Study of IncRNA TUG1/miR-320 in EPC Transplantation to Improve Lower Limb Ischemia and Promote Angiogenesis in Mice

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Research Article

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Abstract

To The aim of the current study was to investigate the changes in lncRNA TUG1/miR-320 and related proteins with ischaemic time in an ischemia model. A nude mouse model of lower limb ischemia was established by ligating the femoral artery, and laser Doppler measurements were used to demonstrate the successful establishment of the ischemia model. The cells were extracted from the bone marrow of nude mice, and the proliferation, migration and vascular-forming ability of the cells were analysed. When transplanted into ischemia model mice, blood flow measurements indicated that EPCs can speed up blood flow recovery. The results of HE staining indicated an improvement in inflammatory damage, and immunohistochemistry revealed an increase in capillaries. RT-PCR and Western blot experiments showed that the improvement of ischemia was related to an increase in lncRNA TUG1 and a decrease in miR-320 and that the expression of the related downstream proteins STAT3, VEGFR-2, Wnt-5a and β-catenin increased gradually. These changes promoted an increase in capillaries, the recovery of blood flow, and the improvement of muscle damage. Therefore, EPC transplantation can improve the inflammatory response of lower limb muscles by increasing the expression of lncRNA TUG1 and thereby accelerate the recovery of ischaemic limbs.

Highlights

- Objective to investigate the role of lncRNA TUG 1 in lower limb ischemic disease, and to prove that it can affect the expression of miRNA-320 and inhibit angiogenesis.
- The role of miRNA-320 in angiogenesis and the pathological development of lower limb ischemia.
- After transplantation of EPCs, the expression of miRNA-320 was affected by lncRNA TUG 1, and VEGFR-2 was increased by STAT3 and Wnt-5a / β-Catenin pathway, which alleviated the symptoms of ischemia.

1. Introduction

As the prevalence of ischaemic cardiovascular disease continues to rise, it is now has one of the world's highest mortality rates. Although medical approaches such as interventional catheter treatment and surgery continue to develop and innovate, the incidence continues to increase. According to the 2018 China Cardiovascular Disease Report, the prevalence of cardiovascular disease in China continues to rise, and the total number of patients is currently 290 million, including 11 million with coronary heart disease. Cardiovascular mortality accounts for more than 40% of all causes of death[1]. Research on stem cell regeneration therapy[2, 3] may bring new hope to patients with ischaemic cardiovascular disease[4]. Endothelial progenitor cells (EPCs) are bone marrow-derived cells that function in the formation of blood vessels, and their proliferation and differentiation can lead to angiogenesis and the growth of the vascular system[5]. Therefore, stem cell transplantation based on EPCs can effectively protect the function of ischaemic tissues and therefore constitutes a new approach for the clinical treatment of ischaemic diseases.
Angiogenesis refers to the formation of new blood vessels in a sprouting or non-sprouting manner based on the original capillaries and/or venules. Angiogenesis is involved in the proliferation and migration of mature vascular endothelial cells and represents one way that a mature body generates new blood vessels[6]. The proliferation and migration of endothelial cells play an essential role in angiogenesis. EPCs are precursors of endothelial cells, which form new blood vessels in situ. This process does not rely on the original vascular system and functions primarily during the embryonic period[7]. EPCs have continuous self-renewal capability and multidirectional differentiation potential, exhibit unique advantages in treating ischaemic diseases, and have a wide range of potential clinical applications[8].

Signal transducer and activator of transcription 3 (STAT3) has been widely proven to promote angiogenesis by promoting the proliferation and migration of vascular endothelial cells and inhibiting their apoptosis. Simultaneously, STAT3 promotes angiogenesis by promoting the proliferation of EPCs, thereby alleviating ischaemic injury to the lower limbs[9]. However, the regulatory role of STAT3 in EPC proliferation and ischaemic diseases remains unclear.

