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Basic Research

ClC-3 Deficiency Impairs the Neovascularization Capacity of Early Endothelial Progenitor Cells by Decreasing CXCR4/JAK-2 Signalling

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ABSTRACT

Background: Endothelial progenitor cell (EPC) therapy has been suggested as a major breakthrough in the treatment of ischemic diseases. However, the molecular mechanism that underlies EPC functional regulation is still unclear.

Methods: We examined the angiogenic capacity of EPCs in a hindlimb ischemia model of wild-type and ClC-3 knockout mice.

Results: Mice lacking of ClC-3 exhibited reduced blood flow recovery and neovascularization in ischemic muscles 7 and 14 days after hind limb ischemia. Moreover, compared with wild-type EPCs, the hindlimb blood reperfusion in mice receiving ClC-3 knockout EPCs was significantly impaired, accompanied by reduced EPC homing and retention. *In vitro*, EPCs derived from ClC-3 knockout mice displayed impaired migratory, adhesive, and angiogenic activity. CXC chemokine receptor 4 (CXCR4) expression was significantly reduced in EPC from ClC-3

RÉSUMÉ

Contexte : Le traitement des maladies ischémiques au moyen de cellules progénitrices endothéliales (CPE) a été présenté comme une percée thérapeutique majeure. Cependant, le mécanisme moléculaire qui sous-tend la régulation fonctionnelle mettant en jeu les CPE n'est toujours pas élucidé.

Méthodologie : Nous avons examiné la capacité angiogénique des CPE dans un modèle d'ischémie des membres postérieurs chez des souris de type sauvage et des souris *knock-out* (KO) ClC-3.

Résultats : Chez les souris KO ClC-3, le rétablissement du débit sanguin et la néovascularisation étaient réduits dans les muscles ischémiques 7 et 14 jours après l'ischémie des membres postérieurs. De plus, une limitation considérable de la reperfusion des membres postérieurs a été observée parallèlement à une diminution de la nostocytose et de la rétention des CPE chez les souris KO ClC-3

Neovascularization is an important repair mechanism for the treatment of ischemic cardiovascular diseases.[1] Since being identified from human peripheral blood in 1997, endothelial progenitor cells (EPCs), despite the controversy on the nomenclature,[2,3] have been widely studied and suggested to play a critical role in postnatal physiologic and pathophysiologic vasculogenesis.[4] By homing to the ischemic sites, EPCs have been shown to promote tissue vascularization and function recovery of heart, brain, wound, and limbs after ischemic injury in numerous EPC-related animal experiments and human clinical studies.[5-8] EPC therapy is now known as an important novel endogenous vascular repair strategy and a major breakthrough in the treatment of ischemic diseases. However, the efficiency of EPC therapy depends largely on the functional activity of EPCs. In the presence of cardiovascular risk factors, impairment in function and number of circulating EPCs has been reported.[9,10] Therefore, identifying the factors affecting the biologic activity of EPCs is essential for development of new therapeutic strategies for ischemic cardiovascular diseases but has thus far been a challenge.

ClC-3, a member of the voltage-gated chloride channel family, has been found to be ubiquitously expressed in almost all eukaryotic cells.[11,12] ClC-3 has been demonstrated to be critical, by acting as Cl^- channel or Cl^-/H^+ antiporter at the cell plasma membrane or intracellular vesicles, in the regulation of a variety of physiologic activities, including cell volume, proliferation, differentiation, migration, and apoptosis.[13,14] Recently, we also found that ClC-3 plays an essential role in the pathologic process of a variety of cardiovascular diseases, such as vascular remodelling, endothelial inflammation, and atherosclerosis.[15-17] However, determining whether ClC-3 has a role in EPC functional activity in response to tissue ischemia in ischemic cardiovascular diseases remains elusive. In the present study, we therefore isolated and cultured EPC from bone marrow of wild-type (WT) and ClC-3 knockout

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knockout mice compared with wild-type. Moreover, the expression and phosphorylation of Janus kinase 2 (JAK-2), a downstream signalling of CXCR4, was also reduced in ClC-3 knockout EPC, indicating that CXCR4/JAK-2 signalling is dysregulated by ClC-3 deficiency. Consistent with this assumption, the migratory capacity of wild-type EPCs was attenuated by either CXCR4 antagonist AMD3100 or JAK-2 inhibitor AG490. More importantly, the impaired migratory capacity of ClC-3 knockout EPCs was rescued by overexpression of CXCR4.

Conclusions: ClC-3 plays a critical role in the angiogenic capacity of EPCs and EPC-mediated neovascularization of ischemic tissues. Disturbance of CXCR4/JAK-2 signalling may contribute to the functional impairment of ClC-3 deficient EPCs. Thus, ClC-3 may be a potential therapeutic target for modulating neovascularization in ischemic diseases.

comparativement aux souris de type sauvage. *In vitro*, les CPE de souris KO ClC-3 présentaient une activité migratoire, adhésive et angiogénique déficiente. L'expression des récepteurs des chimiokines CXC de type 4 (CXCR4) dans les CPE se trouvait significativement réduite dans le cas des souris KO ClC-3 comparativement aux souris de type sauvage. En outre, l'expression et la phosphorylation par les CPE de la Janus kinase 2 (JAK-2), intervenant dans la signalisation en aval des CXCR4, était aussi réduite chez les souris KO ClC-3, ce qui indique qu'une déficience touchant le ClC-3 provoque le dérèglement de la signalisation CXCR4/JAK-2. Conformément à cette hypothèse, la capacité migratoire des CPE chez les souris de type sauvage était atténuée par l'AMD3100, un antagoniste des CXCR4, ou l'AG490, un inhibiteur de la JAK-2. Plus important encore, la surexpression de CXCR4 est venue compenser la déficience migratoire des CPE chez les souris KO ClC-3.

Conclusions : Le ClC-3 joue un rôle essentiel dans la capacité angiogénique des CPE et la néovascularisation des tissus ischémiques mettant en jeu les CPE. La perturbation de la signalisation CXCR4/JAK-2 peut contribuer à l'atteinte fonctionnelle des CPE en présence d'une déficience touchant le ClC-3. Ainsi, le ClC-3 peut être choisi comme cible thérapeutique en vue de moduler la néovascularisation dans les cas de maladies ischémiques.

($ClC\text{-}3^{-/-}$; KO) mice in an attempt to determine the involvement of ClC-3 in the regulation of EPC function. EPC-related angiogenesis *in vivo* was also examined in a hind-limb ischemic model.

