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***Plasminogen repairs abnormal pain perception through improving sensory function recovery and regeneration of peripheral small nerve fiber in db/db mice***

Weiquan Li<sup>1</sup>, Ting Wang<sup>1</sup>, Fen Chen<sup>1</sup>, Chunying Guo<sup>1</sup>, Yanghui Liao<sup>1</sup>, Congcong Quan<sup>1</sup>, Fei Zheng<sup>1</sup>, Jinan Li<sup>1,2,3,\*</sup>

<sup>1</sup> Department of Basic Research, Talengen Institute of Life Sciences, Shenzhen, China,

<sup>2</sup> The First Affiliated Hospital of Shenzhen University, Shenzhen Second People's Hospital, Shenzhen, China, <sup>3</sup> Shenzhen Institute of Geriatrics, Shenzhen, China

\* Corresponding author, Room C602G, 289 Digital Peninsula, Shunfeng Industrial Park, No.2 Red Willow Road, Futian District, Shenzhen, China

*E-mail address:* [jnl@talengen-pharma.com](mailto:jnl@talengen-pharma.com) (J.N. Li)

## 32 **Abstract**

33 Painful diabetic peripheral neuropathy (PDPN) is a devastating complication of  
34 diabetes and severely threatens the health of humankind. The plasminogen activator  
35 system and plasminogen (Plg) have multiple functional roles in tissue regeneration and  
36 extracellular matrix remodeling, which suggests that Plg may have a potentially pivotal  
37 role in anti-PDPN. In the present study, we explore whether an increased level of  
38 circulating Plg has positive effect on repairing abnormal pain perception in diabetic mice  
39 model. Our data demonstrated that additional Plg not only helps healing pain allodynia or  
40 hyperalgesia on the mice at the age of 8 weeks old in early PDPN, but more important,  
41 also has positive effects of regaining normal pain perception from hypoalgesia on the  
42 mice at ages of 14-15 or 24-25 weeks in advanced PDPN. Furthermore, our data also  
43 reveal a possible mechanism for Plg's contribution to rebuilding normal pain perception  
44 among db/db mice by promoting axonal myelination and regeneration of small nerve  
45 fiber in peripheral nervous system. Therefore, our data suggest that Plg show promise to  
46 become a drug candidate for treating diabetic peripheral neuropathic pain.

47

## 48 **Introduction**

49 Painful diabetic peripheral neuropathy (PDPN) is a common complication  
50 accompanying long term Diabetes Mellitus (DM), affecting approximately 50% diabetic  
51 patients [1]. PDPN has been recently defined as a symmetric, length-dependent  
52 sensorimotor polyneuropathy attributable to metabolic and microvascular alterations as a  
53 result of chronic hyperglycemia exposure [2]. Though the specific pathogenesis of PDPN

54 in different stages has not been fully clarified, but it is known that there are two stages  
55 according to the manifestations, the early PDPN and the advanced PDPN. Such  
56 manifestations are 1) thermal hyperalgesia, an equivalent of a clinical phenomenon  
57 described in early PDPN; 2) thermal hypoalgesia, typically present in advanced PDPN; 3)  
58 mechanical hyperalgesia, an equivalent of pain on pressure in early PDPN; 4) mechanical  
59 hypoalgesia, an equivalent to the loss of sensitivity to mechanical noxious stimuli in  
60 advanced PDPN 5) tactile allodynia, a painful perception of a light touch [3]. 20-30% of  
61 patients with PDPN suffer from severe neuropathic pain [4-5], as a leading cause for foot  
62 ulceration and amputation and fall related injury. This may result in withdrawal from  
63 social events there by affecting the quality of life and considerably increasing the  
64 financial burden of treatment [6-8].

65 Unfortunately, so far, there are no effective FDA approved drugs available for  
66 treating PDPN [9-10]. At present, traditional drugs including (1) antiepileptic drugs, such  
67 as gabapentin and pregabalin; (2) analgesics and anesthetics; (3) antidepressants and non-  
68 steroidal anti-inflammatory drugs have been used to treat diabetic neuropathic pain [9-10].  
69 However, only a small portion of PDPN patients (about less than 20%) shows response to  
70 these treatments. Furthermore, the high side effects, high cost and insufficient effects of  
71 these drugs have limited their use in treating PDPN. Therefore, there is an urgent need for  
72 the research and development of effective medications to relieve PDPN.

73 Plasminogen (Plg) is a zymogen mainly produced by the liver and activated to  
74 become the broad-spectrum protease plasmin by either of two physiological plasminogen  
75 activators (PAs): tissue-type PA (tPA) or urokinase-type PA (uPA) [11]. It has been  
76 shown that the extracellular proteolytic activity of plasmin plays a pivotal role in

77 fibrinolysis and extracellular matrix degradation, which is essential during many  
78 damaged tissue remodeling processes, including diabetic wound healing [12–14].  
79 Furthermore, our previous study showed that administering Plg improved diabetic wound  
80 healing by tissue remodelling [14]. Interestingly the research on mice from Seeds' group  
81 has demonstrated that deficiency of any components in the PA system, such as Plg or tPA,  
82 will delay sensational response to external stimuli [15]. However, the roles of Plg on  
83 PDPN is an emerging area.

