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Autophagy of umbilical cord mesenchymal stem cells induced by rapamycin conduces to pro-angiogenic function of the conditioned medium

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ABSTRACT

Angiogenesis is critical for wound healing and tissue repair. Umbilical cord mesenchymal stem cells (UCMSCs)-conditioned medium has certain actions to promote angiogenesis, and is expected for wound healing and tissue repair. However, recent studies showed that the pro-angiogenic efficacy of unprocessed MSCs-conditioned medium is low, and insufficient for tissue repair. Autophagy is a process for protein recycling and a contributor for cell exocrine, which may enhance pro-angiogenic efficacy of the conditioned medium by stimulating cytokine release from UCMSCs. Therefore, in this study we attempted to obtain enhanced autophagy in UCMSCs using different concentrations of rapamycin and compared pro-angiogenic functions of the conditioned media. The *in vitro* data showed that although 100 nM-10 μM rapamycin all could induce autophagy in UCMSCs, 100 nM was the best dose to optimize the angiogenic effect of the conditioned medium. The *in vivo* data also showed that pro-angiogenic effect of the optimized conditioned medium was more obvious than that of the control conditioned medium (0 nM group) in the injected matrigel plaques. Further, the expressions of VEGF, FGF-2, MMP-9, PDGF-α and PDGF-β were markedly increased in UCMSCs treated with 100 nM rapamycin. In conclusion, appropriately enhancing autophagy of UCMSC can improve pro-angiogenic efficacy of the conditioned medium, which may optimize therapeutic applications of UCMSCs-conditioned medium in wound healing and tissue repair.

1. Introduction

Umbilical cord mesenchymal stem cells (UCMSCs) are a type of multipotent stem cells derived from the Wharton's Jelly of human umbilical cords. Like other kinds of mesenchymal stem cells (MSCs), UCMSCs have a high self-renewal ability and multi-potentials to differentiate into functional cells under appropriate conditions [1,2]. Currently, UCMSCs have been the most commonly used seed cells in regenerative medicine due to their special properties such as a wide range of sources, easy of obtaining, low immunogenicity, and a steady genetic background. However, recent studies suggested that the differentiation efficiency of MSCs (including UCMSCs) transplanted into patients and some animal models was very low, and the differentiated cells not enough to complement the lost cells in the injured tissues or organs [3,4].

Growing evidence indicates that the actions of MSCs to repair the damaged tissues or organs mainly result from their strong exocrine functions [5,6]. UCMSCs have been proved to produce a variety of growth factors and cytokines such as fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), epidermal growth factor (EGF), transforming growth factor-β (TGF-β), nerve growth factor (NGF), interleukin (IL)-1β and IL-6 and tumor necrosis factor (TNF-α), as well as other intercellular messengers such as exosomes, circRNAs and microRNAs, which participate in regulating cell proliferation, differentiation, angiogenesis, and so on [6–8]. It has been shown that most of above-mentioned exocrine factors participate in angiogenesis, which is a critical prerequisite for wound healing and tissue repair [9,10].

It is known that the formation speed and amount of newly formed blood vessels in the injured tissues and organs determine the quality of wound healing and the efficiency of tissue and organ repair. A recent study showed that the conditioned medium derived from UCMSC culture contained a certain amount of FGF, VEGF and other angiogenic factors, and had a weak function in promoting the proliferation of vascular endothelial cells but failed to affect angiogenesis [11]. This study indicates that angiogenic function of the conditioned medium

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from the untreated UCMSCs is very limited. To improve pro-angiogenic efficacy of UCMSCs-conditioned medium, some groups have recently attempted to use gene manipulation techniques to enhance the exocrine functions of UCMSCs [12–14]. For instance, Cho et al. applied the targeted genome engineering (transfecting TALEN-L/R targeting vectors containing inducible VEGF gene into UCMSCs) to enhance VEGF secretion and improve the pro-angiogenic efficacy of UCMSCs-derived conditioned medium in repairing myocardial infarction [12]. Xiong et al. utilized adenovirus-associated virus (AAV)-mediated VEGF gene overexpression to improve therapeutic efficacy of UCMSCs in Parkinson's disease [13]. Sharma et al. tried to obtain the increased generation of exosomes from UCMSCs through stimulation with activated T cells [14]. However, there are still some problems such as high cost, complex operation and ethical restriction of using gene manipulation to improve the therapeutic efficacy of UCMSCs. Recently, An et al. enhanced the secretion of VEGF by inducing autophagy of bone marrow mesenchymal stem cells (BMSCs), thereby enhancing the efficacy of conditioned medium to promote angiogenesis [15]. However, compared to UCMSCs, BMSCs are not a kind of seed cells to generate the pro-angiogenic conditioned medium. A study showed that UCMSCs-conditioned medium contained much more growth factors and cytokines than BMSCs-conditioned medium, meanwhile its angiogenic effect was also three times greater than BMSCs-conditioned medium [16]. Therefore, it is necessary to develop an efficient UCMSCs-conditioned medium for promoting angiogenesis through inducing autophagy of the seed cells.

Autophagy is a critical physiological process to maintain cell homeostasis, and plays an important role in a series of cell functions [17–20]. A recent study showed that autophagy is required to maintain the stemness and regenerative potential of MSCs and haematopoietic stem cells (HSCs) [17,18]. The activation of autophagy makes healthier and younger of old HSCs and enhances their metabolism that are closely related to the exocrine functions of stem cells [18,19]. In addition, autophagy could also keep homeostasis of MSCs-derived cells such as adipocytes, chondrocytes and osteocytes [20]. Thus, we hypothesized that strengthening autophagy in UCMSCs may increase their exocrine ability and improve the pro-angiogenic efficacy of their conditioned medium. This study was designed to prove this hypothesis through *in vitro* and *in vivo* experiments.

