Dual efficacy of Fasudil at improvement of survival and reinnervation of flap through RhoA/ROCK/PI3K/Akt pathway

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Funding information
Fujian Natural Science Foundations, Grant/Award Numbers: 2020J01625, 2021J01241, 2021J01666

Abstract
Fasudil is reported to be effective at protecting against ischaemic diseases, and at augmenting axon growth. In this study, we aim to evaluate its efficacy in promoting flap survival and reinnervation. Ninety-two Institute of Cancer Research (ICR) mice were used and divided into the control, Fasudil, LY294002, Fasudil + LY294002 groups, receiving a daily intraperitoneal injection of normal saline, Fasudil (10 mg/kg), LY294002 (5 mg/kg), and Fasudil (10 mg/kg) + LY294002 (5 mg/kg), respectively. On days 0 and 5, the blood perfusion and diameter of the iliolumbar artery in the pedicle of the flaps in the four groups were evaluated using laser speckling contrast imaging (LSCI). On day 5, the flaps were photographed and the necrosis rate of the flaps was calculated using Photoshop CS6. In addition, tissues were harvested from the flaps and divided into two parts. One part underwent routine cryosection and immunofluorescent staining using the antibody against CD31 for evaluation of the microvascular density in the four groups. In the other part, the expression of RhoA, ROCK1+2, p-CPI-17, p-MYPT, p-PTEN, p-PI3K, p-Akt, and vascular endothelial growth factor (VEGF) within the flaps were determined using western blotting. Moreover, at days 0, 7, 15, and 30 after flap surgery, the axons within the flaps were evaluated using immunofluorescent staining with the antibody against Neurofilament-200. It turned out that the necrosis rate was (24.4 ± 7.7)%,(5.2 ± 1.6)%,(29.8 ± 4.2)%,(30.9 ± 7.1)%, respectively, in the control, Fasudil, LY294002, LY294002 + Fasudil groups. There was a significant reduction in the necrosis rate of the flaps in the Fasudil group ($P < .001$). The LSCI and immunofluorescent staining demonstrated that Fasudil could significantly expand the diameter of the iliolumbar artery in the pedicle, boost the overall blood perfusion, and increase the microvascular density of the flaps in the Fasudil group ($P < .05$), which could all be abolished by PI3K inhibitor.
LY294002. On day 5, the expression of p-CPI-17, p-MYPT, and p-PTEN were downregulated, whereas pPI3K, p-Akt, and VEGF were upregulated in the Fasudil group ($P < .001$). As for reinnervation, Neurofilament-200 fluorescent staining revealed that at days 15 and 30 after flap harvest, only in the Fasudil group could new axons be observed. It can be concluded that Fasudil could simultaneously improve the survival and axon growth after flap harvest, a dual efficacy achieved by inhibition of the RhoA/ROCK pathway, which in turn activates /PI3K/AKT pathway.

**KEYWORDS**
Fasudil, flap, PI3K/Akt, PTEN, RhoA/ROCK

**Key Messages**
- Fasudil has an outstanding dual efficacy at promoting survival of and axon growth into the flap
- the dual efficacy can be abolished by inhibition of the PI3K/Akt pathway
- Phosphatase and tensin homologue is possibly involved as an intermediate regulator between rho-associated coiled-coil kinase and PI3K

## 1 | INTRODUCTION

Autologous flap transfer is still the main approach adopted for the reconstruction of defects resulting from traffic accidents, burn, and removal of tumours in the head and neck. After flap survival what concerns the surgeon and patient most is the survival of flap, due to the occurrence of partial or even complete necrosis of flap at various incidences in different applications. Jeffery reported a 3.8% incidence of partial necrosis in flaps transferred to the head and neck; as for flaps transferred for breast reconstruction after mastectomy, a partial necrosis rate ranging from 5% to 30% was reported; and in the reconstruction of the lower limb using distally based sural neurocutaneous flaps, a complete necrosis rate of 3.1%, and a partial necrosis rate of 15.4% was observed. Therefore, finding a reliable pharmaceutical to augment flap survival is an essential task for researchers.