84 In order to make sure the role of Plg in PDPN, a diabetic (db/db) mouse model was  
85 applied to dissect the effects and function of Plg in PDPN and to explore related  
86 underlying mechanism. Our findings support that Plg may play a critical role in  
87 alleviating pain allodynia through rebuilding normal pain perception by significantly  
88 promoting repair and regeneration of damaged small somatosensory nerve fiber.  
89 Therefore, Plg may be a promising therapeutic candidate in treating PDPN and  
90 preventing its related diseases.

## 91 **Materials and methods**

### 92 **Animals**

93 Leptin receptor-deficient db/db mice and their littermates were obtained from the  
94 Animal Research Center of Nanjing University. The animals were kept under standard  
95 laboratory conditions. The Ethics Committee of Talengen Institute of Life Sciences  
96 approved all of the experimental protocols. For studying diabetic peripheral neuropathic  
97 pain, the male animals with age at 8, 14-15 and 25-26 weeks were grouped and treated  
98 with or without Plg protein for indicated time depending on the experimental design. In  
99 the experiments of a burn-wound model, the 16-26 weeks old db/db male mice were

100 grouped and treated with Plg for the experiments. If not mentioned, at least five mice  
101 were included in each experimental group.

## 102 **h-Plg protein administration in diabetic burn-wound healing** 103 **and diabetic peripheral neuropathy study**

104 For the study of diabetic burn-wound healing, the male mice at age 16-26 weeks old  
105 were anesthetized by an intraperitoneal injection of 50 mg/Kg sodium pentobarbital. A  
106 copper rod was heated to 95-100°C by submersion in boiling water. The copper rod was  
107 immediately applied vertically for 6 seconds without additional pressure on the back skin  
108 of mice that had also been depilated before wounding. After wounding, all mice were  
109 individually caged, and wounds were neither sutured nor dressed. The mice received  
110 standardized wounds, and then 2 mg of human Plg was administered daily by 0.2ml IV  
111 injection. In the control group, 0.2 ml of PBS was administered daily by IV injection as a  
112 placebo. The daily treatments were continued for indicated days depending on the  
113 experimental design.

## 114 **h-Plg protein administration in diabetic peripheral neuropathy** 115 **study**

116 As for the study of diabetic peripheral neuropathy, each group of male mice at the age  
117 of 8 weeks, 14-15 and 24-25 weeks were divided into two subgroups, and 2 mg of human  
118 Plg daily was administered directly on Plg-treated group mice by 0.2 ml IV injection, and  
119 the control group mice were administered 0.2 ml of PBS daily by IV injection as a  
120 placebo. The daily treatments were continued for indicated days depending on the  
121 experimental design. Then the animals were used for designed behavior testing.

## 122 **Behavior quantitative sensory testing (QST)**

123 Behavioral signs representing three different components of neuropathic pain were  
124 examined: allodynia, hyperalgesia, and hypoalgesia in response to cold and mechanical  
125 stimuli. To quantify mechanical sensitivity of the foot, the standard quantitative sensory  
126 testing (QST) was used to record numbers of brisk foot withdrawal in response to  
127 normally noxious and innocuous mechanical stimuli as described previously [16-17].  
128 Von Frey fibers are a neurophysiological examination tool used for determining the  
129 mechanical pain threshold in humans and nonhumans [17]. The force applied to the  
130 testing animal is based on the size of monofilament in von Frey used. First, an  
131 intermediate size of monofilament (number 4.31, exerting 2.0 g of force) was gently  
132 applied with enough force to bend it. This was repeated up to three times in distinct areas  
133 along the lateral paw. In the case of a positive response (rapid withdrawal of the paw  
134 within 2 seconds), a smaller filament was tested. If no response was recorded in any of  
135 the three different areas, a larger filament was tested. The frequency of foot withdrawal  
136 was expressed as a percent: (# of trials accompanied by brisk foot withdrawal) X 100 / (#  
137 of total trials). On a given test day and for each hind paw, the same procedure was  
138 repeated by using two different sizes of von Frey filaments. Mechanical sensitivity was  
139 tested on each mouse on day 0 (1 day before administration of Plg) and 3, 4, 6, 7, 9, 11,  
140 12 and 16 days after treating with Plg.