Methods

An extended, detailed *Materials and Methods* section is provided in the online Supplementary Material.

$ClC\text{-}3^{-/-}$ and corresponding $ClC\text{-}3^{+/+}$ control mice of 129/SvJ-C57BL/6 background were kind gifts of Dr Dayue Duan (School of Medicine, University of Nevada, Reno, NV, USA) and bred in house.[18] All experiment strategies, including EPC isolation and culture, EPC migration, adhesion and network formation assay, murine hindlimb ischemia model, immunostaining for CD31, EPC transplantation study, Western blotting, and CXCR4 plasmid transfection, were performed as described previously.[8,19-24]

Statistical analysis

All statistical analyses were performed with the use of GraphPad Prism 5. All data were expressed as mean ± SEM. A 2-tailed Student *t* test for independent samples was used to detect significant differences between 2 groups. One-way analysis of variance followed by Bonferroni multiple comparison test was used to compare differences when there were more than 2 treatment groups. Values of $P < 0.05$ were considered to be statistically significant.

Results

EPC characterization

Recently, 2 distinct populations of EPCs, early and late EPCs, have been described according to their time-dependent appearance in culture.[2,3] Early EPCs, also defined as myeloid angiogenic cells, arise after 3-5 days of culture and grow gradually for 2 weeks with a short lifespan of 3 to 4 weeks. Early EPCs were made up of heterogeneous cells with expressions of both endothelial and monocytic antigens, of which a few clones have been shown to generate late EPC.[2] Late EPCs, now more commonly known as endothelial colony-forming cells, appear after 14-21 days of culture and show characteristics more similar to mature endothelial cells and may reflect the true endothelial progenitors.[3] Despite many questions about what EPCs really are and how they play a role remaining to be explored intensively, both types of EPC have similar potential and are suggested to have a synergistic effect in neovasculogenesis *in vivo*.[25,26]

In the present study, EPCs were cultivated on fibronectin-coated dishes from bone marrow–derived mononuclear cells from mice. After 7 days of cultivation in endothelial differentiation–inducing medium, fluorescent photos showed that the vast majority of adherent cells were double-positive for acetylated low-density lipoprotein (Dil-ac-LDL) and FITC-lectin staining (Fig. 1A), which is consistent with features shared by both early and late EPCs.[2] To further characterize the cells, the immunophenotype of cultured cells were also identified. Immunofluorescence staining revealed that the majority of cells expressed endothelial markers, including von Willebrand factor (vWF) and vascular endothelial growth factor receptor 2 (VEGFR2), as well as the progenitor marker CD34 (Fig. 1B). Meanwhile, monocytic marker CD14 and hematopoietic marker CD45 were also present at similar levels according to flow cytometry (Fig. 1C). According to the culture time (7 days) and the cell characterizations above, the cultured EPCs in our study could be classified as early EPCs.[2,3]

ClC-3 deficiency reduces the functional capacity of EPCs *in vitro*

The critical role for ClC-3 in angiogenic activities of EPC was investigated by comparing the functional capacities of EPCs isolated from WT vs ClC-3 KO mice. Adhesion and