After flap survival, restoration of sensation naturally becomes the next desire from the patient. However, sensation recovery after flap surgery whether neurorrhaphy is performed or not is not ideal in most cases in medium and large-sized flaps due to the fact a skin territory included in the flap can be innervated by more than one cutaneous nerve, which can severely affect the life quality of patients if the flap is located in such areas as the fingers, oral cavity, breasts, and heels. After reconstruction, the fingers need acute sensation to process the daily life routines. Reconstruction of the heel with a senseless flap will seriously attenuate the ability of patients to avoid detrimental friction and constraint conferred by the mismatch between the reconstructed heel and the footwear. In addition, the sensation is the basis of the oral function and plays an important role in chewing, swallowing, velopharyngeal closure, pronunciation, and taste, and its effects on the quality of life of patients after ablation of tongue cancer could not be exaggerated. Moreover, only when reconstructed breasts regain its erogenous sensation can women internalise them as a part of their body. With long-term follow-ups, it has been shown that though flaps can regain a part of the sensation spontaneously, the overall results are not satisfying. Therefore, finding a reliable pharmaceutical to enhance axon growth into the flap is also an essential task.

As a well-known effector of small GTPase RhoA, Rho-associated coiled-coil kinase (ROCK) regulates actin reorganisation during cell adhesion, migration, contraction, and proliferation. Fasudil, a novel inhibitor of ROCK, was first used in patients with subarachnoid haemorrhages to relieve vascular spasms. Since then, many studies have reported additional effects of Fasudil, including anti-inflammation, ameliorating ischaemia-reperfusion, and protection against myocardial infarction or stroke. In addition, Fasudil has been shown to promote axon growth via upregulation of the PI3K/Akt signalling pathway.

Thus, taking into consideration that Fasudil has been successfully used to provide protection against ischaemic diseases and promote axon growth, we speculated that the use of Fasudil could achieve a dual efficacy at improving both flap survival and sensate recovery, which motivated us to conduct this research, the outcome of which then corroborated our speculation.
2 | MATERIALS AND METHODS

2.1 | Exploration of the efficacy of Fasudil at improving flap survival

Thirty-two 5-week-old male Institute of Cancer Research (ICR) mice, weighing 25 ± 3 g, provided by the Experimental Animal Centre of Fujian Medical University were used in this study. The mice were equally divided into the control, Fasudil, LY294002, Fasudil+LY294002 groups, receiving a daily intraperitoneal injection of normal saline, Fasudil (10 mg/kg), LY294002 (5 mg/kg), and Fasudil plus LY294002, respectively. Three days after drug application, the mice underwent intraperitoneal anaesthesia (pentobarbital sodium, 50 mg/kg) and depilation, and then a rectangular peninsula flap measuring 4.5 cm × 1.5 cm pedicled on the iliolumbar vessels was harvested on the right half of the back of each mouse.

Immediately after flap elevation, the mice were placed under the probe of a commercially available laser speckling contrast imaging (LSCI) instrument (Reward Life Technology Co., Ltd.) for measurement of the diameter of the artery in the pedicle, and the perfusion of the proximal and distal areas of the flap. Afterward, the flaps were sutured back in situ. Five days later, the flaps were photographed and photos were imported into Photoshop CS6 for calculation of necrosis rate. After photographing, the flaps were re-elevated for measurement by LSCI, and then tissues were taken from the distal viable part of each flap and divided into two parts: one part placed in 4% paraformaldehyde for immunostaining and the other part stored in liquid nitrogen for western blot analysis (Figure 1).

2.2 | Exploration of the efficacy of Fasudil in improving flap reinnervation

Another 60 mice underwent the same flap harvest and group division as described above. The 15 mice in each group were further equally divided into three time points, that is, 7, 15, and 30 days after flap harvest. At each time point, the mice were sacrificed, and tissues were taken from the distal viable part of each flap, and placed in 4% paraformaldehyde for subsequent immunostaining of axons.

