



Title	Therapeutic Potential of the Prolyl Hydroxylase Inhibitor Roxadustat in a Mouse Hindlimb Lymphedema Model
Author(s)	Hoshino, Yoshitada; Osawa, Masayuki; Funayama, Emi; Ishikawa, Kosuke; Miura, Takahiro; Hojo, Masahiro; Yamamoto, Yuhei; Maeda, Taku
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1 **Therapeutic potential of the prolyl hydroxylase inhibitor roxadustat in a mouse**  
2 **hindlimb lymphedema model**

3

4 Yoshitada Hoshino, Masayuki Osawa, Emi Funayama, Kosuke Ishikawa, Takahiro Miura,  
5 Masahiro Hojo, Yuhei Yamamoto, Taku Maeda

6 Department of Plastic and Reconstructive Surgery, Faculty of Medicine and Graduate School  
7 of Medicine, Hokkaido University, Sapporo, Japan

8

9 Running title: THERAPEUTIC POTENTIAL OF ROXADUSTAT FOR LYMPHEDEMA

10

11 Corresponding author: Taku Maeda

12 Department of Plastic and Reconstructive Surgery, Faculty of Medicine and Graduate School  
13 of Medicine, Hokkaido University, Kita 15, Nishi 7, Kita-ku, Sapporo 060-8638, Japan.

14 Tel: +81-117066978; Fax: +81-117067827

15 Email: takumaeda1105@yellow.plala.or.jp

16

17 Keywords: lymphedema, roxadustat, FG-4592, laboratory animal models

18

19 **Abstract**

20 **Background:** Lymphedema is an intractable disease with no curative treatment available.

21 Conservative treatment is the mainstay, and new drug treatment options are strongly needed.

22 The purpose of this study was to investigate the effect of roxadustat, a prolyl-4-hydroxylase  
23 inhibitor, on lymphangiogenesis and its therapeutic effect on lymphedema in a radiation-free  
24 mouse hindlimb lymphedema model.

25 **Methods and Results:** Male C57BL/6N mice (8–10 weeks old) were used for the  
26 lymphedema model. Mice were randomized to an experimental group receiving roxadustat or  
27 a control group. The circumferential ratio of the hindlimbs was evaluated and lymphatic flow  
28 of the hindlimbs was compared by fluorescent lymphography up to 28 days postoperatively.  
29 The roxadustat group showed an early improvement in hindlimb circumference and stasis of  
30 lymphatic flow. The number and area of lymphatic vessels on postoperative day 7 were  
31 significantly larger and smaller, respectively, in the roxadustat group compared with the  
32 control group. Skin thickness and macrophage infiltration on postoperative day 7 were  
33 significantly reduced in the roxadustat group compared with the control group. The relative  
34 mRNA expression of hypoxia-inducible factor-1 $\alpha$  (*Hif-1 $\alpha$* ), vascular endothelial growth  
35 factor receptor-3 (*VEGFR-3*), vascular endothelial growth factor-C (*VEGF-C*), and Prospero  
36 homeobox 1 (*Prox1*) on postoperative day 4 were significantly higher in the roxadustat group  
37 compared with the control group.

38 **Conclusions:** Roxadustat demonstrated a therapeutic effect in a murine model of hindlimb  
39 lymphedema via promotion of lymphangiogenesis through the activation of HIF-1 $\alpha$ , VEGF-  
40 C, VEGFR-3, and Prox1, suggesting the potential of roxadustat as a therapeutic option in  
41 lymphedema.

42

### 43 **Condensed Abstract**

44 The therapeutic effects of roxadustat were investigated in a murine model of hindlimb  
45 lymphedema. Mice that received roxadustat showed early improvement in hindlimb  
46 circumference and stasis of lymphatic flow. In specimens harvested from the hindlimb, the  
47 number of lymphatic vessels was significantly larger and the area of the lymphatic vessels  
48 significantly smaller in mice that received roxadustat compared with control mice. The  
49 relative mRNA expression of *HIF-1 $\alpha$* , *VEGF-C*, *VEGFR-3*, and *Prox1* was significantly

50 higher compared with control mice, suggesting that roxadustat may be a promising  
51 therapeutic drug for the treatment of lymphedema.

52

### 53 **Introduction**

54 Lymphedema is a refractory condition caused by decreased lymphatic transport capacity and  
55 characterized by local interstitial fluid accumulation, inflammation, and fatty degeneration of  
56 connective tissue.<sup>1</sup> Symptoms include pain, fatigue, and recurrent infections due to  
57 compromised immunity, which significantly reduces patients' activities of daily living. It  
58 affects 200 million people worldwide<sup>2</sup> and is a major complication after treatment of solid  
59 tumors.

60 Current treatments for lymphedema primarily include compression therapy, manual  
61 lymphatic drainage, and other conservative therapies, rather than rebuilding the destroyed  
62 lymphatic system. Although lymphovenous anastomosis and vascularized lymph node  
63 transplantation have been reported to be effective for lymphedema, only specialized centers  
64 are able to perform these procedures and their effectiveness remains controversial.<sup>3</sup> The  
65 pathophysiology of lymphedema remains to be clarified, and there is an urgent need for new  
66 drug therapies.

67 Roxadustat, a novel hypoxia-inducible factor (HIF) prolyl 4-hydroxylase inhibitor, is  
68 of interest as a treatment for renal anemia in patients with chronic kidney disease. In addition,  
69 it has been reported that roxadustat exerts therapeutic effects on cutaneous wound healing,<sup>4,5</sup>  
70 cardiac disease,<sup>6</sup> retinal disease,<sup>7</sup> spinal cord injury,<sup>8</sup> Parkinson's disease,<sup>9</sup> and malignant  
71 tumor growth<sup>10,11</sup> by stabilizing HIF-1 $\alpha$  expression. These findings the great therapeutic  
72 potential of Roxadustat in diseases related to HIF-1 $\alpha$  signaling.

73 In murine lymphedema, lymphatic stasis and inflammation stabilize HIF-1 $\alpha$  and  
74 result in high levels of HIF-1 $\alpha$  expression, indicating that this is required for reparative

75 lymphangiogenesis.<sup>12</sup> HIF-1 $\alpha$  has also been shown to promote lymphangiogenesis by  
76 inducing the expression of vascular endothelial growth factor receptor-3 (VEGFR-3) and  
77 vascular endothelial growth factor-C (VEGF-C) in lymphatic endothelial cells.<sup>12,13</sup> Although  
78 it has been reported that the deletion or inhibition of HIF-1 $\alpha$  exacerbated edema in a murine  
79 model of tail lymphedema postoperatively,<sup>12,14</sup> HIF-1 $\alpha$  stabilization has not been studied in  
80 an animal model of lymphedema. Therefore, in this study, the therapeutic effects of  
81 roxadustat were investigated in a murine model of hindlimb lymphedema that we previously  
82 developed.<sup>15</sup>

83

## 84 **Materials and Methods**

### 85 *Cell culture and treatment*

86 Endothelial Cell Basal Media MV2 (PromoCell, Heidelberg, Germany) supplemented with  
87 and penicillin-streptomycin and Growth Medium MV2 Supplement Mix (PromoCell) was  
88 used to culture human dermal lymphatic endothelial cells (HDLECs) (PromoCell), and  
89 the HDLECs from passages 3–5 were used. Roxadustat (Selleck Chemicals, Houston, TX)  
90 was dissolved in dimethyl sulfoxide (DMSO) and added to the medium at final  
91 concentrations of 0, 5, 10, and 25  $\mu$ M.