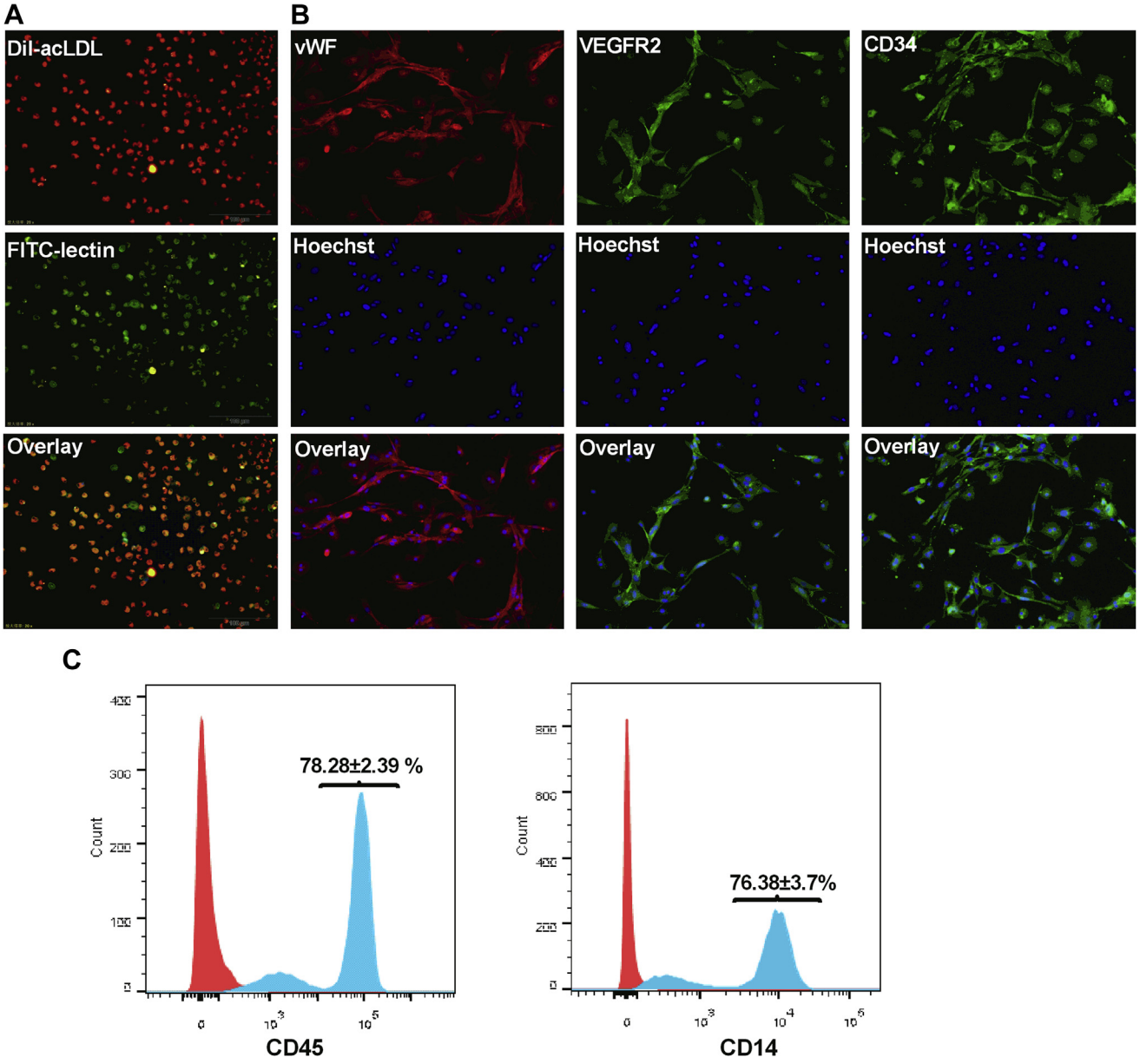

Figure 1. Characterization of cultured endothelial progenitor cells (EPCs). (**A**) Representative fluorescent photographs of EPCs at the 7th day labeled with Dil-acLDL (**red**) and FITC-lectin (**green**), and merged (×200 magnification). (**B**) Immunofluorescent staining of the markers vWF, VEGFR2, and CD34 in cultured EPCs (×400 magnification). The pictures shown were typical of 4 separate experiments. (**C**) Flow cytometry analysis of the expression of CD14 and CD45. Numbers are the percentage of positive cells determined by comparison with corresponding negative control labeling.

migration are very important steps in EPC homing to injured tissues. In a modified Boyden chamber assay, deficiency of ClC-3 significantly reduced SDF-1−induced EPC migration by about 30% (Fig. 2, A and B). Moreover, the number of adherent EPCs to fibronectin from ClC-3 KO mice was also less than that from WT mice (Fig. 2, C and D). In line with the reduced level of migratory and adhesive ability, EPC-induced angiogenesis in an *ex vivo* assay was also significantly decreased with ClC-3 KO (Fig. 2, E-H). The culture of EPCs on a Matrigel matrix revealed that more network structures, as determined by the tube length, tube number, and tube closed area, were developed in WT EPCs than in EPCs derived from ClC-3 KO mice. These results indicated that EPCs from the ClC-3 KO mice have an impaired angiogenic capacity.

Impaired *in vivo* neovascularization in ClC-3 deficient mice

Having demonstrated that ClC-3 plays a crucial role in EPC function *in vitro*, we next investigated whether ClC-3 KO affects the neovascularization process *in vivo* in the hindlimb ischemia model. Hindlimb perfusion was evaluated after surgery with the use of serial laser Doppler perfusion

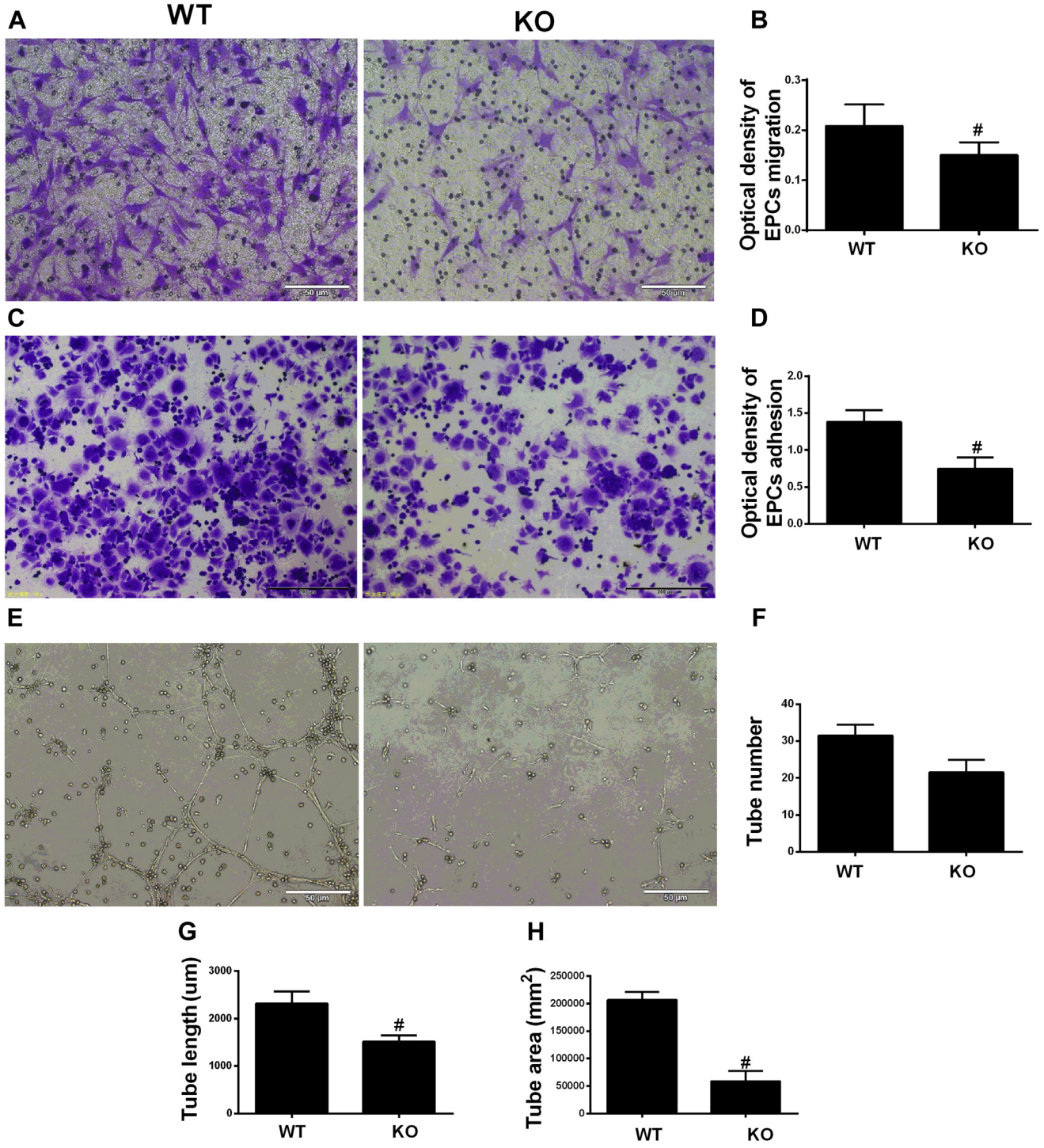

Figure 2. Effects of ClC-3 on the migratory, adhesive, and angiogenic activity of endothelial progenitor cells (EPCs) *in vitro*. (**A**, **B**) Representative photographs and quantification analysis of SDF-1α–induced migration of wild-type (WT) and ClC-3 knockout (KO) EPCs (×200 magnification; #$P <$ 0.05 vs WT; n = 7 mice per group; *t* test). (**C**, **D**) Representative photographs and quantification analysis of EPC adhesion to fibronectin (×200 magnification; #$P <$ 0.01 vs WT; n = 8 mice per group; *t* test). (**E-H**) Representative photographs and quantification analysis of EPC network formation in an *ex vivo* angiogenesis assay. Angiogenesis was quantified by measuring the total length, number, and closed area of the tube-like structures with the use of a computer-assisted microscope (#$P <$ 0.05 vs WT; n = 6 mice per group; *t* test).