141 To quantify cold sensitivity of the foot, brisk foot withdrawal in response to acetone  
142 application was measured. The mice were placed under a transparent plastic dome on a  
143 metal mesh floor, and acetone was applied to the plantar surface of the foot. To do this,  
144 an acetone bubble was formed at the end of a small polyethylene tube that was connected

145 to a syringe. The bubble was then gently touched to the heel. The acetone quickly spread  
146 over the proximal half of the plantar surface of the foot. The acetone was applied five  
147 times (once every 5 min) to each paw. The frequency of foot withdrawal was again  
148 expressed as a percent: (# of trials accompanied by brisk foot withdrawal) X 100 / (# of  
149 total trials). Cold sensitivity was tested on each mouse on day 0 (1 day before  
150 administration of Plg) and 3, 4, 6, 7, 11, 12, and 16 days after treating with Plg.

151 To quantify the pain sensation evoked by pin-prick, the 27-gauge needle was used to  
152 stimulate the foot of the mouse but not to penetrate the dermis. The db/db mice were  
153 stimulated on the soles of their left and right feet every 3 minutes for a total of 10 times,  
154 and the number of paw withdrawal reactions was counted. The frequency of foot  
155 withdrawal was again expressed as a percent: (# of trials accompanied by brisk foot  
156 withdrawal) X 100 / (# of total trials). Pin-prick test was performed on each mouse on  
157 day 0 (1 day before administration of Plg) and 3, 4, 6, 7, 11, 12, and 16 days after treating  
158 with Plg.

## 159 **H&E staining**

160 The sciatic nerve tissues were fixed in 4% paraformaldehyde, embedded in paraffin  
161 and sectioned 3  $\mu$ m thick. The sections were stained for morphological analysis using an  
162 H&E staining kit. The slides were examined by light microscopy under a Nikon  
163 microscope, and images were recorded digitally using a camera connected to a computer.

## 164 **Immunohistochemical analyses**

165 The paraffin-embedded sections were rehydrated and then treated with antibodies  
166 against Fibrin (cat# ab27913), or PGP9.5 (cat# ab10404) purchased from Abcam  
167 (Cambridge, UK). The signal intensity was detected immunohistochemically by the

168 peroxidase anti-peroxidase method. In brief, the antigens were first retrieved by treatment  
169 with Citrate buffer at high temperature for 20 min, and then the tissue sections were  
170 blocked with 5% non-immunized goat serum (Vector laboratories, USA, cat# SK-1012-  
171 50) and incubated with the antibodies against Fibrin or PGP9.5 diluted in PBS. After this  
172 procedure, an anti-rabbit link antibody was applied, followed by a rabbit PAP complex.  
173 The staining was visualized through a diaminobenzidine (DAB) reaction, and the sections  
174 were counterstained with hematoxylin.

## 175 **Light microscopic examination**

176 The slides were examined by light microscopy under a Nikon microscope (C-SHG1),  
177 and images were recorded digitally using Nikon DS-Fi3 connected to a computer. When  
178 needed, IPP6.0 pathological image analysis software was used to determine the integral  
179 optical density (IOD) or average optical density (AOD) of immuno-positive products for  
180 each group and area of interest. The integrated optical density (IOD) is the sum of the  
181 individual pixels within the field of view of the specimen. Average optical density (AOD)  
182 is the total value of pixels in the area of interest (IOD SUM) divided by the area to be  
183 tested. Both IOD and AOD represent the intensity of protein expression.

## 184 **Results**

### 185 **Effect of Plg on small sensory nerve fiber in wounds sites in** 186 **diabetic mice.**

187 To explore the effect of Plg on peripheral nerve fiber in diabetic wound sites, the  
188 diabetic db/db mice were treated daily for 3, 7 or 14 days by intravenous (IV) injections  
189 of 2 mg/0.2 ml human Plg or 0.2 ml PBS after burning injury. PGP9.5



190 immunohistochemical staining of a tissue section from wounded sites was performed.  
191 The results (Fig.1A-B) showed that the expression of PGP9.5 in the wound sites of the  
192 Plg-treated group is higher than the control group without Plg administration on day 3  
193 and 7, and with a significantly difference on day 14 ( $p < 0.05$ ). The results indicated that  
194 Plg treatment enhances the regeneration of injured small nerve fiber while the healing of  
195 diabetic wounds was improved.

### 196 **Effect of Plg on sciatic nerve fiber in the diabetic mice**

197 To further investigate the effect of Plg on sciatic nerve fiber in the diabetic mice, the  
198 db/db mice at the age of 24-25 weeks old were injected intravenously with 2 mg Plg  
199 protein daily for 15 days. The H&E staining of sciatic nerve tissue shows that a large  
200 number of the myelin sheath of axons are swollen, demyelinated and disintegrated in the  
201 control group without administration of Plg (as shown in figure 2A(a)). In contrast,  
202 sciatic nerve fibers of Plg-treated mice were morphologically closely arranged, and the  
203 myelin sheath of the axonal structure was well maintained, and few of them are  
204 disintegrated as shown in figure 2A(b). Moreover, increased expression of PGP9.5 of  
205 sciatic nerve tissue in the Plg-treated group was observed by PGP9.5  
206 immunohistochemical staining test (Fig. 2B). These data indicate that the number and  
207 density of sciatic nerve fibers with intact axonal structures are increased and better  
208 maintained in the Plg-treated group.