92

### 93 *Proliferation assay*

94 To assess the effect of roxadustat on HDLEC proliferation, a Cell Counting Kit-8 assay  
95 (CCK-8; Dojindo Molecular Technologies, Japan) was performed, following a previously  
96 reported protocol.<sup>4</sup> Briefly, cells were seeded on a 96-well plate at a density of  $2 \times 10^3$   
97 cells/well with 0, 5, 10, or 25  $\mu$ M of roxadustat added to the medium and incubated for 5  
98 days. From days 0–5, 10  $\mu$ L of CCK-8 solution was added to each well, after which the

99 samples were incubated for 60 min at 37°C. The absorbance was detected at 450 nm on the  
100 Infinite 200 PRO microplate reader (Tecan Japan, Kawasaki, Japan).

101

### 102 ***Scratch wound assay***

103 HDLECs were seeded in six-well plates at a density of  $5.0 \times 10^5$  cells/well and cultured at  
104 37°C for 24 h. After a confluent monolayer formed, a sterile pipette tip was used to make a  
105 linear scratch. Phosphate-buffered saline was used to wash away the cellular residue, after  
106 then 2 mL of medium containing 0, 5, 10, or 25  $\mu\text{M}$  roxadustat was added to each well and  
107 the plates were incubated at 37°C. Photographs were taken at 0 and 24 h, and the amount by  
108 which each scratch had closed and the migration rate were determined using ImageJ software  
109 (National Institutes of Health, Bethesda, MD).

110

### 111 ***Tube formation assay***

112 Pretreatment of HDLECs with 0, 5, 10, or 25  $\mu\text{M}$  roxadustat was carried out for 24 h. Then,  
113 the HDLECs were mixed with medium and seeded in a 24-well plate precoated with 100  $\mu\text{L}$   
114 of Geltrex (Life Technologies, Grand Island, NY) at  $8 \times 10^4$  cells/well and incubated at 37°C  
115 for 6 h. After microscopic observation, the total length of each tube was calculated using  
116 ImageJ software (National Institutes of Health).<sup>4</sup>

117

### 118 ***Animals***

119 All experiments involving animals in this study were approved by the Hokkaido University  
120 Institutional Animal Care and Use Committee. Male C57BL/6N mice, 8–10 weeks of age  
121 (SLC, Tokyo, Japan), were kept in a room maintained at 24°C under a 12-h light/dark cycle  
122 with free access to food and water.

123

124 ***Murine Model and Drug Administration***

125 The murine model of hindlimb lymphedema was created using our previously established  
126 method.<sup>15</sup> Briefly, a circumferential incision was made in the left inguinal skin, followed by  
127 resection of the inguinal lymph node and surrounding fat pad. After tying the prenodal and  
128 postnodal lymphatic vessels, the popliteal lymph node and surrounding fat pad were resected.  
129 A 1-mm-thick silicone sheet was used to fashion a 3-mm-wide rectangular splint, which was  
130 then placed in the inguinal wound and affixed to the skin and underlying muscle. The mice  
131 were randomly assigned to either an experimental group receiving 25 mg/kg roxadustat  
132 (Selleck Chemicals) in DMSO<sup>4,7</sup> or a control group receiving only DMSO and were given  
133 intraperitoneal injections every 2 days, including the day of surgery, for up to 2 weeks. A total  
134 of 36 mice were used and sacrificed on day 28 (12 mice for edema assessment and  
135 fluorescence lymphatic imaging), on day 7 (12 mice for histology), and on day 4 (12 mice for  
136 RNA isolation).

137

138 ***Edema Assessment***

139 Lymphedema formation in the hindlimb was evaluated quantitatively on days 0, 2, 4, 7, 10,  
140 14, 17, 21, 24, and 28 after surgery ( $n = 6$  per group), at which time the circumference of the  
141 musculotendinous junction of the gastrocnemius muscle was measured bilaterally, and the  
142 circumference ratio was calculated according to the following formula.

143 Circumference ratio = (Treated hindlimb circumference / Untreated hindlimb circumference)  
144  $\times 100\%$ .

145

146 ***Fluorescence Lymphatic Imaging***

147 Lymphatic structures in the hindlimbs of the control and roxadustat groups were compared by  
148 fluorescent lymphography every week for 4 weeks postoperatively ( $n = 6$  per group). Prior to

149 imaging, isoflurane was used to anesthetize the mice and their residual fur was removed. A 5-  
150  $\mu\text{L}$  volume of indocyanine green solution (2.5 mg/mL Diagnogreen in distilled water; Daiichi  
151 Sankyo Company, Ltd., Tokyo, Japan;) was subcutaneously injected into both paws with a  
152 26-gauge needle. Imaging was performed indocyanine green using a near-infrared  
153 fluorescence camera system (Photodynamic Eye; Hamamatsu Photonics, Hamamatsu, Japan)  
154 15 min after indocyanine green injection. The coverage of the fluorescent area (i.e., the  
155 dermal backflow at the thigh) was measured using ImageJ software (National Institutes of  
156 Health).

157

### 158 *Histology*

159 Six mice per group were sacrificed at 7 days after surgery and skin samples were obtained.  
160 To minimize the impact of inflammation due to wound healing, the samples were taken from  
161 an area 6 mm distal to the inguinal wound. Then, skin sections were fixed in 4%  
162 paraformaldehyde, embedded in paraffin, and subjected to immunohistochemical or Elastica–  
163 Masson staining. Immunohistochemical staining was carried out to evaluate lymphatic  
164 endothelial cells and macrophage infiltration. Sections were incubated overnight with  
165 antibodies against LYVE-1 (Abcam, Inc., Cambridge, MA) and F4/80 (Cedarlane,  
166 Burlington, Canada). Histofine (Nichirei, Tokyo, Japan), which was developed using 3,3'-  
167 diaminobenzidine, was used as the secondary antibody. Elastica–Masson staining was  
168 performed to evaluate skin thickness. A whole-slide scanner (Nano Zoomer Digital  
169 Pathology; Hamamatsu Photonics) was used to capture digital images of the slides, which  
170 were then visualized using NDP.view2 software (Hamamatsu Photonics). In accordance with  
171 a previous report,<sup>16</sup> lymphatic vessels were defined as vessels with an immunopositive  
172 endothelium and a vascular lumen. Lymphatic vessels were identified by two examiners other  
173 than the first author after the specimens were randomized, and those identified by both



174 examiners were designated as lymphatic vessels. LYVE-1-stained sections were scanned at  
175 low magnification (40×) and “hot spots” with the greatest number of lymphatic vessels were  
176 noted. Then, five of these areas in each section were observed at high magnification (200×)  
177 and the average number of lymphatic vessels within the hot spots in each field of view was  
178 calculated.<sup>17</sup> Additionally, the average area of the lymphatic lumen in each field of view was  
179 calculated using ImageJ software (National Institutes of Health). Quantification of the F4/80  
180 positive area was performed using ImageJ software (National Institutes of Health) by  
181 observing 10 randomly selected high-magnification fields of view (400×). The thickness of  
182 skin in the roxadustat and control groups was measured as the distance from the epidermis to  
183 the dermal-fat junction by two examiners other than the first author, using ImageJ software  
184 (National Institutes of Health) after the specimens were randomized.

