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研究課題名（和文）Inhibiting Scar Development for Promoting Stem Cell Engraftment in Chronic Spinal Cord Injuries.

研究課題名（英文）Inhibiting Scar Development for Promoting Stem Cell Engraftment in Chronic Spinal Cord Injuries.

研究代表者

ルノー三原　フランソワ（Renault-Mihara, Francois）

慶應義塾大学・医学部（信濃町）・特任講師

研究者番号：２０５７０４２７

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研究成果の概要（和文）：＃1：私たちは、分子XがSCI後に病変のリモデリングを誘発することを発見しましたが、移植されたNSPCの生存率と分布は変わらず、それは私たちがテーマを放棄することにつながりました。 ＃2瘢痕形成反応性星状細胞の遺伝子発現プロファイルの特徴付けは、他のグループによって報告されています。＃3：STAT3が反応性星状細胞およびグリア瘢痕形成の動態を調節するメカニズム（JCBに発表）を説明しました。
＃４：病理学的神経瘢痕の新規マーカーを特徴付けるために２つの異なる実験モデルが使用された。 我々の仮説は、これらの遺伝子は治癒期における不完全な治癒と関連しているということです。

研究成果の学術的意義や社会的意義
Spinal cord injury is a major medical problem as all the surviving patients inevitably become chronic patients. Our research focusing on the mechanisms of scar formation and stabilization in the subacute and chronic phases respectively may help design new therapeutic strategies.

研究成果の概要（英文）：#1: We found that molecule X, regulates the expression of several proteases and induces lesion remodeling after SCI, but the survival rate and distribution of transplanted neural stem cells are unchanged, which led us to abandon the theme. #2 The characterization of the gene expression profile of scar-forming reactive astrocytes has been reported by another research group (Hara M. et al., Nature Medicine, 2017). #3: We found that the transcription factor STAT3 regulates the dynamics of reactive astrocytes in vitro and glial scar formation in vivo. This study was published in the Journal of Cell Biology (Renault-Mihara et al., 2017).#4: Two different experimental models were used to characterize novel markers of pathological nerve scars. Our hypothesis is those genes are associated with incomplete healing in the central nervous system. This study is still in progress.

研究分野：spinal cord injury

キーワード：　Spinal cord injury　reactive astrocytes　glial scar　chronic scar　stem cell　STAT3　RhoA

１．研究開始当初の背景

In response to various insults of the central nervous system (CNS), astrocytes adjacent to the lesion become reactive, start to proliferate, polarize towards the lesion and assemble into a scar border that progressively secludes the lesion core. Seclusion of the lesion core during the subacute phase of spinal cord injury (SCI) exerts a beneficial action by limiting tissue damage, restricting inflammation, and preserving locomotor function. Understanding the characteristics and mechanisms of lesion proximal scar-forming astrocytes is therefore crucial to exploit these beneficial mechanisms of the subacute phase. Based on our prior characterization a novel transgenic reporter mouse (Nestin-CCE) showing that specific fluorescent labeling of scar-forming reactive astrocytes (RA) was possible, we had initially planned to characterize the gene expression pattern of these scar-forming RA, however we have been scooped and have therefore decided to re-orient this research on the mechanisms that trigger the polarization of scar-forming RA (**Aim#1**). While the depletion of the macrophages that invade the lesion center after SCI was indeed known to prevent the polarization of RA, the mechanisms involved were unclear. Based on the report by our group that the transcription factor STAT3 was crucial in RA for the seclusion of the lesion core (Okada et al., *Nature Medicine*, 2006), we have also completed a project that focus on the intracellular mechanisms whereby STAT3 regulates RA' dynamics after injury (**Aim#2**).