STAT3 is an essential member of the STATs family. It is widely expressed in different cells and tissues and participates in regulating physiological and pathological processes such as cell growth, proliferation, apoptosis, and malignant tumour transformation. Several studies have reported that activated STAT3 directly affects the proliferation, migration, and apoptosis of vascular endothelial cells by directly regulating VEGFR-2 expression, thereby promoting angiogenesis[10]. VEGFR-2 is the most critical growth factor in inducing EPCs to proliferate and differentiate into endothelial cells[11]. These results suggest that STAT3 promotes the proliferation and differentiation of EPCs by upregulating VEGFR-2 expression and participating in vascular repair. In addition, the Wnt/β-catenin signalling pathway plays an essential role in the angiogenic activity of endothelial cells and promotes angiogenesis by inducing the activation of STAT3[12]. Studies have shown that the STAT3 and the Wnt/β-catenin pathways are cross-promoting and jointly regulate retinal pigment epithelial cells[13]. These results suggest that cross-promotion of the STAT3 and Wnt/β-catenin pathways affects vascular endothelial cells and promotes angiogenesis. In a mouse model of lower limb ischemia, activation of the JAK2/STAT3 signalling pathway was shown to increase the proliferative activity of EPCs and assist in the repair of EPCs in blood vessels[9].

MicroRNAs (miRNAs) are a class of noncoding small RNAs that play an essential regulatory role in many human diseases by participating in important physiological activities such as cell growth, differentiation, and apoptosis. Numerous studies have confirmed that miRNAs are closely related to the development of ischaemic diseases and can therefore serve as important diagnostic or therapeutic targets. Various studies have shown that miR-320 significantly inhibits the proliferation, migration and angiogenesis of vascular endothelial cells[14] and is regulated by hypoxia[15]. Inhibiting the expression of miR-320 alleviates the nerve damage caused by spinal cord ischemia in rats and significantly improves the motor function of the hind limbs. Members of the miR-320 family include miR-320a/b/c/d/e, among which miR-320a/b/c/d are highly conserved in humans and other animals. miR-320a/b significantly inhibits endothelial cell growth and proliferation[16] and exhibits antiangiogenic effects[17].
Long noncoding RNAs (lncRNAs) are a class of noncoding RNA molecules that are longer than 200 nt and are located in the cytoplasm of the nucleus[18]. Recent studies have shown that lncRNAs are highly expressed in tissues and participate in various physiological and pathological processes at the transcription, translation, and epigenetic levels. lncRNAs affect the pathological development of many diseases by regulating angiogenesis[19]. Taurine-upregulated gene 1 (TUG1) is a lncRNA that is conservatively expressed in vascular endothelial cells from different sources. TUG1 significantly promotes tumour angiogenesis in human hepatoblastoma[20] and glioblastoma[21] by promoting the proliferation and migration of vascular endothelial cells and has a positive regulatory effect on Wnt/β-catenin signalling[22]. In mice with low-temperature-induced liver injury, the level of lncRNA TUG1 is significantly decreased; furthermore, the overexpression of lncRNA TUG1 alleviates the low-temperature-induced apoptosis of hepatic sinusoidal endothelial cells[23]. These studies show that lncRNA TUG1 plays a vital role in promoting vascular endothelial cell proliferation and migration and inhibiting their apoptosis by promoting angiogenesis. However, its effect on the proliferation and differentiation of EPCs and its role in ischaemic diseases have not been reported.

Therefore, we used a nude mouse model and established and cultivated EPCs to explore the roles of lncRNA TUG1, miR-320 and STAT3 expression and the VEGFR-2, Wnt-5a and β-catenin pathways in lower limb ischemia. We also examined the in vitro proliferation, differentiation and angiogenesis potential of EPCs derived from mouse bone marrow. Finally, the relationship between observed improvements in lower limb ischemia in model mice after EPC transplantation and lncRNA TUG1, miR-320, STAT3, Wnt-5a and β-catenin was verified in vivo. This study identified a new target and plan for the treatment of ischaemic cardiovascular disease.

2. Materials And Methods

2.1 Cell culture

The femur and tibia of nude mice (Beijing Vital River, China) were aseptically removed, washed with sterile PBS (Shanghai Qiagen, China), and then immersed in ethanol (Shanghai Titan, China) for disinfection. A syringe was used to aspirate the basal medium (Shanghai iCell Bioscience, China) and flush the bone marrow until the bones turned white. After collecting the washing solution, it was centrifuged at 1000g for 5 min, the cells were resuspended in EPC unique medium (Shanghai iCell Bioscience, China) and then cultured in a 37°C/5% CO₂ incubator (Thermo Fisher Scientific, USA).