185

#### 186 ***RNA Isolation and Real-Time Polymerase Chain Reaction***

187 To isolate the RNA, 6 mice per group were sacrificed at 4 days after surgery. Skin and  
188 subcutaneous tissue samples were collected from the hindlimb at an area 6 mm distal to the  
189 inguinal wound and frozen. RNeasy Fibrous Tissue Mini Kit (Qiagen, Hilden, Germany) was  
190 used to extract total RNA from the skin samples, after which a High Capacity RNA-to-cDNA  
191 Kit (Applied Biosystems, Foster City, CA) was used to reverse-transcribe the RNA to cDNA.  
192 Next, Power SYBR Green PCR Master Mix and a StepOnePlus Real-Time PCR System  
193 (Applied Biosystems) were used to perform real-time quantitative reverse transcription  
194 polymerase chain reaction (PCR) and the relative levels of PCR products were calculated  
195 using the  $\Delta\Delta C_t$  method.<sup>15</sup> Examinations of each sample was were performed three times.  
196 Primers for reverse transcription PCR are listed in Table 1.

197

#### 198 ***Statistical Analysis***

199 Differences between the two groups were analyzed by Student's *t*-test, while differences  
200 among three or more groups were analyzed by one-way analysis of variance followed by a  
201 Tukey–Kramer multiple comparisons test. Results from multiple experiments are expressed  
202 as mean values  $\pm$  standard error. All statistical analyses was performed using JMP software  
203 ver. 16.0.0 (SAS Institute, Inc., Cary, NC), with  $p < 0.05$  considered to indicate statistical  
204 significance.

205

## 206 **Results**

### 207 *In vitro* assays

208 Roxadustat was found to stimulate the proliferation of HDLECs in a dose-dependent manner  
209 (Fig. 1A) and significantly enhanced their motility ( $p < 0.05$ ) (Fig. 1B, C). Roxadustat also  
210 significantly enhanced HDLEC tube formation ( $p < 0.05$ ) (Fig. 1D, E).

### 211 *Edema Assessment*

212 Edema disappeared grossly on days 21 and 28 in the roxadustat and control groups,  
213 respectively (Fig. 2A). The hindlimb circumference ratio reached a maximum on day 4 in  
214 both groups and then gradually approached 100%. As in our previous study,<sup>15</sup> the hindlimb  
215 circumference ratio reached 100% on day 28 in the control group and on day 21 in the  
216 roxadustat group. The circumference ratio was significantly lower in the roxadustat group  
217 compared with the control group from postoperative days 2–24 (Fig. 2B).

218

### 219 *Fluorescence Lymphatic Imaging*

220 The control group showed diffuse dermal backflow throughout the treated hindlimb from 1–4  
221 weeks postoperatively. In contrast, the roxadustat group showed dermal backflow, but with  
222 lower fluorescence compared with the control group (Fig. 3A). Compared with the control

223 group, the coverage of the fluorescent area at the thigh at 2 weeks postoperatively was  
224 significantly reduced in the roxadustat group ( $*p < 0.05$ ) (Fig. 3B).

225

## 226 ***Histology***

227 The roxadustat group had more lymphatic vessels (Fig. 4A, B) and a smaller lymphatic  
228 lumen area (Fig. 4A, C) compared with control group. There were also significantly more  
229 LYVE-1-positive lymphatic vessels in the roxadustat group ( $8.5 \pm 0.5$ ) compared with the  
230 control group ( $5.2 \pm 0.4$ ) (Fig. 4B,  $p < 0.05$ ). The area of lymphatic lumen in the roxadustat  
231 group ( $334 \pm 38 \mu\text{m}^2$ ) was significantly less than that in the control group ( $1,385 \pm 163 \mu\text{m}^2$ )  
232 (Fig. 4C,  $p < 0.05$ ). Furthermore, the F4/80-positive area was significantly smaller in the  
233 roxadustat group ( $1.9 \pm 0.3 \%$ ) compared with the control group ( $3.1 \pm 0.4 \%$ ) ( $*p < 0.05$ )  
234 (Fig. 5A, B). Skin thickness in the roxadustat group ( $240 \pm 18 \mu\text{m}$ ) was significantly less than  
235 that in the control group ( $296 \pm 12 \mu\text{m}$ ) ( $*p < 0.05$ ) (Fig. 5C, D).

236

## 237 ***Real-Time PCR***

238 The relative mRNA expression levels of *Hif-1 $\alpha$* , *VEGFR-3*, *VEGF-C*, and Prospero  
239 homeobox 1 (*Prox1*) were significantly higher in the roxadustat group ( $1.6 \pm 0.2$ ,  $4.3 \pm 0.7$ ,  
240  $2.5 \pm 0.3$ , and  $2.8 \pm 0.4$ , respectively) compared with the control group (Fig. 6,  $p < 0.05$ ).

241

## 242 **Discussion**

243 In this study, we used a novel radiation-free murine model of hindlimb lymphedema, which  
244 we had previously developed.<sup>15</sup> The lack of appropriate animal models is one of the reasons  
245 why the elucidation of the pathogenesis of lymphedema has been hampered. Many studies of  
246 lymphedema in animal models have been conducted mainly in mouse-tail<sup>12,14</sup> and hindlimb  
247 edema models,<sup>18,19</sup> but because the mouse-tail edema model does not involve lymph-node

248 excision and many murine models of hindlimb edema use radiation, a suitable clinical  
249 lymphedema animal model for molecular biological studies did not exist. Our lymphedema  
250 model does not use radiation, thus eliminating its effects. In addition, the lymph node  
251 excision model more closely mimics clinical lymphedema.

252         Although there have been several reports on the most appropriate circumferential  
253 measurement site for hindlimb lymphedema, including a point located at 5 or 6 mm from the  
254 heel,<sup>15,18</sup> we took circumferential measurements at the musculotendinous junction of the  
255 gastrocnemius muscle to ensure anatomic consistency at each time point,<sup>17</sup> taking into  
256 account the growth of the mice during the experiment, and evaluated it in comparison with  
257 the untreated side.

258         The circumferential ratio, which indicates the degree of edema, improved faster in  
259 the roxadustat group compared with the control group. Fluorescent imaging evaluation of  
260 lymphatic flow showed significantly lower fluorescence after 2 weeks in the roxadustat  
261 group, whereas dermal backflow, which is indicative of lymphatic stasis, was diffuse  
262 throughout the hindlimb over the 4-week postoperative period in the control group,  
263 suggesting that lymphatic stasis was improved in the roxadustat group. Yamamoto et al.<sup>20</sup>  
264 reported that the degree of dermal backflow was positively correlated with clinical severity,  
265 and the present results are consistent with theirs. The formation of lymphatic collateral  
266 vessels and enhanced function of the draining collecting vessels were considered as possible  
267 reasons for the reduced dermal backflow in the roxadustat group, but neither could be  
268 confirmed in our study due to the low resolution of the fluorescence camera system.