2.3 | Immunofluorescence

The flap tissues taken at each time point were immersed in 4% paraformaldehyde for 24 hours before being moved to 30% glucose for dehydration. Afterward, routine cryosection with a thickness of 15μm was performed, and immunofluorescent staining was carried out with the following protocol: the frozen sections were rinsed two times with phosphate buffer saline (PBS) for 10 minutes each, and permeated with 0.1% triton x-100 in PBS for 10 minutes, and then blocked in 5% normal goat serum in PBS (pH 7.4) for 1 hour. Afterward, sections were incubated with primary antibodies at 4°C overnight, and then with secondary antibodies for 1 h at room temperature. Washing was

![Diagram of experimental design](image-url)

**Figure 1** Experimental design of the study. Ninety-two ICR mice were used in the study, and divided into four groups, receiving daily intraperitoneal (I.P) injection of normal saline, Fasudil, LY294002, and Fasudil+LY294002, respectively. Three days later, a flap based on the iliolumbar artery was harvested on the right half of the back of each mouse. The mice received continued I.P injection of the corresponding agents and were assigned into five-time points. At each time point, various procedures were carried out. I.F, immunofluorescence; I.P., intraperitoneal; LSCI, laser speckling contrast imaging; WB, western blotting;
performed in PBS (pH 7.4) between all steps. Primary antibodies used included antibodies against CD31, Neurofilament-200, and α-smooth muscle actin (SMA). The detailed information of the antibodies was listed in Table 1.

The anti-CD31 antibody was used to mark the microvessels in tissues on day 5. The anti-Neurofilament-200 and α-SMA antibodies were used to mark the axons and vessels in flap tissues at days 0, 5, 7, 15, and 30. Photomicrographs from three random fields were taken at /C20 magnifications from the flap tissue of each mouse. The number of microvessels in each field was counted by a third person blind to the group division. The average number of microvessels from the three fields represented the microvascular density of the flap at day 5.

### 2.4 Western blotting

The flap tissues were lysed with radio-immunoprecipitation assay lysis buffer (Beyotime, Shanghai, China), and the protein concentrations were measured using an enhanced bicinchoninic acid protein assay kit (Beyotime, Shanghai, China). Equal amounts of protein (20 or 30 μg) were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis gels and were then transferred onto poly(vinylidene fluoride) membranes (Millipore, Massachusetts). The membranes were blocked with 5% non-fat milk at 37°C for 2 hours and were then incubated overnight with primary antibodies at 4°C. After rinsing three times with 1 × Tris-HCl buffer solution-Tween, the membranes were then incubated with a radish peroxidase-bound secondary antibody for 2 hours. Bands were visualised by enhanced chemiluminescence and quantified using Image J (National health institute). The details of the antibodies used were listed in Table 1.

