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GATA2 participates in the recanalization of lymphatic vessels after surgical lymph node extirpation

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Abstract (175 words)

Lymphatic recanalization failure after lymphadenectomy constitutes a major risk of lymphedema in cancer surgery. It has been reported that GATA2, a zinc finger transcription factor, is expressed in lymphatic endothelial cells and is involved in the development of fetal lymphatic vessels. GATA3, another member of the GATA family of transcription factors, is required for the differentiation of lymphoid tissue-inducer (LTi) cells and is essential for lymph node formation. However, how GATA2 and GATA3 function in recanalization after the surgical extirpation of lymphatic vessels has not been elucidated. Employing a new model of lymphatic recanalization, we examined the lymphatic reconnection process in *Gata2* heterozygous deficient (*Gata2*$^{+/-}$) and *Gata3* heterozygous deficient (*Gata3*$^{+/-}$) mice. We found that lymphatic recanalization was significantly impaired in *Gata2*$^{+/-}$ mice, while *Gata3*$^{+/-}$ mice rarely showed such abnormalities. Notably, the perturbed lymphatic recanalization in the *Gata2*$^{+/-}$ mice was partially restored by crossing with the *Gata3*$^{+/-}$ mice. Our results demonstrate for the first time that GATA2 participates in the regeneration of damaged lymphatic vessels and the unexpected suppressive activity of GATA3 against lymphatic recanalization processes.

Introduction

Lymphatic capillaries collect interstitial fluid and converge into the collecting lymphatic vessels. The collected fluid, i.e., lymph, drains into the lymph node through the afferent lymphatic vessels and flows out from the efferent lymphatic vessel. Thereafter, the lymph fluid flows through the thoracic duct and enters into the venous blood flow (Tammela et al., 2010). Multiple genes have been identified to be involved in lymphatic development, and their mutations are often associated with refractory lymphedema, which is typically characterized by severely swollen extremities (Brouillard et al., 2014). Among them, the zinc finger transcription factor GATA2 has been recognized as a key regulator of lymphatic development.

The GATA family of transcription factors contains two C_4 zinc fingers that serve as its DNA binding domain and recognize the cognate consensus motif (A/T)GATA(A/G) (Yamamoto et al., 1990; Ko and Engel, 1993). The zinc finger domain of the GATA factors is conserved among the six members (*Gata1~6*) that constitute this multigene family. A series of clinical studies revealed a causal role of germline heterozygous *GATA2* mutations in Emberger syndrome, which manifests as primary lymphedema associated with a predisposition to acute myeloid leukemia (Ostergaard et al., 2011, Polat et al., 2018). Consistent with these clinical findings, lymphatic vasculature-specific conditional *Gata2* knockout embryos exhibited pronounced edema with striking defects in lymphatic vessel structure, underscoring the essential role of GATA2 in developmental lymphangiogenesis (Kazenwadel et al., 2015). Therefore, accumulating studies have established that GATA2 plays a key role in lymphatic development, while the roles of GATA2 in the adult lymphatic vasculature and recanalization after disruption of the lymphatic vessels have not been fully characterized.

GATA3 has been established as a master regulator of T cell development (Ho et al., 2009). Additionally, recent studies have revealed that GATA3 plays an essential role in the differentiation of lymphoid tissue inducer (LTi) cells that constitute a subset of innate lymphoid cells (ILCs) and support lymph node development (Yagi et al., 2014; Bovay et al., 2018). Indeed, it was reported that hematopoietic lineage-specific conditional deletion of *Gata3* led to the absence of lymph nodes primarily due to loss of LTi cells (Oosterwegel et al., 1992; Yagi et al., 2014; Bovay et al., 2018). While both GATA2 and GATA3 are involved in lymphatic development, whether they cooperatively or distinctively function in the regeneration of lymphatic structures remains elusive.

In the present study, we demonstrate that *Gata2* heterozygous deficient mice showed impaired recanalization after lymph node extirpation, while *Gata3* heterozygous deficient mice rarely showed such delays in lymphatic recanalization. Notably, the perturbed lymphatic recanalization in the *Gata2* heterozygous deficient mice was partially restored by crossing with the *Gata3* heterozygous deficient mice. We demonstrate for the first time that GATA2 participates in the recanalization of lymphatic vessels in adult mice and that GATA3 potentially suppresses lymphatic recanalization processes.

Materials and Methods

Mutant mice

Gata2 eGFP knock-in (*Gata2*$^{+/-}$) and *Gata3 LacZ* knock-in (*Gata3*$^{+/-}$) mice carry eGFP and *LacZ* reporter genes that are inserted into the translational initiation site of the *Gata2* and *Gata3* alleles, respectively (van Doorninck et al., 1999; Minegishi et al., 2003; Moriguchi et al., 2006). The primers used for genotyping are listed in Table 1. Experiments were performed using 6–8-week-old mice. All experiments were performed in accordance with Japanese laws and guidelines for the care of experimental animals according to the Animal Experiment Enforcement Rule of Tohoku Medical and Pharmaceutical University. The protocol was approved by the Committee on the Ethics of Animal Experiments of Tohoku Medical and Pharmaceutical University (Permit Number: 19017-cn). All surgeries were performed under a combination anesthetic (M/M/B: 0.3/4/5) prepared with 0.3 mg/kg medetomidine, 4.0 mg/kg midazolam, and 5.0 mg/kg butorphanol by intraperitoneal injection (Kawai et al., 2011), and all efforts were made to minimize suffering.

Evans Blue staining for detection of lymphatic vessels

To improve the visibility of the popliteal lymph node with minimal toxicity, 0.1% Evans Blue (EB) dye was injected into the mouse footpad subdermal space (Figures 1A and B). Since EB dye binds to albumin in the tissue and is sent to the lymph vessels, the lymph nodes turned blue by massaging the footpads. After footpad massage, the popliteal lymph node receiving the injected EB became bluish. The popliteal lymph node on both sides was excised, and then the skin incision was closed (Figures 1C – H).