269         Microscopically, dilation of lymphatic vessels has been reported in the early stages  
270 of lymphedema,<sup>21</sup> and our previous study involving a murine model of hindlimb lymphedema  
271 revealed cutaneous and subcutaneous lymphatic dilation.<sup>15</sup> In the present study, we found that  
272 on postoperative day 7, the number of cutaneous and subcutaneous lymphatic vessels was

273 significantly larger and their lumen area was significantly smaller in the roxadustat group  
274 compared with the control group. This suggests that lymphangiogenesis was more  
275 pronounced in the roxadustat group and that the dilation of lymphatic vessels, which  
276 indicates lymphatic stasis, was improved. In addition, skin thickness in the roxadustat group  
277 was significantly less than that in the control group. Furthermore, significantly less  
278 macrophage infiltration was found in the roxadustat group than in the control group, which is  
279 consistent with previous reports.<sup>22</sup>

280 HIF-1 is a transcriptional activator of various genes that play a role in the adaptive  
281 response of cells to hypoxia. Dysfunction in systems that regulate HIF-1 activity has been  
282 implicated in the pathogenesis of malignancies and other diseases.<sup>23</sup> HIF-1 has a  
283 heterodimeric structure comprising oxygen-sensitive HIF-1 $\alpha$  and constitutively expressed  
284 HIF-1 $\beta$  subunits. Under normoxic conditions, the ubiquitin–proteasome pathway rapidly  
285 degrades the HIF-1 $\alpha$  subunit following hydroxylation of proline residues by HIF prolyl-4-  
286 hydroxylases.<sup>7</sup> The HIF system targets a wide range of genes, and in particular, increased  
287 HIF expression via prolyl hydroxylation domain protein inhibitors such as roxadustat has  
288 been reported to have therapeutic effects in cutaneous wound healing,<sup>4,5</sup> cardiac disease,<sup>6</sup>  
289 retinal disease,<sup>7</sup> spinal cord injury,<sup>8</sup> Parkinson's disease,<sup>9</sup> and malignant tumors,<sup>10,11</sup> and is  
290 attracting attention as a new pharmacological target.

291 HIF-1 $\alpha$  promotes lymphangiogenesis by inducing VEGFR-3 and VEGF-C  
292 expression in lymphatic endothelial cells<sup>12,13</sup> and by increasing VEGFR-3 expression through  
293 the transcriptional activation of *Prox1*.<sup>24</sup> It has also been reported that inhibition or deletion  
294 of HIF-1 $\alpha$  exacerbates edema in a murine model of tail lymphedema postoperatively.<sup>12,14</sup>  
295 These findings suggest that HIF-1 $\alpha$  expression may be involved in postoperative lymphatic  
296 regeneration. In the present study, *Hif-1 $\alpha$* , *VEGFR-3*, *VEGF-C*, and *Prox1* mRNA expression  
297 levels were significantly elevated in the roxadustat group, suggesting that roxadustat

298 stabilizes *Hif-1α* expression and promotes lymphangiogenesis via the increased expression of  
299 *VEGFR-3*, *VEGF-C*, and *Prox1*.

300 VEGF-C is a critical regulator of lymphangiogenesis and controls numerous  
301 lymphatic cell processes via VEGFR-3 signaling, including differentiation, migration, and  
302 survival.<sup>25</sup> It has been shown that the local administration of VEGF-C or gene therapy  
303 involving adenoviral vectors significantly increases lymphangiogenesis and decreases  
304 swelling in animal models of both primary and secondary lymphedema.<sup>26,27</sup> However, the  
305 application of VEGF-C therapy in the clinical setting has yet to be realized because VEGF-C  
306 acts as a key regulator of the tumor microenvironment, potentially increasing the risk of  
307 recurrence and metastasis.<sup>25,28</sup>

308 Previous studies have reported that Roxadustat is noncarcinogenic<sup>29</sup> and it was found  
309 not to promote the initiation, progression, or metastasis of tumors in a VEGF-sensitive  
310 spontaneous breast cancer model.<sup>30</sup> Meanwhile, a phase 2 study is being conducted to  
311 evaluate roxadustat for the treatment of anemia in patients receiving chemotherapy for non-  
312 myeloid malignancies (NCT04076943). In addition, there are reports that roxadustat inhibits  
313 tumor growth by enhancing macrophage phagocytosis of malignant tumors<sup>10</sup> and by  
314 increasing sensitivity to chemotherapy and inhibiting tumor growth in a murine model of  
315 malignancy.<sup>11</sup> Although roxadustat is reported to have anti-tumor effects as described above,  
316 an increase in *VEGF-C* mRNA expression was also found in the present study, and thus its  
317 effects on malignant tumors warrant further study.

318 The present study has some limitations that merit consideration. First, the murine  
319 model of hindlimb lymphedema examined in this study does not address chronic  
320 lymphedema and is based on acute lymphedema. Human lymphedema progresses slowly and  
321 does not improve spontaneously, whereas in animal models, it often resolves spontaneously.  
322 In this model, gross edema resolves in approximately 1 month. Therefore, although this

323 model anatomically approximates human lymphedema, it is not a perfect mimic, including  
324 the course of the disease. Second, although data in numerous animal studies have  
325 demonstrated that roxadustat does not induce carcinogenesis<sup>29</sup> or promote cancer,<sup>30</sup> further  
326 safety verification through clinical trials is needed to ensure its safety against malignant  
327 tumors in the clinical context.

328

### 329 **Conclusions**

330 In summary, roxadustat exerted a therapeutic effect in a murine model of hindlimb  
331 lymphedema by promoting lymphangiogenesis via activation of HIF-1 $\alpha$ , VEGFR-3, VEGF-  
332 C, and Prox1. This suggests that roxadustat induces reparative lymphangiogenesis against  
333 impaired lymphatic function and thus shows promise as a therapeutic option for lymphedema.

334

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337 study.

338

### 339 **Author Contributions**

340 Yoshitada Hoshino: Conceptualization, Investigation, Data curation, Writing- Original draft  
341 preparation

342 Masayuki Osawa: Methodology, Funding acquisition

343 Emi Funayama: Methodology, Validation

344 Kosuke Ishikawa: Supervision, Data curation

345 Takahiro Miura: Validation, Visualization

346 Masahiro Hojo: Data curation, Formal analysis

347 Yuhei Yamamoto: Conceptualization, Project administration

348 Taku Maeda: Conceptualization, Investigation, Supervision, Validation

349

### 350 **Author Disclosure Statement**

351 No competing financial interests exist.

352

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434

435 **Figure Legends**

436 **FIG. 1.** Effects of roxadustat on human dermal lymphatic endothelial cells (HDLECs) *in*  
437 *vitro*. **(A)** Roxadustat stimulated HDLEC proliferation in a dose-dependent manner. **(B)**  
438 Effects of roxadustat on HDLEC migration. *Scale bar* = 200  $\mu\text{m}$ . **(C)** Roxadustat  
439 significantly enhanced HDLEC migration in a dose-dependent manner. *Error bars* = standard  
440 error of the mean. **(D)** Effects of roxadustat on the HDLEC tube formation. *Scale bar* = 200  
441  $\mu\text{m}$ . **(E)** Roxadustat significantly enhanced HDLEC tube formation at concentrations of 10  
442 and 25  $\mu\text{M}$ . *Error bars* = standard error of the mean. **(A, C, E)**  $*p < 0.05$  compared with the  
443 control group,  $\dagger p < 0.05$  compared with the 5- $\mu\text{M}$  roxadustat treatment group,  $\ddagger p < 0.05$   
444 compared with the 10- $\mu\text{M}$  roxadustat treatment group.

445

446 **FIG. 2.** Effects of roxadustat on edema. **(A)** Representative images of the murine model of  
447 hindlimb lymphedema in both groups. Hindlimb circumference was measured at the  
448 musculotendinous junction of the gastrocnemius muscle (arrowhead). **(B)** Edema assessment  
449 demonstrated that the roxadustat group had a lower circumference ratio than the control  
450 group from postoperative days 2 to 24 ( $*p < 0.05$ ). *Error bars* = standard error of the mean.