209 Increased deposition of fibrin at the injured site is a common feature of diabetic  
210 injury. In turn, the reduction of fibrin deposition may contribute to the healing of injured  
211 tissue. To investigate the effect of Plg on fibrin in wound sciatic nerve tissue, the 24-25  
212 weeks old db/db mice were injected intravenously with 2 mg Plg daily for 15 days. Then

213 the samples of injured sciatic nerve tissues from these mice were subjected to detect the  
214 degree of fibrin deposition by fibrin immunohistochemical staining test. As shown in  
215 figure 2C, the deposition of fibrin in sciatic nerve tissue in the Plg-treated group was  
216 significantly lower compared to the control group. This result suggests that increased Plg  
217 in circulation facilitates fibrin degradation to clear its deposition, which may be  
218 beneficial for axonal regeneration in damaged sciatic nerve tissue of db/db mouse model.

### 219 **Effect of Plg on both hyperalgesia and hypoalgesia to pain** 220 **sensation in diabetes mice**

221 The db/db mice develop diabetes at 4 weeks of age, exhibit allodynia and  
222 hyperalgesia between 8 and 12 weeks of age, and exhibit hypoalgesia after 12 weeks of  
223 age [18]. In the present study, we followed a protocol to quantify the degree of  
224 mechanical allodynia in response to pressure from a von-Frey filament. From Fig.3A, the  
225 results showed that the pain threshold of the group from 8 week old mice administrated  
226 with Plg after 9 days is significantly increased ( $P < 0.05$ ), indicating the symptom of  
227 diabetic hyperalgesia is improved.

228 In contrast, the results from 14-15 week old mice with advanced PDPN representing  
229 hypoalgesia showed that the pain threshold of the group administrated with Plg gradually  
230 reduced and exhibited significant difference on days 3 and 12 compared with the control  
231 group without Plg treatment (Fig. 3B,  $P < 0.05$ ). Similarly, the results from 24-25 week  
232 old mice with advanced PDPN showed that the pain threshold of the group administrated  
233 with Plg gradually reduced and exhibited significant difference on day 16 (Fig. 3C,  $P <$

234 0.05). Together, Plg can increase the pain threshold of diabetes mice at early PDPN and  
235 decrease the pain threshold of diabetes mice at advanced PDPN.

236 **Effect of Plg on abnormal thermal perception to cold**  
237 **stimulation in diabetic mice.**

238 Damaged peripheral small sensory nerve systems due to diabetic hyperglycemia can  
239 also change the thermal perception resulting in cold allodynia in response to cold stimuli.  
240 To explore if Plg treatment has effects on thermal perception to cold stimuli, two groups  
241 of diabetic mice treated with Plg at different ages were subjected to cold stimulation  
242 behavior test with acetone. The results of the QST from the Plg-treated 14-15 week old  
243 mice showed that a significant increase of sensational response to cold stimulation on  
244 days 6 ( $P < 0.05$ ) and 12 ( $P < 0.01$ ) compared with the control group without Plg treatment  
245 (Fig. 4A). Similarly, the results from the Plg-treated 24-25 week old mice show a  
246 significantly increased percentage of sensational response on days 4 and 16 of treatment  
247 (Fig. 4B). It should also be noted that in all days with non-significant results, the mean  
248 values of the treated group still trend in the same direction as the significant ones. Our  
249 results indicate that Plg treatment may repair diabetic-induced somatosensory  
250 dysfunction to relieve pain and cold allodynia in db/db mouse model with advanced  
251 PDPN.

252 **Effect of Plg on abnormal pain sensation evoked by pinprick**  
253 **test in diabetic mice**

254 To further investigate the effect of Plg on somatosensory nervous system in the  
255 diabetic mice model, two groups of diabetic mice treated with Plg protein at different

256 ages were subjected to pin-pricking behavior tests. The results from the Plg-treated 14-15  
257 week old mice showed that the percentage of sensational response to pin-prick test was  
258 significantly increased on days 6 ( $P < 0.05$ ) and 12 ( $P < 0.001$ ) compared with the control  
259 group without Plg treatment (Fig. 5A). Similarly, the results from the Plg-treated 24-25  
260 week old mice with advanced PDPN showed a significant increase of sensational  
261 response on days 7 ( $P < 0.01$ ) and 16 ( $P < 0.05$ ) (Fig. 5B). Just as mentioned above, the  
262 mean values of the treated group on all non-significant days trend to the same direction as  
263 the significant ones. The results indicate that Plg treatment may alleviate pain  
264 hypoalgesia in the diabetic mouse model with advanced PDPN.