Evaluation of lymphatic vessel recanalization

To increase the visibility of the connected lymphatic vessels around the popliteal space, 0.5% EB dye was used for the evaluation. To confirm recanalization after popliteal lymph node extirpation, we also observed collateral lymphatic vessels (Figure 4A). The iliac lymph node, which is located at the ascending side of the popliteal lymph node, was used as an indicator of successful recanalization of the popliteal lymphatic vessel. The ventrolateral subcutaneous lymph node served as an indicator of the collateral lymphatic flow that usually emerged when the recanalization was unsuccessful (Figures 4B – D).

Scoring the recanalization of popliteal lymphatic vessels

The progression of lymphatic recanalization was evaluated by scores ranging from 0 to 2 based on the two verification points: the existence of efferent lymphatics and the shape of the recanalization area (Figure 3A). The scoring was as follows: satisfactory (2): lymphatic vessels that were reconnected in a line shape and scored two points (Figure 3A-a); good (1): lymphatic vessels that were reconnected but with crumply reticulated lymphatic vessels and scored a single point (Figure 3A-b); and fail (0): lymphatic vessels that were not connected and scored no points (Figure 3A-c).

RT-PCR analysis

The mRNA expression of genes related to lymphangiogenesis was evaluated by quantitative RT-PCR (qRT-PCR). Total RNA was extracted from the granulation tissues around the popliteal lymph vessels using TRIzol™ reagent (Thermo Fisher Scientific, Tokyo, Japan). The extracted RNA was subjected to reverse transcription with a High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific). Next, 1.0 μg of total RNA was used to synthesize cDNA. The primers used for genotyping and qRT-PCR are listed in Table 1. cDNA was diluted twenty-fold before PCR amplification. qRT-PCR was performed using a QuantStudio 3 (Thermo Fisher Scientific). Negative controls were included in each reaction, and PCR products obtained with each primer pair were subjected to a melting curve or analysis by the ΔΔCt method. The data were analyzed with QuantStudio Design & analysis Software v1.2.

Histological analysis

The granulation tissues were fixed overnight in 4% PFA (paraformaldehyde) at 4°C and then processed for immunostaining as paraffin-embedded sections (4 μm). For immunofluorescence analysis, co-localization of podoplanin (PDPN) and Prox1 was performed using rat anti-mouse PDPN antibody (MBL D321-3) and rabbit anti-Prox1 antibody (AngioBio 11-002P) as primary antibodies and Chicken anti-Rat IgG Alexa Fluor 488 (Thermo Fisher Scientific A21470) and Goat anti-Rabbit IgG Alexa Fluor Plus 555 (Themo Fisher Scientific A32732) as secondary antibodies for signal detection. Fluorescence of GFP protein was diminished during the paraffin embedding process. Fluorescence was visualized using a Zeiss AxioImager M2 upright microscope. Separate images were taken and merged using ZEN 2.1 software.

Statistical analysis

The means, standard deviations were determined. The data were subjected to one-way analysis of variance using Microsoft Excel software (Microsoft Co., Redmond, WA, USA) at the 0.05 significance level. Analyses were performed using Student's *t* test and the chi-square test as appropriate.

Results

Lymphatic vessels were reconnected 3 weeks after popliteal lymph node extirpation in mice.

The recanalization process of lymph vessels after popliteal lymph node extirpation in rabbits was described previously (Ikomi et al., 2006). In mouse experiments, it was demonstrated that lymphatic reconnection emerged from a mesh-like structure after cutting the collecting lymph vessels, and subsequently the primitive lymphatic connections gave rise to a single main trunk (Ikomi et al., 2008). Exploiting these ideas and procedures, we established a new model of lymphatic recanalization by excising the popliteal lymph nodes in mice (Figure 1). Employing this model, we examined the time course of lymphatic recanalization after popliteal lymph node extirpation in wild-type mice. We found that the connection of the lymphatic vessels was clearly detected by EB staining at 3 weeks after extirpation (Figure 2A). This result indicates that recanalization was almost completed approximately 3 weeks after extirpation of the popliteal lymph node.

Recanalization of the lymphatic vessels was impaired in Gata2 heterozygous deficient mice.

Considering the critical role of GATA2 in developmental lymphangiogenesis, we hypothesized that GATA2 plays a role in adult lymphatic recanalization. To address this issue, we examined recanalization after popliteal lymph node extirpation in adult *Gata2* heterozygous deficient (*Gata2*$^{+/-}$) mice (6 – 8 weeks old). Notably, the *Gata2*$^{+/-}$ mice showed obvious retardation in recanalization in comparison to the wild-type control mice, as reconnection was detected only faintly at 3 weeks after the lymph node extirpation in the *Gata2*$^{+/-}$ mice (Figure 2B). Subsequently, the lymphatic vessels were reconnected to a level comparable to the control mice at 4 weeks after extirpation (Figure 2E and F). These results clearly indicate that heterozygous *Gata2* deficiency diminishes lymphatic regeneration and thereby delays recanalization of popliteal lymphatics in adult mice.

Recanalization of the lymphatic vessels was normal in Gata3 heterozygous deficient mice.

Given the essential role of GATA3 in the differentiation of LTi cells and lymph node development, we next addressed whether GATA3 plays a role in lymphatic regeneration. To this end, we examined lymphatic recanalization after popliteal lymph node extirpation in *Gata3* heterozygous deficient (*Gata3*$^{+/-}$) mice. We found that the *Gata3*$^{+/-}$ mice showed almost normal recanalization processes. In the *Gata3*$^{+/-}$ mice, the connected lymphatics appeared as a single blue-stained line at 3 weeks after extirpation (Figure 2C). These results indicate that recanalization of the lymphatic vessels was hardly affected in the *Gata3*$^{+/-}$ mice.