451

452 **FIG. 3.** Effects of roxadustat on lymphatic flow. **(A)** Representative images of fluorescent  
453 lymphography in both groups at postoperative weeks 1–4. In the treated hindlimb,  
454 fluorescence was observed extensively at the thigh (*area outlined in yellow*). The popliteal  
455 lymph node (*arrowhead*) was identified in the untreated hindlimb. **(B)** The coverage of the  
456 fluorescent area at the thigh in the roxadustat group was significantly less than that in the  
457 control group at postoperative weeks 2–4 ( $*p < 0.05$ ). *Error bars* = standard error of the  
458 mean.

459

460 **FIG. 4.** Effects of roxadustat on lymphatic vessels. **(A)** Representative photomicrographs of  
461 LYVE-1 immunohistochemical staining in the control group (*left*) and the roxadustat group  
462 (*right*) at postoperative day 7. *Scale bars* = 250  $\mu\text{m}$ . **(B)** The average number of lymphatic  
463 vessels was significantly larger in the roxadustat group compared with the control group ( $*p$   
464  $< 0.05$ ). *Error bars* = standard error of the mean. **(C)** The average area of lymphatic vessels  
465 in the roxadustat group was smaller than that in the control group ( $*p < 0.05$ ). *Error bars* =  
466 standard error of the mean.

467

468 **FIG. 5.** Effects of roxadustat on macrophage infiltration and skin thickness. **(A)**  
469 Representative photomicrographs of F4/80 immunohistochemical staining in the control  
470 group (*left*) and the roxadustat group (*right*) at postoperative day 7. *Scale bars* = 250  $\mu\text{m}$ . **(B)**  
471 The F4/80-positive area in the roxadustat group was significantly less than that in the control  
472 group ( $*p < 0.05$ ). *Error bars* = standard error of the mean. **(C)** Representative  
473 photomicrographs of Elastica–Masson staining in the control group (*left*) and the roxadustat  
474 group (*right*) at postoperative day 7. *Scale bars* = 250  $\mu\text{m}$ . **(D)** Skin thickness in the  
475 roxadustat group was significantly less than that in the control group ( $*p < 0.05$ ). *Error bars*  
476 = standard error of the mean.

477

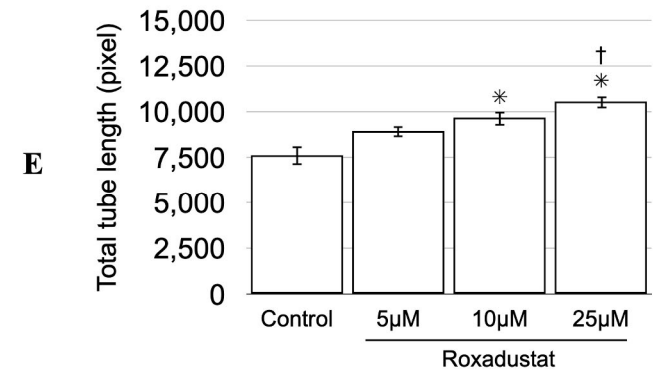
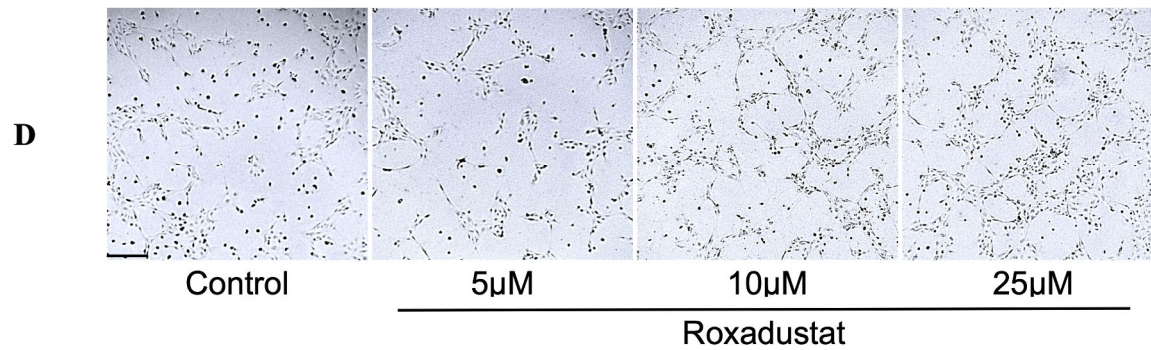
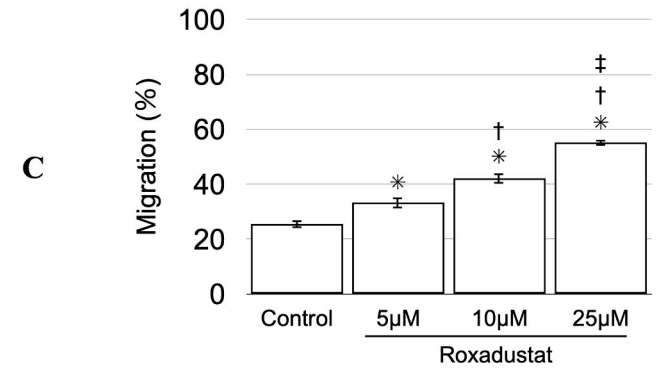
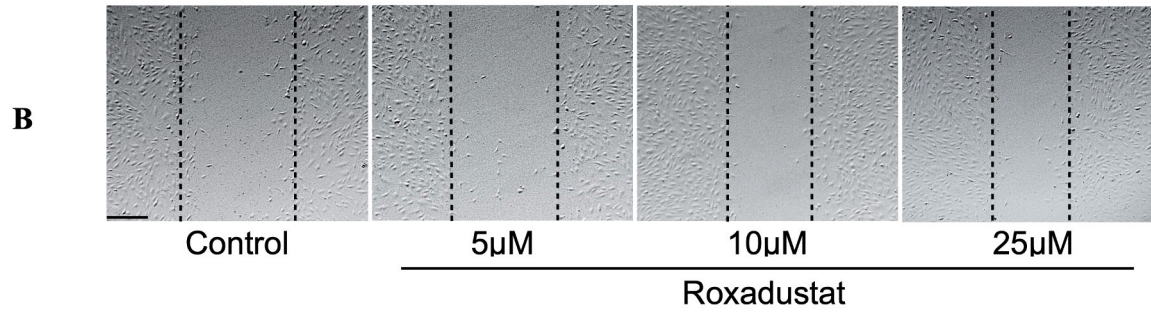
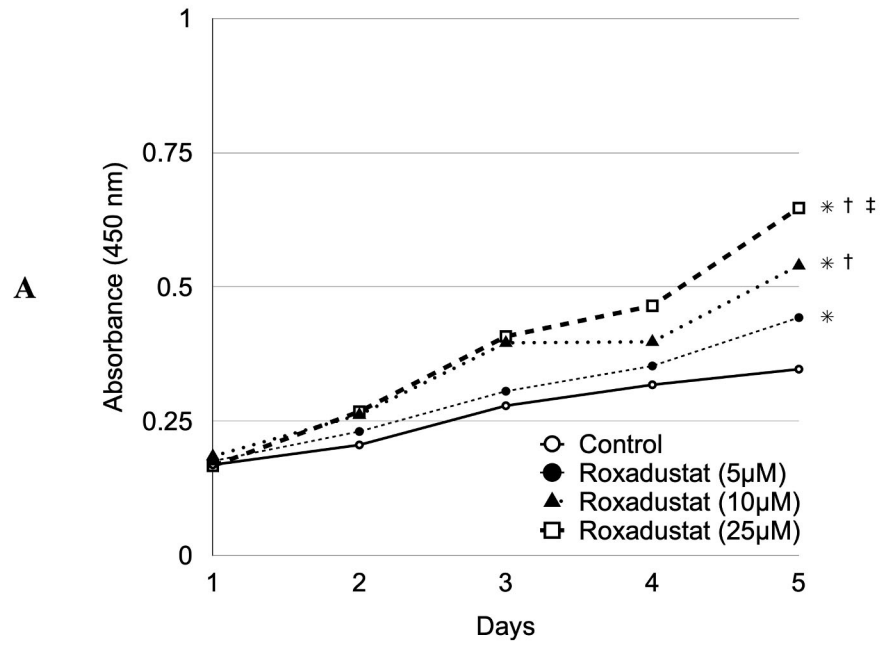
478 **FIG. 6.** Real-time reverse transcription PCR analysis of the skin and subcutaneous tissues  
479 demonstrated that *Hif-1 $\alpha$* , *VEGF-C*, *VEGFR-3*, and *Prox1* were expressed at significantly  
480 higher levels in the roxadustat group compared with the control group at postoperative day 4  
481 ( $*p < 0.05$ ). *Error bars* = standard error of the mean.