| Antibody                                      | Company                       | Catalogue number | Comments                          |
|------------------------------------------------|-------------------------------|------------------|-----------------------------------|
| Anti-RhoA antibody                            | BOSTER                        | BM4479           | Rabbit polyclonal, panaxonal marker 1:500 |
| Anti-ROCK1+ROCK2 antibody                     | Abcam                         | ab45171          | Rabbit polyclonal, panaxonal marker 1:1000 |
| Anti-PI3 kinase p85 (19H8) rabbit              | Cell Signaling Technology     | #4257            | Rabbit monoclonal, panaxonal marker 1:1000 |
| Anti-Phospho-PI3 kinase p85 (Tyr458)/p55 (Tyr199) antibody | Cell Signaling Technology     | #4228            | Rabbit polyclonal, panaxonal marker 1:1000 |
| Anti-Phospho-Akt (Ser473) antibody            | Cell Signaling Technology     | #9271            | Rabbit polyclonal, panaxonal marker 1:1000 |
| Anti-AKT1+AKT2+AKT3 antibody [EPR16798]       | Abcam                         | ab179463         | Rabbit monoclonal, panaxonal marker 1:500 |
| Phospho-MYPT1 (Thr696) antibody               | Cell Signaling Technology     | #5163            | Rabbit monoclonal, panaxonal marker 1:1000 |
| Mouse anti-SMA antibody                       | Sigma                         | C6798            | Mouse polyclonal, panaxonal marker 1:500 |
| Purified rat anti-mouse CD31 antibody         | BD Biosciences                | 550274           | Rat polyclonal, panaxonal marker 1:500 |
| Anti-neurofilament (NF) 200-FITC antibody     | Sigma                         | SAB4200811       | Mouse monoclonal, panaxonal marker 1:500 |
| Goat anti-rabbit IgG H&L (DyLight 488) antibody | Abcam                         | ab96883          | Goat polyclonal, panaxonal marker 1:500 |
| Goat anti-rabbit IgG H&L (CY3) antibody       | Beyotime                      | ab0516           | Goat polyclonal, panaxonal marker 1:500 |
| Rabbit anti-rat IgM/Alexa Fluor 488 antibody  | BIOSS Antibody                | bs-0346R-AF488   | Rabbit polyclonal, secondary antibody 1:500 |
| HRP-conjugated affinipure goat anti-mouse IgG (H+L) | Proteintech                  | SA00001-1        | Goat polyclonal, secondary antibody 1:500 |
| HRP-conjugated affinipure goat anti-rabbit IgG (H+L) | Proteintech                  | SA00001-2        | Goat polyclonal, secondary antibody 1:1000 |

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| Anti-ROCK1+ROCK2 antibody                     | Abcam                         | ab45171          | Rabbit polyclonal, panaxonal marker 1:1000 |
| Anti-PI3 kinase p85 (19H8) rabbit              | Cell Signaling Technology     | #4257            | Rabbit monoclonal, panaxonal marker 1:1000 |
| Anti-Phospho-PI3 kinase p85 (Tyr458)/p55 (Tyr199) antibody | Cell Signaling Technology     | #4228            | Rabbit polyclonal, panaxonal marker 1:1000 |
| Anti-Phospho-Akt (Ser473) antibody            | Cell Signaling Technology     | #9271            | Rabbit polyclonal, panaxonal marker 1:1000 |
| Anti-AKT1+AKT2+AKT3 antibody [EPR16798]       | Abcam                         | ab179463         | Rabbit monoclonal, panaxonal marker 1:500 |
| Phospho-MYPT1 (Thr696) antibody               | Cell Signaling Technology     | #5163            | Rabbit monoclonal, panaxonal marker 1:1000 |
| Mouse anti-SMA antibody                       | Sigma                         | C6798            | Mouse polyclonal, panaxonal marker 1:500 |
| Purified rat anti-mouse CD31 antibody         | BD Biosciences                | 550274           | Rat polyclonal, panaxonal marker 1:500 |
| Anti-neurofilament (NF) 200-FITC antibody     | Sigma                         | SAB4200811       | Mouse monoclonal, panaxonal marker 1:500 |
| Goat anti-rabbit IgG H&L (DyLight 488) antibody | Abcam                         | ab96883          | Goat polyclonal, panaxonal marker 1:500 |
| Goat anti-rabbit IgG H&L (CY3) antibody       | Beyotime                      | ab0516           | Goat polyclonal, panaxonal marker 1:500 |
| Rabbit anti-rat IgM/Alexa Fluor 488 antibody  | BIOSS Antibody                | bs-0346R-AF488   | Rabbit polyclonal, secondary antibody 1:500 |
| HRP-conjugated affinipure goat anti-mouse IgG (H+L) | Proteintech                  | SA00001-1        | Goat polyclonal, secondary antibody 1:500 |
| HRP-conjugated affinipure goat anti-rabbit IgG (H+L) | Proteintech                  | SA00001-2        | Goat polyclonal, secondary antibody 1:1000 |

### 2.5 Statistical analysis

All data in this study were expressed in the form of mean ± SD. One-way analysis of variance (ANOVA) was adopted for detection of statistical difference among the
four groups with least significant difference or Dunnett's T3 adopted for post-hoc comparisons depending on the homogeneity of variance. A $P$ value lesser than .05 was considered as statistically significant.