Gata3 heterozygous deficiency partially restored the impaired recanalization of lymphatic vessels in Gata2 heterozygous deficient mice.

To determine whether GATA2 and GATA3 cooperatively function in the lymphatic recanalization process, we next subjected compound heterozygous mutant (*Gata2*$^{+/-}$::*Gata3*$^{+/-}$) mice to the same series of analyses. *Gata2*$^{+/-}$::*Gata3*$^{+/-}$ mice showed variable results in the recanalization of the lymphatic vessels; some were reconnected clearly (Figure 2D), while others were occasionally not reconnected. We therefore categorized the recanalization status into three groups and scored them as 0 (fail), 1 (good) or 2 (satisfactory) according to the designated criteria (Figures 3A-a – c, see materials and methods). The recanalization of the control and *Gata3*$^{+/-}$ mice 3 weeks after popliteal lymph node extirpation scored 1.90 ± 0.30 and 1.83 ± 0.39 on average, respectively, while *Gata2*$^{+/-}$ mice scored 0.63 ± 0.57, consistent with the obvious retardation in lymphatic recanalization (Figure 3B-a, $p \leq 0.001$). Notably, the average recanalization score in the *Gata2*$^{+/-}$::*Gata3*$^{+/-}$ mice was improved to 1.20 ± 0.92. In the breakdown of the recanalization score, the vast majority of the *Gata2*$^{+/-}$ mice were categorized as “fail (0)” (40.7%) or “good (1)” (55.6%), whereas only 3.7% of the *Gata2*$^{+/-}$ mice achieved a “satisfactory (2) score” (Figure 3B-b). Although the *Gata2*$^{+/-}$::*Gata3*$^{+/-}$ mice showed variable results in the recanalization of the lymphatic vessels, 50% of them were “satisfactory (2)”. These results indicate that lymphatic recanalization failure in the *Gata2*$^{+/-}$ mice was partially rescued by crossing with *Gata3*$^{+/-}$ mice. The recanalization of the *Gata2*$^{+/-}$ mice 4 weeks after the popliteal lymph node extirpation scored 1.75 ± 0.46 on average and 75% of the *Gata2*$^{+/-}$ mice reached “satisfactory (2)” (Figure 3B-a and -b). These results indicate that the lymphatic vessels were recanalized to almost normal level at 4 weeks in the *Gata2*$^{+/-}$ mice.

Examination of the iliac and subcutaneous lymph nodes by EB staining confirmed the recanalization status of popliteal lymphatic vessels.

The iliac lymph node is located downstream of the popliteal lymphatic vessels. Therefore, successful recanalization of the popliteal lymph vessels was assumed to result in dense blue staining in the iliac lymph node (Figures 4A and B). In the next series of analyses, the reconnection of lymphatic vessels was further evaluated by inspecting EB staining in the iliac lymph node as well. The *Gata3*$^{+/-}$ mice exhibited normal reconnection of the popliteal lymphatic vessels, so that the iliac lymph node was stained blue, as in the control mice (Figures 4E and G). In contrast, the *Gata2*$^{+/-}$ mice failed recanalization of the popliteal lymphatic vessels; therefore, the iliac lymph node was not stained with EB dye (Figure 4F). When the popliteal lymphatic vessels were successfully reconnected in *Gata2*$^{+/-}$::*Gata3*$^{+/-}$ mice, their iliac lymph nodes were stained with EB dye (Figure 4H).

Failure in popliteal recanalization often induced outgrowth of the collateral lymphatic tract through which the lymph drained into the abdominal subcutaneous lymph node instead of the iliac LNs (Figures 4A, C and D). Given this, we next examined EB staining in the subcutaneous lymph node. We found that the *Gata2*$^{+/-}$ mice that failed recanalization of popliteal lymph vessels exhibited robust growth of collateral lymphatic tracts in the hind limb (arrow in Figure 2B and F). As anticipated, the *Gata2*$^{+/-}$ mice showed brightly EB-stained subcutaneous lymph node, indicating that unsuccessful popliteal recanalization evoked collateral lymph flow draining into the subcutaneous lymph node (Figures 4I and J). In contrast, the control, *Gata3*$^{+/-}$ and *Gata2*$^{+/-}$::*Gata3*$^{+/-}$ mice with successful recanalization showed unstained subcutaneous lymph node (Figures 4I, K and L). These results suggested that the lymphatic recanalization failure altered lymphatic circuitry in the *Gata2*$^{+/-}$ mice.

Gene expression profile of lymphatic endothelial cells during the recanalization process

Prox1, a homeobox transcription factor, which is expressed in lymphatic endothelial cells (LECs) and their progenitors plays an essential role for LEC development (Srinivasan et al., 2007). *Flt4*/*Vegfr3* (Fms Related Receptor Tyrosine Kinase 4/Vascular endothelial growth factor receptor 3) and *Pdpn* (podoplanin) are specifically expressed in LECs and serve as genetic markers for LECs (Kaipainen et al., 1995, Uhrin et al., 2010). Given these, we examined the expression pattern of these three LEC-marker genes in the lymph node-excised site at 3 weeks after lymph node extirpation when the reconnection of popliteal lymphatic vessels was almost finished. We

found that there were no statistically significant differences in the *Pdpn, Prox1* and *Flt4* mRNA expression levels among the four genotypes of mice, suggesting that the populations of LECs and their progenitor cells were not significantly diminished in either *Gata2*$^{+/-}$, *Gata3*$^{+/-}$ or *Gata2*$^{+/-}$::*Gata3*$^{+/-}$ mice (Figures 5A – C). These results suggest that the impaired recanalization of the *Gata2*$^{+/-}$ mice was not due to potential differentiation defect of the LECs.