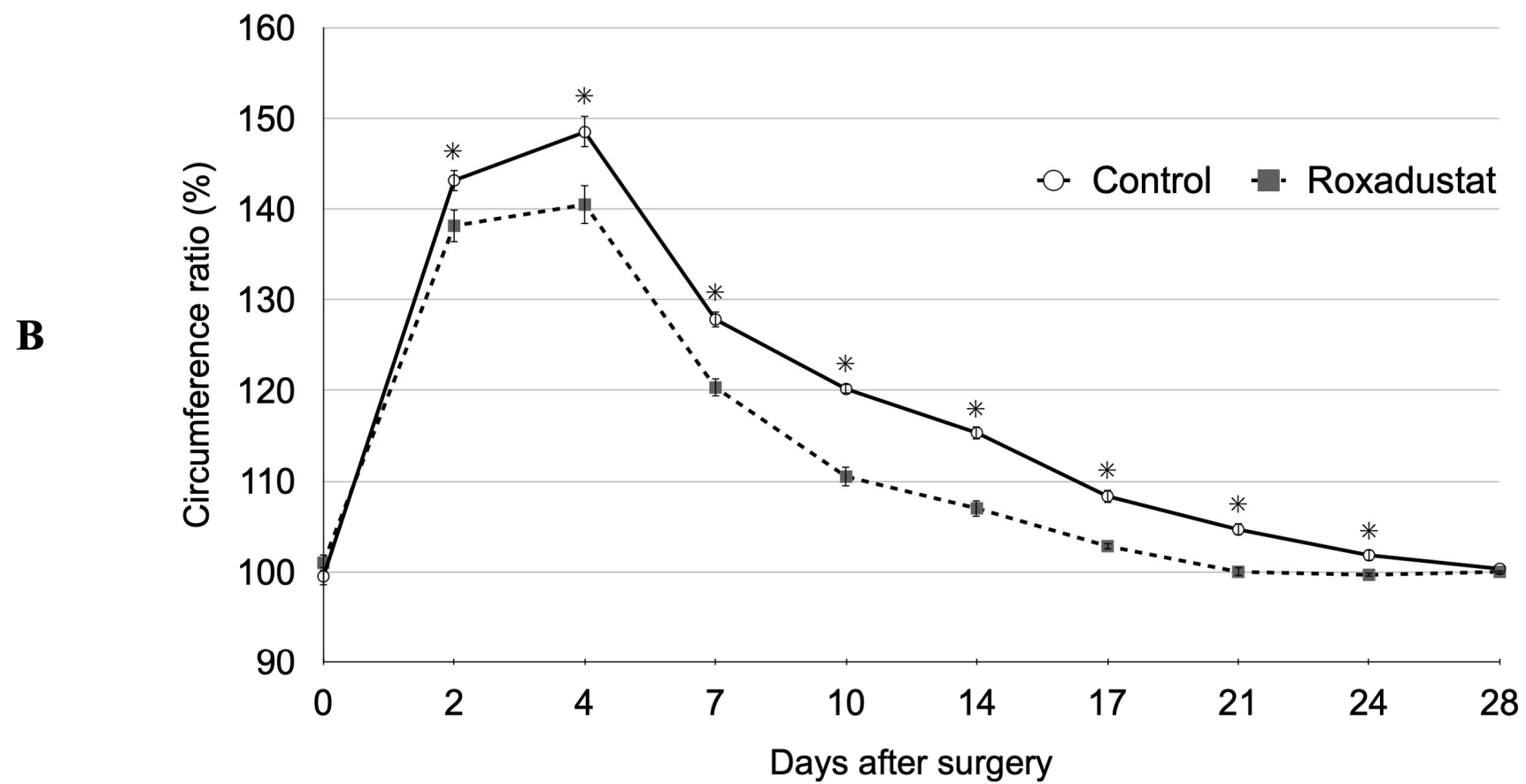
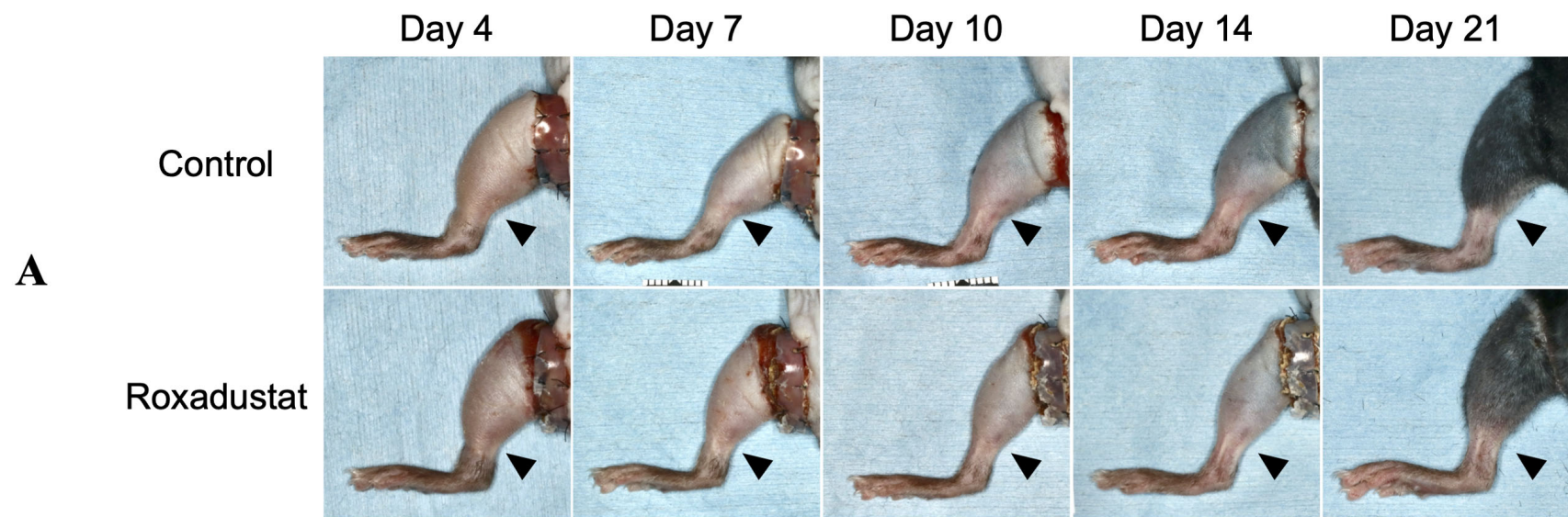
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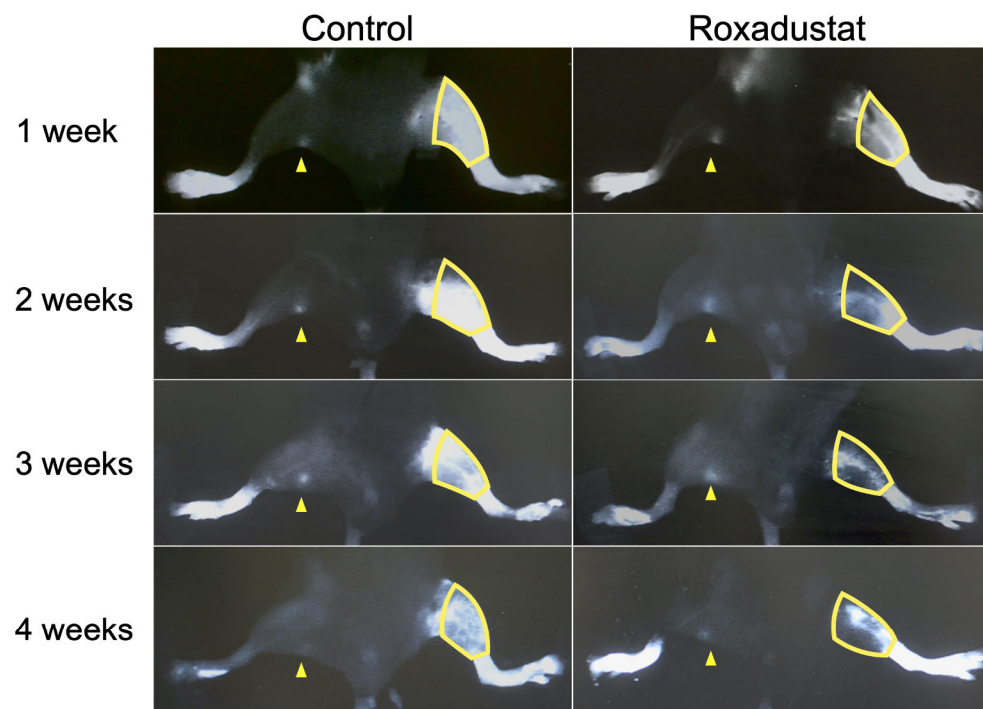
483 **Table 1. Reverse-transcription PCR primer sequences and product size**