## 265 **Discussion**

266 Painful diabetes peripheral neuropathy (PDPN) is a subtype of sensorimotor  
267 neuropathy and is the most commonly acquired neuropathy in diabetes mellitus (DM)  
268 [19-20]. Its pathomechanism has not yet been completely clarified, but the neuronal  
269 hyperexcitability and sensitization in early stage of DPN and hypoalgesia in late stage of  
270 DPN are widely accepted as playing an important role.

271 Allodynia and hyperalgesia at early stage of DPN characterized by spontaneous or  
272 evoked pain is described as electric shock-like or burning after nerve dysfunction and  
273 damage in the peripheral sensory nerve system induced by diabetic hyperglycemia. In the  
274 peripheral nervous system (PNS), there are more thin ( $< 1 \mu\text{m}$ ) unmyelinated axons,  
275 known as C-fiber axons or small fibers, than myelinated axons [21]. Therefore, the  
276 earliest changes of DPN occur at the level of unmyelinated C fibers, with initial  
277 imbalance between degeneration and regeneration of C fiber resulting in pain, allodynia,  
278 and hyperalgesias [22]. In the present study, we observed that Plg treatment is able to

279 correct abnormal pain sensitization by increasing the pain threshold on the db/db mice at  
280 early stage of PDPN after 9 days of Plg supplementation when symptom of hyperalgesia,  
281 exhibiting hypersensitive to mechanic pressure, is developed (Fig. 3A). It is known that  
282 the impairment of small nerve fibers results in the loss of normal thermal and pain  
283 perception, whereas large nerve fiber impairment results in loss of normal touch and  
284 vibration perception [23-25]. Small nerve fibers constitute 70 to 90% of peripheral nerve  
285 fibers and are functionally classified into somatic sensory, somatic motor, and autonomic  
286 fibers, which regulate several key functions such as tissue blood flow, temperature, and  
287 pain perception as well as sweating [26-27]. Expression of PGP9.5 in the diabetic wound  
288 skin is increased after administration with Plg for 14 days (Fig.1). This results indicated  
289 that Plg promoted small nerve fibers regeneration at the wounded site in the diabetic mice.  
290 Blasi and Mignatti have disclosed that the PA system was implicated in various tissue  
291 remodelling processes as early as 1990s' [28-29]. That is to say Plg can improve  
292 allodynia and hyperalgesia of early stage of PDPN through promoting the regeneration of  
293 small nerve fibers to elevate the pain perception threshold.

294 As the disease course progresses, mild segmental axonal demyelination then occurs,  
295 followed by frank axonal degeneration of myelinated fibers as demyelination surpasses  
296 remyelination[30]. These changes lead to a progressive loss of distal sensation in a distal-  
297 to proximal course along the nerve that defines diabetic peripheral neuropathy [1]. This  
298 phase is defined as the advanced stage of DPN. At this stage, hypoalgesia is developed  
299 and exhibiting hyposensitive to external mechanic, cold stimuli and mechanical noxious  
300 stimuli. Surprisingly, we observed that Plg treatment could reverse pain insensate state to  
301 normal pain response by lower the pain perception threshold of diabetic mice at the age

302 of 14-15 weeks and 24-25 weeks that has lost sensation to the pain and developed  
303 advanced DPN (Fig.3B-C, Fig.4, and Fig.5). Advanced DPN is associated with elevated  
304 vibration and thermal perception thresholds that progress to sensory nerve loss, occurring  
305 in conjunction with degeneration of all fiber types [3]. In the current study, H&E staining  
306 result shown that the nerve fibers in sciatic nerve of diabetic mice administrated with Plg  
307 were wrapped by epineurium and closely arranged, while a large number of myelin axons  
308 in sciatic nerve tissue of diabetic mice without Plg treatment are swelling, demyelinated  
309 and collapsed (Fig. 2A). Meanwhile, increased expression of PGP9.5 in sciatic nerve  
310 tissue of Plg-treated group indicated that the number and density of small nerve fibers  
311 with intact axonal structures are increased and better maintained in the Plg-treated group  
312 (Fig. 2B). In addition, the deposition of fibrin in sciatic nerve tissue of the Plg-treated  
313 group was significantly decrease (Fig.2C). These results revealed that Plg can improve  
314 hypoalgesia by promoting sciatic nerve remyelination of diabetic mice to lower sensory  
315 nerve damage and loss,