Lymphatic endothelial cells in the granulation tissue failed to form vessel structure in the Gata2 heterozygous deficient mice.

Finally, we evaluated regenerating lymphatic vessels in the granulation tissue by anti-PDPN and anti-Prox1 immunohistochemistry at 3 weeks after the surgery. In the control mice, PDPN and Prox1-double positive LECs forming the small lymphatic vessels were detected (inlet in Figure 5D). In contrast, *Gata2*$^{+/-}$ mice showed only scattered LECs without tubular structure which were stained for PDPN- and Prox1-immunoreactivity (Figure 5E). Some of *Gata2*$^{+/-}$ mice showed abundant number of scattered PDPN-positive LECs (Figure 5F white arrowheads), suggesting that lymphatic vessel formation was diminished in *Gata2*$^{+/-}$ mice, albeit differentiated identity of LECs was maintained.

Discussion

In the present study, we established a novel evaluation method for lymphatic recanalization in mice and demonstrated that *Gata2* heterozygous deficiency caused a marked delay in the recanalization of lymph vessels. Furthermore, simultaneous *Gata3* heterozygous deficiency partially improved the recanalization status of *Gata2*$^{+/-}$ mice.

In this study, we improved the lymphatic recanalization method that was previously used in rabbits and mice (Ikomi et al., 2006, 2008) and established a novel quantitative evaluation system by scoring recanalization status upon popliteal lymph node extirpation in mice. This evaluation system enabled us to demonstrate the statistically significant defects in lymphatic recanalization in *Gata2*$^{+/-}$ mice. This simple and accurate evaluation method for popliteal lymphatic recanalization would be useful for screening of therapeutic candidates such as VEGF-C (Ikomi et al., 2008). Future analyses with this method will verify potential efficacy of VEGF-C or other drugs for the lymphatic recanalization in the *Gata2*$^{+/-}$ mice.

It has been known that human *GATA2* heterozygous deficiency leads to hereditary diseases, including Emberger syndrome (Ostergaard et al., 2011; Polat et al., 2018; Zawawi et al., 2018; Mishra et al., 2020). Recently, a series of studies regarding the development of lymphatic vessels and valves have reported that GATA2 directs the migration of LECs from veins to lymphatic vessels during embryogenesis (Yang et al., 2014; Kazenwadel et al., 2015; Frye et al., 2018; Mahamud et al., 2019). In the present study, we found that *Gata2*$^{+/-}$ mice showed proper development of lymph nodes and almost normal levels of *Prox1* expression, a marker for LECs during the recanalization process. These results suggest that a haploinsufficiency of *Gata2* occurred in the lymphatic regeneration process but is sufficient for normal differentiation and proliferation of LECs and subsequent proper lymphangiogenesis in adult mice. Moreover, the significant delays in lymphatic recanalization in *Gata2*$^{+/-}$ mice indicate that haploinsufficiency of *Gata2* diminishes lymphatic recanalization even if developmental lymphangiogenesis is maintained. Therefore, lymphedema manifested in Emberger syndrome was expected to be exacerbated by physical damage or lymph node biopsy due to the reduced reconnection ability of the lymphatic vessels.

GATA3 is essential for the expression of a series of inflammatory cytokines in Th2 cells, including IL-4, IL-5, and IL-13 (Barnes, 2008). Indeed, local administration of GATA3 antisense oligonucleotides ameliorates pulmonary inflammation associated with a murine model of asthma (Finotto et al., 2001). Considering the proinflammatory activity of GATA3, the inflammatory

cytokine level might be subtly decreased in the *Gata3*$^{+/-}$ background, and therefore, the inflammatory response around the damaged lymphatic vessels might be diminished. Such an anti-inflammatory local microenvironment might ameliorate recanalization failure in *Gata2*$^{+/-}$::*Gata3*$^{+/-}$ mice.

Recently, it has been reported that the hardness (or softness) of connective tissue, which depends on the amount of surrounding collagen fibers, affects the migration of LECs (Yang et al., 2014; Frye et al., 2018). Moreover, a transgenic mouse line forcibly expressing GATA3 in the T-cell lineage is prone to developing chemically induced fibrosis with enhanced collagen III expression, suggesting that GATA3 regulates collagen levels (Yoh et al., 2015). Given these findings, addressing whether GATA2 and GATA3 regulate the plasticity of surrounding connective tissue and the subsequent mobility of LECs by regulating collagen levels is of keen interest.

Diminished connection of lymph vessels after lymphadenectomy can cause lymphedema (Campisi, 1999). Patients who suffer from lymphedema have a poor prognosis and require lifelong treatments, such as lymph drainage. Although metastatic lymph nodes have to be radically resected, surgeons are asked to minimize the resection area to reduce the probability of postoperative lymphedema and preserve the quality of life of cancer patients (Ahmed et al., 2016). The development of a new system facilitating recanalization of lymphatic vessels would lead to new therapeutic avenues for lymphedema. Analyses following this research will provide a mechanistic basis for lymphedema treatments.

Acknowledgments

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Figure legends

Figure 1. Procedure for popliteal lymph node extirpation.

(A) Diagram of the subdermal injection of Evans Blue (EB) dye. The popliteal lymph node (LN) turns blue after massage of the injected footpad when lymph flows normally. (B) EB dye was injected into the footpad (white arrow in panel B). To perform popliteal lymph node extirpation, the popliteal skin was incised (C and D; area of dashed rectangle in panel C) and denuded of subcutaneous tissues (E). The popliteal lymph node was excised out (F; white dotted circle, bottom-left bracket; excised lymph node). G: Confirmation of EB dye leakage in the excised area after lymph node extirpation. H: The skin was closed after the surgery. LN: lymph node. Scale bars indicate 5 and 1 mm in panels (B and C) and (D – H), respectively.