2.2 Flow cytometry identification

Cell morphology and growth density were observed, and then the cells were resuspended in PBS and centrifuged at 300g for 5 min, suspended at density of 2×10⁶ cells/ml, and placed in a refrigerator at 4°C for 30 min. The cell suspension was transferred to a flow test tube, and 10 µl each of fluorescently labelled anti-mouse monoclonal antibodies CD14-FITC and CD34-APC was added (Shanghai Rebiosci Biotech, China). The cells were placed in a refrigerator at 4°C and incubated in the dark for 90 min, after
which 500 µl of PBS was added to each tube and centrifuged at 1000g for 10 min. The supernatant of each tube was then discarded, 300 µl of PBS was added, and the samples were stored in a refrigerator at 4°C in the dark. Flow cytometry (BD FASaria Cell Sorter, USA) was used to detect the expression of cell surface antigens.

### 2.3 CCK-8

EPCs were inoculated into a 96-well culture plate at a concentration of 3,000 cells/well and cultured overnight in an incubator. During 1-6 days of culture, 10 µl of CCK-8 (Shanghai Dojindo, China) was added to each well, and culture medium was used as a blank control. After mixing, the cells were incubated for 2 h then placed in a microplate reader (Thermo Fisher Scientific, USA) to measure the absorbance at 450 nm. The OD values of the test sample and blank control wells at 450 nm were recorded, and the final OD was determined using the following equation: OD value of the test sample – OD value of the control sample.

### 2.4 Transwell cultivation

The concentration of EPCs was adjusted to 80 000/ml. The Transwell cocultivation chamber (Corning, USA) was placed into the culture plate, 20 µl of PBS was added to immerse the chamber and the plate was incubated at room temperature for 30 min. A total of 0.1 ml of cell suspension was pipetted into the chamber, and 0.6 ml of culture medium was added to the lower well plate. The chamber was carefully placed into the well plate and incubated at 37°C. Migration was regularly observed, and cell status was recorded. After washing twice with PBS, 0.5 ml of 4% paraformaldehyde (Bio Biotech, USA) was drawn into a clean hole, placed in a small chamber and fixed for 15 min at room temperature. A Crystal Violet Staining Kit (Genemed Biotechnology, USA) was used to wash and stain the chamber, and the chamber was placed at room temperature for 20 min. One millilitre of Regent A was placed in the washing well, and the chamber was put back for washing three times until the Regent A became a colourless and transparent liquid. Images were acquired and recorded using an electron microscope (Nikon, USA).

### 2.5 Matrigel assay

Matrigel (B.D. Co, USA) was dissolved and added at 50 µl per well into a 96-well plate (kept on ice) and allowed to fix in a 37°C incubator for 1 h. The concentration of EPCs was then adjusted to $6 \times 10^5$/ml. To each well of a 96-well plate containing Matrigel, 100 µl of EPC suspension was added. The formation of blood vessels was observed via electron microscopy 24 h and 48 h after EPC addition.

### 2.6 Establishment of a nude mouse model of lower limb ischemia

Animal experiments were approved by the Ethics Committee of Nanchang University. Six-week-old NU/NU nude mice were purchased from Beijing Weitonglihua Laboratory Animal Technology Co., Ltd. Intraperitoneal injection of 4% chloral hydrate was used for anaesthesia, and the dose was 40 mg/kg per animal. The lower limbs of each nude mouse were exposed, and the skin was gently cut with a sterile scalpel. Tissue blood vessels were separated under a microscope at 15× magnification, the femoral artery
of the lower limbs was exposed and the tissue ligaments between the femoral artery, femoral vein and nerves of the lower limbs were separated. The two ends of the femoral artery were ligated with 10/0 nylon thread, the femoral artery was cut off in the middle the skin of the lower extremity was intermittently closed with 3/0 nylon thread; the incision was sterilized with iodophor. A laser Doppler blood flow metre (Beijing Jiandel Technology Co., Ltd.) was used to check the blood flow velocity of the ischaemic limb.

2.7 Cell transplantation

Endothelial progenitor cells were collected at a density of 15×10^6/ml. After 48 h of ischemia, 200 µl of EPCs was injected into the tails of nude mice. The control group was injected with the same amount of NS, and the normal control group was not treated. On the third day and the seventh day, laser Doppler was used to check the blood flow recovery of the lower limbs. The gastrocnemius muscle was used for the next experiment.

2.8 HE staining

The gastrocnemius muscle was placed in 10% paraformaldehyde fixative (Beijing Solarbio Co., China) for more than 24 h, and the edges were trimmed smoothly and rinsed with double-distilled water. After 24 h, conventional gradient ethanol dehydration was applied. The tissue was embedded in paraffin to form a wax block. Slices were cut to a thickness of 4-5 µm, attached to the slide and placed in a 65°C constant-temperature drying oven for 2 h. The baked slides were then dewaxed, placed in hematoxylin dye solution for 10 min and rinsed with running water. The sections were placed into 0.5% eosin solution for staining for 1 min and then rinsed with running water for 1-2 min. The slides were dehydrated and mounted with different concentrations of ethanol successively and then cleared with xylene. Finally, the slides were dried at room temperature and observed with neutral gum.