316 The pathogenesis of DPN is considered to be multifactorial. A combination of chronic  
317 hyperglycemia, oxidative stress, inflammation, microangiopathy of nerve blood vessels  
318 and a reduction in nerve fiber repair result in damage to the vasa nervorum, the  
319 microvessels that supply blood to neural tissue[31-32]. Chronic hyperglycaemia and  
320 hyperlipidemia exposure in diabetes mellitus cause mitochondrial dysfunction and  
321 inflammatory signals activation, further leading to metabolic injury and cell apoptosis,  
322 ultimately resulting in microvascular and neuronal damage. The unmyelinated C fibers  
323 are more susceptible to metabolic injury in large part because they lack the degree of  
324 protection and nutrient supplementation afforded to myelinated axons by Schwann cells

325 (SCs) [33]. Furthermore, high glucose increases ROS and inflammatory factor level  
326 through the TLR4/NF $\kappa$ B pathway in Schwann cells (SCs) [34]. All of these will cause the  
327 peripheral nerve cell death and demyelination as lacking nutrient supplementation of SCs  
328 and microvascular. Recently, Juliana' group revealed that Plg/Pla and the receptor Plg-  
329 R $\kappa$ T could mediate macrophage polarization to the M2 phenotype via STAT3 signaling,  
330 playing a key role in anti-inflammatory [35]. Meanwhile, PA system/plasminogen is  
331 reported to regulate expression and activation of some growth factors including  
332 transforming growth factor (TGF- $\beta$ ), nerve growth factor (NGF), vascular endothelial cell  
333 growth factor (VEGF) and fibroblast growth factor (FGF), and other growth factors for  
334 tissue regeneration and extracellular matrix remodeling [36]. Further studies revealed that  
335 activated TGF- $\beta$  stimulates Schwann cell proliferation and differentiation which is  
336 essential for the formation of axonal myelin sheaths and axonal support for nerve  
337 regeneration and functional recovery in the peripheral nervous system after injury [37-  
338 38]. Zou's study have showed that tPA or tPA/Plg promotes axonal remyelination and  
339 regeneration of injured sciatic nerve tissue[39]. Therefore, Plg can promote peripheral  
340 nervous axons remyelination and regeneration by multiple pathway to repair allodynia  
341 and hyperalgesia and reverse hypoalgesia of PDPN in diabetes mellitus.

342

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## 347 **Conflicts of interest**

348 J.L has patented the usage of Plg for the treatment of wound healing and holds stock in a  
349 start-up company that owns the right to develop Plg for wound healing purposes. The  
350 remaining authors state no conflict of interest.

351

## 352 **References**

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354 1. Caitlin W. Hicks & Elizabeth Selvin. Epidemiology of Peripheral Neuropathy and Lower  
355 Extremity Disease in Diabetes. *Current Diabetes Reports*. 2019, 19:86.

356 2. Tesfaye S, Boulton AJ, Dyck PJ, et al. Diabetic neuropathies: update on definitions, diagnostic  
357 criteria, estimation of severity, and treatments. *Diabetes Care* 2010;33:2285-93.

358 3. Irina G. Obrosova. Diabetic Painful and Insensate Neuropathy: Pathogenesis and Potential  
359 Treatments. *Neurotherapeutics: The Journal of the American Society for Experimental  
360 NeuroTherapeutics*. 2009, 6: 638-647.

361 4. Tesfaye S, Vileikyte L, Rayman G, Sindrup SH, Perkins BA, Baconja M, Vinik AI, Boulton AJ.  
362 Toronto Expert Panel on Diabetic Neuropathy. Painful diabetic peripheral neuropathy: consensus  
363 recommendations on diagnosis, assessment and management. *Diabetes/metabolism research and  
364 reviews* 2011 Oct;27(7):629e38.

365 5. Callaghan BC, Cheng HT, Stables CL, Smith AL, Feldman EL. Diabetic neuropathy: clinical  
366 manifestations and current treatments. *Lancet Neurol* 2012 Jun 1;11(6):521e34.

367 6. Larsen PR, Kronenberg HM, Melmed S, et al. Williams, Textbook of endocrinology. tenth ed.  
368 Philadelphia, Pa, USA: Saunders; 2002.

369 7. Quattrini C, Tesfaye S. Understanding the impact of painful diabetic neuropathy.  
370 *Diabetes/metabolism research and reviews* 2003 Jan 1;19(S1).

371 8. Argoff CE, Cole BE, Fishbain DA, Irving GA. Diabetic peripheral neuropathic pain: clinical and  
372 quality-of-life issues, In *Mayo Clinic Proceedings* 81. Elsevier; 2006 Apr 1. p. S3e11. 4.

373 9. Schreiber AK. Diabetic neuropathic pain: Physiopathology and treatment. *World J Diabetes*.  
374 2015;

375 10. Papanas N, Ziegler D. Emerging drugs for diabetic peripheral neuropathy and neuropathic pain.  
376 *Expert Opin. Emerg. Drugs*. 2016.

377 11. Irigoyen JP, Muñoz-Cánoves P, Montero L, Koziczak M, Nagamine Y. The plasminogen  
378 activator system: Biology and regulation. *Cell. Mol. Life Sci*. 1999.