imaging studies. Similar low levels of blood flow were shown in the WT and ClC-3 KO groups immediately after surgery. However, as demonstrated in Figure 3, A and B, recovery of blood flow in ischemic legs was significantly reduced in ClC-3 KO mice compared with WT littermates. In WT mice, hindlimb perfusion recovered to about 50% of the nonischemic limb by day 7 and ultimately returned to 64% of the nonischemic limb by day 14 (Fig. 3B). Compared with WT mice,

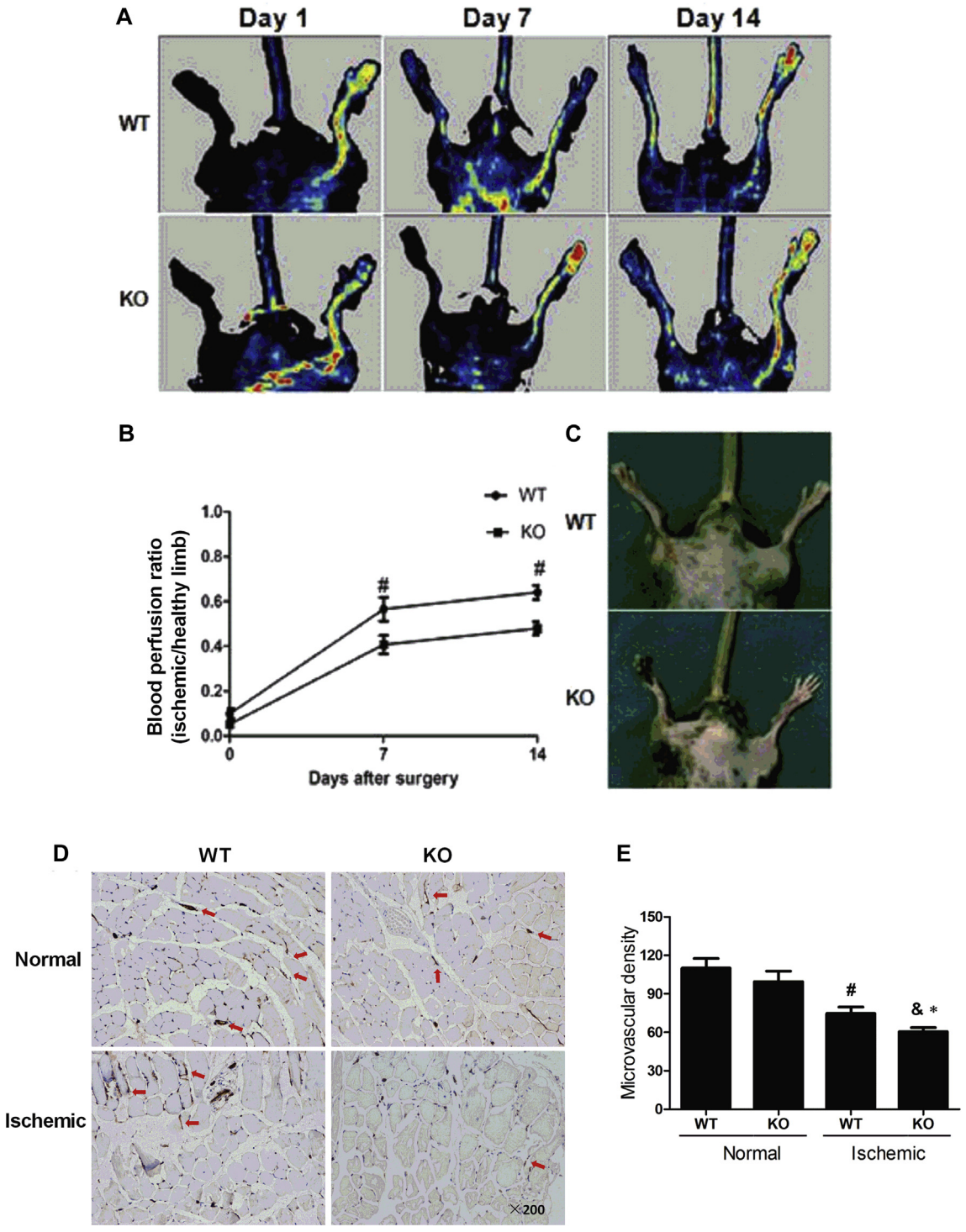

Figure 3. Effect of ClC-3 on *in vivo* neovascularization. (**A**) Representative photographs from laser Doppler perfusion imaging at days 1, 7, 14 of ischemic (**left**) and nonischemic (**right**) limbs in wild-type (WT) and ClC-3 knockout (KO) mice after ligation of femoral artery. (**B**) Quantification of laser Doppler–derived blood flow. Hindlimb perfusion was significantly impaired in ClC-3 KO mice compared with WT (#$P < 0.05$ vs WT; n = 6 mice each group; *t* test). (**C**) Hindlimb ischemia model in WT and ClC-3 KO mice. The ClC-3 KO mice were more susceptible to gangrene. (**D**) Representative immunohistochemical staining of ischemic gastrocnemius tissues from WT and ClC-3 KO mice with anti-CD31 monoclonal antibody (**brown**) on postoperative day 14. Arrows indicate CD31-positive capillaries. (**E**) Quantitative analysis of capillary density in muscle sections on postoperative day 14. Immunohistochemistry results suggested that the CD31-positive capillary number of the ClC-3 KO mice was significantly less than that of WT mice (×200 magnification; #$P < 0.05$ vs WT normal; &$P < 0.05$ vs KO normal; *$P < 0.05$ vs WT ischemic; n = 5 mice each group; 1-way analysis of variance). (**F**) Representative bioluminescence images of mice after injection of EPCs labeled with CM-DiI for 3 days. The pictures shown are typical of 5 mice. (**G-H**) Representative photographs and quantification from laser Doppler perfusion imaging at days 1, 3, and 7 of ischemic (left) and nonischemic (right) limbs in WT mice receiving intravenous injections of 5×10^5 WT or ClC-3 KO EPCs (*$P < 0.05$ vs KO; n = 5 mice per group; *t* test). (**I**) Representative photographs of CD31 immunoflurescene staining and DiI-positive cells in ischemic muscle sections on day 7 of EPC injection. The pictures shown are typical of 4 separate experiments.