Gene	Accession number	Primer sequences	Size (bp)
<i>Hif-1<math>\alpha</math></i>	NM_010431.2	MA216271-F: TGCGTGCATGTCTAATCTGTTCC MA216271-R: AAGATTCTGACATGCCACATAGCTC	102
<i>VEGF-C</i>	NM_009506.2	MA241730-F: TGCTGCTGCACATTATAACACAGA MA241730-R: CGGACACACATGGAGGTTTAAAGA	149
<i>VEGFR-3</i>	NM_008029.3	MA227448-F: TGGGCGACAGGGTTCTCATA MA227448-R: GACATGGTGGCTCTGGTCTAACTC	85
<i>Prox1</i>	NM_008937.3	MA209433-F: AGCCAGTGTTTAATCTTTGCATCC MA209433-R: AACCATTTGCCTGCTCATTCC	145
<i>GAPDH</i>	NM_008084.3	MA050371-F: TGTGTCCGTCGTGGATCTGA MA050371-R: TTGCTGTTGAAGTCGCAGGAG	150

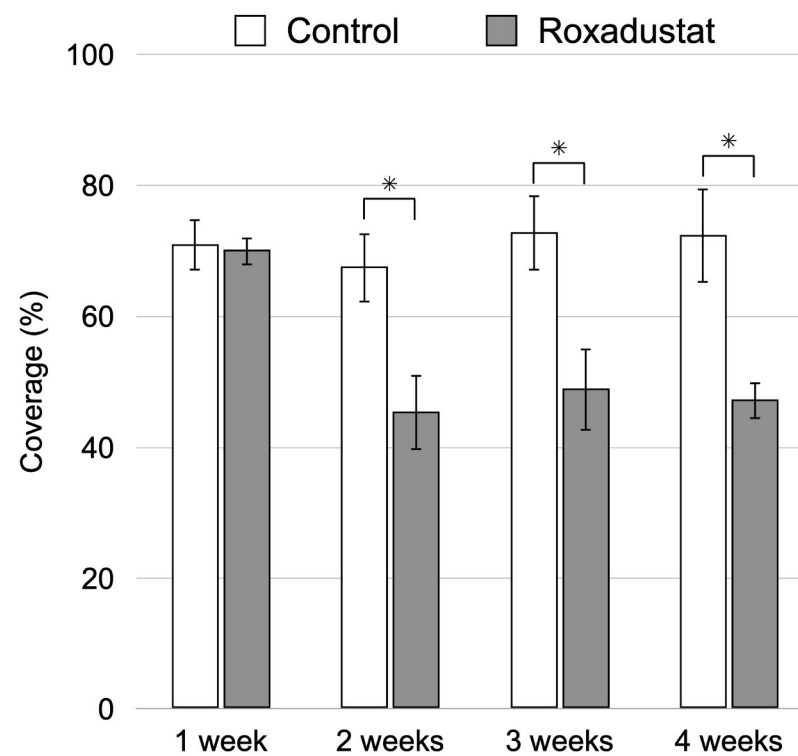
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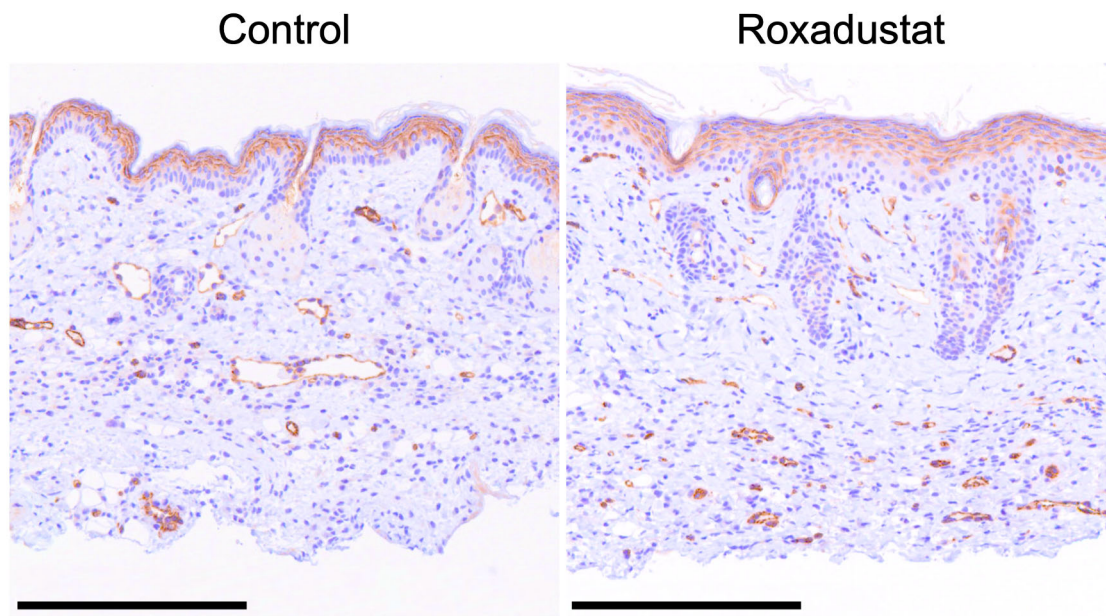


