Figure 7. Schematic summary of the present findings. The Lin$^-$/ckit$^-$/CD106$^+$/CD44$^+$ BMC population abundantly migrates into damaged muscles to suppress inflammation and activate the muscle regeneration processes in Duchenne muscular dystrophy, in part via the TSG-6-mediated pathway. Chronic injury/regeneration cycles drain the numbers of both Lin$^-$/ckit$^-$/CD106$^+$/CD44$^+$ and Lin$^-$/ckit$^-$/CD106$^+$/CD44$^-$ populations in BM. Abbreviation: BM-MSCs, bone marrow-mesenchymal stromal cells.

Hagiwara et al. proposed that BMT did not significantly improve the muscle function of mdx mice [43] compared with non-BMT mdx mice, which is consistent with this study. For BMT, the recipient mice receive high-dose irradiation that ablates not only bone marrow cells but also other cells to worsen the regeneration ability. Therefore, in our study, we first conducted the 10-week-old mdx or WT mouse BMC transplantation into 3–4-week-old mdx mice (Mdx-wt vs. Mdx-mdx) to compare these two BMT models on mdx mouse pathology. Although muscle differentiation from BMCs was almost undetectable, we observed muscle function and pathological differences between these mice. From that point, we hypothesized and investigated whether the differences in the WT and mdx BM-MSC populations and the alteration of BM-MSC populations could be related to muscle pathological conditions in mdx mice.

Several possibilities could account for the reduction of BM-MSC populations in 10-week-old mdx mice. We previously reported that transient reduction of the CD45$^-$/CD44$^+$/CXCR4$^+$ cells in BM with appearance of this population in peripheral blood during ectopic bone formation [8]. In addition, during the inflammation phase in multiple sclerosis mice, interferon-gamma secreted from activated T cells decreased the number of CFU-Fs and CD45$^-$ cells in BM [44]. These data suggest that induction of direct or indirect stimuli in pathological conditions can mobilize MSC populations from BM to contribute to tissue regeneration at a distant location. In mdx mice, these stimuli from degenerated muscles are expected to chronically continue because the muscle pathological symptoms have progressed. Thus, the continuous requirement to contribute to muscle regeneration from BM might be responsible for the reduction in the BM-MSC population in mdx mice (Fig. 7). Previously, we reported that HMGB1 is abundantly released from necrotic epithelial cells of the mouse skin graft model and mobilizes PDGFR$^+$ cells from BM into the circulation [9]. In DMD mice, however, we did not observe HMGB1 elevation in the serum (data not

shown), indicating another unknown mechanism underlying the mobilization of BM-MSCs from BM into circulation and damaged muscles.

Surface marker analyses have suggested the existence of multiple subpopulations in BM-MSCs [15, 16], and the roles of each population in vivo have remained unclear. Here, we demonstrated that BM-MSCs can be subdivided into Lin$^-$/ckit$^-$/CD106$^+$/CD44$^+$ and Lin$^-$/ckit$^-$/CD106$^+$/CD44$^-$ cells. Our whole-transcriptome analysis demonstrated that the Lin$^-$/ckit$^-$/CD106$^+$/CD44$^-$ cells preferentially expressed SDF-1α in BM. SDF-1α-expressing stromal cells in BM contribute to niche formation for HSCs [10, 13, 14, 45]. In addition, there are some similarities in the expression profiles of other genes between Lin$^-$/ckit$^-$/CD106$^+$/CD44$^-$ BMCs and CXCL12 (SDF-1α)-abundant reticular cells or niche-maintaining cells in BM [10–12, 14, 46]. From this context, we estimate that Lin$^-$/ckit$^-$/CD106$^+$/CD44$^-$ cells in BM may be involved in this niche formation mechanism for HSCs. Further precise investigations are necessary to elucidate the roles and functions of this mesenchymal cell population in BM.

Similar to HSC, heterogeneous MSC populations are also hierarchically organized at the apex, from stem-like cells with self-renewing capacity to more differentiated cells with limited lineage potentials [15, 16]. The stem cell populations in various tissues are generally thought to be in a slow cycling or quiescence state under physiological conditions [47–49]. In this study, the Lin$^-$/ckit$^-$/CD106$^+$/CD44$^+$ population was proliferative and dominantly accumulated in damaged muscles. Conversely, the Lin$^-$/ckit$^-$/CD106$^+$/CD44$^-$ population in BM was shown to be a slow-cycling population. Thus, it appears that the Lin$^-$/ckit$^-$/CD106$^+$/CD44$^+$ population includes effecter cells that egress into the circulation in response to injury and serve to support regeneration through their anti-inflammatory activity and resident stem-cell activation, whereas the Lin$^-$/ckit$^-$/CD106$^+$/CD44$^-$ population in BM includes a stem-like cell population. However, it is still unclear whether a hierarchical relationship between Lin$^-$/ckit$^-$/

CD106$^+$/CD44$^-$ and Lin$^-$/ckit$^-$/CD106$^+$/CD44$^+$ BMCs (Fig. 7) exists, and further studies are warranted to clarify the in vivo relationship of these two populations.

In a recent study, CD106 marked an MSC subpopulation with unique and powerful immunomodulatory activities in vitro [6]. However, no report is available for the in vivo function of CD106$^+$ MSCs. This study showed that recruited Lin$^-$/ckit$^-$/CD106$^+$/CD44$^+$ BMCs highly expressed TSG-6 in damaged muscle. TSG-6 is a strong anti-inflammatory protein that is secreted from MSCs in culture [3, 42, 50–52]. We also showed that the TSG-6- or TSG-6/CD44-mediated pathway activates myoblast and satellite cells in vitro and in vivo. In addition, we observed the acceleration of muscle regeneration as well as the suppression of inflammation following the treatment of freshly isolated Lin$^-$/ckit$^-$/CD106$^+$/CD44$^+$ BMCs, in part through the TSG-6-mediated pathway. However, we cannot yet determine the benefits of Lin$^-$/ckit$^-$/CD106$^+$/CD44$^-$ BMCs for damaged muscles. Although the accumulation of Lin$^-$/ckit$^-$/CD106$^+$/CD44$^-$ BMCs is low in mdx muscles, these cells may have a high impact on the muscle regeneration process. Therefore, in future studies, it would be of interest to investigate whether this small BM-MSCs fraction (Lin$^-$/ckit$^-$/CD106$^+$/CD44$^-$) has the same effect on muscle regeneration as observed in Lin$^-$/ckit$^-$/CD106$^+$/CD44$^+$ BMCs or whether they exhibit different activities, such as muscle differentiation.

In this study, we did not consider the potential effect of Lin$^-$/ckit$^-$/CD106$^+$/CD44$^+$ BMCs or TSG-6 on muscle resident fibro/adipocyte progenitors (FAPs). Recent studies have indicated that sufficient FAPs activities are important for muscle regeneration [53–55]. It is also known that BM-MSCs and FAPs have similar surface markers [54, 55] and express CD44 [56, 57], thus suggesting that TSG-6 secreted from Lin$^-$/ckit$^-$/CD106$^+$/CD44$^+$ BMCs also has effects on FAP proliferation and activities to facilitate muscle regeneration. The interaction of BM-MSCs with FAPs during muscle regeneration needs to be investigated precisely in the future. Together, these findings illustrate the putative scheme for the roles of the BM-derived Lin$^-$/ckit$^-$/CD106$^+$/CD44$^+$ BMCs, which dominantly migrate in damage muscles and release TSG-6, with other trophic factors, to suppress inflammation/fibrosis and promote muscle regeneration processes in vivo. With future studies, the local/systemic administration of TSG-6 may become a new candidate strategy for improving DMD pathology.

CONCLUSIONS

In conclusion, it is clear that the normalization of dystrophin gene expression is necessary for the ultimate cure of DMD. Nevertheless, we consider that the series of results shown here have a significant impact on the muscle repair mechanisms by the endogenous BM-MSC population. This study also provides a novel concept that the normalization of MSC populations in BM may prevent the secondary exacerbation of inflammatory/fibrotic damages and improve clinical manifestations. A chronic exhaustion of MSC populations in BM appears to occur in other intractable hereditary or nonhereditary diseases besides DMD if persistent injury and inflammation continuously activate Lin$^-$/ckit$^-$/CD106$^+$/CD44$^+$ cells in BM. Further precise analyses of heterogeneous BM-MSC populations to understand their roles and mechanisms in various in vivo pathological settings may provide novel therapeutic strategies by targeting intrinsic homeostatic maintenance mechanisms driven by BM-MSCs in vivo.

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AUTHOR CONTRIBUTIONS

R.F.: conception and design, performed experiments, collection and/or assembly of data, data analysis and interpretation, financial support, and manuscript writing; K.T.: conception and design, manuscript writing, financial support, and final approval of manuscript; E.A.: collection and/or assembly of data and data analysis and interpretation; K.N. and S.I.: data analysis and interpretation; Y. Kikuchi: administrative support and provision of study material; Y. Kaneda: conception and design, financial support, and final approval of manuscript.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicate no potential conflicts of interest.