2.9 RT-PCR

After weighing the gastrocnemius muscle tissue, RNA was extracted by the TRIzol method (Beijing TransGen Biotech, China), and 1 µl was used to determine the OD value with a nucleic acid analyser (G.E. Medical Company, USA); the remainder of the sample was stored at −80°C. A cDNA reverse transcription kit (Beijing TransGen Biotech, China) was used in the PCR amplification instrument (Thermo Fisher Scientific, USA) to synthesize cDNA as follows: 42°C for 15 min, 85°C for 5 s, 4°C ∞ and storage at −20°C until further use. A two-step cycle protocol was used to measure mRNA expression, and a PCR amplification kit (Beijing TransGen Biotech, China) was used for amplification on a fluorescent quantitative PCR instrument (ABI 7500, ABI Inc., USA). The data were analysed and the relative TUG1 and miR-320 levels calculated using the comparison 2 − ΔΔCq method. Primers used were as follows: TUG1 forward 5'-CTGAAGAAAGGCAACATC-3', reverse 5'-GTAGGCTACTACAGGATTG-3', miRNA320 forward 5'-AAAAGCUGGGUUGAGAGCGA-3'.

2.10 Western blot analysis

Gastrocnemius muscle tissue was cut into small pieces, and the protein components were extracted with a protein extraction kit (Beijing TransGen Biotech, China) and centrifuged at 14,000g for 10 min at 4°C.
The supernatant was carefully collected. Samples were incubated in a water bath at 99°C for 10 min and then stored at −20°C for later use.

The SDS-PAGE gel was prepared, wrapped with plastic wrap to exclude exposure to the air, and placed in a refrigerator at 4°C for later use. The prepared SDS-PAGE gel (Beijing Boster Biological Technology, China) was placed into the electrophoresis tank, and the 50-µl protein samples were added to the gel lanes in the electrophoresis tank. After electrophoresis, the glass plate was transferred to a NC membrane (Thermo Scientific Company, USA). The NC membrane was removed after electroporation and sealed at room temperature for 1 h. The membrane was washed once with PBST (Beijing Solarbio Co, China) for 10 min, after which the following corresponding primary antibodies were added: anti-VEGFR-2 (CST, USA, 55B11), anti-Wnt-5a (Novus Biologicals, USA, NBP2-24752), anti-STAT3 (CST, USA, 79D7), anti-β-catenin (CST, USA, D10A8), and anti-β-tubulin (CST, USA, 9F3). The membrane was placed in a refrigerator at 4°C overnight. The membrane was washed three times with PBST, and secondary antibody (CST, USA) was added. The ratio of exposure liquid (Beijing Solarbio Co, China) was A:B = 1:1, and an appropriate amount was placed on the film so as to completely cover it. Proteins were detected based on the intensity of grey colour, as determined using Image J software.

2.11 Statistical analysis

The experimental data were analysed using GraphPad Prism 7.0 statistical software, and the values are expressed as the mean ± standard deviation (SD). ANOVA was used to compare data between multiple groups, with p<0.05 indicating that a difference was statistically significant. A t-test was used for comparisons between two groups, and p<0.05 was considered statistically significant.

3. Results

3.1 Lower limb blood flow velocity after ischemia

To study the success of establishment of the lower limb ischemia model, we ligated the femoral artery of nude mice and examined the blood flow in the lower limbs using a laser Doppler flowmeter, as shown in Figure 1(A). Within 48 h of ischemia, the blood flow velocity of the lower limbs was significantly decreased after femoral artery ligation compared with the contralateral nonischaemic lower limbs and the normal control group, as shown in Figure 1(B). The nude mouse lower limb ischemia model was therefore successfully constructed.