The healing of CNS lesions is systematically incomplete in mammalians, resulting in chronic scars that hinder significant axonal regeneration and are considered to reduce the efficacy of cell transplantation in animal models of chronic SCI. Among other inhibitory molecules that constitute the chronic scars, the chondroitin sulfate proteoglycans (CSPGs) represent an attractive therapeutic target for this chronic phase of SCI. Indeed, the degradation of the sugar chains of CSPGs by the Chondroitinase ABC (cABC) has been shown to promote axonal growth and plasticity, as well as motor recovery to some extent in various animal models. Based on our preliminary data indicating that treatment of primary culture of astrocytes with a pharmacological analogous of the molecule X is associated with the down-regulation of key genes involved in chondroitin sulfate biosynthesis and with the up-regulation of relevant proteases, we have decided to examine if the molecule X could therefore induce a functional remodeling of the lesion scar (**Aim#3**).

Due to the relatively disappointing results of the **Aim#3**, we have developed a novel research on the specific markers of the chronic scars (**Aim#4**). Tissue wound response and healing are dynamic processes, where four main phases are usually distinguished: immediate hemostasis, inflammation, followed by repair and remodeling. A meta-analysis of gene expression data comparing the responses to injury in different tissues showed that the remodeling phase is selectively reduced in the spinal cord (Sass *et al.*, *BMC Genomics*, 2017), suggesting that the incomplete scar healing in CNS tissues results mostly from poor remodeling. Our hypothesis is that the natural progression of healing through the 4 phases described above is specifically stopped in the CNS at the subchronic stage through an unknown mechanism.

２．研究の目的

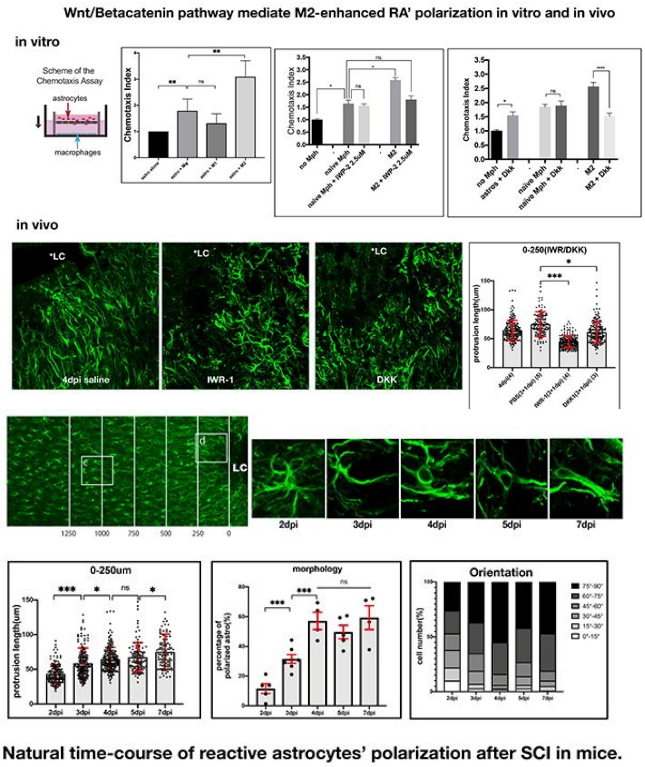
Aim#1: Whereas astrocytes have proved to be an excellent cellular model for in vitro studies focusing on the intracellular mechanisms of polarization, surprisingly the molecules that initiate and induce RA' polarization after CNS injury are still unknown. Following injury, RA' polarization is the first step in the acute reaction of RA that allows secluding the lesion site. It seems reasonable to think that by boosting RA' polarization it may be possible to accelerate lesion seclusion, and thereby promote functional recovery after SCI. **Aim#2:** While STAT3 has been reported to control cell migration through a variety of molecular mechanisms in various cells and various patho-physiological contexts, the mechanisms whereby STAT3 regulates the dynamics of reactive astrocytes and glial scar formation was unknown. Deciphering these mechanisms may prove useful in the therapeutic management of CNS lesions' healing. **Aim#3:** is to evaluate the effect of the molecule X regarding the remodeling of the lesion scar and its consequences regarding the distribution and functional integration of stem/progenitor cells grafted at the chronic stage of mouse spinal cord injury. **Aim#4**: We have tried to identify molecular markers of the speculated defective healing progression, in order to be able, in a next stage, to monitor the changes in healing upon various experimental conditions (genetic background, drugs…). Our ultimate goal is to determine if by using the newly identified markers it is possible to promote the completion of CNS lesion healing, and thereby prevent the development of chronic scars.