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Functional analysis of the nuclear localization signal of the POU transcription factor Skn-1a in epidermal keratinocytes

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Abstract. POU domain proteins are a family of critical regulators of development and differentiation due to their transcriptional activity in the nucleus. Skn-1a, a member of the POU domain protein family, appears to be expressed predominantly in epidermal keratinocytes and is thought to play a critical role in keratinocyte differentiation and proliferation. In this study, we examined the mechanisms involved in the nuclear localization of Skn-1a. We transiently expressed enhanced green fluorescent protein (EGFP) reporter constructs encoding EGFP fusions with Skn-1a deletion and mutation proteins in normal human epidermal keratinocytes (NHEKs). The experiments clearly demonstrated that Skn-1a contained a functional nuclear localization signal (NLS) domain, and that the smallest domain necessary for Skn-1a nuclear transport was the GRKRKKR sequence located within amino acids 279-285. Previous studies have shown that the phosphorylation of specific amino acids neighboring the NLS may regulate nuclear transport and that the amino acid residues threonine (Thr) and serine (Ser) have the potential to undergo phosphorylation. We examined whether the amino acids Thr286 and Ser287, which reside adjacent to the NLS at the carboxy-terminal side, play a role in Skn-1a nuclear localization. For this purpose, we generated three EGFP-Skn-1a mutation constructs, in which Thr286, Ser287, or both Thr286 and Ser287 residues were replaced with alanine, respectively. The results showed that the Thr286 and Ser287 residues were involved in the regulation of nuclear localization as well as epidermal differentiation. These results suggested that the epidermal differentiation signaling pathway, involving kinase and phosphatase activation, may regulate the NLS activity of Skn-1a in keratinocytes. Collectively, these data contribute to understanding the mechanisms of nuclear translocation of POU domain proteins and epidermal differentiation.

Introduction

The epidermis is a stratified squamous epithelium in which viable cells move outwardly from the basal layer to become terminally differentiated keratinocytes that eventually constitute the *stratum corneum*. Keratinocyte proliferation and differentiation are closely regulated by cellular transcription factors, including activator protein-1 family proteins (1), nuclear factor-κB family proteins (2), cytidine-cytidine-adenosine-adenosine-thymidine/enhancer-binding proteins (3), p53-related proteins (4), and POU transcription factors (5). POU transcription factors are characterized by a bipartite POU domain in which a homeodomain is connected by a short linker region to an N-terminally located POU-specific domain (6). The POU-specific and POU homeodomain are DNA-binding domains of the helix-turn-helix type.

Skn-1a, a member of the POU domain transcription factor family, appears to be expressed predominantly in the epidermal keratinocytes and is thought to play a critical role in keratinocyte differentiation and proliferation (7-9). Skn-1a transactivates the expression of the genes encoding K10 and SPRP2A, which are expressed during keratinocyte differentiation, suggesting that Skn-1a promotes keratinocyte differentiation. Furthermore, we have previously reported that the mRNA expression of Skn-1a increases in cultured normal human keratinocytes subsequent to calcium-induced differentiation (9,10).

Similar to POU domain proteins characterized thus far (2), Skn-1a exerts its function in the nucleus. Nuclear proteins enter this cellular compartment via the nuclear pore complex after being synthesized in the cytoplasm, and usually, they are actively transported through the nuclear pore (11). Such active transportation requires energy, transport receptors, and an endogenous nuclear localization signal (NLS) within the cargo protein.

NLSs have been identified in a variety of nuclear proteins ranging in size from <100 to >1,000 amino acids,

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including polymerases, kinases, phosphatases, transcription factors, histones, tumor suppressor molecules, and various viral proteins. In the present study, we characterized the NLS of Skn-la, which was shown to be localized within its DNA-binding domain as a motif highly conserved among the POU domain proteins. Its identification enhances our understanding of the evolution of POU domain proteins and the mechanisms involved in regulating the access of POU domain proteins to the nucleus.

Materials and methods

Enhanced green fluorescent protein (EGFP)-Skn-la deletion and mutation reporter constructs. The EGFP-Skn-la plasmid was constructed as described below (7). First the Skn-la coding region, which contains the canonical NLS consensus sequence, GRKRKKR (aa279-285), was amplified by performing PCR and subcloned into the C-terminus of pEGFP (Clontech Laboratories, Palo Alto, CA, USA). The primers were used for PCR, *Bsr*GI-207; 5'-GGGTGTACA AGTTCACACAGGGAGATGGGCTGGCGA-3' and *Not*I-295; 5'-TATGCGGCCGCAGTCAGGCGGATGTTG GTCTC-3' for the 207-295 fragment, *Bsr*GI-207 and *Not*I-287; 5'-GGGGCGGCCGCTTTAGCTGGTCCGTTTCTTTCT CTTCCTACCAAA-3' for the 207-287 fragment, *Bsr*GI-258; 5'-GGGTGTACCCTCTCCGTCAGA CCCCTCAGTG-3' and *Not*I-287 for the 258-287 fragment, *Bsr*GI-269; 5'-GGGTGT ACACCTCCTACCCCAGCCTCAGTGAA-3' and *Not*I-287 for the 269-287 fragment. The 273-287, 279-287 and 279-285 fragments were directly employed as the following linkers: 5'-GTACAGGCTCAGTGAAGTATTTGCTAGGAAGAG AAAGAAACGGACCAGCGC-3' and 5'-GGCCGCGCT GGTCCGTTTCTTTCTCTTCGTACCAAATACTTCACT GAGCCT-3' for the 273-287 fragment, 5'-GTACAC CGGTAGGAAGAGAAAGAAACGGACCAGCGC-3' and 5'-GGCCGCGCTGGTCCGTTTCTTTCTCTTCCTAC CGGT-3' for the 279-287 fragment, 5'-GTACACCGGTAG GAAGAGAAAGAAACGGACCAGCGC-3' and 5'-GGCCG CCCGTTTCTTTCTCTTCCTACCGGT-3' for the 279-285 fragment. The EGFP-Skn-la deletion constructs pEGFP-Skn-la 207-295, pEGFP-Skn-la 207-287, pEGFP-Skn-la 258-287, pEGFP- Skn-la 269-287, pEGFP-Skn-la 273-287, pEGFP-Skn-la 279-287 and pEGFP-Skn-la 279-285 were generated by cloning the corresponding segments into pEGFP as *Bsr*GI-*Not*I fragments. We also constructed the mutation constructs pEGFP-Skn-la 279-285m in which both Arg282 and Lys283 were replaced by alanines using the following linkers: 5'-GTACACCGGTAGGAAGGCTGCTAAACG GACCAGCGC-3' and 5'-GGCCGCCCGTTTAGCAGCCTT CCTACCGGT-3'.

Plasmids for the EGFP-Skn-la mutation reporter constructs EGFP-Skn-la 207-287m1 in which threonine (Thr) 286 was replaced with alanine, EGFP-Skn-la 207-287m2 in which serine (Ser) 287 was replaced with alanine, and EGFP-Skn-la 207-287m3 in which both Thr286 and Ser287 were replaced with alanine, were constructed from pEGFP1-Skn-la 258-287 by PCR with mutagenetic primers (forward for all, *Bsr*GI-207; 5'-GGGTGTACAAGTTCACACAGGGAG ATGGGCTGGCGA-3'; reverse, 5'-GGGGCGGCCGCTT TAGCCGGTCCGTTTCTTTCTCTTCCTACCAAA-3' for

m1, 5'-GGGGCGGCCGCTTTAGCTGCCCCGTTTCTTTC TCTTCCTACCAAA-3' for m2, and 5'-GGGGCGGCCG CTTTAGCCGCCCCGTTTCTTTCTCTTCCTACCAAA-3' for m3).

Cell culture. Normal human epidermal keratinocytes (NHEKs) from neonatal foreskin were obtained commercially (Clonetics, San Diego, CA, USA). Cultures were grown in a 60-mm culture dish in keratinocyte growth medium containing human recombinant epidermal growth factor (0.1 ng/ml), insulin (5 ng/ml), hydrocortisone (0.5 ng/ml), gentamicin (50 ng/ml), and amphotericin-B (50 ng/ml). Phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich, St. Louis, MO, USA) was used to induce keratinocyte differentiation.

Transfection and microscopy. The plasmid constructs described above were used in transient transfection studies in cultured NHEKs (9,10). After the cells had grown to ~70% confluence, they were transfected with 5 ng of the EGFP-Skn-la expression vector using the DOTAP Liposomal Transfection Reagent (Roche Applied Science, Indianapolis, NJ, USA). The pEGFP plasmid was used as the control. The cells expressing EGFP were analyzed by fluorescence microscopy of 10 randomly selected fields. The cells were considered positive when the nuclear fluorescence signal was clearly stronger that the cytoplasmic fluorescence signal. The total number of cells expressing EGFP (A) and the number of the positive cells in which EGFP was localized in the nucleus (B) were measured, and the rate of B/A was calculated. The rate of nuclear translocation of each construct was expressed as a percentage relative to that of EGFP-Skn-la 279-285 which contained the most minimum component of the DNA fragment.