3.2 Gastrocnemius muscle cells after ischemia

Damage to gastrocnemius muscle cells in the lower limbs of ischaemic nude mice was assessed because the gastrocnemius muscle tissue is independently supplied by the femoral artery. Therefore, gastrocnemius muscle tissue was stained with HE to observe the inflammatory response of muscle cells after ischemia. As shown in Figure 1(C), the gastrocnemius muscle cells in the ischaemic group had a disordered arrangement of muscle fibres, decreased continuity, and widened gaps relative to the
contralateral nonischaemic limb and the normal control group; the model group had no obvious changes from 6 to 12 h. However, from 12 to 24 h and 24 to 48 h, cytoplasmic eosinophilia gradually increased, cell necrosis increased, cytoplasmic redness deepened, and a nearly granular phenomenon appeared. The gastrocnemius muscle exhibited no obvious collateral circulation within 48 h of femoral artery ischemia.

3.3 The degree of gastrocnemius muscle cell damage is related to a decrease in IncRNA TUG1

To study the expression of IncRNA TUG1 in the gastrocnemius muscle cells of the ischaemic limb, we used RT-PCR to quantify the expression of IncRNA in each group. The results are shown in Figure 2(A). As the ischaemic time was prolonged, the expression of IncRNA TUG1 declined, and the expression of miRNA-320 increased.

3.4 Cell damage-related protein analysis

To study protein expression in the gastrocnemius muscle cells of the ischaemic limb, we used Western blotting, as shown in Figure 2(B). Compared with the normal control group, expression of STAT3, Wnt-5a, β-catenin, and VEGFR-2 decreased after ischemia, and the degree of decrease was greater as the ischaemic time was prolonged.

3.5 Nude mouse bone marrow cells contain EPC

We cultured cells extracted from the bone marrow of nude mice, and the morphology of these cells was observed under a microscope. As shown in Figure 3(A), electron microscopy revealed the presence of irregular cell structures, such as strips and spheres. These cells were identified by flow cytometry, as shown in Figure 3(B). The rate of CD14 positivity in bone marrow–derived cells of nude mice was 99.9%, and the positivity rate of CD34 was 86.4%, indicating that the cell population included EPCs.

3.6 EPC extracted from bone marrow have proliferative potential

The proliferation capability of the cells extracted from the bone marrow of nude mice was examined using a CCK-8 kit. As shown in Figure 4(A), over time, the cell proliferation rate increased.

3.7 EPC extracted from bone marrow can migrate

To assess the ability of bone marrow–derived EPCs cells to migrate, we used a Transwell box. As shown in Figure 4(B), the EPCs exhibited an ability to migrate over time.

3.8 EPC extracted from bone marrow exhibit angiogenic activity

To assess the angiogenic potential of EPCs derived from the bone marrow of nude mice, we used Matrigel culture. As shown in Figure 4(C), EPCs formed cell clusters at 24 h, and this cell cluster formation reflected the sprouting capability of EPCs during blood vessel formation[24, 25]. After 48 h, the
angiogenic activity increased significantly; after 72 and 96 h, the vascular morphology of the cells became more obvious, indicating that the cells extracted from bone marrow exhibit a certain degree of angiogenic activity.

3.9 EPC transplantation can improve lower limb ischemia

To assess the improvement in blood flow in the lower limbs of ischaemic nude mice following EPC transplantation, we analysed blood flow using a laser Doppler flowmeter. As shown in Figure 5(A), nude mice exhibited obvious ischemia in the left limb (mark 2) compared with the normal side limb (mark 1) on the second day of ischemia. On the fifth day, the collateral recovery effect of the EPC-injection group was better than that of the NS-injection control group, and there was no significant difference in the subsequent two tests. On the ninth day of ischemia, the recovery of the EPC-injection group was still better than that of the control group. The lower limbs of nude mice thus began recovering by the fifth day of ischemia and were markedly improved by the seventh day after transplantation, and blood flow recovery was greatly improved compared with that of the contralateral limb control group. Therefore, transplantation of EPCs is very helpful for improving ischemia of the lower limbs of nude mice.

3.10 Transplantation of EPC reduces gastrocnemius muscle cell damage after ischemia

To further verify that transplanted EPCs can ameliorate damage to the gastrocnemius muscle cells of ischaemic nude mice, we stained muscle tissue with HE, as shown in Figure 5(B). The gastrocnemius muscle cells of the normal control group exhibited the largest cross-section and were arranged in an orderly manner. The gastrocnemius muscle cell area of the NS-injected control group and the EPC-injected experimental group exhibited a certain degree of atrophy, and that of the control group was more significant, with increased cell necrosis. The cell arrangement of the experimental group was more orderly than that of the control group. Therefore, the gastrocnemius muscle cells of nude mice transplanted with EPCs were effectively restored, and cell damage was reduced.