３．研究の方法

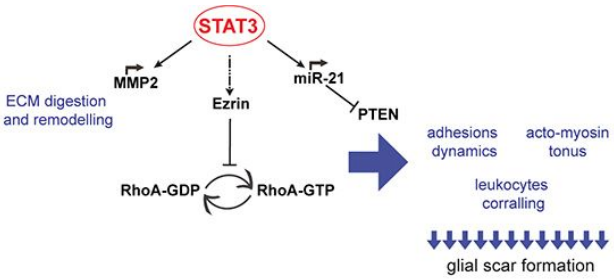
Aim#1: Using trans-well co-culture system in which inflammatory cells (microglia or bone marrow-derived macrophages) are seeded into lower wells, we have evaluated the chemotaxis of RA towards macrophages upon various conditions (blocking antibodies, specific inhibitors..). We have used spinal cord crush injury model in mice to confirm the relevancy of our findings. **Aim#2**: in order to study STAT3 mechanisms in RA' dynamics, we have combined in vitro studies using primary astrocytes from various genetic backgrounds (wild-type, STAT3 knock-out, MMP2KO and PTENKO) and in vivo examination of glial scar formation after SCI in these various mice. **Aim#3**: The compound X (or saline) was administered twice a day for one week starting from 6 weeks after moderate (60 kDyn) contusive SCI at the thoracic level (T10) of 8 week-old wild-type C57Bl/6J mouse. The eventual remodeling of the lesion center was evaluated by combining gene expression analysis by qPCR, immunostaining, western-blotting and dot-blot. The functional effect of the treatment on the integration of grafted cells was evaluated by combining in a second stage of the study the treatment X with the transplantation two weeks later of mouse neural stem / neuroprogenitor cells (NSPCs). NSPCs were expressing both Venus fluorescent protein and Luciferase, wich allowed monitoring the survival of transplanted cells using intravital bioluminescent imaging (IVIS), and the engraftment rate of transplanted cells through fluorescence. The motor function was evaluated using Basso Mouse Score (BMS) scale. **Aim#4**: the study was based on immunostaining, Western-blotting and qPCR analyses of lesion center samples of various mice at different time points after contusive thoracic SCI.

４．研究成果

Aim#1: We have observed a strong chemotaxis of RA towards macrophages, and have shown that macrophage-induced polarization of RA involves B1-integrin signaling. Moreover we have found that alternatively activated macrophages (M2) are stronger inducers of RA' polarization compared to naïve macrophages or classically activated macrophages (M1). We found that M2 enhance RA' polarization through Wnt/β-catenin pathway. Next, we have characterized quantitatively the natural time course of RA'polarization after crush injury in mice and have further observed that blocking Wnt/β-catenin pathway with two different drugs can reduce RA's polarization in vivo. Conversely, the administration of a canonical Wnt ligand (Wnt3a) can accelerate RA' polarization in vivo. We are currently preparing for submission the corresponding manuscript.