Western blot analysis. Western blot analysis was performed following a routine method (9,10). Briefly, the transfected cells were lysed by the sample buffer, electrophoresed on 15% polyacrylamide gels, and then transferred onto a polyvinylidene difluoride membrane. The membranes were incubated with monoclonal anti-GFP antibodies (Roche Diagnostic Corp., Indianapolis, IN, USA), followed by incubation with a horseradish peroxidase-labelled secondary anti-mouse antibody. Immunocomplexes were visualized using visualized using the Image Lab System (Bio-Rad, Hercules, CA, USA). Anti-β-actin antibodies were used as a control.

Statistical analysis. The Student's t-test was used to determine the level of significance of differences in sample means. $P<0.01$ was considered significant. Data were shown as mean ± SD.

Results

Nuclear translocation of the EGFP-Skn-la deletion proteins. To identify the regions of the Skn-la protein required for its transport into the nucleus, we generated reporter constructs for Skn-la deletion proteins fused to EGFP at their N-termini (Fig. 1). The putative NLS consensus sequence, GRKRKKR is located between amino acids 279 and 285. We transfected the constructs into NHEKs and analyzed the EGFP fusion protein localization 24 h later by fluorescence microscopy. Western blot analysis was also performed to
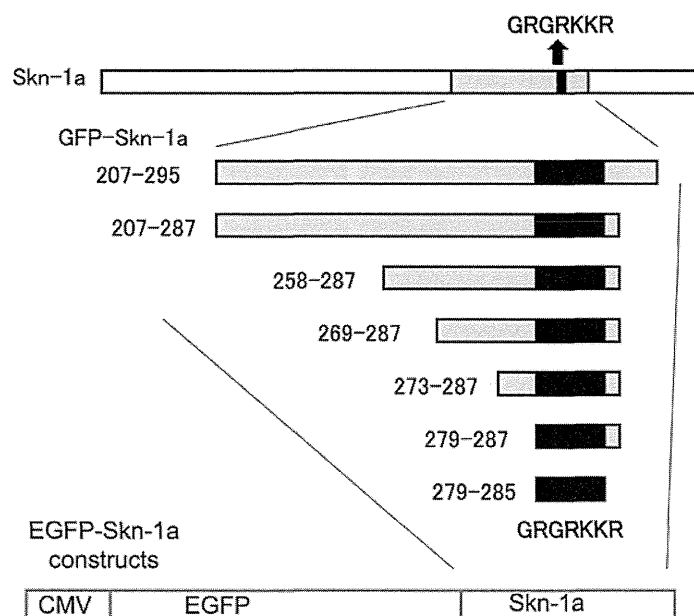
Figure 1. Schematic representation of the enhanced green fluorescent protein (EGFP)-Skn-1a deletion reporter constructs. General structure of the EGFP-Skn-1a constructs. The constructs contain the cytomegalovirus (CMV) promoter and the enhanced green fluorescent protein (EGFP) gene. Skn-1a fragments were fused with the C-terminal amino acid of EGFP. GRGRKKR (aa279-285) is the putative consensus nuclear localization signal (NLS) of Skn-1a.
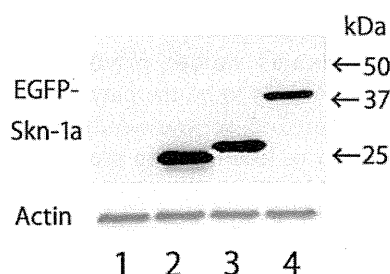

Figure 2. Western blot analysis of the fusion proteins. We transfected the constructs and western blot analysis was performed. Typical experiment was shown in the figure. 1, no transfection; 2, pEGFP, basic construct; 3, enhanced green fluorescent protein (EGFP)-Skn-1a 279-285, the shortest EGFP construct; 4, EGFP-Skn-1a 207-287, the longest EGFP construct. Actin, Anti-β-actin antibodies were used as control.

confirm production of the EGFP fusion protein. The result of western blot analysis revealed that each construct exhibited a band with an estimated size (Fig. 2). Typical positive cells are shown in Fig. 3. The nuclear fluorescence signal was clearly stronger than the cytoplasmic fluorescence signal. Results of the immunofluorescence experiments showed that the rates of nuclear translocation of the fusion proteins from pEGFP-Skn-1a 258-287, 269-287, 273-287, 279-287 and 279-285 were almost identical (Fig. 4). On the other hand, rates for the fusion proteins from the constructs 207-295 and 207-287 were relatively low. The control plasmid pEGFP had little or no ability to enter the nucleus. We also examined the mutation constructs pEGFP-Skn-1a 279-285m in which both Arg282 and Lys283 were replaced by alanines. The result showed the rate to be identical to that of control pEGFP (data not shown).

Nuclear translocation of the EGFP-Skn-1a mutation proteins. The amino acids Thr286 and Ser287 reside adjacent to the NLS on its carboxy-terminal side. We examined their role

in Skn-1a nuclear localization by generating three mutation reporter constructs based on the pEGFP-Skn-1a 207-287 construct. Thr286 was replaced with alanine to generate the construct pEGFP-Skn-1a 207-287m1, Ser287 with alanine for the construct pEGFP-Skn-1a 207-287m2, and both Thr286 and Ser287 residues were replaced with alanine for the construct pEGFP-Skn-1a 207-287m3 (Fig. 5). We then compared the nuclear translocation rates of the pEGFP-Skn-1a mutation proteins. The nuclear translocation rates of the pEGFP-Skn-1a207-287m1 and pEGFP-Skn-1a207-287m2 mutation proteins were identical to that of pEGFP-Skn-1a 207-287, whereas the pEGFP-Skn-1a 207-287m3 mutation protein showed a significantly higher rate compared to the other three constructs (Fig. 6).

Effect of PMA treatment on nuclear translocation. PMA has been well characterized as a protein kinase C activator as well as an inducer of late differentiation marker expression and cornified envelope assembly *in vitro*. In the subsequent series of experiments we examined the rate of nuclear translocation of the reporter proteins from pEGFP-Skn-1a 207-287, pEGFP-Skn-1a 207-287m1, pEGFP-Skn-1a 207287m2, and pEGFP-Skn-1a 207-287m3 in NHEKs treated for 24 h with 10^{-8}-10^{-6} M PMA. The nuclear translocation rates of proteins from pEGFP-Skn-1a 207-287, pEGFP-Skn-1a 207-287m1, and pEGFP-Skn-1a 207-287m2 were enhanced by PMA. However, the rate of the pEGFP-Skn-1a 207-287m3 mutation protein was independent of PMA concentration, and following treatment with 10^{-8} M PMA all four proteins showed essentially the same translocation rate (Fig. 7).

Discussion

The transport of proteins between the nucleus and cytoplasm occurs primarily through the nuclear pore complex where
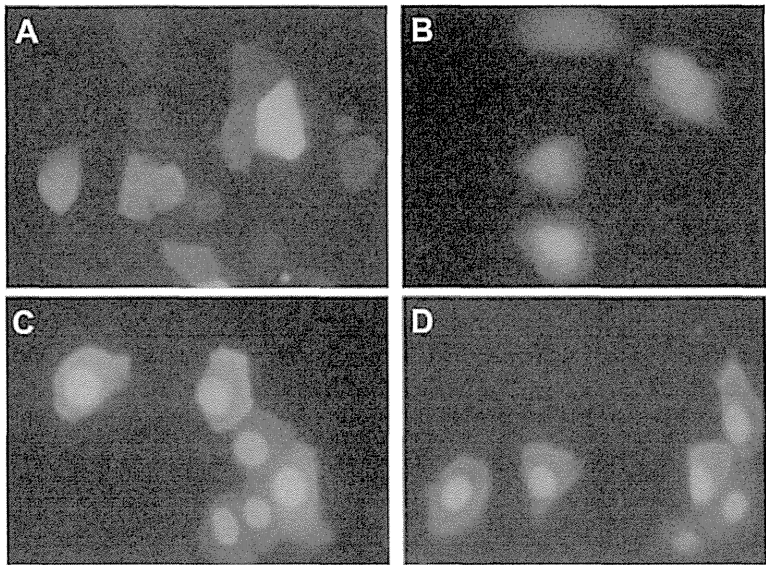
Figure 3. Nuclear translocation of enhanced green fluorescent protein (EGFP)-Skn-1a fusion proteins. We transfected the constructs into normal human epidermal keratinocytes (NHEKs) and analyzed the EGFP fusion protein localization 24 h later by fluorescence microscopy. Typical positive cells for nuclear translocation are shown. The specimen of pEGFP (A and B) shows no or few positive cells and (C) the majority of cells are positive in the sample of pEGFP-Skn-1a 279-285. (D) Half of the cells are positive in pEGFP-Skn-1a 207-287.