3 | OUTCOMES

3.1 | Significant improvement of flap survival by Fasudil abolished by LY294002 for inhibition of PI3K pathway

The necrosis rate was $(24.4 \pm 7.7\%)$, $(5.2 \pm 1.6\%)$, $(29.8 \pm 4.2\%)$, and $(30.9 \pm 7.1\%)$, respectively, in the control, Fasudil, LY294002, LY294002+Fasudil groups. (Figure 1). One-way ANOVA showed that the necrosis rate of the Fasudil group was significantly less than that of the other three groups ($P < .001$). Dunnett's T3 post-hoc comparisons showed no significant difference in necrosis rate among the other three groups ($P > .05$) (Figure 2 and 3).

3.2 | Significant improvement of flap perfusion by Fasudil abolished by LY294002 for inhibition of PI3K pathway

As could be observed from the speckling images on day 0, 3 days of medication by Fasudil before surgery could significantly increase the diameter of the iliolumbar artery in the pedicle in comparison to the other three groups ($P = .018$), thus resulting in augmented perfusion in the proximal part, which prompted weak blood perfusion to traverse cross the choke vessels, leading to slightly, but nevertheless significantly increased perfusion in the distal part of the flap (Figure 4B, $P = .013$). Five days after flap harvest, though the iliolumbar artery in the other three groups diluted in comparison to that on day 0, the diameter of it in the Fasudil group was still larger in diameter than in the other groups ($P = .001$), resulting in again more intensive perfusion in the proximal part of the flap ($P = .001$). Moreover, considerable dilation of ‘choke vessels’ in tortuous form as indicated by green arrowheads could be observed in the Fasudil group (Figure 4F), enabling seemingly undiminished propagation of blood flow to the distal part, resulting in remarkably more intensive perfusion in the distal part of the flap in comparison to other three groups, ensuring near-complete survival of the flap ($P < .001$) (Table 2).
3.3 | Activation of PI3K/AKT pathway and inhibition of PTEN by Fasudil

For RhoA and ROCK1+2, there were no significant differences concerning the expression of RhoA and ROCK 1+2 among the four groups ($P > .05$). The expression of p-PI3K and p-Akt in the Fasudil group was significantly higher than that in the other three groups ($P < .001$). In Contrast, the expression of p-CPI-17 and p-MYPT was distinctly higher in the Fasudil group than in the other three groups ($P < .001$). Moreover, the expression of p-PTEN was significantly lower in the Fasudil and Fasudil +LY294002 groups than in the other three groups ($P < .001$), and no significant difference in the expression of p-PTEN could be detected.
between the Fasudil and Fasudil+LY294002 groups ($P > .05$), and between the other two groups ($P > .05$). In addition, the expression of vascular endothelial growth factor (VEGF) was higher in the Fasudil group in comparison to other groups ($P < .001$) (Figure 5).

3.4 Promotion of angiogenesis by Fasudil abolished by inhibition of PI3K

The average number of microvessels per field was 47.2 ± 5.0, 65.0 ± 6.3, 41.6 ± 4.5, and 49.4 ± 3.2 in the control, Fasudil, LY294002, and Fasudil+LY294002 groups, respectively (Figure 6). The vascular density within the flaps in the Fasudil group was significantly larger than that in the other three groups ($P < .001$). There is no significant difference in vascular density among the other three groups ($P > .05$).

3.5 Enhancement of axon growth into the flap by Fasudil abolished by LY294002 for inhibition of PI3K/Akt pathway

On day 0, rich nerve fibres could be observed in all four groups. On day 5, obvious Wallarian degeneration
appeared in our groups. On day 7 after flap harvest, nerves within the flaps underwent complete Wallarian degeneration accompanied by negative immunopositive staining of NF-200. 15 and 30 days after flap harvest, immunopositive staining of NF-200 re-emerged in the Fasudil group, which was otherwise not visible in the other three groups (Figure 7).