379 12. Romer J, Bugge TH, Pyke C, Lund LR, Flick MJ, Degen JL, et al. Impaired wound healing in  
380 mice with a disrupted plasminogen gene. *Nat Med*. 1996;

381 13. Romer J, Bugge TH, Pyke C, Lund LR, Flick MJ, Degen JL, et al. Plasminogen and wound  
382 healing . *Nat. Med*. 1996.

383 14. Shen Y, Guo Y, Mikus P, Sulniute R, Wilczynska M, Ny T, et al. Plasminogen is a key



- 384 proinflammatory regulator that accelerates the healing of acute and diabetic wounds. *Blood*.  
385 2012;
- 386 15. Siconolfi LB, Seeds NW. Mice lacking tPA, uPA, or plasminogen genes showed delayed  
387 functional recovery after sciatic nerve crush. *J Neurosci* [Internet]. 2001;21:4348-4355.
- 388 16. Obrosova IG. Diabetic Painful and Insensate Neuropathy: Pathogenesis and Potential  
389 Treatments. *Am Soc Exp Neurother*. 2009;
- 390 17. Fa F, Nurrochmad A, Ae N, Susilowati R. Optimization of Mice Model of Painful Diabetic  
391 Neuropathy ( PDN ). 2017;49:97–105.
- 392 18. Sullivan K, Lentz S, Roberts JJ, Feldman E. Criteria for creating and assessing mouse models of  
393 diabetic neuropathy. *Curr Drug Targets*. 2008;
- 394 19. Hartemann A, Attal N, Bouhassira D, et al. Painful diabetic neuropathy: diagnosis and  
395 management. *Diabetes Metab* 2011; 37: 377-388.
- 396 20. Fateh HR, Madani SP, Heshmat R, et al. Correlation of Michigan neuropathy screening  
397 instrument, United Kingdom screening test and electrodiagnosis for early detection of diabetic  
398 peripheral neuropathy. *J Diabetes Metab Disord* 2015; 15: 18.
- 399 21. Feldman EL, Hughes RA, Willison HJ. Progress in inflammatory neuropathy-the legacy of Dr  
400 Jack Griffin. *Nature Reviews Neurology*. 2015; 11:646-650.
- 401 22. Sajic M. Mitochondrial dynamics in peripheral neuropathies. *Antioxid Redox Signal*.  
402 2014;21(4):601-20.
- 403 23. Kles K, Vinik A. Pathophysiology and Treatment of Diabetic Peripheral Neuropathy: The Case  
404 for Diabetic Neurovascular Function as an Essential Component. *Curr Diabetes Rev*. 2006;2:131-  
405 45.
- 406 24. Lee-Kubli CA, Calcutt NA. Painful neuropathy: Mechanisms [Internet]. 1st ed. *Handb. Clin.*  
407 *Neurol*. Elsevier B.V.; 2014.
- 408 25. Spallone V, Greco C. Painful and painless diabetic neuropathy: One disease or two? *Curr Diab*  
409 *Rep*. 2013;13:533-49.
- 410 26. Malik RA, Veves A, Tesfaye S, Smith G, Cameron N, Zochodne D, et al. Small fibre neuropathy:  
411 Role in the diagnosis of diabetic sensorimotor polyneuropathy. *Diabetes. Metab. Res. Rev*. 2011.
- 412 27. Javed S, Petropoulos IN, Tavakoli M, Malik RA. Clinical and diagnostic features of small fiber  
413 damage in diabetic polyneuropathy [Internet]. 1st ed. *Handb. Clin. Neurol*. Elsevier B.V.; 2014.  
414 Available from: <http://dx.doi.org/10.1016/B978-0-444-53480-4.00019-9>.
- 415 28. Blasi F. Proteolysis, cell adhesion, chemotaxis, and invasiveness are regulated by the u-PA-u-  
416 PAR-PAI-1 system. *Thromb Haemost* 1999; 82: 298-304.
- 417 29. Mignatti P, Rifkin DB. Biology and biochemistry of proteinases in tumor invasion. *Physiol Rev*  
418 1993; 73: 161-195.
- 419 30. Mizisin AP. Mechanisms of diabetic neuropathy: Schwann cells. *Handbook of Clinical*