EGFP-Skn-1a constructs
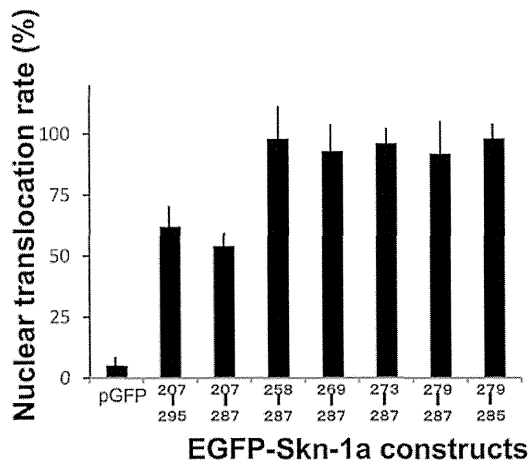
Figure 4. Rates of nuclear translocation of the enhanced green fluorescent protein (EGFP)-Skn-1a deletion proteins in normal human epidermal keratinocytes (NHEKs). Several plasmid constructs, which express Skn-1a deletions as fusion proteins with EGFP were developed and transfected into NHEKs. EGFP-Skn-1a deletion protein localization was analyzed using fluorescence microscopy at 24 h following transfection. The total number of cells expressing EGFP (A) and the number of the positive cells in which EGFP was localized in the nucleus (B) were measured, and the rate of B/A was calculated. The rate of nuclear translocation of each construct was expressed as a percentage relative to that of EGFP-Skn-1a 279-285, which contained the most minimum component. The indicated values show the mean rate ± SD.

proteins can enter the nucleus either by diffusion or by signal-mediated transport (11). The structural constraints of the nuclear pore complex established that only proteins with a molecular mass of <40 kDa are able to enter the nucleus by passive diffusion. However, many proteins are imported by signal-mediated transfer, among them Skn-1a, which is ~48 kDa in size (12). Based on previous observations, we hypothesized that the regulation of nuclear localization of

Skn-1a serves as a molecular switch to control the transcription of various epidermal genes.

The NLStradamus model predicts the sequence GRKRKKR (aa279-285) to be the putative consensus NLS of Skn-1a (13). Therefore, we transiently expressed in keratinocytes a number of EGFP fusion proteins containing the putative NLS with deletions or mutations. The results clearly demonstrate that Skn-1a contains a functional NLS domain, and that the smallest domain necessary for Skn-1a transport is located within amino acids 279-285 (Fig. 2), suggesting that this region of Skn-1a functions as an NLS. Substitution of alanines for both Arg282 and Lys283 eliminated NLS activity of the sequence GRKRKKR (aa279-285). We also found that the deletion proteins from the constructs 207-295 and 207-287 showed a relatively low rate compared with the other shorter constructs, suggesting the presence of a region spanning from 207-287 that potentially inhibits or counteracts the NLS function. It is possible that Ser and Thr residues, prone to phosphorylation, are present in the region 207-258. The computer program NetPhos 2.0 (14), which is utilized to identify potential phospholylation sites, showed that the value of Ser230 was relatively as compared to that of Ser287. Thus, studies targeting Ser230 are to be conducted.

The three POU transcription factors Oct-1, Oct-6, and Skn-1a are expressed in the epidermis. The corresponding NLS regions of these human POU transcription factors Oct-1, Oct-6 and Skn-1a are GLSRRRKKRTSIET, AQGRKRKKRTSIEV, VFGRKRKKRTSIET, respectively (the NSL sequence is underlined) (7,15,16). Notably, all three POU factors contain the identical TSIE amino acid sequence situated at the C-terminus of the NLS. This binding suggests that this area may be important in keratinocyte growth and differentiation. Findings of previous studies have shown that the phosphorylation of particular amino acids neighboring the NLS may regulate NLS activity (17), and that the amino acid residues Thr and Ser are potential candidates for undergoing phosphorylation.
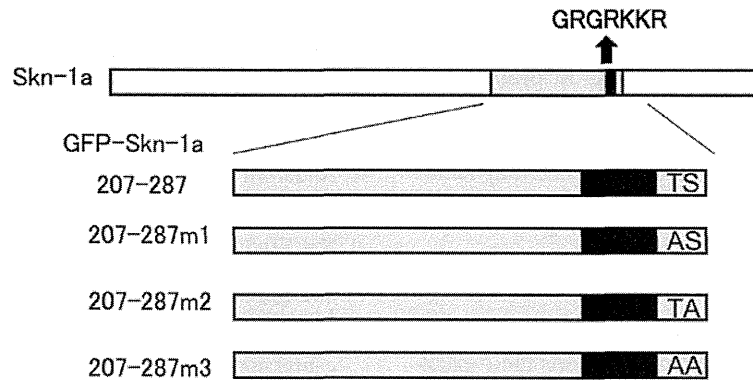
Figure 5. Schematic representation of the enhanced green fluorescent protein (EGFP)-Skn-1a mutation reporter constructs. The fusion protein EGFP-Skn-1a 207-287 contains threonine (Thr) 286 and serine (Ser) 287, which reside adjacent to the nuclear localization signal (NLS) at the carboxy-terminal side. EGFP-Skn-1a 207-287m1 encodes a mutation protein in which Thr286 is replaced with alanine; EGFP-Skn-1a 207-287m2 encod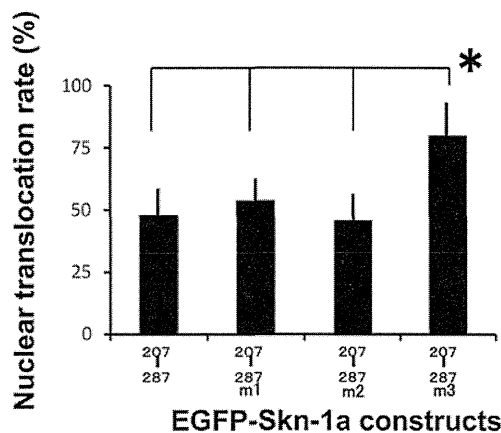es a mutation protein in which Ser287 is replaced with alanine; EGFP-Skn-1a 207-287m3 encodes a mutation protein in which both Thr286 and Ser287 are replaced with alanines.

Figure 6. Rates of nuclear translocation of the mutant constructs. We generated enhanced green fluorescent protein (EGFP)-Skn-1a mutation constructs, then transfected them into normal human epidermal keratinocytes (NHEKs), and analyzed the translocation rates. The indicated values are the mean translocation rate ± SD. Significant differences (*p<0.01) were found between 207-287 and m3, m1 and m3, and m2 and m3.

Figure 7. Effect of phorbol 12-myristate 13-acetate (PMA) treatment on the nuclear translocation rate of enhanced green fluorescent protein (EGFP) The extent of nuclear translocation of the deletion and mutation proteins encoded by the EGFP-Skn-1a 207-287, EGFP-Skn-1a 207-287m1, EGFP-Skn-1a 207-287m2 and EGFP-Skn-1a 207-287m3 constructs prior to and following treatment with various concentrations of PMA. Prior to treatment, the calcium concentration in the medium was adjusted to 0.03 mM to maintain a basal cell-like phenotype of undifferentiated cells. The indicated values are the mean translocation rate ± SD.

Consequently, we generated three mutation reporter constructs derived from EGFP-Skn-1a 207-287, in which Thr286 was replaced with alanine (EGFP-Skn-1a 207-287m1), Ser287 with alanine (EGFP-Skn-1a 207-287m2), or both Thr286 and Ser287 with alanine (EGFP-Skn-1a 207-287m3) and expressed them in keratinocytes. Results of the present study demonstrate that the nuclear translocation rate of construct m3 was higher than those of EGFP-Skn-1a 207-287, and constructs m1 and m2, suggesting that Thr286 and Ser287 play modulating negative roles in NLS function. We also hypothesize that the nuclear accumulation of Skn-1a correlates with the dephosphorylation of both Thr286 and Ser287.

In the presence of low calcium concentration in the medium, keratinocytes are maintained phenotypically as a basal cell-like population of undifferentiated cells. Under these conditions, the proteins from EGFP-Skn-1a 207-287, EGFP-Skn-1a 207-287m1 and EGFP-Skn-1a 207-287m2 translocate to the nucleus with low efficiency, compared to EGFP-Skn-1a 207-287m3. PMA has been well characterized as an activator of protein kinase C as well as an

inducer of late differentiation marker expression and cornified envelope assembly *in vitro*. Therefore, we examined the translocation rates of the mutation reporter proteins in differentiated keratinocytes induced by PMA. As a result of PMA treatment, the nuclear translocation rate of proteins from EGFP-Skn-1a 207-287, EGFP-Skn-1a 207-287m1, and EGFP-Skn-1a 207-287m2 increased. However, the rate of EGFP-Skn-1a 207-287m3 was independent of PMA concentration, and following 10^{-8} M PMA treatment, all four proteins showed essentially the same translocation rate. These data indicate that Thr286 and Ser287 residues play a role in keratinocyte differentiation, suggesting that the epidermal differentiation signaling pathway, involving kinase and phosphatase activation, regulates the NLS activity of Skn-1a in the epidermal keratinocytes.

