Aging in the peripheral nervous system (PNS) leads to its dysfunction and lowers regenerative capacity after injury. Based on the pro-regenerative effect of hepatocyte growth factor (HGF) in injured peripheral nerves, we investigated whether this growth factor is involved in the age-related degeneration of the PNS. We observed that the capacity for nerve regeneration was significantly reduced under aging conditions as indicated by the decreased level of SCG10-positive axons. Functional recovery was also impaired. We further tested whether the HGF/c-Met pathway was involved, and the activation of the c-Met receptor upon nerve injury was significantly reduced, whereas the production of HGF protein was still comparable to that in young mice. Moreover, the phosphorylation and expression of c-Jun, a key regeneration-associated gene, was also lowered in aged animals. In addition, exogenous administration of the HGF expressing plasmid DNA significantly ameliorated the pain-like behavior in young animals, however, such analgesic activity was impaired in aged mice. These data suggested that the HGF/c-Met pathway might be involved in the age-related impairment of regenerative capacity in the PNS.
plasmid DNA was highly hindered in aged animals. These data suggest that the c-Met receptor is critically involved in age-related impairment of the peripheral nerve regeneration process.

2. Materials and Methods

2.1. Animals

All experimental protocols for the animal studies were performed based on the approval of the Seoul National University (SNU) Institutional Animal Care and Use Committee (IACUC). Nine-week-old, one-year-old, and two-year-old male C57BL/6 mice were purchased from Orient Bio Inc. (Gyeonggi-do, Korea). They were housed at 22 °C with a 12-hour light-dark cycle and were given ad libitum access to food and water.

Sciatic nerve crush injury was performed as described previously [3, 4]. The animals were anesthetized with isoflurane, and the right thigh was shaved and disinfected by applying povidone and 70% EtOH sequentially. The sciatic nerve was exposed by making an incision (~5 mm), and the exposed nerve was crushed for 15 s using fine hemostatic forceps (FST, British Columbia, Canada) unilaterally. After the nerve crush, the transparence of the sciatic nerve was observed to confirm the sciatic nerve injury. Incisions were then closed by giving 2–3 sutures, and the recovery of the anesthetized animals was monitored. For intramuscular administration of plasmid DNA, 200 μg of pCK or pCK-HGF-X7 were injected to the thigh muscle near the sciatic nerve.

To analyze the sciatic nerve and DRG at the histological and molecular level, these tissues were prepared as previously described [3, 4, 8]. After euthanizing, a full laminectomy process was performed to expose the spinal cord, DRG, and sciatic nerve. The L4-L6 DRGs connected to the sciatic nerve was isolated from the proximal to the distal region of the injury site with a total length of 4 mm. The collected tissues were then homogenized using polypropylene pestles for further process.

2.2. Behavior test

Seven days after the nerve crush injury, nerve pinch tests were performed as previously described [4]. The mice were anesthetized with a relatively low level of isoflurane. A small incision was made, followed by the exposure of the injured sciatic nerve. The sciatic nerve was then pinched starting from the distal region of the sciatic nerve to the proximal direction. When mice show a withdrawal reflex after the pinch stimulation, the distance between the pinch site and the injury site was measured.

The development of mechanical allodynia was assessed at relevant time points using the von Frey filament tests. After nerve crush injury, plasmid DNAs were injected into the bicep femoris muscle with 200 μg/ head. To perform the von Frey filament tests, animals were placed in the cylinder individually for at least 4 h prior to the test. The 0.16g filament was chosen to stimulate the hind paw because this thickness showed a minimal withdrawal behavior in normal mice but highly exhibited the pain-like behavior in the nerve injured group. More than ten independent stimulations were applied, and the withdrawal behaviors were counted. The frequency of withdrawal activity was then calculated.