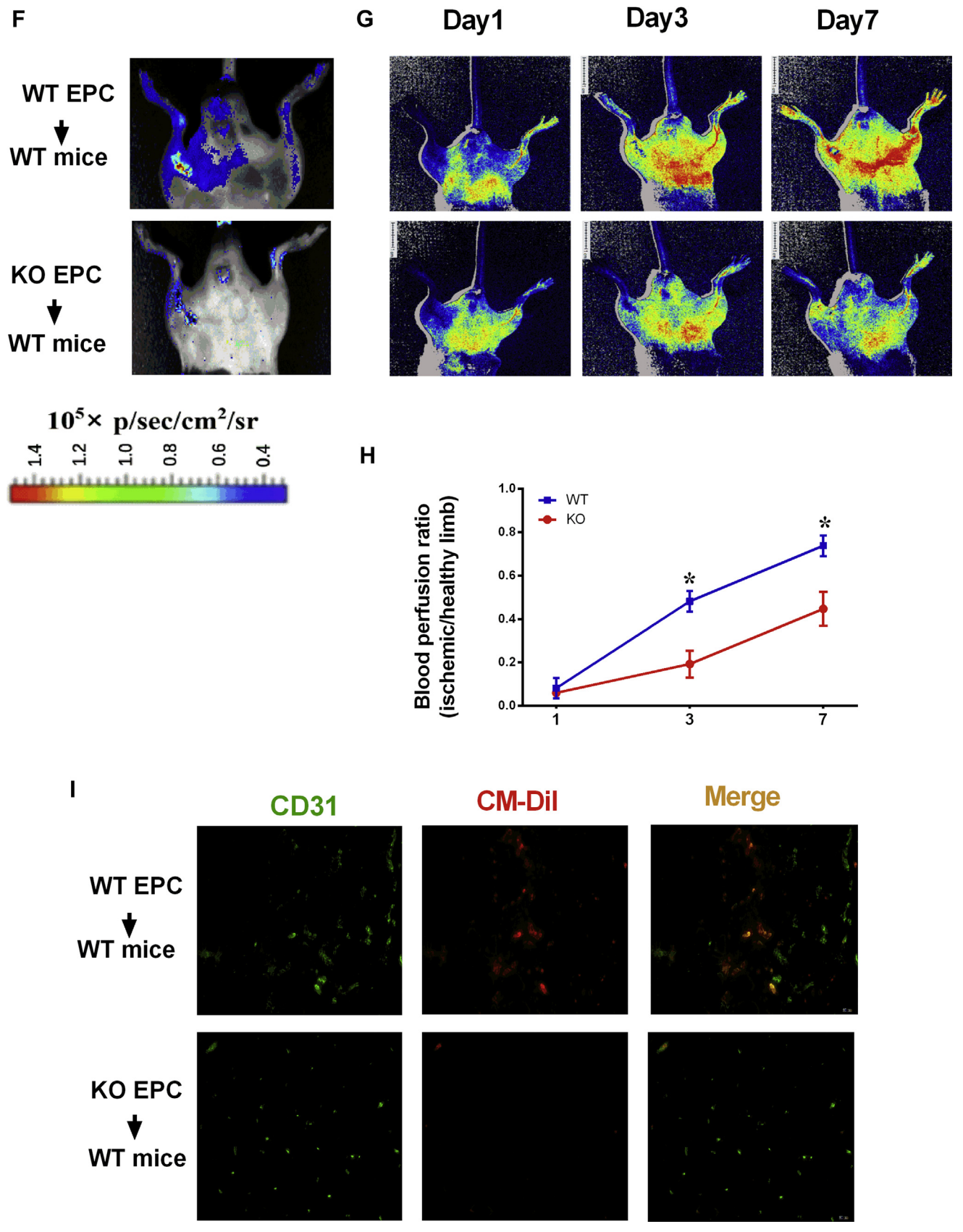

Figure 3. Continued.

flow recovery in ClC-3 KO mice was significantly impaired (37.4% at day 7 and 48.0% at day 14; $P < 0.05$). Moreover, the ischemic hindlimbs of ClC-3 KO mice were more susceptible to gangrene, with 50% incidence (3 mice with tip necrosis among the 6 ClC-3 KO mice). There was another ClC-3 KO mouse displaying severe gangrene and autoamputation of the ischemic limb. Considering that the latter serious gangrene may have been caused by various factors and that the ischemic leg was gone in this mouse, it was excluded in data analysis. A representative image of gangrene with tip necrosis is shown in Figure 3C. We did not observe gangrene in WT mice. These data were further corroborated by a quantitative analysis of capillary density in histologic tissue sections from the ischemic gastrocnemius muscle on day 14 after surgery. As shown in Figure 3, D and E, immunostaining of CD31-positive cells demonstrated a significant decrease in capillary density of ischemic muscle in ClC-3 KO compared with WT mice.

In a separate experiment to assess whether the impaired function of EPCs contributes to the reduced neovascularization in ClC-3 KO mice, the ability of WT and ClC-3 KO EPCs to enhance angiogenesis and restore perfusion *in vivo* was further investigated. WT mice underwent unilateral hindlimb ischemia surgery. One day after surgery, mice received an intravenous infusion of either ClC-3 KO or WT EPCs. To visually confirm cell accumulation in the ischemic limb, delivery of CM-Dil–labeled EPCs was first analyzed with the use of noninvasive *in vivo* bioluminescence imaging. By day 3, EPCs demonstrated preferential accumulation in the ischemic hindlimbs compared with the contralateral limb. Moreover, ischemic hindlimbs of mice intravenously injected with WT EPCs showed greater cell homing and localization compared with ClC-3 KO EPCs (Fig. 3F). Blood perfusion was assessed by means of laser Doppler analysis. As expected, flow recovery of mice was more robust with delivery of WT EPCs than with ClC-3 KO EPCs. In mice that received WT EPCs, the blood flow ratio in the ischemic limb reached 73.8 ± 4.7% of the contralateral limb by 7 days. At the same time point, the perfusion for mice that received ClC-3 KO EPCs reached only 44.7 ± 5.8% (Fig. 3, G and H). Histologic analysis revealed that CD31-positive capillary density was higher in the ischemic muscle of recipient mice injected with WT EPCs than with ClC-3 KO EPCs (Fig. 3I). In addition, mice with WT EPC injection showed more Dil-labeled EPC retention in the ischemic muscle at day 7, whereas only a few KO EPCs could be observed. Taken together, these data suggest that ClC-3 KO EPCs are functionally impaired in their ability to promote neovascularization and flow recovery *in vivo*.