3.11 EPC transplantation can increase gastrocnemius microvessel density after ischemia

To determine the microvessel density in the lower limbs of nude mice transplanted with EPCs, we used immunohistochemistry analysis. As shown in Figure 5(C), many dark-blue cell nuclei were observed in the experimental group injected with EPCs. The capillary density (CD31-positive area) of the gastrocnemius muscle tissue on the ischaemic side of the lower limbs of nude mice was slightly higher than that of the mice in the control group injected with NS and higher than that of the normal control group. The density of microvessels is a good indicator of the recovery of muscle blood flow. These results demonstrate that EPC injection can promote the renewal of capillaries.

3.12 EPC transplantation to improve lower limb ischemia is related to an increase in IncRNA TUG1 expression
To evaluate the expression of lncRNA TUG1 in gastrocnemius muscle cells transplanted with EPCs in ischaemic limbs, we used RT-PCR analysis. The results are shown in Figure 6(A). Comparison of gene expression between nude mice transplanted with EPCs and the control group after 48 h of ischemia showed that the expression of lncRNA TUG1 increased, whereas the expression of miRNA-320 decreased.

### 3.13 Analysis of related proteins after ischemia improvement

To study the effect of inhibiting EPCs on protein expression in ischaemic gastrocnemius cells, we detected EPCs using Western blotting, as shown in Figure 6(B). The results showed that compared with the NS-injected control group, the transplantation of EPCs increased the expression of STAT3, Wnt-5a, β-catenin, and VEGFR-2 protein.

### 4. Discussion

The animal ischemia model used in our study was generated by ligating the femoral artery alone. Using this method, some scholars have analysed blood flow velocity using laser Doppler after ligation of the femoral artery at the groin and have found that blood flow can be reduced to 10–20% of the normal level after ligation. However, this method has faster blood flow recovery[26, 27] and is only suitable for experimental studies within 72 h[28]. Our study sought to analyse the effects of EPC transplantation after ischemia for 6 h, 12 h, 24 h, 48 h and other times in comparison with controls. Nude mice were selected to facilitate subsequent transplantation of autologous EPCs to assess the improvement in ischemia and avoid autoimmune reactions leading to the death of the mice.

Studies have shown that lncRNA TUG1 is abnormally expressed in a variety of malignant tumours. The abnormal expression of TUG1 is closely related to tumour cell proliferation, migration, cell cycle changes, apoptosis inhibition and drug resistance[29], and it also plays an important role in the growth of aneurysms[30]. This shows that the high expression of lncRNA TUG1 has a significant effect on promoting angiogenesis. In human gliomas, the downregulation of miRNA-320c expression promotes tumour growth and metastasis[31]. Additionally, miRNA-320 regulates the radiosensitivity of C33AR cervical cancer cells by targeting β-catenin[32]. Studies have found that inhibiting the expression of miR-320 in myocardial microvascular endothelial cells (MMVECs) of diabetic rats promotes angiogenesis and enhances the proliferation and migration capabilities of MMVECs[15]. This indicates that the expression of miRNA-320 plays an important role in inhibiting angiogenesis. Therefore, we know from the results of PCR analyses of gastrocnemius muscle cells subjected to different ischemia times that the expression of lncRNA TUG1 gradually decreases as the expression of miRNA-320 gradually increases as the ischaemic time increases. In the bioinformatics analysis, we found that the conserved sequence of miR-320 contains a binding site for lncRNA TUG1. This demonstrates that there is mutual inhibition between TUG1 and miR-320, but the specific regulatory mechanism needs further study.

The latest research has found that the expression of STAT3 is closely related to breast cancer tumour growth and metastasis[33], and it has also been found that the JAK2/STAT3 signalling pathway has a neuroprotective effect on focal cerebral ischemia[34]. This shows that STAT3 plays an important role in
angiogenesis; therefore, we examined the expression of STAT3 protein in ischaemic mice using Western blotting. The expression of STAT3 protein in the lower limbs of the ischaemic nude mice gradually decreased as the ischaemic time was prolonged. In hepatic stellate cells, the α-Wnt/β-catenin axis was shown to promote the progression of hepatocellular carcinoma[35]. Some studies have reported that IncRNA ASB16-AS1 promotes the proliferation, migration and invasion of cervical cancer cells by regulating the miR-1305/Wnt/β-catenin axis[36]. STAT3 can interact with the wnt/β-catenin pathway. Therefore, to determine whether IncRNA TUG1 also promotes cell growth and proliferation by regulating STAT3 and the Wnt/β-catenin axis, further research is needed to clarify this pathway. The results of our Western blotting experiments showed that the expression of Wnt-5a and β-catenin proteins in gastrocnemius muscle cells gradually decreases with an increasing ischaemic time, and the trend is the same as that for STAT3. VEGFR-2 is a growth factor that plays an important role in angiogenesis. Our Western blotting results showed that as the ischaemic time was prolonged, the expression of VEGFR-2 gradually decreased. The key to treating ischaemic diseases therefore is to increase VEGFR-2 expression. STAT3 directly regulates the expression of VEGFR-2 through activation, and STAT3 interacts with wnt/β-catenin. Whether STAT3 and wnt/β-catenin can jointly promote the expression of VEGFR-2 and improve the damage associated with ischaemic diseases will require further research.