Figure 2. Recanalization after popliteal lymph node extirpation in the control, *Gata2*$^{+/-}$, *Gata3*$^{+/-}$ and *Gata2*$^{+/-}$::*Gata3*$^{+/-}$ mice.

Recanalization of the popliteal lymphatic vessels in the control (A), *Gata2*$^{+/-}$ (B), *Gata3*$^{+/-}$ (C) and *Gata2*$^{+/-}$::*Gata3*$^{+/-}$ (D) mice at 3 weeks after lymph node extirpation. Recanalization at 4 weeks after lymph node extirpation in the control (E) and *Gata2*$^{+/-}$ (F) mice. Blue lines or spots indicate staining with subdermal injection of EB dye into the footpad. White solid arrowheads indicate the afferent lymphatic vessels. Open arrowheads indicate the efferent lymphatic vessels. Dotted circles indicate the recanalization area. Black arrow indicates collateral lymphatic tract. Scale bars indicate 1 mm.

Figure 3. Evaluation and scoring of the recanalization after popliteal lymph node extirpation in the control, *Gata2*$^{+/-}$, *Gata3*$^{+/-}$ and *Gata2*$^{+/-}$::*Gata3*$^{+/-}$ mice.

(A) Evaluation and scoring of the recanalized lymph vessels after popliteal lymph node extirpation. (a) Satisfactory (2 points): lymphatic vessels were reconnected in a line shape; (b) good (1 point): lymphatic vessels were reconnected but with crumply reticulated lymphatic vessels; and (c) fail (0 points): lymphatic vessels were not connected. White solid arrowheads indicate the afferent lymphatic vessels. Open arrowheads indicate the efferent lymphatic vessels. Dotted circles indicate the recanalization area. Scale bars indicate 1 mm.

(B) The recanalization score of the lymphatic vessels 3 weeks after popliteal lymph node extirpation in the four genotypes of mice and 4 weeks in *Gata2*$^{+/-}$ mice. (a) Average recanalization score in the four genotypes of mice. The data are presented as the means ± S.D. The recanalization

score was in accordance with (A). The numbers in brackets were indicated the number of trials in each genotype. (b): The 100% stacked bar graph of the recanalization score in the four genotypes of mice. Point 0 (black solid column), point 1 (gray column) and point 2 (open column). The numbers indicate the percentage of each score.

Figure 4. Evaluation of popliteal lymph vessel recanalization with iliac LN and S.C. LN staining in control, *Gata2*$^{+/-}$, *Gata3*$^{+/-}$ and *Gata2*$^{+/-}$::*Gata3*$^{+/-}$ mice.

(A) Diagram of the visualization of successful (left) and failed (right) recanalization after popliteal lymph node (LN) extirpation using EB dye injection into the footpads. (B – D): Representative pictures of the iliac LN (B) and subcutaneous (S. C.) LNs (C and D) 3 weeks after popliteal LN extirpation. (B) The iliac LNs were stained with EB when the lymph vessel was recanalized (left) but not stained when recanalization failed (right). (C) Clear colored S. C. LNs were observed when successful recanalization was achieved. (D) Blue-stained S. C. LNs were observed in the failed recanalization. (E – H) Iliac lymph nodes were stained blue when the corresponded side of lymphatic recanalization proceeded properly. Iliac lymph nodes remained unstained in cases of unsuccessful recanalization (F). (I – L) Abdominal S. C. LNs were stained once the collateral lymphatic tract was formed (J). Control (E and I), *Gata2*$^{+/-}$ (F and J), *Gata3*$^{+/-}$ (G and K) and *Gata2*$^{+/-}$::*Gata3*$^{+/-}$ (H and L). LN: lymph node. White dotted circles indicate transparent LNs. Scale bars indicate 1 mm.

Figure 5. Expression patterns of lymphatic endothelial markers after popliteal lymph node extirpation in control, *Gata2*$^{+/-}$, *Gata3*$^{+/-}$ and *Gata2*$^{+/-}$::*Gata3*$^{+/-}$ mice.

The mRNA expression of *Pdpn* (A), *Prox1* (B) and *Flt4* (C) in the granulation tissues of the lymph node-excised region was evaluated 3 weeks after popliteal lymph node extirpation in each mouse genotype. There were no significant differences in mRNA expression level of the four genes among the mouse genotypes by one way ANOVA analysis (A – C). The error bars indicate S.D. $n \geq 8$.

(D – F): Representative images of regenerated lymphatic vessels in the granulation tissues. The PDPN (Green) and Prox1 (Red) were co-expressed in the LECs. PDPN- and Prox1- double positive LECs formed tube structure in the control mice (lower right rectangle in D, enlarged view). (E and F) *Gata2*$^{+/-}$ mice showed only scattered LECs without tube structure that were

stained for either PDPN or Prox1. White arrowheads indicate scattered LECs. ad: adipose tissue, mu: muscles, v: vein. Scale bars are 100 μm.