Reduced CXCR4 expression contributes to impaired neovascularization capacity of ClC-3 KO EPCs

There is increasing evidence that CXC chemokine receptor 4 (CXCR4) is a key regulator of migration, homing, and retention of EPCs at the sites of injured tissue. We postulated that the limited angiogenic potential of EPCs from ClC-3 KO mice might be related to dysfunctional CXCR4. To address this issue, we first examined CXCR4 expression level in cultured EPCs. As shown in Figure 4, A and B, there was abundant CXCR4 expression in EPCs isolated from bone marrow of WT mice, as shown by Western blot analysis after 7 days in culture. Importantly, surface expression of CXCR4 was significantly reduced in EPCs from ClC-3 KO mice. Meanwhile, the CXCR4 protein level in ischemic muscle of ClC-3 KO mice was also lower than that of WT mice, indicating an impaired homing of CXCR4-positive EPCs (Fig. 4, C and D).

Dysregulation of JAK-2 signalling contributes to impaired neovascularization capacity

It is well known that Janus kinase 2 (JAK-2) is one of the downstream targets of CXCR4 signalling,[22,24] so we further investigated whether CXCR4-mediated JAK-2 signalling is dysregulated in EPCs from ClC-3 KO mice. Consistent with the reduced expression of CXCR4, both total protein and phosphorylation levels of JAK-2 were significantly reduced in ClC-3 KO EPCs compared with WT (Fig. 5). Therefore, the decreased activation of the downstream signal of the CXCR4 receptor, JAK-2, may contribute to the functional impairment of EPC by ClC-3 deficiency.

To further investigate the relationship between dysregulation of CXCR4/JAK2 signalling pathway and impaired angiogenic capacity of ClC-3 KO EPCs, we incubated EPCs from WT mice with CXCR4 inhibitor AMD3100 and JAK-2 inhibitor AG-490 to block the CXCR4 and JAK-2 signalling, respectively. Pretreatment with AMD3100 or AG490 profoundly reduced the migratory capacity of EPCs, but combined JAK-2 and CXCR4 blockade did not result in an additive effect (Fig. 6A), indicating that the effect of CXCR4 on angiogenic activity of EPC is mediated by JAK-2. More importantly, when CXCR4 expression in ClC-3 KO EPCs was rescued by cell transfection with CXCR4 cDNA plasmid (Fig. 6B), the impaired cell migratory ability was significantly reversed (Fig. 6C), suggesting that the dysfunctional CXCR4/JAK2 pathway accounts for the reduced EPC function in ClC-3 KO mice.

Discussion

This study provides the first evidence that deficiency of ClC-3 results in impaired *in vitro* angiogenic capacity of EPCs and *in vivo* neovascularization after hindlimb ischemia. It was also shown that the impairment of EPC function in ClC-3 KO mice is closely correlated with the down-regulation of CXCR4/JAK-2 signalling. These data suggest that ClC-3 is essential in the regulation of EPC-mediated angiogenic function. Targeting ClC-3 may be a potential therapeutic strategy for postnatal ischemic diseases.

Neovascularization is an important adaptive response and repair mechanism to rescue tissue from critical ischemia in vascular occlusive diseases.[1] In our previous studies, we showed that ClC-3, a member of the voltage-gated ClC family, plays an important role in vascular remodelling and atherosclerosis,[16,17] the most common risk factors for ischemic cardiovascular diseases. However, whether ClC-3 has a direct contribution to tissue ischemia remained to be determined. To illustrate this issue, we examined the blood flow in a hindlimb ischemic model in ClC-3 KO mice. We found that lack of ClC-3 resulted in significantly reduced ischemia-induced neovascularization, as demonstrated by a slower rate of blood flow reperfusion after ischemia and a

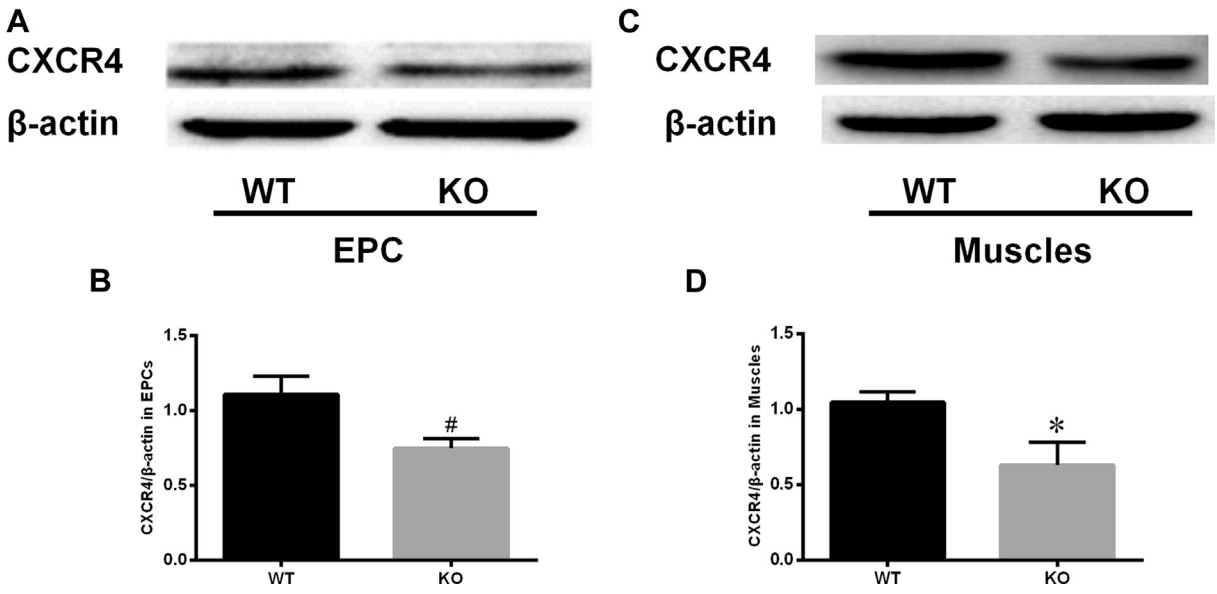

Figure 4. CXCR4 protein expression in (**A, B**) bone marrow–derived EPCs or (**C, D**) ischemic hindlimb muscle from WT and ClC-3 KO mice. ClC-3 deficiency down-regulated the expression level of CXCR4 in both EPCs and ischemic muscles (#$P < 0.01$ vs WT-EPCs [n = 8 per group; *t* test]; *$P < 0.05$ vs WT-muscles [n = 5 per group; *t* test]).

decreased capillary density in ischemic muscles, strongly suggesting that ClC-3 is an essential factor to modulate the course of ischemic disease.