4 | DISCUSSION

Three major novel findings from this study included: (a) Fasudil has an outstanding dual efficacy at promoting survival of and axon growth into the flap; (b) the dual efficacy can be abolished by LY294002 for inhibition of the PI3K/Akt pathway; (c) PTEN is possibly involved as an intermediate regulator between ROCK and PI3K. From a preclinical perspective, our study demonstrates that Fasudil, already approved for clinical treatment of cerebral vasospasm caused by subarachnoid haemorrhage, should be further clinically trialled as a medication after flap harvest.

Though there is already a report indicating the beneficial efficacy of Fasudil on flap survival, the exact underlying mechanism remains elusive. Existent researches pointed out that in the immediate post-elevation period,
The hyperadrenergic state is thought to be a significant contributor to ischaemia. This state results from division of the sympathetic nerves, releasing norepinephrine, which activates GEF that transforms inactivated RhoA-GDP to activated RhoA-GTP. The activated RhoA then regulates ROCK to phosphorylate CPI-17. Phosphorylated CPI-17 or ROCK can both phosphorylate MYPT1, the regulatory subunit of MLCP, to inhibit MLCP activity. Inhibited MLCP favours interaction between phosphorylated myosin and actin, leading to contraction of vascular smooth muscles. ROCK also favours the separation of myosin and actin, which causes relaxation of vascular smooth muscles and extension of axonal growth cone. Also, our study demonstrates that inhibition of ROCK by Fasudil can phosphorylate and activate PI3K and Akt, which can also lead to dephosphorylation of MYPT1. This indirect pathway seems to override the direct pathway, because inhibition of PI3K/Akt pathway results in total abolition of the efficacy of Fasudil and upregulation of p-MYPT1.

Since significant upregulation of p-PTEN can be caused by Fasudil, it indicates the potential involvement of p-PTEN serving as an intermediate factor between ROCK and PI3K. Phosphorylation of MYPT1 at Thr-696 or Thr-853 by ROCK exerts inhibitory effect on MLCP activity, potentiating the Ca^2+ sensitivity of the contractile apparatus. Another inhibitor of MLCP is the 17-kDa protein phosphatase-1, CPI-17. CPI-17 can

![Diagram showing the probable involvement of RhoA/ROCK/PI3K/Akt pathway underlying the efficacy of Fasudil at simultaneously improving flap survival and reinnervation.](image-url)
phosphorylated by PKC or ROCK at Thr38 to inhibit the MLCP complex so crosslinking between actin and myosin can be sustained.25

Therefore, as mentioned above, increased release of vasoconstrictors, including norepinephrine, ensues immediately after flap harvest, which then activates the RhoA/ROCK pathway, leading to reduced activity of MLCP by phosphorylation of either MYPT-1 or CPI-17, resulting in cross bridging between the myosin and actin, and therefore, contraction of vascular smooth muscles in the flap. As demonstrated by western blotting at day 5, after intraperitoneal use of Fasudil, a potent primary inhibitor of ROCK, though the overall expression of RhoA and ROCK was unaffected, the expression of p-MYPT and p-CPI-17 was significantly reduced, which combined to enhance the activity of MLCP, resulting in unlocking the cross bridging between the myosin and actin and then relax of vascular smooth muscles, as evidenced by dilation of the iliolumbar artery in the pedicle and choke vessels in the middle of the flap at the two time points measured using LSCI. In our opinion, this is the most important factor that leads to the significantly augmented perfusion and the near-complete flap survival after Fasudil application.

Several studies reported that PI3K/Akt was involved in downstream signalling of RhoA/ROCK pathway.17,18 In this study, we found that after application of Fasudil, p-PI3K and p-Akt was significantly upregulated, indicating that inhibition of the Rho/ROCK pathway could result in activation of the PI3K/Akt pathway. Since activation of PI3K/Akt pathway is implicated in increased secretion of VEGF and promotion of angiogenesis in numerous studies, we investigated whether this phenomenon occurs in our study. It turned out that significantly increased expression of VEGF alongside enhanced angiogenesis could be observed in the Fasudil group. Hence, we opined that dilation of vessels importing more blood and denser capillaries allowing quicker blood distribution to cells coordinated the dramatic augmentation in perfusion and flap survival following Fasudil application.