Results of the present study suggest that the transcriptional regulation of various epidermal genes provides a differentiation-specific phenotype of the epidermis. At present, the precise mechanism of transcription factor nuclear translocation in keratinocytes remains to be characterized. Further characterization of the transportation mechanisms may facilitate an understanding of signal transduction into the nucleus.

Acknowledgements

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自家嗅粘膜移植による脊髄再生医療

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Spinal Cord Regeneration with Olfactory Mucosa Autografts

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The efficacy of olfactory mucosa autografts for chronic spinal cord injury has been previously reported. The new voluntary activity in response to voluntary effort has been documented by EMG, but the emergence of motor evoked potentials, which reflects the status of electrophysiological conductivity including the corticospinal pathway after olfactory mucosa autograft treatment, has not been ascertained. We report herein, the emergence of motor evoked potentials after olfactory mucosa autograft.

はじめに

神経系は情報の伝達を役目とし，神経細胞は興奮を多数の筋細胞，またはさらに他の神経細胞に伝えるという神経網を形成している．ヒトの体内では，すべての内臓，血管および腺は，そのヒト固有の神経網によって支配されており，これが自律神経系である．脊椎動物では自律神経系のほかに，中枢神経系と末梢神経系からなる体性神経系があり，そのヒト固有の意識的な知覚，随意運動および情報の統合を行っている．よってこれら神経系は，そのヒト固有の径路，また神経網が存在するという面があり，一度損傷され修復される場合は，その径路や神経網が，その人なりに再構築されなければならない．よって神経系の特に中枢神経系における再生医療においては，単に細胞や組織移植が生着しても，それがただちに再生となるわけではなく，粘り強いリハビリテーションによって新たな神経径路または神経網が再獲得されて，初めて再生医療の成功に繋がる．したがって神経再生は，新しい神経径路や神経網の再獲得のための契機をいかにして創造し，そしていかに効果的なリハビリテーションを実施するかという，総合的見地に立って考えなくてはならない．

脊髄再生研究の歴史

依然として困難とされる中枢神経再生であるが，基礎研究においてはブレイクスルーとなる研究が 1970 年代後半から出されている．その一つが胎児脊髄移植である[4]．脳損傷モデルにおいては，移植されたグラフトからの神経分布，ホスト脳の障害された領域からの神経伝達物質の放出が確認され，軸索成長の促進と機能回復が認められた[1)4)13)32]．これによる神経軸索再建機序は，移植された胎生神経組織が neuron を含むことから，ホスト脊髄と双方向性のシナプス結合を形成するためと考えられ[5]，この成功を基に脊髄損傷基礎研究は臨床への新しい方向性を得た．しかしながら，これをヒトの治療に

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応用することはできなかった．一人の脊髄損傷の患者さんの移植には 10～15 体の胎児が必要であり，倫理的に許容されることはなかったのである．そこで注目されたのがその胎生組織中の神経幹細胞または胚性幹細胞であった．

脊髄損傷においても多くの幹細胞による細胞移植療法が検討されており，特に iPS 細胞や ES 細胞には大きな期待が寄せられている．脊髄損傷動物に対する神経幹細胞移植による機能回復のメカニズムとして，移植した神経幹細胞由来の介在ニューロンによる損傷脊髄内の局所神経回路網の部分的な修復，移植した神経幹細胞由来の幼弱なアストロサイトが産生する神経栄養因子，サイトカイン，細胞増殖因子や細胞外マトリックスによる宿主神経軸索の再生誘導，血管新生誘導などが想定されている[42]が，切断された神経径路の再構築は証明されていない．また幹細胞をはじめとした細胞療法では，移植後のそれら細胞の生存率は低く，多くは移植後 24 時間で死滅してしまうことが明らかとなっており[27][41][59]，最近の研究はこれら細胞を生存させるための足場の開発が一つの大きな研究動向となっている[51]．

脊髄損傷の急性期と慢性期

脊髄損傷の急性期から亜急性期と慢性期ではその病態がまったく異なり，よってその治療戦略もまったく異なったものとなる．

損傷を受けた脊髄では細胞が破壊され，神経線維と血管が剪断される．これがいわゆる一次損傷で，これに引き続いて炎症など細胞障害性の二次損傷が続発する[50]．しかしながらこの二次損傷とされる一次損傷後の炎症反応は，損傷の修復に重要であるとの報告もあり[11][45][46]，急性期についてはどのようなサイトカインや細胞などが，病態の増悪もしくは修復にかかわっているのかを解明する研究が進められている[2][19][26][60]．

損傷部位において神経細胞は脱落し，二次損傷後の慢性期においては軸索再生を許容しがたいグリア瘢痕組織が周囲を被う[16][17]．よって慢性期の治療のためには，失われた神経細胞の補塡と軸索伸長の促進，そして軸索再生に拒絶的なグリア瘢痕組織と生来再生に拒絶的な脊髄微小環境[37][52]を改善する必要がある．つまり軸索伸長のための足場を作る必要がある[30]．

急性期から亜急性期の細胞移植療法

期待される ES 細胞，iPS 細胞といった幹細胞もしく

はこれらを神経系細胞に分化させた細胞による移植療法においては，軸索の再髄鞘化等による機能回復が報告され，大きな期待が寄せられている[6][25][33][36][40][56]．米国のベンチャー企業 Geron 社が，ヒト ES 細胞を用いた脊髄損傷治療の開発を行い，昨年 FDA より承認を得て，昨年 10 月に第 1 例目が施行されている．しかしながら日本国内においては，ヒト ES 細胞を臨床研究や治療に応用することについて，安全性の問題のみならず中絶胎児同様倫理的問題がいまだ解決をみていない．自家細胞を用いるヒト iPS 細胞は倫理的問題が少ないとされ，臨床応用に向けた研究が精力的に行われているが，しかしその効果発現機序は基本的に ES 細胞と同じと考えられており，損傷後 8 日目あたりに限定される．

骨髄間質細胞は，その豊富な神経栄養因子が神経保護効果を示し[43]，古くから数カ国で臨床研究が実施された．韓国やチェコスロバキアでの急性期から亜急性期における骨髄間質細胞移植の臨床研究では，神経学的機能回復が認められたと報告されている[54][55][61]．日本では関西医科大学附属病院にて急性期の脊髄損傷患者 3 名に行われている．現時点で重篤な有害事象の報告はなく，今後の発展が期待される．

これら臨床研究は細胞の移植時期が急性期から亜急性期であり，完全麻痺であっても自然経過で回復が少なからず認められる時期に相当するため，有効性を論じるのが難しいが今後の研究の進展が期待される．

慢性期の移植療法

脊髄損傷慢性期においては，神経細胞の脱落，神経軸索の断裂，さらにグリア瘢痕が生じている．よって神経細胞の補塡，神経ネットワーク構築のための軸索伸長因子，さらに軸索伸長が許容される足場が必要となる．末梢神経や培養したシュワン細胞を移植すれば，中枢神経軸索がそれを許容的な足場としてよく伸びてくることは多くの研究者によって確認されている．Cheng ら[9]が成熟ラットの脊髄髄節を切除し，その空隙を複数の肋間神経で架橋したのは拒絶的環境の回避を意図したものであったし，Li ら[29]の行った嗅神経鞘細胞の移植も拒絶的環境を許容的に変える試みといってよいであろう．

ⓛ 神経幹細胞
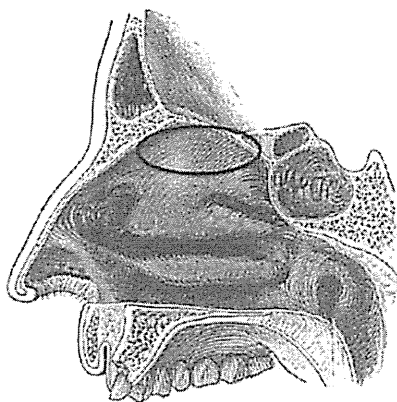失われた神経細胞の補塡については神経幹細胞がその候補となるが，幹細胞は単に移植されたのみでは長期間生存できない[41]．しかしながら近年の研究で脊髄損傷局所の微小環境が改善されれば，神経や軸索はその適切な

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Fig. 1 The location of olfactory mucosa which was removed and transplanted

A|B

Fig. 2 A : T1 weighted MR Image of thoracic spinal cord injury.
B : Gd-enhanced image on T1 weighted MR image.