Table 1. Sequences of primers used for genotyping

Gene	Sense primer	Antisense primer	Size (bp)	Assay
$Gata2^{GFP}$	CTGAAGTTCATCTGCACCACC	GAAGTTGTACTCCAGCTTGTGC	306	genotyping
$Gata3^{LacZ}$	TTCGCCAGCTGCGTAATAGCGAAGAGGC	TAGGTCACGTTGGTGTAGATGGGCGCATCG	200	genotyping
Pdpn	GGGATGAAACGCAGACAACAG	TTTAGGGCGAGAACCTTCCA	150	qPCR
Prox1	ATTCAGGAAGCGCAATGCAG	AACCACTTGATGAGCTGCGA	160	qPCR
Flt4	CTGGCAAATGGTTACTCCATGA	ACAACCCGTGTGTCTTCACTG	121	qPCR
β-actin	TATAAAACCCGGCGGCGCA	ATCCATGGCGAACTGGTGG	115	qPCR

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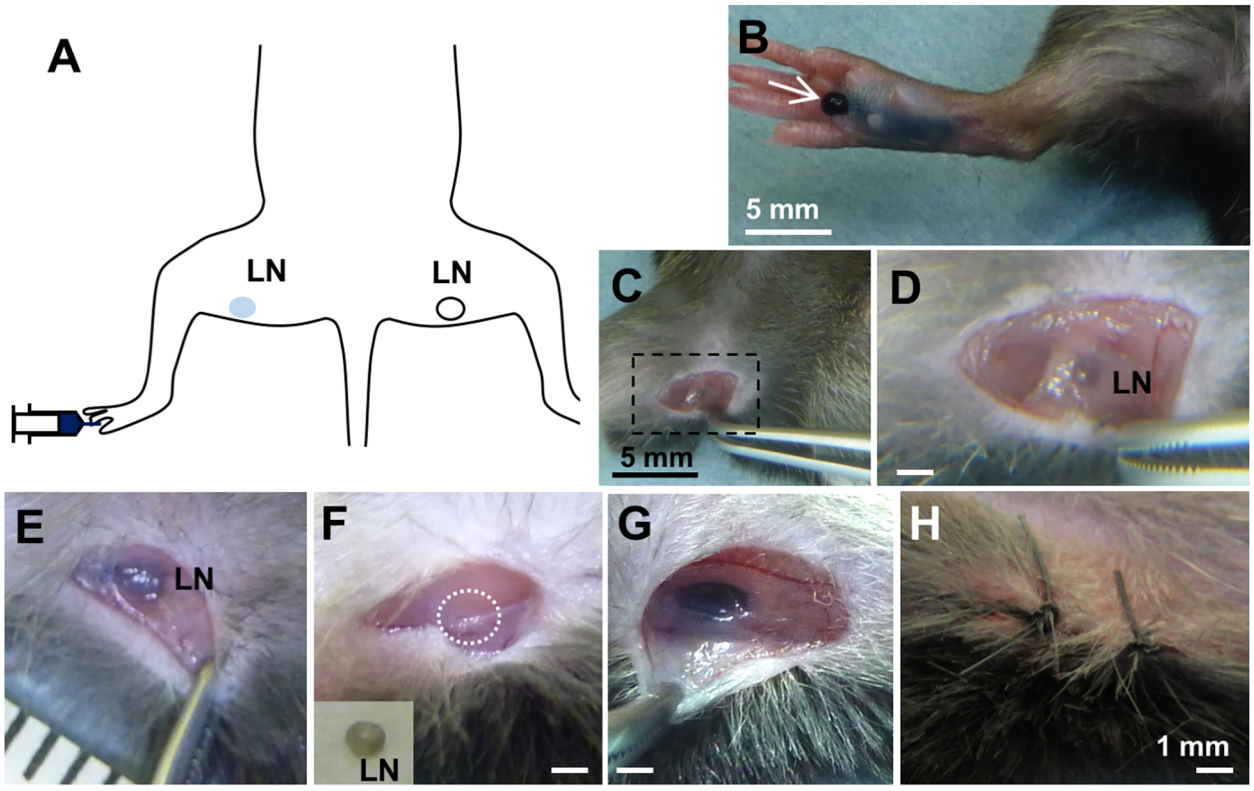

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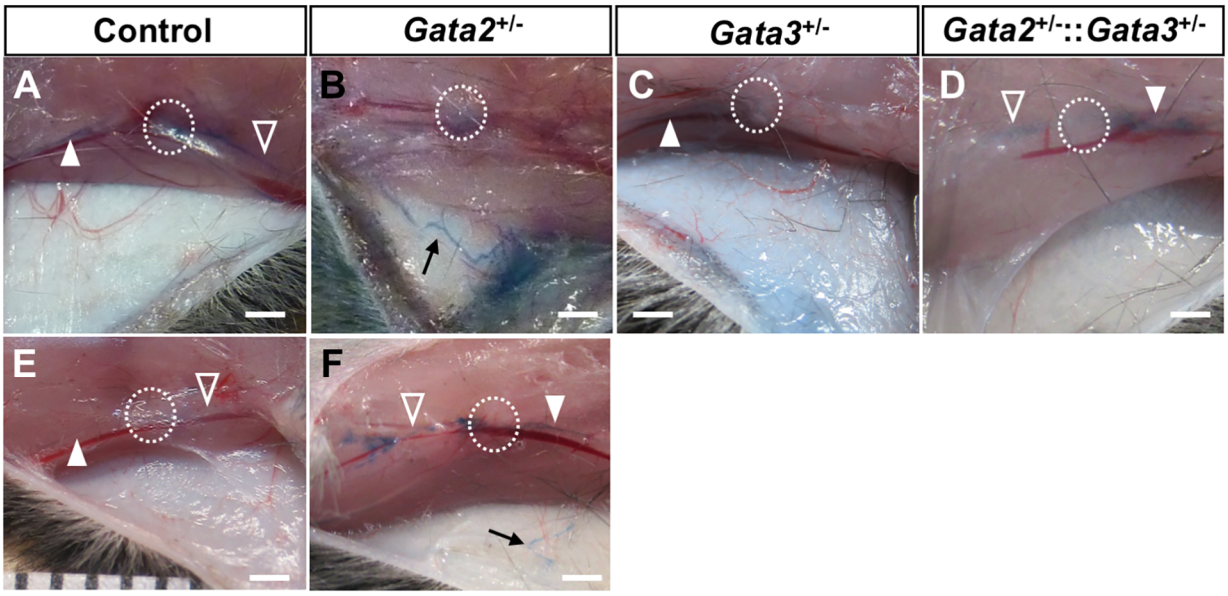

