DBA+ cells. F. Dynamic model of cardiac stem cells with KADL method. PASC: primitive stem cell, secondary-PASC: secondary primitive stem cell, ASC: adult stem cell, committed adult stem cell.

Chapter 6: Conclusions

The study here is a meaningful and promising attempt on identification of tissue-residential adult stem cell by using GalNAc carbohydrate binding lectin bioprobe combined with pulse-chase post injury model. 5-fluorouracil was used to kill the proliferating cells, and then activates the surviving tissue-residential adult stem cells; making the further label possible. In low cellular turnover tissue (liver, heart) of mice, tissue-residential adult stem cells (hepatic stem cell, cardiac stem cell) can be labeled and keep these labels through the asymmetrical division. For further identifying the heterogeneous and primitive adult stem cell, by introducing the GalNAc carbohydrate binding bioprobe (dolichos biflorus agglutinin: DBA) as an indicator of the pluripotency and the monitor of the early differentiation undergoing in living cell. Ligand of DBA bioprobe probably is the characteristics molecular expressed in surface membrane of adult stem cells, and the expression of the ligand will gradually declined when they move towards to differentiation. The ligand of DBA bioprobe can be used to discriminate pluripotent cells from mixed cell populations. Therefore, in this study, an innovative method can be used as a reliable, valuable tool to identify and isolate the adult stem cells. It is also an exploration and understanding for dynamic gradient development process of tissue-residential adult stem cell, which will lead to the new direction and basis for further detailed research and application depend on different types of adult stem cells.

論文審査結果の要旨

　成体幹細胞は、様々な組織の中で死傷した細胞を補充し、恒常的な細胞数の維持を保つために重要な細胞である。幹細胞の分裂と分化は速度が遅く、かつ、非対称性な分化であることなどに特徴づけられるが、細胞回転や再生能力は細胞によって異なっている。すでに細胞回転が速い成体幹細胞は同定されているが、成体幹細胞やそれらが局在する場所の同定を正確にかつ確実に同定できる手法は未だ開発の余地がある。本論文は、GalNAc糖鎖結合蛋白質をバイオプローブとして用いて、組織中の成体幹細胞を同定する新規手法を提案した論文であり、6章から構成されている。

　第1章は序論であり、本研究の背景および目的について述べている。

　第2章は、ブロモデオキシウリジン標識を用いてマウスの私生児に内在している肝幹細胞を同定している。5-フルオロウラシルを導入すると、成体増殖細胞は傷害する一方、肝幹細胞は残存する。5-フルオロウラシル導入して4日経過後、抗ブロモデオキシウリジン抗体を用いて、ブロモデオキシウリジンを取り込んで分化した肝幹細胞を検出すると門脈周辺や中心部に局在化していることが観測された。

　第3章では、心臓中の幹細胞の再生機能と細胞回転性をパスルチェイス実験により評価した。心臓中の細胞は、抗ブロモデオキシウリジン抗体で染色され、心筋や内皮中の染色細胞には幹細胞マーカーであるSca-1やc-Kitを発現しているものがあった。5-フルオロウラシルを導入したところ、成体増殖細胞は完全に傷害された一方、活性化された幹細胞が数多く観測され、細胞傷害後の心筋組織再生に幹細胞が重要な役割を果たしていることが示された。ブロモデオキシウリジンを取り込んで分化した肝幹細胞でSca-1もしくはc-Kitを発現していた割合は、傷害後の時間経過とともに増加しており、いくつかの幹細胞がブロモデオキシウリジンを取り込んだ形で分化し組織を再生していくことが示された。

　第4章は、抗ブロモデオキシウリジン抗体と共にDBAと呼ばれる凝集素である糖タンパク質も幹細胞検出のプローブとして用いることで、幹細胞の不均一な非対称性分化を評価している。パルス導入の肝臓組織の経過を両プローブで追跡したところ、どちらにおいても染色強度が異なる母集団が観測され、始原幹細胞が非対称に分化していることが示された。そして、DBAがより陽性な細胞は、細胞管を含んでいる管状構造周辺に局在しており、ブロモデオキシウリジンがより陽性な細胞は、DBA陽性細胞よりも数が多く、より初期な幹細胞であることが示され、この二つのバイオプローブを使うことによって、幹細胞の不均一な非対称性分化を追跡できている。

　第5章では、心臓組織を用いて、パスルチェイス実験における抗ブロモデオキシウリジン抗体とDBAのバイオプローブによる幹細胞追跡を行った。その結果、DBA陽性細胞とブロモデオキシウリジン陽性細胞の増殖は真逆に挙動することが示され、その相関から、幹細胞は、始原幹細胞から二次始原細胞を経て成体幹細胞に分化していることが示唆された。

　第6章では本論文を総括し、今後の展望について述べた。

　以上、本論文では、今後の再生医療に重要な成体幹細胞の同定を、ブロモデオキシウリジン標識や糖タンパク質のバイオプローブを用いて行う手法を開発し、幹細胞の分化について重要な知見を得ており、バイオ工学の発展に寄与することが少なくない。

　よって、本論文は博士(工学)の学位論文として合格と認める。