2.3. Immunohistochemistry (IHC)

The fixation of sciatic was performed using 10% formalin. After the overnight incubation, tissues were washed with 0.1 M PBS, followed by sequential immerse in 15% sucrose at 4 °C (8 h) and 30% sucrose at 4 °C (overnight). Samples were then cryo-preserved in OCT compound (Sakura Tissue Tek, CA, United States). Slides were generated by sectioning the OCT-embedded tissues (12 μm). To perform immunostaining, slides were incubated in the blocking solution (2% BSA with 0.1% Triton X-100 in 0.1 M PBS) for an hour, followed by the treatment with primary antibodies specific to SGC10 (or STMN2, Novus, CO, United States) and βIII tubulin (BioLegend, CA, United States) at 4 °C (overnight). After three washes with 0.1 M PBS, secondary antibodies specific to anti-rabbit IgG (Invitrogen, MA, United States) or anti-mouse IgG (Invitrogen, MA, United States) were treated at room temperature (2 h). After the additional DAPI staining (Vectorshield, PA, United States), immunofluorescence was measured using an IN Cell Analyzer 2000 (GE Healthcare, MA, United States).

2.4. ELISA

ELISAs were performed according to the manufacturer's instruction. Briefly, one and three days after injury, total proteins were extracted from the injured sciatic nerve using RIPA Buffer (Cell Signaling Technology, MA, United States) in the presence of Protease/Phosphatase Inhibitor Cocktail (Cell Signaling Technology, MA, United States). The level of each protein was then analyzed using Mouse/Rat HGF Quantikine ELISA Kit (R&D, MN, United States) or Mouse HGFR/c-MET DuoSet ELISA Kit (R&D, MN, United States).

2.5. Western blot

Total protein extracted from tissues was subjected to electrophoresis using a 4–12% Bis-Tris gradient gel (Invitrogen, MA, United States) for 50 min. After transferring the gel to PVDF membranes (GE Healthcare, MA, United States), membranes were then blocked with Blocker Casein in PBS (Thermo Fisher, MA, United States) at room temperature (45 min), followed by the incubation with primary antibodies specific to c-Met (Sigma, MO, United States), phosphorylated c-Met (Cell Signaling Technology, MA, United States), c-Jun (Cell Signaling Technology, MA, United States), phosphorylated c-Jun (Cell Signaling Technology, MA, United States), and GAPDH (Cell Signaling Technology, MA, United States) 4 °C (overnight), respectively. Membranes were washed three times TBST (Invitrogen, MA, United States) and then subjected to incubation with a secondary antibody specific to anti-rabbit IgG (Cell Signaling Technology, MA, United States) at room temperature (1 h). Immuno-stained membranes were exposed to Super Signal West Femto Maximum Sensitivity Substrate (Thermo Fisher, MA, United States) or Super Signal West Pico Chemiluminescent Substrate (Thermo Fisher, MA, United States), and signal intensities were visualized using ImageQuant Las 4000 (GE Healthcare, MA, United States), followed by the quantification using ImageJ software (NIH).

2.6. Statistical analysis

For statistical analysis, GraphPad Prism was used. Data are shown as the mean ± standard deviation (SD) or the mean ± standard error of the mean (SEM). Statistical significance was evaluated using one-way ANOVA followed by Tukey’s multiple comparison testing or two-way ANOVA followed by Bonferroni’s multiple comparison testing (*p < 0.0001).
growth of SCG10-positive axons was significantly hindered, suggesting impairment of the nerve regeneration process (Figure 1A).

Next, we investigated whether aging affected the functional recovery of injured nerves using the nerve pinch test [4]. Sciatic nerve crush was induced, and the distance between the injury site and distal region where animals showed a withdrawal response was measured to assess the functionally regenerated axons. Consistent with the histological analysis, the length of the regenerated nerve was significantly downregulated in the injured sciatic nerve in aged mice (two-year-old) compared to that in young animals (nine-week-old) (Figure 1B). These data suggested that the regenerative capacity of peripheral nerves is significantly hindered by aging.