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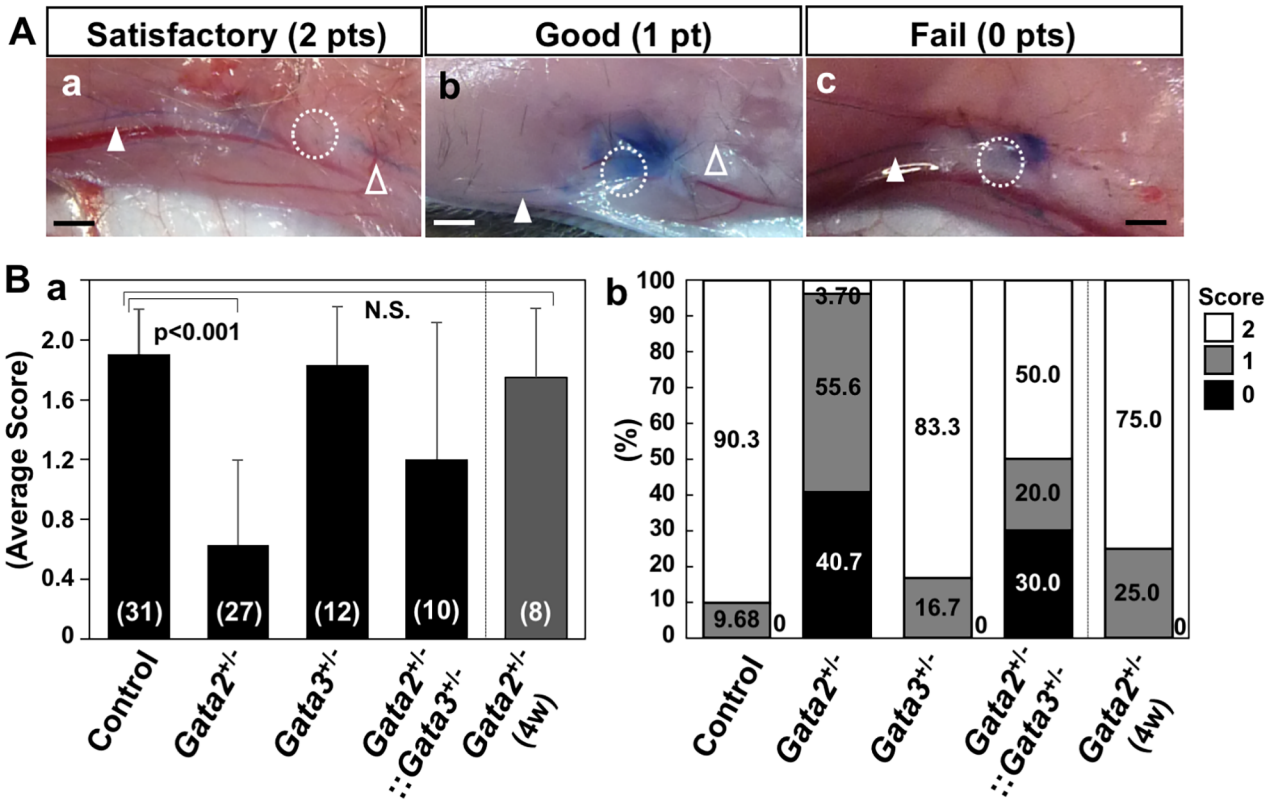

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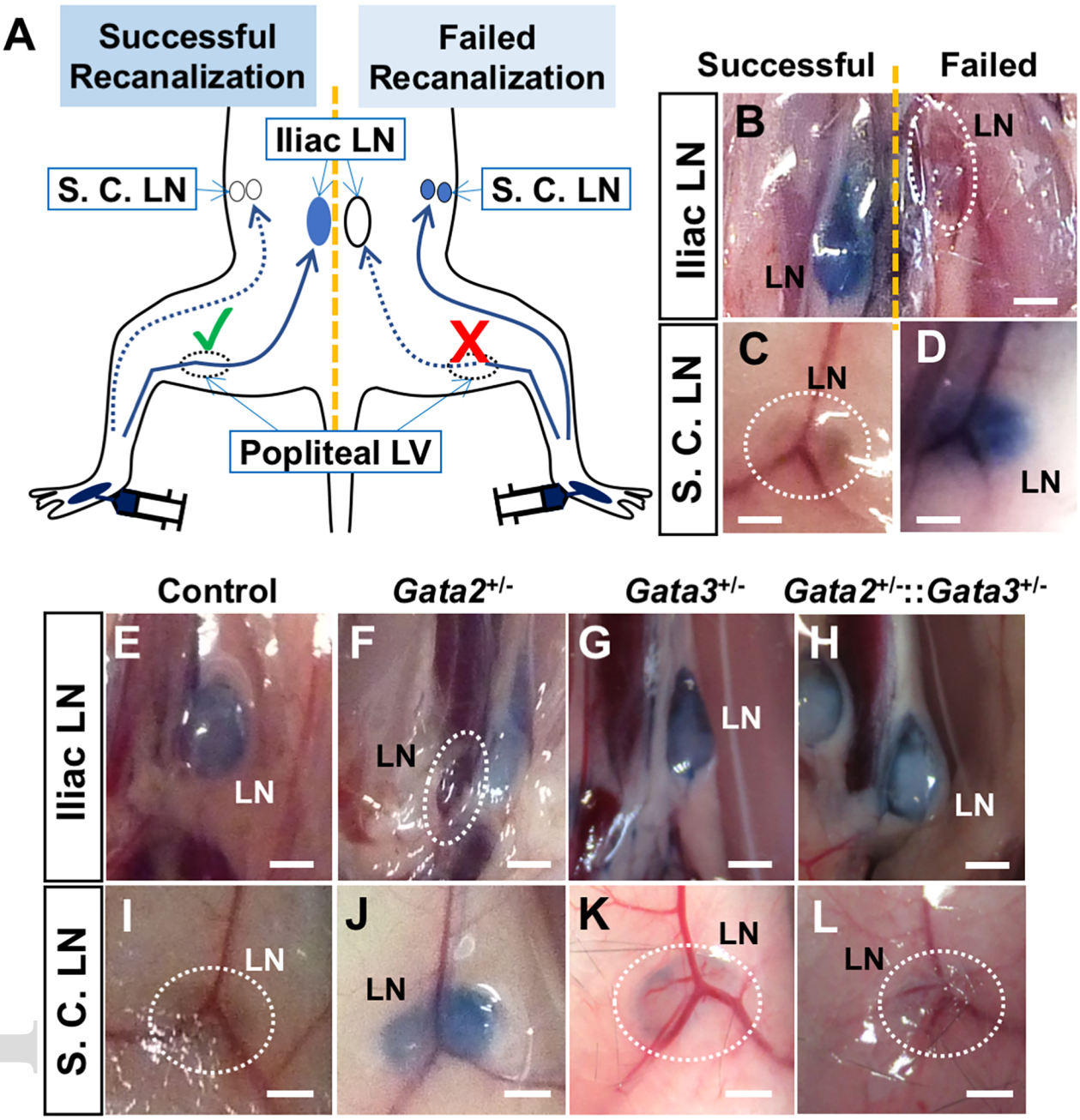