EPCs are circulating precursor cells[37] that originate from the bone marrow[38] and have the ability to differentiate into mature endothelial cells (ECs) [39]. To facilitate the identification of EPCs in peripheral blood, studies have proposed methods to identify cell surface antigens, such as CD14, CD34 and kinase insertion domain receptor (KDR), which is also known as VEGFR-2 [40]. Therefore, we directly extracted the EPCs in the bone marrow of the nude mice used in our experiments. EPCs are bone marrow–derived stem cells that participate in the formation of blood vessels, and their proliferation and differentiation can lead to angiogenesis and growth of the vascular system[5]. Therefore, we experimentally verified that EPCs exhibited growth, proliferation, migration and angiogenesis capabilities and that the cells extracted from the bone marrow of nude mice have potential value for improving ischemia of the animals’ own lower limbs.

However, a disadvantage of our study was that we did not detect the expression of VEGFR-2 in the extracted EPCs, and we did not process the EPCs. By upregulating or downregulating the expression of VEGFR-2, we would be able to examine the mechanism of cell growth and blood vessel formation. PCR analyses alone would not elucidate the regulatory pathway of the mechanism whereby EPCs improve ischemia. Such analyses could only show that the improvement of ischemia is related to changes in the expression of IncRNA TUG1/miR-320. However, it is still unknown whether TUG1 regulates protein changes in downstream pathways. It would be necessary to further increase TUG1 expression or downregulate miR-320 expression to clarify this specific pathway sequence. Whether miR320 has an effect on the expression of STAT3 protein is also unknown. It is currently known that miR-320 participates in the JAK/STAT signalling pathway to regulate inflammation[41]. Through our Western blotting results, we confirmed that increased expression of STAT3 and wnt-5a/β-catenin protein induces VEGFR-2–mediated improvement in ischemia, but the genes that dominate upstream regulation are still unknown. Therefore, further experiments will be needed to elucidate the downstream regulation of IncRNA
TUG1/miR-320 and completely define the upstream regulatory mechanism involving the joint effects of the STAT3 and Wnt/β-catenin pathways and EPCs in the ischemia model.

5. Conclusion

The proliferation and migration of endothelial cells play an essential role in angiogenesis, and EPCs are the precursor cells of endothelial cells. EPCs have a continuous self-renewal function and multidirectional differentiation potential and exhibit unique advantages in treating ischaemic diseases. The transplantation of EPCs can effectively protect the integrity and function of ischaemic tissue. Through in vivo experiments, we demonstrated that cell damage occurs within 48 h of inducing ischemia in the lower limbs of nude mice by cutting the femoral artery by ligation, and expression of the IncRNA TUG1 gene, which promotes angiogenesis, decreases, whereas expression of miR-320, which inhibits angiogenesis, increases. This leads to a decline in the expression of the STAT3 and Wnt-5a/β-catenin proteins that promote the differentiation of ECs, and the decrease in VEGFR-2 expression temporarily inhibits the collateral vessels. We found that EPCs were contained among the bone marrow–derived cells extracted from nude mice, and under standard culture conditions, these cells could grow, proliferate, migrate and form blood vessels. Therefore, we transplanted them into ischaemic animals to further demonstrate the ability to improve ischemia, alleviate muscle cell injury and that the increase in microvessel density of muscle tissue is related to the increase in IncRNA TUG1 expression, which promotes angiogenesis, and to the decrease in angiogenesis-inhibiting miR-320 expression. Ischemia contributes to the increase in STAT3 and Wnt-5a/β-catenin protein expression to promote the differentiation of EPCs and finally causes an increase in VEGFR-2 expression to promote collateral circulation.