環境下で再生しうることが明らかになってきている[5)15)]．移植された神経幹細胞は神経系細胞に分化するのみでなく，軸索伸長を促進するさまざまなまた多くの因子を分泌する[20)]．神経幹細胞移植は細胞補填と軸索伸長の観点から有望であるが，脊髄損傷局所の微小環境の改善を図ることなく，神経幹細胞移植のみでは十分な再生効果は得られないことも明らかとなっている[8)20)]．

② 嗅神経鞘細胞（OEC）

　嗅粘膜から嗅球という中枢神経に至る嗅神経系は，ほぼ生涯を通して生理的条件下で神経と軸索再生が認められる例外的な部位である[14)]．この例外的神経再生効果は，主に嗅粘膜上皮に含まれる神経幹細胞と OEC によるものと考えられている[47)]．このうち OEC は脊髄損傷慢性期に軸索伸長効果を示す細胞であり[35)]，髄鞘細胞として，中枢神経におけるオリゴデンドロサイトや末梢神経におけるシュワン細胞とは異なり，嗅神経から嗅球という末梢から中枢にまで至る神経軸索を伸長させうるものである[12)47)]．成体の OEC はシュワン細胞とは異なり，中枢神経に分類される網膜神経節細胞からの軸索を長く伸長させ[28)]，海馬神経細胞と共培養するとシュワン細胞存在下よりもよくアストロサイトが結合することなどが明らかとなっている[57)]．成体中枢神経中の軸索阻害因子から伸長軸索を保護する作用を有し[12)]，損傷脊髄に移植されると，豊富な神経軸索伸長因子を放出するとともに，軸索伸長のための足場を形成し[24)29)]，加えて軸索の髄鞘化を図り神経伝導速度を向上させ[10)18)22)34)53)]，切断された下向路を再生し機能回復をもたらすことが明らかにされている[29)48)]．最も有意義な点は，ある程度グリア瘢痕組

織中を浸潤し，各種栄養因子や接着因子を放出して軸索伸長を促進することである[24)]．Griffith University の Mackay-Sim ら[35)]は OEC を 6 名の慢性期脊髄損傷患者に移植し，移植後 3 年間で重篤な有害事象が発生していないことを報告している．この臨床研究報告では 1 名に神経学的機能の回復がみられたとしている．

③ 嗅粘膜

　生来脊髄は軸索伸長に拒絶的であり[37)52)]，移植された幹細胞の生存のためにもまた軸索伸長のためにも再生のための足場を作ることが必要である[30)51)]．嗅粘膜中には神経細胞を補填しうる神経幹細胞と神経軸索伸長作用を有する嗅神経鞘細胞が存在し，また嗅粘膜中で神経再生が活発に起こっていることから，嗅粘膜自身が神経軸索再生の足場として有用であると考えられる[14)]．ラットを用いた基礎的研究でもその有効性が確認されており[3)23)]，嗅粘膜は慢性期脊髄損傷に対する理想的な移植片であると考えられている[30)31)]．

自家嗅粘膜移植法

　完全対麻痺の脊髄損傷慢性期患者に対する自家嗅粘膜移植法は，2001 年にポルトガルはリスボンの Egas-Moniz 病院で Lima ら[30)31)]によって始められた．嗅粘膜（Fig. 1）は頭蓋外において唯一生理学的条件下で神経再生が認められる部位であり[14)]，脊髄損傷の修復機転に寄与すると考えられている神経幹細胞と嗅神経鞘細胞を含
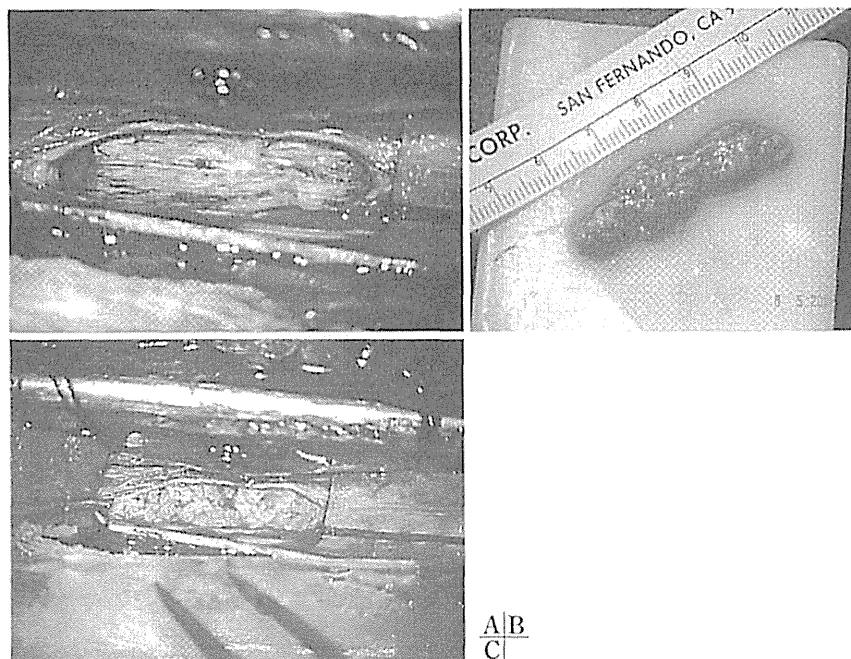
Fig. 3　A : Removal of intra-spinal cord scar tissue.
B : Minced olfactory mucosa.
C : Transplantation of minced olfactory mucosa.

んでいる[7)21)38)49)]．特に患者自身の鼻腔において内視鏡的に採取されうる自己移植片であることから[58)]，拒絶反応や倫理的問題が俎上にあがらない．

　脊髄損傷に対し患者の嗅粘膜そのものを移植する嗅粘膜移植法は，現在脊髄損傷後 6 カ月以上を経過した慢性期患者に対し（Fig. 2），損傷部位の髄内瘢痕組織を摘出した後に（Fig. 3A），自家嗅粘膜を内視鏡下に摘出細切し（Fig. 3B），脊髄瘢痕組織摘出腔に移植するものである（Fig. 3C）．瘢痕組織の摘出は正常脊髄組織を障害しないように部分的に行うが，損傷部位の吻側尾側では，正常脊髄との神経架橋のため十分な後正中溝の開放を必要としている[30)31)]．Lima ら[30)31)]は，年齢 19〜37 歳の男性 17 名，女性 3 名の合計 20 名の両下肢完全運動麻痺の脊髄損傷慢性期患者に自家嗅粘膜移植を施行し，集中的なリハビリテーションを施行している．術後 12〜45 カ月（平均 27.7 カ月）の観察期間で，ASIA (American Spinal Injury Association) 分類において 6 名が A から C へ，3 名が B から C へ，そして 2 名が A から B へと回復し，15 名で下肢筋電図における随意性の収縮がみられ，さらに 5 名で膀胱機能検査上の改善も得られたと報告している．嗅覚については全症例で回復をみており，95％で 2 カ月以内に回復している．有害事象については，3 名で皮下髄液の貯留を認めたが，経過観察あるいは簡単な

縫合処置で回復している．1 名で術後 1 年を経過した時点で過敏性腸炎がみられ，5 年間継続したと報告している．これは内臓性神経因性疼痛と考えられている．また 1 名で MRSA による細菌性髄膜炎を併発し，バンコマイシン等の化学療法で治癒したが，ASIA 分類で B から A へと悪化．2 カ月後再び B へと回復している．

　Detroit Medical Center のチームは嗅粘膜移植を受けた 38 名の患者と，受けていない 22 名の脊髄損傷患者に同様のリハビリを行い，統計学的な有意差には至らなかったものの運動機能については非移植群で 27％に改善がみられたのに対し，移植群で 58％に認められたと報告している（2008 年 7 月の The 26th Annual National Neurotrauma Society Symposium）．

　われわれは 2002 年から自家嗅粘膜移植による損傷脊髄機能再生法の開発として臨床研究を施行している．対象基準は，脊髄損傷後 6 カ月以上が経過し，年齢 40 歳以下の Frankel または ASIA 分類 A，B における下肢完全運動麻痺で，MRI における損傷部位の長さが 3 cm 以内，また鼻腔に感染症を認めないものとしている．2010 年 4 月までに自家嗅粘膜移植を施行した患者は 4 名である．2008 年 2 月 7，8 日に 2 例，2009 年 7 月 17 日に 1 例，2010 年 3 月 19 日に 1 例，両下肢完全運動麻痺を呈する胸髄損傷患者に対し，自家嗅粘膜移植術を施行し

た．これら4例において，これまで当研究と関連がある
と判断される感染症，悪性新生物の発生を認めていない．
有害事象として嗅覚低下や，頭痛および脊損領域の痛み
が出現した症例もあるが，いずれも重篤に至らずに回復
しており，研究の継続に影響を与えるような安全性の問
題は発生していない．評価検査項目の一つである筋電図
測定では，4例中1例で下部腹直筋，傍脊柱筋と大腿筋
膜張筋で筋電図波形が出現し，別の1例では下部腹直筋
の筋電図波形の出現を認めている．またもう1例では大
腿四頭筋で筋電図波形の出現を認めている．これらの結
果より自家嗅粘膜移植法は，2011年末，国の先進医療の
認定を受けている．

Lima ら[30)31)]は嗅粘膜移植と同時に，長期にわたるリハ
ビリテーションの重要性を強調している．嗅粘膜移植単
独またリハビリテーション単独では回復を期待できず，
骨格筋，血管さらに神経の再構築のためのリハビリテー
ションが必要であるとしている．どのようなリハビリ
テーションが理想的であるかはまだ不明としながらも，
特に重力をかけた歩行リハビリテーションが重要で，こ
れを BIONT（Brain-initiated overground nonrobotic/non-
weight supported training）として提唱している．