A large body of evidence indicates that EPCs derived from bone marrow play a critical role in postnatal neovascularization in response to tissue ischemia.[4-8] To date, 2 distinct populations of EPCs have been observed, early and late EPCs.[2] Although clear differences have been shown between these 2 endothelial progenitors, it has been demonstrated that both subsets contribute to angiogenesis in ischemic diseases.[2,3] In this regard, we speculated that the impairment in reperfusion in mice lacking ClC-3 may be related to EPC dysfunction and their inability to augment the neovascularization process. Consistent with this hypothesis, we found that in our cultured early EPCs, ClC-3 deficiency resulted in impaired EPC abilities to migrate toward stromal cell–derived factor 1, adhere to fibronectin, and form network structures, suggesting that impaired EPC function

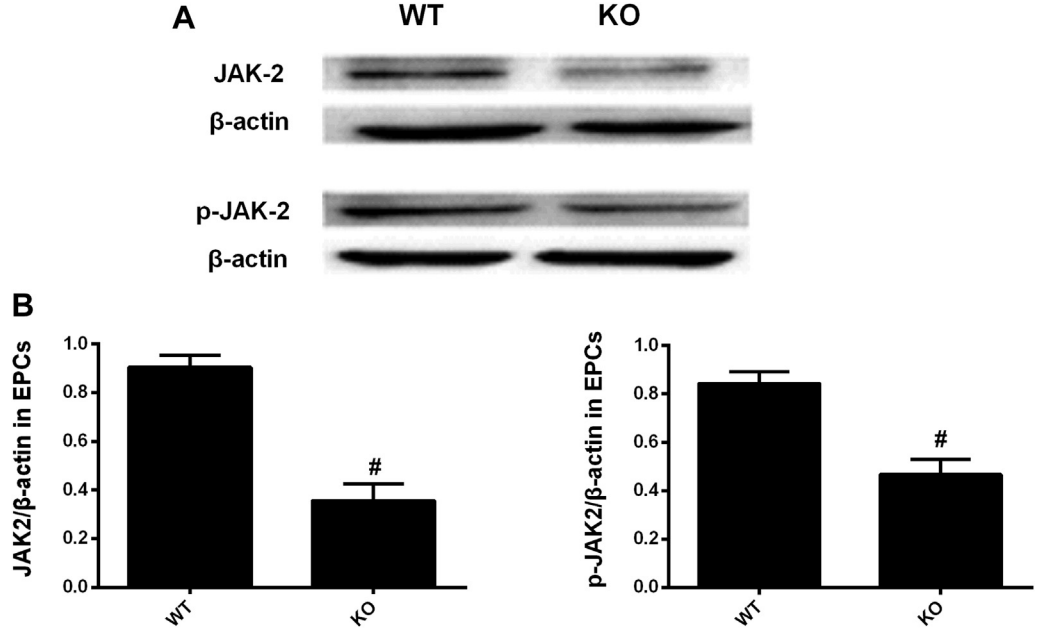

Figure 5. ClC-3 knockout reduced the expressions of JAK-2 and p-JAK-2 in mouse BM-EPCs: (**A**) representative photographs and (**B**) quantification analysis. #$P < 0.01$ vs the WT group (n = 8 per group; *t* test).