Abbreviations
Declarations

Conflicts of interest: The authors have no conflicts of interest to declare.

Ethics approval and consent to participate: Not applicable.

Consent for publication: Not applicable.

Availability of data and materials: Not applicable.

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(VII) Final approval of manuscript: All authors

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Figures
Figure 1

Establishment of a lower limb ischemia model and muscle inflammatory injury at different ischaemic times. A. The blood flow velocity of the lower limbs of the ischaemic group mice was compared with that of the contralateral nonischaemic lower limbs and the normal control group mice using laser Doppler blood flow measurement. B. Differences in laser Doppler blood flow velocity measurements of ischaemic nude mice in each group were compared using ANOVA (P<0.01). Blood flow velocity between the
ischaemic lower limbs and contralateral nonischaemic limbs at different times showed statistically significant differences (**p<0.01, ***p<0.001). C. HE staining (1000×) of the lower limbs of mice in the ischaemic group compared with the contralateral nonischaemic lower limbs and the lower limbs of the normal control group.
Muscle gene and protein expression at different ischaemic times. A. ANOVA indicated a significant difference in muscle gene and protein expression with increasing ischaemic time between each group (p<0.0001). The expression of IncRNA TUG1 in the gastrocnemius muscle of each group was significantly different compared with the normal control group (*p<0.05, **p<0.01, ***p<0.001). Compared with the normal control group, expression of the miRNA-320 gene in the gastrocnemius muscle of each group was also significantly different (*p<0.05, **p<0.01, ***p<0.001). B. Left: Grey values of STAT3, wnt-5a, β-catenin and VEGFR-2 protein bands in lower limb muscles at different ischaemic times. Right: Expression of STAT3, wnt-5a, β-catenin, and VEGFR-2 protein differed significantly with increasing ischaemic time (p<0.0001, ANOVA); expression at different ischaemic times also differed significantly with the normal control group, as indicated by t-tests (*p<0.05, **p<0.01, ***p<0.001).
Figure 3

Cell morphology and flow cytometric identification of cells extracted from bone marrow. A. Cell morphology and structure were examined under an electron microscope (200×). B. The results of flow cytometric identification of cells extracted from bone marrow of nude mice.
Figure 4

Cells extracted from bone marrow exhibit proliferation, migration, and vascularization capabilities. A. Histogram of OD value of each sample (**)p<0.01). B. The cells were observed under a microscope (200x) through the Transwell culture chamber, and a microplate reader was used to analyse the absorbance at 570 nm, and the cell migration rate was then calculated (*p<0.05). C. As observed under a microscope
(24h, 48h for 200×), 72h, 96h for 100×, the growth state of EPCs on Matrigel gradually increased with the number of cell clusters (*p<0.05) until the cells assumed a clear vascular shape.

**Figure 5**

Changes in blood flow of lower limbs after transplantation of EPCs cells, as well as inflammatory damage and microangiogenesis of muscle tissue. A. Mice with lower limb ischemia for 48 h were injected with EPCs as the experimental group or injected with NS as a comparison of blood flow velocity in the
control group at different times. B. Mice with lower limb ischemia for 48 h were variously treated with EPCs or NS, and HE staining (100×) of the normal control group was performed. C. CD31-positive area determined by immunohistochemical staining of mice with lower limb ischemia for 48 h after injection of EPCs or NS, relative to the normal control group (100×).

**Figure 6**
Gene and protein expression in lower limb muscles after different treatments relative to the normal control group after 48 h of ischemia. A. Forty-eight hours after ischemia, different treatments were administered, and gene expression in the lower limb muscles differed significantly compared with that of the normal control group (\(p<0.0001\), ANOVA). Gastrocnemius IncRNA TUG1 gene expression in the experimental group and the control group differed significantly compared with the normal control group (\(**p<0.01, ***p<0.001\)). Compared with the normal control group, expression of the miRNA-320 gene in gastrocnemius muscle of each group also differed significantly (\(**p<0.01, ***p<0.001\)). B. Left: Effect of different treatments on the grey values of STAT3, wnt-5a, \(\beta\)-catenin and VEGFR-2 protein bands in the normal control group after 48 h of ischemia. Right: Analysis of the protein expression of STAT3, wnt-5a, \(\beta\)-catenin and VEGFR-2 relative to the normal control group after 48 h of ischemia after different treatments (\(p<0.001\), ANOVA). The experimental group, control group and normal control group differed significantly by t-test as well (\(*p<0.05, **p<0.01, ***p<0.001\)).