- 420 Neurology. 2014; 126:401-428.
- 421 31. Boulton AJ. Diabetic neuropathy: classification, measurement and treatment. *Curr Opin*  
422 *Endocrinol Diabetes Obes.* 2007, 14: 141-145.
- 423 32. West XA, Malinin NL, Merkulova AA, Tischenko M, Kerr BA. Oxidative stress induce  
424 angiogenesis by activating TLR2 with novel endogenous ligands. *Nature* 2010, 467: 972-976.
- 425 33. Malik RA, Tesfaye S, Newrick PG, Walker D, Rajbhandari SM, Siddique I, Sharma AK, Boulton  
426 AJ, King RH, Thomas PK, Ward JD. Sural nerve pathology in diabetic patients with minimal  
427 but progressive neuropathy. *Diabetologia.* 2005; 48:578-585.
- 428 34. Wei Tang, Xiangfang Chen, Haoqi Liu, et al. Expression of Nrf2 Promotes Schwann Cell-  
429 Mediated Sciatic Nerve Recovery in Diabetic Peripheral Neuropathy. *Cell Physiol Biochem.*  
430 2018; 46: 1879-1894.
- 431 35. Juliana P V, et al. Plasminogen and the Plasminogen Receptor, Plg-RKT, Regulate Macrophage  
432 Phenotypic, and Functional Changes. *Frontiers in Immunology.* 2019, 10:01458.
- 433 36. Rifkin DB, Mazziere R, Munger JS, Noguera I, Sung J. Proteolytic control of growth factor  
434 availability. *Apmis.* 1999;107:80-85.
- 435 37. Sulaiman WAR. Transforming growth factor-b promotes axonal regeneration after chronic nerve  
436 injury. *Spine (Phila Pa 1976).* 2016;41:S29.
- 437 38. Li M, Zhang P, Li H, Zhu Y, Cui S, Yao D. TGF- $\beta$ 1 is critical for Wallerian degeneration after  
438 rat sciatic nerve injury. *Neuroscience.* 2015;284:759-67.
- 439 39. Zou T, Ling C, Xiao Y, Tao X, Ma D, Chen ZL, et al. Exogenous tissue plasminogen activator  
440 enhances peripheral nerve regeneration and functional recovery after injury in mice. *J*  
441 *Neuropathol Exp Neurol.* 2006;

442

## 443 **Figure legends**

444

### 445 **Fig. 1. Plg treatment promotes the regeneration of small nerve fiber in diabetic** 446 **wounds**

447 A: Representative image of PGP9.5 immunohistochemical staining of wounded skin of  
448 db/db mice on days 3, 7, 14 after injury respectively. (a-c): Control group, (d-f): Plg-  
449 treated group, Magnification is 400x.

450 B: Quantitative analysis of PGP9.5 expression in figure 1A. n = 5 mice used in each  
451 group. \* P < 0.05 vs. Control group.

452

453 **Fig. 2. Plg treatment promotes regeneration of injured sciatic nerve in the diabetic**  
454 **mice**

455 **A:** Representative image of H&E staining of sciatic nerve tissue from db/db mice at the  
456 age of 24-25 weeks treated with Plg for 15 days, (a): Control group, (b): Plg-treated  
457 group, Magnification is 400x.

458 **B:** Representative image of PGP.95 immunohistochemical staining of injured sciatic  
459 nerve of db/db mice at age of 24-25 weeks treated with Plg for 15 days, (a): Control  
460 group, (b): Plg-treated group, Magnification is 400x.

461 **C:** Representative image of fibrin immunohistochemical staining of injured sciatic nerve  
462 tissue of db/db mice at the age of 24-25 weeks treated with Plg for 15 days, (a): Control  
463 group, (b): Plg-treated group, Magnification is 400x.

464

465 **Fig. 3. Plg treatment alleviates mechanical allodynia by behavioral von-Frey**  
466 **filament test of diabetic mice**

467 **A:** Quantitative analysis of mechanical allodynia and hyperalgesia on eight-week-old  
468 db/db mice with early-stage DPN treated by Plg supplementation for the indicated  
469 number of days.

470 **B:** Quantitative analysis of mechanical allodynia and hypoalgesia on 14-15 week old  
471 db/db mice with middle stage of DPN treated by Plg supplementation for the indicated  
472 number of days.

473 **C:** Quantitative analysis of mechanical allodynia and hypoalgesia on 24-25 week old  
474 db/db mice with advanced DPN treated by Plg supplementation for the indicated number  
475 of days.

476

477 **Fig. 4. Plg treatment enhances sensational response to cold stimulation by acetone in**  
478 **diabetic mice**

479 **A:** Quantitative analysis of cold allodynia and hypoalgesia on 14-15 week old diabetic  
480 mice treated by Plg supplementation for the indicated number of days.

481 **B:** Quantitative analysis of cold allodynia and hypoalgesia on 24-25 week old diabetic  
482 mice treated by Plg supplementation for the indicated number of days.

483

484 **Fig. 5. Plg treatment enhances sensational response to pain stimulated by pin-prick**  
485 **test in diabetic mice**

486 A: Quantitative analysis of mechanical allodynia and hypoalgesia on 14-15 week old  
487 diabetic mice treated by Plg supplementation for the indicated number of days.

488 B: Quantitative analysis of mechanical allodynia and hypoalgesia on 24-25 week old  
489 diabetic mice treated by Plg supplementation for the indicated number of days.