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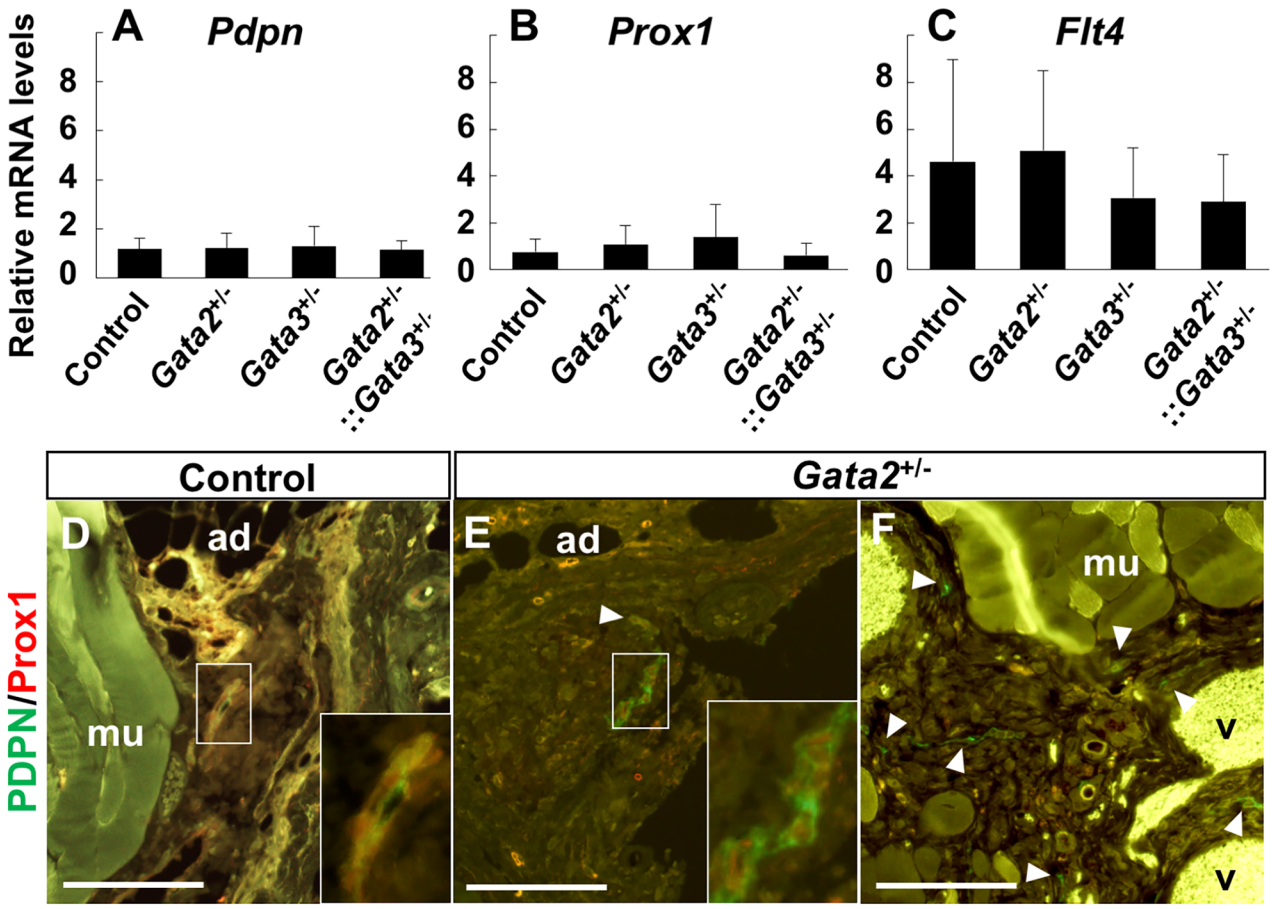

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