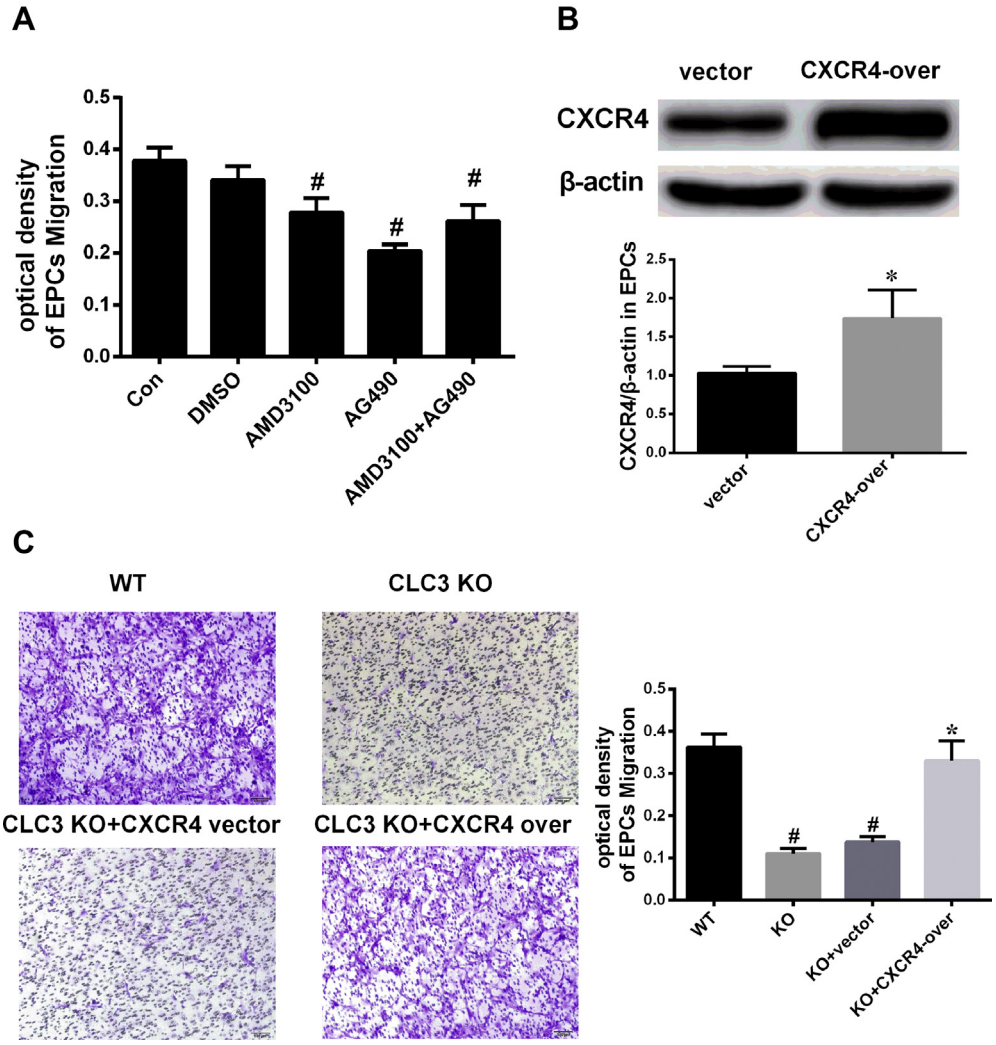

Figure 6. (**A**) CXCR4/JAK-2 signalling blockade inhibited the migratory capacity of EPCs from WT mice. EPCs were preincubated with the CXCR4 inhibitor AMD3100, the JAK-2 inhibitor AG490, or both (#$P < 0.05$ vs control; n = 4 per group; 1-way analysis of variance). (**B**) The expression of CXCR4 protein in EPCs transfected with empty vector or CXCR4 cDNA plasmid was measured by means of Western blot (*$P < 0.01$ vs vector; n = 5 per group; *t* test). (**C**) Representative photographs and quantification analysis of SDF-1α—induced migration of ClC-3 KO EPCs with or without CXCR4 transfection. CXCR4 overexpression rescued the impaired migratory capacity of ClC-3 KO EPCs (#$P < 0.01$ vs WT; *$P < 0.01$ vs KO; n = 4 separate experiments; 1-way analysis of variance).

may be an important contributor to the reduced perfusion recovery after injury in ClC-3 KO mice.

It is worth noting that although these results support a contribution of EPCs to blood reperfusion in ischemic tissue, other cell types lacking ClC-3 might also participate in the disrupted recovery process owing to it global knockout. To minimize this criticism, the direct role of EPCs in blood restoration was tested by EPC transplantation. We found that mice treated with WT EPCs had significantly improved blood reperfusion in ischemic hindlimb, whereas transplantation of EPCs from ClC-3 KO mice to WT mice impaired the neovascularization process and blood flow restoration after hindlimb ischemia.

An issue of concern is that ClC-3 KO mice were more susceptible to gangrene despite the difference in blood perfusion recovery between WT and ClC-3 KO mice being modest. The possible reason why ClC-3 KO mice are particularly vulnerable to ischemia-induced necrosis may be that although the difference in flow restoration in WT and KO mice is modest, the reduced blood perfusion may be sufficient to induce tissue gangrene in KO mice. In a previous study, early EPCs have been linked to alternatively activated M2 macrophages.[27] It is well known that M2 macrophages have an important function to modulate inflammatory and immune responses. Thus, the impairment in antiinflammatory function of early EPCs acting as M2 macrophages, if any, may also contribute to the vulnerability of ClC-3 KO mice to ischemia. In addition, the global knockout of ClC-3 may also affect other inflammatory cells directly, thereby contributing to tissue necrosis. Another possibility is that ClC-3 may also affect the recovery process by acting on vascular smooth muscle cells (VSMCs), a key element of blood vessels. It is well known that ClC-3 plays an important role in VSMC function.[13] Silence of ClC-3 has been shown to inhibit cell proliferation in VSMCs.[28,29] This could provide an alternate explanation for the worse tissue

gangrene observed in ClC-3 KO mice. Delineation of the influence of ClC-3 in other cell types after ischemia is worthy of further study.

The molecular mechanisms by which ClC-3 KO impairs neovascularization after ischemia are potentially diverse, because various molecules may be involved in the regulation of EPC function. Among them, the importance of CXCR4 is increasingly highlighted. It has been demonstrated that CXCR4 plays a key role in neovascularization by modulating EPC migration and homing.[30,31] We therefore hypothesized that abnormality in CXCR4 signalling may result in reduced EPC function in ClC-3 KO mice. To address these assumptions, the CXCR4 expression in EPC was examined. Consistent with the impaired EPC function, CXCR4 expression was significantly reduced in EPCs from ClC-3 KO mice compared with WT. More importantly, the "rescue" experiment showed that the negative impact of ClC-3 deficiency on cell migration in ClC-3 KO EPCs was significantly overcome by overexpression of CXCR4, supporting that CXCR4 contributes to the functional effects of ClC-3 on EPCs. Our results are also in accordance with previous investigations finding that stimulation of CXCR4-mediated signalling can facilitate EPC-mediated neovascularization[32] whereas CXCR4 blockade attenuated EPCs angiogenic activity and homing to sites of ischemic tissue.[22] In addition, phosphorylation of JAK-2, a well known CXCR4 downstream signalling molecule,[22,24,33] was significantly reduced in EPCs derived from ClC-3 KO mice. The importance of CXCR4/JAK-2 signalling in EPC function was further supported by the finding that the migratory capacity of EPCs from WT mice was profoundly inhibited by either CXCR4 or JAK-2 blockade. Our data suggest that diminished CXCR4/JAK2 signalling is, at least in part, responsible for the reduction in EPC-mediated angiogenic capacity in ClC-3 KO mice. However, whether other angiogenic factors besides CXCR4 signalling may also have an impact on EPC-mediated neovascularization deserves further exploration. In fact, in our pilot study, we found that ClC-3 KO does not affect the 7-day cultured EPC expression of VEGFR2, another well known critical factor for vasculogenesis (data not shown). Delineation of other mechanisms by which ClC-3 regulates angiogenesis should be the focus of future research.

Conclusion

In summary, we demonstrate that deficiency of ClC-3 attenuates the *in vitro* functional properties of EPCs and impairs EPC-mediated *in vivo* neovascularization by decreasing CXCR4/JAK-2 signalling. This study may provide novel insight into the impact of ClC-3 on cardiovascular diseases associated with ischemia. Targeting ClC-3 may be a potential intervention strategy for combating disorders of EPC function in ischemic diseases.

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Disclosures

The authors have no conflicts of interest to disclose.

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Supplementary Material

To access the supplementary material accompanying this article, visit the online version of the *Canadian Journal of Cardiology* at www.onlinecjc.ca and at https://doi.org/10.1016/j.cjca.2019.08.009.