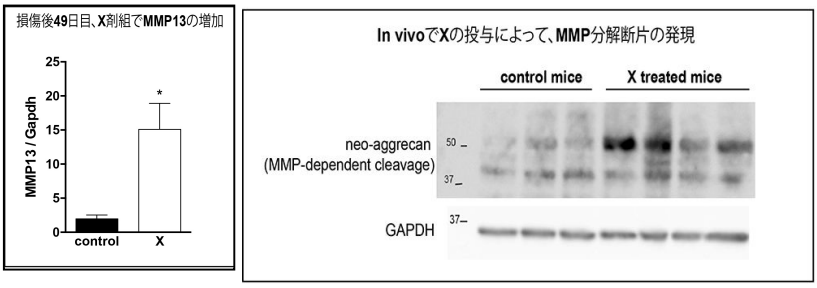


Aim#2: We found that the transcription factor STAT3 regulates the dynamics of reactive astrocytes in vitro, and glial scar formation in vivo. We have shown that regulation of the GTPase RhoA by STAT3 modulates glial scar formation in vivo. The figure below details the molecular effectors involved in the control of RA' dynamics by STAT3. This study has been published in the *Journal of Cell Biology* (Renault-Mihara et al., 2017, PMID: 28642362).

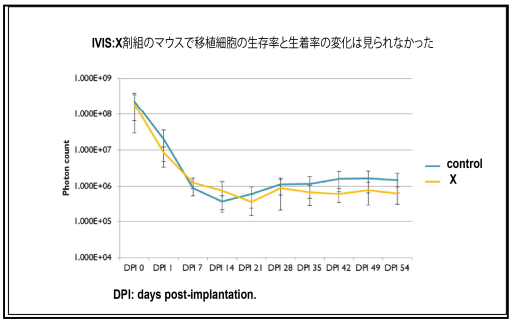


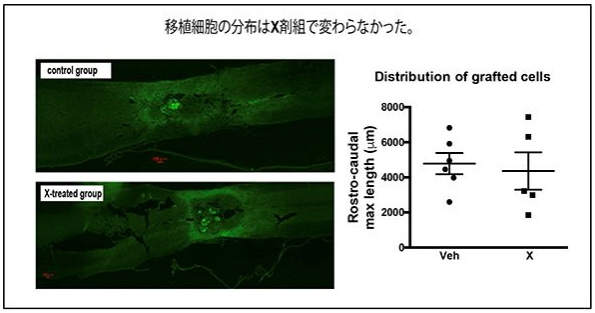
Model for the regulation of reactive astrocytes dynamics and glial scar formation by STAT3.

Aim#3: We have found that the molecule X regulates the expression of several proteases (including MMP13) in the injured spinal cord and induces lesion remodeling, as seen by the advent of digested fragments of aggrecan.

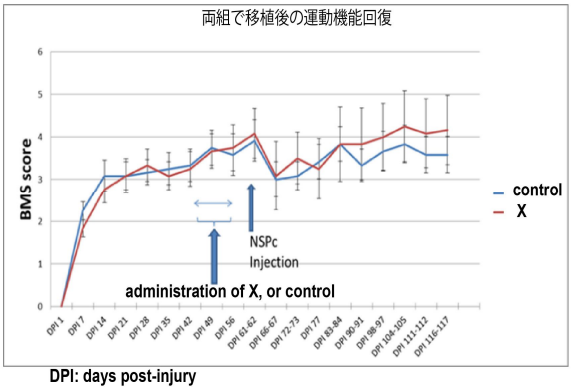


However, the survival rate and distribution of the transplanted NSPCs were unchanged in the group of mice that had received the molecule X before the transplantation.





Finally, the functional recovery of the transplanted mice was not significantly ameliorated by the administration of the molecule X before transplantation of NSPCs, which led us to abandon this research theme.



Aim#4: Using two different models of mouse SCI where lesion healing is deteriorated compared to normal mice, I have identified by western blotting a novel molecular marker that appears early after injury, while it is absent in normal mice at this stage. These two mouse models of deteriorated spinal cord scars are characterized by defective lesion compaction with reduced glial scars and enhanced fibrotic scars. The advent of the marker in the chronic phase of injured normal mice suggests that the marker is linked with the maturation of the fibrotic scar and may be related to the pause in the healing process of CNS lesions. By comparing the gene expression levels in the different mouse models at various stages, I have identified candidate biological pathways for the advent of this marker. Consequently, I have identified reagents that increase the expression of the marker in the two deteriorated models of SCI. Furthermore, I have observed that one reagent can induce the expression of the marker X in the normal mice at the subacute phase, where it is normally not observed. Our preliminary studies suggest that inducing the advent of this marker in the subacute phase of SCI normal mice is associated with larger lesion scar and deteriorated functional recovery, which seems to confirm our initial hypothesis.