おわりに

脊髄損傷においては，細胞療法等の単独の移植療法よ
り他の因子等とのコンビネーション療法のほうが効果的
であるとの知見が出されており[39)44)]，特定の細胞等の単
一の因子で成功を収めることは困難であろう．嗅粘膜は，
細胞，軸索伸長因子，再生のための足場という3因子を
兼ね備えており，現在のところ理想的な移植材料の一つ
と考えられる．また移植によって獲得される神経系の再
構築は生来のものではなく，骨格筋，血管そして神経ネッ
トワーク構築の remodeling が必要であり，そのためのリ
ハビリテーションが移植と並んで重要であるといえよ
う．

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————— 要　旨 —————

自家嗅粘膜移植による脊髄再生医療

岩月　幸一

　完全脊髄損傷慢性期に対する嗅粘膜移植法においては，運動機能の部分的回復およびそれに伴う随意性の筋電図の出現が報告されている．しかし筋電図の出現は，電気生理学的に皮質脊髄路の再構築を証明するものではない．われわれは完全脊髄損傷慢性期患者4名に対し本法を施行したが，4例中2例において6カ月後より運動機能の改善がみられ，うち1例では装具を用いた立位保持や歩行器を用いた歩行が可能となった．4名いずれの患者においても，日常生活上何らかの運動機能改善がみられた．また1例において，大脳運動野の経頭蓋磁気刺激により下肢筋において運動誘発電位が認められ，錐体路の接続性を世界で初めて電気生理学的に明らかにした．

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■今後の研究の展開

嗅粘膜移植による脊髄再生医療
Spinal Cord Regeneration with Olfactory Mucosa Autograft

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慢性期完全脊髄損傷に対する嗅粘膜移植法は，嗅粘膜が脊髄損傷の修復機転に寄与すると考えられている神経幹細胞と嗅神経鞘細胞を含み，これの移植により脊髄瘢痕組織を，軸索再生可能な組織に改変することを目的としている．2011年までに4症例施行している．2症例でASIA motor scoreの改善を認め，また随意性の筋電図の発現を認めている．うち1症例で下肢筋において，運動誘発電位（motor evoked potential：MEP）の発現を認めた．2011年度末先進医療の指定を受け，新たな臨床研究を開始する．

はじめに

　再生医療は未分化の細胞を用い，人体の一部を再生させる医療とされている．完成した他者の臓器を移植する臓器移植医療とは異なり，少数の未分化な細胞から病態に応じて適切な組織や臓器を再生できれば，極めて広範囲の疾病や外傷に対する治療効果が期待される．さらにそれが患者本人に由来する細胞であれば，倫理的また拒絶反応のリスクも回避できることから，これまでの医療を根本的に変えてしまう可能性を秘めている．

　神経疾患においても，神経難病や神経損傷の切り札として再生医療は大きな期待がかけられている．しかし中枢神経系における再生医療は，他臓器のそれとは根本的に異なる点があることを理解しておく必要がある．

　神経系は情報の伝達を役目とし，神経細胞は興奮を多数の筋細胞，またはさらに他の神経細胞に伝えるという神経網を形成している．ヒトの体内では，全ての内臓，血管および腺は，そのヒト固有の神経網によって支配されており，これが自律神経系である．脊椎動物では自律神経系の他に，中枢神経系と末梢神経系からなる体性神経系があり，そのヒト固有の意識的な知覚，随意運動および情報の統合を行っている．よってこれら神経系は，そのヒト固有の径路，また神経網が存在するという面が

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あり，一度損傷され修復される場合は，その径路や神経網が，そのヒト固有のものとして再構築されなければならない．よって神経系の特に中枢神経系における再生医療においては，単に細胞や組織移植が成功しても，それが直ちに再生につながるわけではなく，粘り強いリハビリテーションによって新たな神経径路または神経網が形成されて，初めて再生医療の成功につながる．したがって神経再生は，新しい神経径路や神経網の再獲得のための契機をいかにして創造し，そしていかに効果的なリハビリテーションを実施するかという総合的見地に立って考えなくてはならない．

脊髄再生研究の歴史

困難とされてきた脊髄再生であるが，基礎研究においては革新的な研究が1970年代後半から発表されている．その1つが胎児脊髄移植である．脳損傷モデルにおいては，移植されたグラフトからの神経分布，ホスト脳の障害された領域からの神経伝達物質の放出が確認され，軸索成長の促進と機能回復が認められた[1]．これによる神経軸索再建機序は，移植された胎生神経組織が神経細胞を含むことから，ホスト脊髄と双方向性のシナプス結合を形成するためと考えられ，この成功を基に脊髄損傷基礎研究は臨床への新しい方向性を得た．しかしながら，これをヒトの治療に応用することはできなかった．1人の脊髄損傷の患者の移植には10～15体の胎児が必要であり，倫理的に許容されることはなかったのである．そこで注目されたのが，その胎生組織中の神経幹細胞または胚性幹細胞であった．

脊髄損傷においても多くの幹細胞による細胞移植療法が検討されており，特にiPS細胞やES細胞には大きな期待が寄せられている．脊髄損傷動物に対する神経幹細胞移植による機能回復のメカニズムとして，移植した神経幹細胞由来の介在ニューロンによる損傷脊髄内の局所神経回路網の部分的な修復，移植した神経幹細胞由来の幼若アストロサイトが産生する神経栄養因子，サイトカイン，細胞増殖因子や細胞外マトリックスによる宿主神経軸索の再生誘導，血管新生誘導などが想定されている[2]が，切断された神経径路の再構築は証明されていない．また幹細胞をはじめとした細胞療法では，移植後のそれ

ら細胞の生存率は低く，多くは移植後24時間で死滅してしまうことが明らかとなっており[3]，最近の研究はこれら細胞を生存させるための足場の開発が1つの大きな研究動向となっている[4]．

脊髄損傷の急性期と慢性期

脊髄損傷の急性期から亜急性期と慢性期ではその病態が全く異なり，よってその治療戦略も全く異なったものとなる．

損傷を受けた脊髄では細胞が破壊され，神経線維と血管が剪断される．これがいわゆる一次損傷で，これに引き続いて炎症など細胞障害性の二次損傷が続発する[5]．しかしながら，この二次損傷とされる一次損傷後の炎症反応は，損傷の修復に重要であるとの報告もあり[6]，急性期についてはどのようなサイトカインや細胞などが，病態の増悪もしくは修復に関わっているのかを解明する研究が進められている[7]．

損傷部位において神経細胞は脱落し，二次損傷後の慢性期においては軸索再生を許容しがたいグリア瘢痕組織が損傷部位を満たす[8]．したがって慢性期の治療のためには，失われた神経細胞の補填と軸索伸長の促進，そして軸索再生に拒絶的なグリア瘢痕組織と生来再生に拒絶的な脊髄微小環境[9]を改善する必要がある．つまり軸索伸長を可能とする足場を作る必要がある[10]．

1. 慢性期の移植療法

脊髄損傷慢性期においては，神経細胞の脱落，神経軸索の断裂，さらにグリア瘢痕が生じている．よって神経細胞の補填，神経ネットワーク構築のための軸索伸長因子，さらに軸索伸長が許容される足場が必要となる．末梢神経や培養したシュワン細胞を移植すれば，中枢神経軸索がそれを許容的な足場としてよく伸びてくることは多くの研究者によって確認されている．Chengらが成熟ラットの脊髄髄節を切除し，その空隙を複数の肋間神経で架橋したのは拒絶的環境の回避を意図したものであった[11]．よって慢性期においては，脱落した神経細胞を補填し得る細胞，軸索伸長因子，そして軸索伸長を許容する足場の供給といった3つの因子が必要となる．

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2. 神経幹細胞

失われた神経細胞の補填については神経幹細胞がその候補となるが，幹細胞は単に移植されただけでは長期間生存できない[12]．しかしながら近年の研究で脊髄損傷局所の微小環境が改善されれば，神経や軸索はその適切な環境下で再生し得ることが明らかになってきている[13]．移植された神経幹細胞は神経系細胞に分化するだけでなく，軸索伸長を促進する様々な，また多くの因子を分泌する[14]．神経幹細胞移植は細胞補填と軸索伸長の観点から有望であるが，脊髄損傷局所の微小環境の改善を図ることなく，神経幹細胞移植だけでは十分な再生効果は得られないことも明らかとなっている[14]．