By application of PI3K inhibitor LY294002 together with Fasudil, the upregulation of p-PI3K and p-Akt was abolished, which lead to upregulation of p-MYPT and p-CPI-17, resulting in eventual inhibition of the activity of MLCP and thus contraction of vascular networks of the flaps in the LY294002 group and the Fasudil+LY294002 group. This phenomenon implies that PI3K/Akt pathway could regulate the action of ROCK on phosphorylation of MYPT and CPI-17, though the exact mechanism underlying this regulation remains to be elucidated. The upregulation of p-MYPT and p-CPI-17 leads to inhibition of the dilation of the vascular network and the collapse of the growth cones of the axons that tend to grow into the flaps. Furthermore, inhibition of the PI3K/Akt pathway also offset the increased secretion of VEGF caused by Fasudil, resulting in abated angiogenesis in the LY294002 group and the Fasudil+LY294002 group (Figure 8B).

PTEN (phosphatase and tensin homologue deleted on chromosome 10) was discovered in 1997 as a tumour suppressor of which the expression is often lost in tumours.29 Recent evidence revealed that PTEN is a new ROCK1 substrate that is involved in the regulation of cell death and survival.30 Numerous studies showed that ROCK1 activation enhances the activity of PTEN and inhibits the activation of Akt.31 For instance, PTEN phosphorylation induced by ROCK1 decreases the phosphorylation of Akt in HEK cells.32,33 In the present study, we found that after application of Fasudil, p-PTEN was indeed significantly downregulated, indicating the potential involvement of PTEN as a crucial link in the downstream the PI3K/Akt pathway activated after Fasudil application. Therefore, PTEN serves as another potential therapeutic target, and whether PTEN inhibition for activation of PI3K/Akt pathway can promote flap survival and reinnervation remains to be investigated.

Though inhibition of RhoA or ROCK to promote axon growth after injury in the peripheral and central nerve systems has been widely reported, our study is the first to explore whether inhibition of ROCK could augment reinnervation after flap harvest. It turned out that without using Fasudil there were no detectable axons in the flap 30 days after surgery. After Fasudil intervention, as soon as 15 days after surgery could abundant axons be detected, and the sprouting of axons into the flap enhanced by Fasudil was again reversed by LY294002. Since only in the Fasudil group can axons be observed, no statistical analysis was attempted. It has been clear that activation of the RhoA/ROCK pathway phosphorylates MYPT-1, LIM kinase, and collapsing response mediator protein-2 can regulate the cytoskeleton dynamics and cause growth cone collapse, inhibiting neurite outgrowth.34,35 Since the dorsal root ganglia in the mice are two small for either analysis by immunofluorescence and western blotting, the exact and most important molecules involved in propulsion of axons into the skin after flap harvest is very challenging to be investigated in the mice, which is the major limitation of this study. Using larger experimental animals in a future study might address the problem.

In conclusion, our study first demonstrates the excellent dual efficacy of Fasudil at enhancing flap survival and reinnervation through inhibition of the RhoA/ROCK pathway and activation the PI3K/Akt pathway in a mouse flap model.

ACKNOWLEDGEMENTS
We are deeply grateful for the funding from Fujian Natural Science Foundations (Grant No. 2020J01625, 2021J01666, and 2021J01241).
CONFLICT OF INTEREST
The authors declare no conflicts of interest.

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**How to cite this article:** Wang H, Fang F, Chen S, Jing X, Zhuang Y, Xie Y. Dual efficacy of Fasudil at improvement of survival and reinnervation of flap through RhoA/ROCK/PI3K/Akt pathway. *Int Wound J*. 2022;19(8):2000-2011. doi:10.1111/iwj.13800