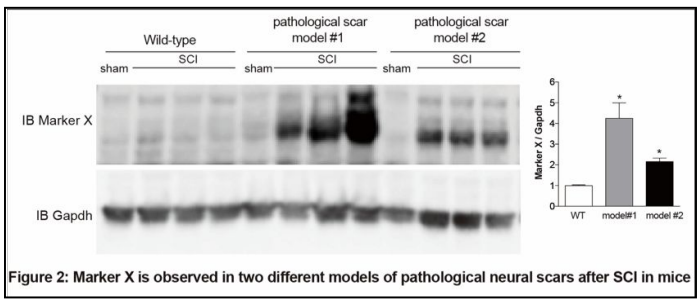


Figure 2: Marker X is observed in two different models of pathological neural scars after SCI in mice

５．主な発表論文等

〔雑誌論文〕（計　3 件）all 3 articles were peer reviewed.

(1) STAT3: Down the R(h)oAd. Renault-Mihara F, Okano H. *Cytokine*. 2018 Feb;102:149-150. doi: 10.1016/j.cyto.2017.08.003. Epub 2017 Aug 10. PMID: 28803695.

(2) STAT3-regulated RhoA drives reactive astrocyte dynamics. Renault-Mihara F, Okano H. *Cell Cycle*. 2017;16(21):1995-1996. doi: 10.1080/15384101.2017.1377032. PMID: 28933592.

(3) Regulation of RhoA by STAT3 coordinates glial scar formation. Renault-Mihara F, Mukaino M, Shinozaki M, Kumamaru H, Kawase S, Baudoux M, Ishibashi T, Kawabata S, Nishiyama Y, Sugai K, Yasutake K, Okada S, Nakamura M, Okano H. *J Cell Biol*. 2017 Aug 7;216(8):2533-2550. doi: 10.1083/jcb.201610102. Epub 2017 Jun 22. PMID: 28642362

〔学会発表〕（計　3　件）

(1) ISG (Interdisciplinary Science Group) meeting, Keio University, 2017 December 26th. **Oral presentation** (60'). Renault-Mihara Francois. Regulation of RhoA by STAT3 coordinates glial scar formation.

(2) ASCB/EMBO (American Society of Cell Biology/European Molecular Biology Organization) 2017 meeting. Philadelphia, USA • 2017 December 2-**6. Poster**. Renault-Mihara Francois, Masahiko Mukaino, Munehisa Shinozaki, Hiromi Kumamaru, Satoshi Kawase, Mathieu Baudoux, Toshiki Ishibashi, Soya Kawabata, Yuichiro Nishiyama, Keiko Sugai, Kaori Yasutake, Seiji Okada, Masaya Nakamura and Hideyuki Okano. Regulation of RhoA by STAT3 coordinates glial scar formation.

(3) CiRA/ISSCR International Symposium 2016. Pluripotency: From Basic Science to Therapeutic Applications. Celebrating 10 years of iPS Cell Technology, 2016 22-24 March, 2016 , Kyoto, Japan. **Poster**. Francois Renault-Mihara, Masahiko Mukaino, Seiji Okada, Masaya Nakamura and Hideyuki Okano. Visualization of Reactive Astrocytes' Migration Towards a Spinal Cord Lesion.

６．研究組織

(1)**研究分担者**

研究分担者氏名：

ローマ字氏名：

所属研究機関名：

部局名：

職名：

研究者番号（8 桁）：

(2)**研究協力者**

研究協力者氏名：孫 怡姬

ローマ字氏名：SON, Iki (M2 student, for **aim#1**)

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