3.2. Activation of c-Met receptor is suppressed in aged animals

The c-Met signaling pathway plays a critical role in the regeneration of injured nerves. Previous studies suggested that axon outgrowth is hindered by the inhibition of the c-Met receptor [4]. Based on these results, we hypothesized that c-Met might not be properly activated in the injured nerves of aged animals. Sciatic nerves from young and aged mice were prepared to analyze the level of phosphorylated c-Met. As shown in Figure 2A, nerve crush injury highly induced the phosphorylation of the c-Met receptor, however, this increase was not observed in injured nerves from aged animals. The expression of c-Met was also impaired by aging. Compared to young mice (nine-week-old), the induction of the c-Met protein by nerve injury was significantly inhibited in the aged group (one-year-old) (Figure 2A). The ELISA data also demonstrated that the protein level of c-Met in the sciatric nerves was not induced in aged animals, consistent with the Western blot analysis (Figure 2B). The quantification of bands from Western blot analysis showed that the ratio between phosphorylated c-Met and c-Met was highly induced in the young injured sciatic nerve, however, this induction was significantly downregulated in the aged sciatic nerve (Figure 2A). These data suggested that the induction of both expression and phosphorylation of c-Met receptor are impaired in aged condition.

To further investigate whether the level of HGF, a ligand for the c-Met receptor, was also affected by aging, we analyzed the protein level of HGF in the injured sciatic nerve. It has been reported that HGF is upregulated upon nerve injury [3, 4]. Consistent with this previous report, the HGF protein significantly increased in the injured nerves of young animals, and sciatic nerve from aged mice still showed high levels of HGF protein (Figure 2C), suggesting that aging minimally affects the production of HGF protein.

3.3. Nerve injury induces the expression of c-Jun in young, but not in aged animals

To further investigate the effect of aging on peripheral nerve injury at the molecular level, we tested whether the induction of nerve injury-related proteins was affected in aged animals. Upon nerve injury, the activation of the HGF/c-Met pathway mediates the induction of c-Jun protein, a key regeneration-associated transcription factor [4]. In sciatic nerves from young mice, the level of phosphorylated c-Jun was increased...
Injured nerves and total protein was also induced. In aged animals, however, nerve injury-mediated induction of both c-Jun phosphorylation and expression were significantly hindered (Figure 3A). We further analyzed the level of c-Jun in the DRG. Consistent with the data from the sciatic nerve, nerve injury highly induced the level of both phosphorylation and expression of c-Jun, which was impaired under aging conditions (Figure 3B). Other signaling pathways such as Akt and Erk remained unchanged. In the case of Stat3, the activity of this transcription factor was induced upon nerve injury and further upregulated in aged conditions (Figure 3B). These data suggested that the activation of the HGF/c-Met/c-Jun pathways is significantly hindered in aged animals, leading to the inefficient regeneration of injured peripheral nerves.

### 3.4. HGF expressing plasmid DNA showed minimal analgesic efficacy in aged animals

To further investigate whether the c-Met signaling pathway was functionally impaired in the aged mice, we tested the efficacy of HGF-
expressing plasmid DNA (pCK-HGF-X7) as this plasmid DNA has been reported to show analgesic effects in a murine peripheral neuropathic pain model through c-Met receptor [8, 10]. We induced sciatic nerve injury by crushing the sciatic nerve in both young and aged animals. After nerve injury, pCK-HGF-X7 was administered through intramuscular (i.m.) injection into the bicep muscle. As shown in Figure 4A, the frequency of withdrawal behavior in the nerve-injured young animals was significantly ameliorated in pCK-HGF-X7 i.m. injected mice, consistent with the analgesic effects reported in the previous studies [8, 10]. When the HGF-expressing plasmid DNA was injected into 1-year-old aged animals, the level of frequency was comparable between the group administered with pCK (control vector) and pCK-HGF-X7, indicating that the HGF protein expressed from pCK-HGF-X7 had a minimal analgesic effect in aged condition (Figure 4B). Taken together, these data suggested that the biological activities of both endogenous and exogenous HGF protein were significantly hindered in aged animals due to the reduced expression and activation level of c-Met protein.

4. Discussion

In this study, we investigated the possible involvement of the HGF/c-Met pathway in the age-related impairment of peripheral nerve regeneration. Consistent with previous results [6], the capacity of axon outgrowth and functional recovery of peripheral nerves were significantly suppressed in aged animals. Moreover, the activation of the HGF/c-Met/c-Jun pathway involved in peripheral regeneration [4] was also impaired. Finally, the HGF-expressing plasmid DNA showed minimal analgesic effect in aged animals. These data suggested that the HGF/c-Met/c-Jun pathway is involved in the age-mediated dysfunction of the axon regeneration process.