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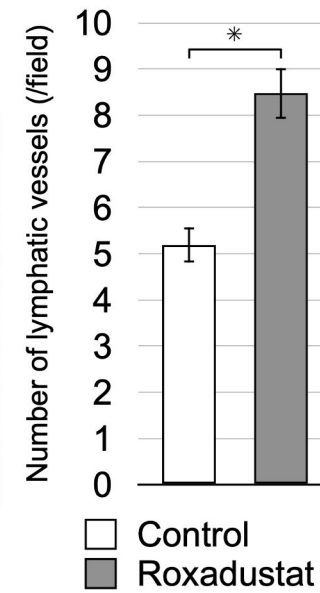


**B**

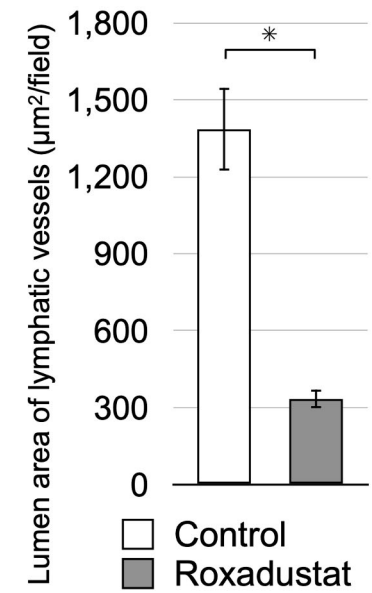




**A**



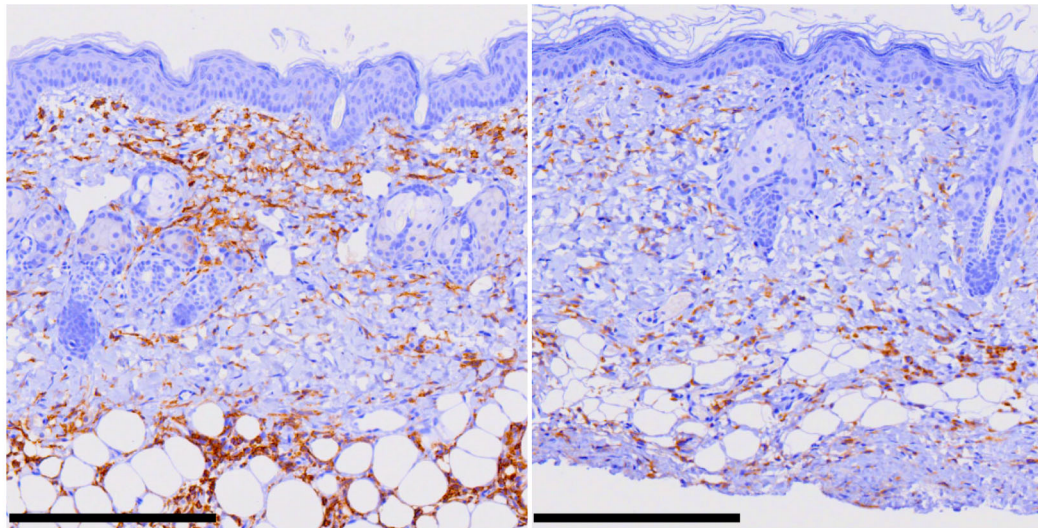
**B**



**C**

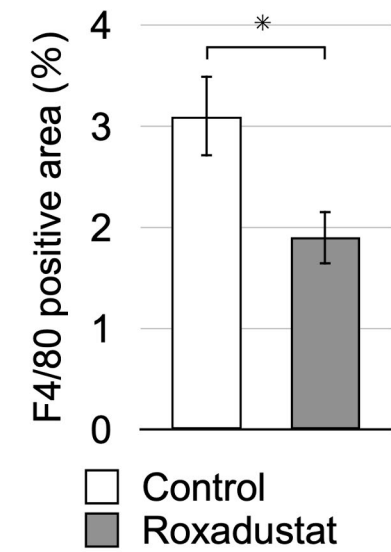
**A**

F4/80



Control

Roxadustat

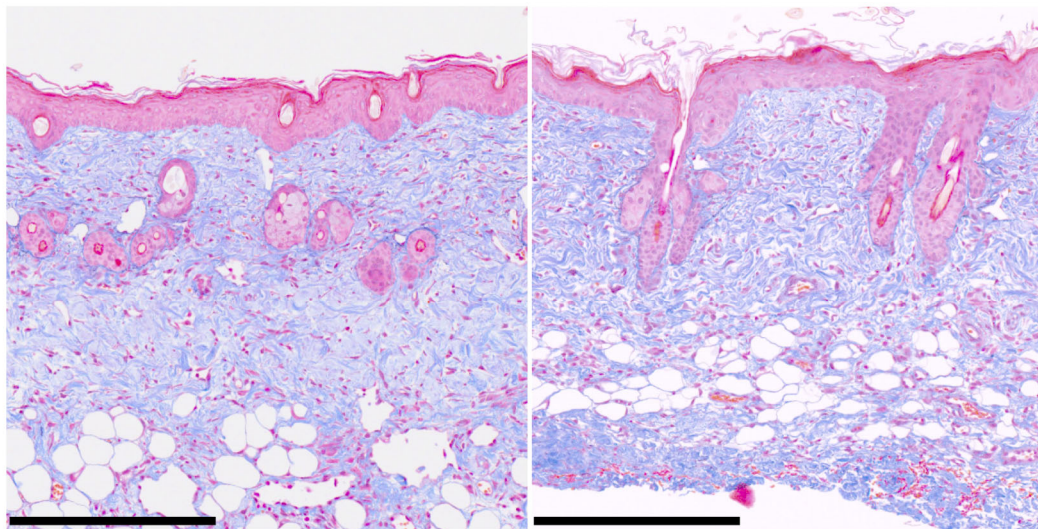
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Control

Roxadustat

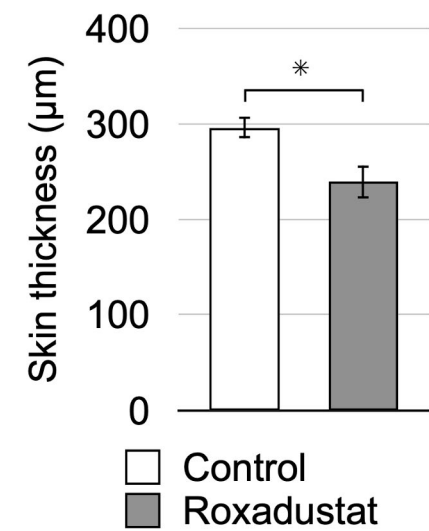
**C**

Elastica-Masson



Control

Roxadustat

**D**

Control

Roxadustat