3. 嗅神経鞘細胞（OEC）

嗅粘膜から嗅球という中枢神経系に至る嗅神経系は，ほぼ生涯を通して生理的条件下で神経と軸索再生が認められる例外的な部位である[15]．この例外的神経再生効果は，主に嗅粘膜上皮に含まれる神経幹細胞と嗅神経鞘細胞（olfactory ensheathing cell：OEC）によるものと考えられている[16]．このうちOECは脊髄損傷慢性期に軸索伸長効果を示す細胞であり[17]，髄鞘細胞として，中枢神経系におけるオリゴデンドロサイトや末梢神経におけるシュワン細胞とは異なり，嗅神経から嗅球という末梢から中枢にまで至る神経軸索を伸長させ得るものである[18]．成体のOECはシュワン細胞とは異なり，中枢神経系に分類される網膜神経節細胞からの軸索を長く伸長させ[19]，海馬神経細胞と共培養するとシュワン細胞存在下よりもアストロサイトがよく結合することなどが明らかとなっている[20]．成体中枢神経系中の軸索阻害因子から伸長軸索を保護する作用を有し[18]，損傷脊髄に移植されると，豊富な神経軸索伸長因子を放出するとともに，軸索伸長のための足場を形成し[21,22]，加えて軸索の髄鞘化を図り神経伝導速度を向上させ，切断された下向路を再生し機能回復をもたらすことが明らかにされている．最も有意義な点は，ある程度グリア瘢痕組織中を浸潤し，各種栄養因子や接着因子を放出して軸索伸長を促進することである[21]．Griffith大学のMackay-SimらはOECを慢性期脊髄損傷6症例に移植し，移植後3年間で重篤な有害事象が発生していないことを報告している[17]．この臨床研究報告では1症例に神経学的機能の回復がみられたとしている．

4. 嗅粘膜

生来脊髄は軸索伸長に拒絶的であり[9]，移植された幹細胞の生存のためにも，また軸索伸長や再生のための足場を作ることが必要である[4]．嗅粘膜中には神経細胞を補填し得る神経幹細胞と神経軸索伸長作用を有する嗅神経鞘細胞が存在し，また嗅粘膜中で神経再生が活発に起こっていることから，嗅粘膜自身が神経軸索再生の足場として有用であると考えられる[15]．ラットを用いた基礎的研究でもその有効性が確認されており[23]，嗅粘膜は慢性期脊髄損傷に対する理想的な移植片であると考えられている[10]．

自家嗅粘膜移植法

完全対麻痺の脊髄損傷慢性期症例に対する自家嗅粘膜移植法は，2001年にリスボンのEgas-Moniz病院でLimaらによってはじめられた[10]．嗅粘膜（図1）は頭蓋外において唯一生理学的条件下で神経再生が認められる部位であり[15]，脊髄損傷の修復機転に寄与すると考えられている神経幹細胞と嗅神経鞘細胞を含んでいる．特に患者自身の鼻腔において内視鏡的に採取され得る自己移植片であることから，拒絶反応や倫理的問題がない．

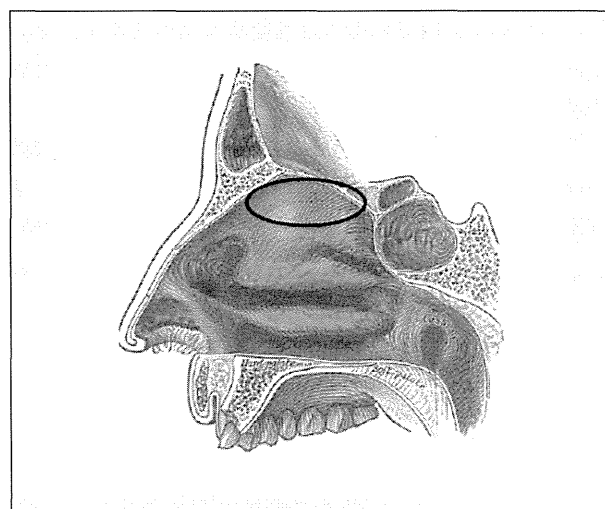脊髄損傷に対し患者の嗅粘膜そのものを移植する嗅粘膜移植法は，現在脊髄損傷後6カ月以上を経過した慢性期患者に対し，損傷部位の髄内瘢痕組織を摘出した後に，

図1 移植に用いる嗅粘膜の場所

自家嗅粘膜を内視鏡下に摘出，細切し，脊髄瘢痕組織摘出腔に移植するものである（図2）．瘢痕組織の摘出は正常脊髄組織を障害しないように部分的に行うが，損傷部位の吻側尾側では，正常脊髄との神経架橋のため十分な後正中溝の開放を必要としている[10]．Carlos Limaら

図2 嗅粘膜移植法
(a)脊髄損傷部位の瘢痕組織の摘出，(b)細切された嗅粘膜，
(c)瘢痕組織の摘出腔に移植された嗅粘膜

は，19〜37歳の男性17症例，女性3症例の合計20症例の両下肢完全運動麻痺の脊髄損傷慢性期患者に自家嗅粘膜移植を施行し，集中的なリハビリテーションを施行している．術後12〜45カ月（平均27.7カ月）の観察期間で，ASIA（American Spinal Injury Association）分類において6症例がAからCへ，3症例がBからCへ，そして2症例がAからBへと回復し，15症例で下肢筋電図における随意性の収縮がみられ，さらに5症例で膀胱機能検査上の改善も得られたと報告している．嗅覚については全症例で回復をみており，95％で2カ月以内に回復している．有害事象については，3症例で皮下髄液の貯留を認めたが，経過観察あるいは簡単な縫合処置で回復している．1症例で術後1年を経過した時点で過敏性腸炎がみられ，5年間継続したと報告している．これは内臓性神経障害性痛と考えられている．また1症例でMRSAによる細菌性髄膜炎を併発し，バンコマイシン等の化学療法で治癒したが，ASIA分類でBからAへと悪化．2カ月後再びBへと回復している[10,24]．

Detroit医療センターのチームは嗅粘膜移植を受けた38症例と，受けていない22症例の脊髄損傷患者に同様のリハビリを行い，統計学的な有意差には至らなかったものの，運動機能については非移植群で27％に改善がみられたのに対し，移植群で58％に認められたと報告している（2008年7月のThe 26th Annual National Neurotrauma Society Symposium）．

われわれは2002年から自家嗅粘膜移植による損傷脊髄機能再生法の開発として臨床研究を施行している．対象基準は，脊髄損傷後6カ月以上が経過し，40歳以下のFrankelまたはASIA分類A，Bにおける下肢完全運動麻痺で，MRI画像における損傷部位の長さが3cm以内，また鼻腔に感染症を認めないものとしている．2010年4月までに自家嗅粘膜移植を施行したのは4症例である（表1）．2008年2月7，8日に2症例，2009年7月17日に1症例，2010年3月19日に1例症，両下肢完全運動麻痺を呈する胸髄損傷患者に対し，自家嗅粘膜移植術を施行した．これら4症例において，これまで当研究と関連があると判断される感染症，悪性新生物の発生を認めていない．有害事象として嗅覚低下や，頭痛および脊損領域の痛みが出現した症例もあるが，いずれも重篤に至らず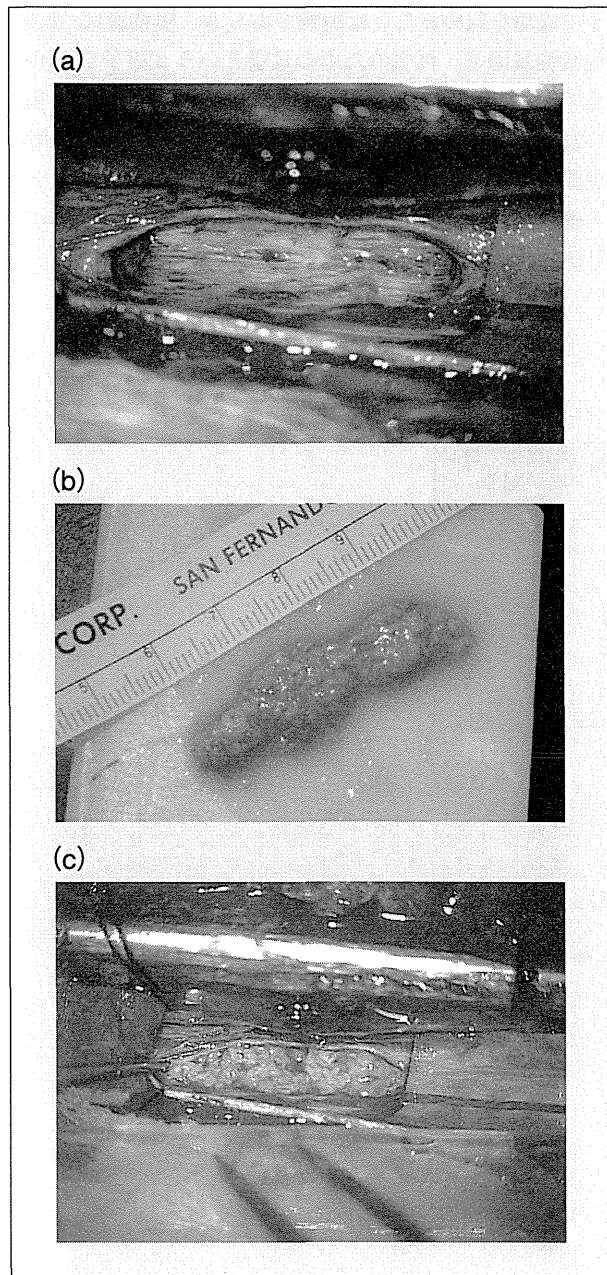に回復しており，研究の継続に影響を与えるような安全性の問題は発生していない．評価検査項目の1つ