In recent studies, HGF has been reported to be involved in the regeneration of peripheral nerves, and supplementation with this gene shows therapeutic effects in various peripheral neuropathy models [3, 4, 8, 11]. HGF promoted the differentiation of repair-type Schwann cells by inducing AP-1 transcription factors and accelerated the outgrowth of injured axons through c-Jun protein [3, 5]. Moreover, administration of the HGF gene enhanced the regeneration of injured peripheral nerves and ameliorated pain behaviors [3, 8]. In the current study, we observed that the activation of the c-Met receptor and c-Jun was significantly inhibited in aged conditions (Figures 2 and 3), in accordance with the impairment of nerve repair. Therefore, these data collectively suggested that the HGF/c-Met/c-Jun pathway plays an important role in the damaged peripheral nerve to accelerate its regeneration but was insufficient to activate the regeneration programs in aged conditions.

Aging affects the function of various organs in humans, leading to the development of numerous diseases [12]. More specifically, inefficient regenerative capacity with aging has been reported in the PNS [6]. In motor neurons, the phosphorylation and expression of c-Jun are significantly hindered under aging conditions [13]. In this study, we demonstrated that the p-c-Jun and c-Jun levels in the sciatic nerve and DRG, a cell body of sensory neuron, were decreased in aged animals. These data collectively suggested that c-Jun might be an important indicator of injury-mediated nerve damage related to aging conditions. Additionally, the c-Met pathway might be an upstream factor controlling the expression of c-Jun [4].

It has also been reported that HGF induces the re-myelination of the injured sciatic nerve in a c-Met-dependent manner [3, 5]. As shown in Figure 2A, the protein levels of phosphorylated c-Met and total c-Met were significantly suppressed in the injured sciatic nerve prepared from aged animals. Although the current study mainly focused on the

Figure 4. Analgesic effect of plasmid DNA expressing HGF in nerve-injured young and aged mice. (A, B) To assess the pain-relieving effect of pCK-HGF-X7, the von Frey filaments test was performed. Upon nerve injury, 200 μg of plasmid DNA was intramuscularly injected into the bicep femoris muscle. The withdrawal behavior after the mechanical stimulation (more than 10 times for each animal) to the hind paw was counted, followed by the calculation of withdrawal frequency. Values in the graphs are shown as the mean ± SEM (N = 6 for each group). Statistical analysis was performed using two-way ANOVA, followed by Bonferroni’s multiple comparison testing (*, p < 0.05; **, p < 0.01; ***, p < 0.001; ****, p < 0.0001).
age-related impairment of the axonal outgrowth process, it is highly possible that the Schwann cell-mediated repair process may also be hindered in aged conditions. Therefore, it will be interesting to investigate whether the aging process affects the activity of Schwann cells mediated by the HGF/c-Met/c-Fos pathway [3, 5].

The impairment of regeneration in aged PNS leads to peripheral neuropathy associated with pain [6]. There are several treatment options such as gabapentinoids, opioids, and NSAIDS with limited efficacy or undesirable side effects [14]. Moreover, agents that promote the regeneration or functional recovery of the injured PNS are currently not available. A gene therapy approach to supply the HGF gene has been reported to show therapeutic effects in patients with diabetic peripheral neuropathic pain (DPN) [15]. As shown in Figure 2, the activation of c-Met was hindered in aged conditions implying that this receptor might not be properly activated in elderly people. Therefore, the detailed mechanism regarding the inhibition of the c-Met receptor in aged animals should be investigated to enhance the efficacy of HGF gene therapy in elderly people with peripheral neuropathies.

Declarations

Author contribution statement

Nayeon Lee; Junghun Lee: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.
Sunyoung Kim: Conceived and designed the experiments; Analyzed and interpreted the data.

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The authors declare the following conflict of interests: All authors are employee of Helixmith Co. Ltd.

Additional information

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References

[1] T. Nakamura, S. Mizuno, The discovery of hepatocyte growth factor (HGF) and its significance for cell biology, life sciences and clinical medicine, Proc. Jpn. Acad. Ser. B Phys. Biol. Sci. 86 (2010) 588–610.
[2] S.L. Organ, M.-S. Tao, An overview of the c-MET signaling pathway, Ther. Adv. Med. Oncol. 5 (2011) S7–S19.
[3] K.R. Ko, J. Lee, D. Lee, B. Nho, S. Kim, Hepatocyte growth factor (HGF) promotes peripheral nerve regeneration by activating repair Schwann cells, Sci. Rep. 8 (2018) 8316.
[4] N. Lee, S.H. Lee, J. Lee, M.-Y. Lee, J. Lim, S. Kim, S. Kim, Hepatocyte growth factor is necessary for efficient outgrowth of injured peripheral axons in vitro culture system and in vivo nerve crush mouse model, Biochem. Biophys. Rep. 26 (2021), 100779.
[5] K.R. Ko, J. Lee, B. Nho, S. Kim, c-Fos is necessary for HGF-mediated gene regulation and cell migration in Schwann cells, Biochem. Biophys. Res. Commun. 503 (2018) 2855–2860.
[6] N. Salvadores, M. Sanhueza, P. Manque, F.A. Court, Axonal degeneration during aging and its functional role in neurodegenerative disorders, Front. Neurosci. 11 (2017).
[7] M.W. Painter, Aging Schwann cells: mechanisms, implications, future directions, Curr. Opin. Neurobiol. 47 (2017) 203–208.
[8] B. Nho, J. Lee, J. Lee, K.R. Ko, S.J. Lee, S. Kim, Effective control of neuropathic pain by transient expression of hepatocyte growth factor in a mouse chronic constriction injury model, Faeeh. J. 32 (2018) 5119–5131.
[9] J.E. Shin, S. Geisler, A. DiAntonio, Dynamic regulation of SCG10 in regenerating axons after injury, Exp. Neurol. 252 (2014) 1–11.
[10] B. Nho, K.R. Ko, S. Kim, J. Lee, Intramuscular injection of a plasmid DNA vector expressing hepatocyte growth factor (HGF) ameliorated pain symptoms by controlling the expression of pro-inflammatory cytokines in the dorsal root ganglion, Biochem. Biophys. Res. Commun. 607 (2022) 60–66.
[11] S.H. Lee, S. Kim, N. Lee, J. Lee, S.S. Yu, J.H. Kim, S. Kim, Intrathecal delivery of recombinant AAV1 encoding hepatocyte growth factor improves motor functions and protects neuromuscular system in the nerve crush and SOD1-G93A transgenic mouse models, Acta Neuropathol. Commun. 7 (2019) 96.
[12] F. Roselli, D. Jurk, J.F. Passos, F. d’Adda di Fagagna, Telomere dysfunction in ageing and age-related diseases, Nat. Cell Biol. 24 (2022) 135–147.
[13] Q. Yuan, H. Su, J. Guo, K.Y. Tsang, K.S.E. Cheah, K. Chiu, J. Yang, W.-M. Wong, K.-F. Su, J.-D. Huang, W. Wu, Z. Lin, Decreased c-Jun expression correlates with impaired spinal motoneuron regeneration in aged mice following sciatic nerve crush, Exp. Gerontol. 47 (2012) 329–336.
[14] L. Colloca, T. Ludman, D. Bouhassira, R. Baron, A.H. Dickenson, D. Yarnitsky, R. Freeman, A. Truini, N. Attal, N.B. Finnerup, C. Eccleston, E. Kalso, D.L. Bennett, R.H. Dworkin, S.N. Raja, Neuropathic pain, Nat. Rev. Dis. Prim. 3 (2017), 17002.
[15] J.A. Kessler, A. Shabani, C.N. Sang, M. Christiansen, D. Kudrow, A. Vinik, N. Shin, Gene therapy for diabetic peripheral neuropathy: a randomized, placebo-controlled phase III study of VM202, a plasmid DNA encoding human hepatocyte growth factor, Clin. Transl. Sci. 14 (2021) 1176–1184.