2. Materials and methods

2.1. Sources of cells and animals

UCMSCs were kindly gifted from the Stem Cell and Biotherapy Engineering Research Center of Henan Province, and primary human umbilical vein endothelial cells (HUVECs) were purchased from the Kunming Cell Bank of Chinese Academy of Science (Kunming, China). C57BL/6 mice (male) were obtained from Beijing Vital River Laboratory Animal Technology Co., Ltd (Beijing, China). The animal study protocol was approved by the Ethics Committee of Xinxiang Medical University and conformed to the Guide for Care and Treatment of Experimental Animals published by the Ministry of Science and Technology of the People's Republic of China (Beijing, China).

2.2. Cell culture

The 2nd generation UCMSCs were cultured in DMEM with 10 % FBS, 100 U/mL penicillin and 100 U/mL streptomycin and maintained in a humidified incubator with 5 % CO_2 at 37 °C. The first replacement of medium was performed following 24-h culture, and then the medium was replaced every three days. When the cells grew to 80 % confluence, they were digested and seeded into 24-well plates, and then cultured in DMEM with different concentrations (0, 100 nM, 1 μM and 10 μM) of rapamycin for 6 h to induce autophagy. Thereafter, the medium was replaced following washing with PBS for 3 times, incubated with UCMSCs for an additional 24 h, and collected for the following experiments. The autophagy levels of different groups of UCMSCs were detected by immunofluorescent LC-3 staining.

HUVECs were cultured in DMEM with 10 % FBS, 100 U/mL penicillin and 100 U/mL streptomycin, and maintained at 37 °C with 5 % CO_2. The medium was replaced every three days. The cells at passages 3–5 were used in this study.

2.3. The in vitro tube formation assay

Matrigel Matrix (BD Biosciences, San Jose, CA, USA) was thawed on ice and plated into 96-well plates (100μL/well) overnight. HUVECs were cultured with different conditioned media for 24 h, digested with 0.25 Trypsin-EDTA, and washed with PBS twice. Cells (1×10^4/well) were then seeded into the matrigel-coated plates, and cultured with different conditioned medium for an additional 3 h. The tube formation was viewed and imaged under an invert microscope (Olympus, Tokyo, Japan). The length of tube-like networks was calculated using Image *J* software.

2.4. The in vivo tube formation assay

C57BL/6 mice were anesthetized with sodium pentobarbital (80 mg/kg, i.p.), and 200 μL of matrigel mixed with HUVECs (5×10^4/100 μL) was subcutaneously injected into mouse inguinal areas as previously published protocols [21]. The conditioned medium (200 μL) was injected into mouse groins around matrigel plaques every day until sacrifice. One week later, the mice were euthanized under anesthesia, and the matrigel plaques were harvested and frozen at −80 °C. The frozen plaques were sectioned and stained with H&E following standard protocols. The immunostaining of CD31 was performed on matrigel plaque sections as previously described [22]. The animal use in this study was reviewed and approved by the Experimental Animal Ethics Committee of Xinxiang Medical University. All animal experiments complied with the ARRIVE guidelines, and were performed according to the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978).

2.5. Quantitative reverse-transcriptase polymerase chain reaction

UCMSCs were cultured in DMEM with or without rapamycin (100 nM) for 6 h. Total RNA was extracted from different groups of UCMSCs using TRIzol™ Reagent (Takara, Beijing, China). cDNA was synthesized with a PrimeScript™ RT Kit (Takara) according to the manufacturer's instructions and PCR reactions were performed using HieffTM qPCR SYBR Green Master Mix (Takara) on a Fast 7500 real-time PCR machine (Applied Biosystems, Carlsbad, CA, USA). The relative mRNA expressions were quantified using the comparative threshold cycle method. The PCR primers were designed and synthesized by GeneCreate Biological Engineering Co., Ltd (Shanghai, China). The primers listed as follows: VEGF-A forward: AGGGCAGAATCATCACGAAGT, reverse: AGGGTCTCGATTGGATGGCA; FGF-1 forward: GCCCTGACCGAGAAGTTTAATC, reverse: CCCCGTTGCTACAGTAGAGG; FGF-2 forward: AGAAGAGCGACCCTCACATCA, reverse: CGGTTAGCACACACTCCTTTG; TGF-α forward: AGGTCCGAAAACACTGTGAGT, reverse: AGCAAGCGGTTCTTCCCTTC; MMP-3 forward: CTGGACTCCGACACTCTGGA, reverse: CAGGAAAGGTTCTGAAGTGACC; MMP-9 forward: TGTACCGCTATGGTTACACTCG, reverse: GGCAGGGACAGTTGCTTCT; PDGF-α forward: GCAAGACCAGGACGGTCATTT, reverse: GGCACTTGACACTGCTCGT; PDGF-β forward: CTCGATCCGCTCCTTTGATGA, reverse: CGTTGGTGCGGTCTATGAG; HIF-1α forward: CACCACAGGACAGTACAGGAT, reverse: CGTGCTGAATAATACCACTCACA; Ang II forward: CTCGAATACGATGACTCGGTG, reverse: TCATTAGCCACTGAGTGTTGTTT; GAPDH forward: GGCTGTTGTCATACTTCTCATGG, reverse: GGAGCGAGATCCCTCCAAAAT.

2.6. Statistical analysis

Statistical analysis was performed with SPSS 15.0 software. Data are presented as means ± standard deviations (SDs). The univariate comparisons of means were evaluated with Student's *t* tests or one-way ANOVA with Tukey's post-hoc adjustment for multiple comparisons when appropriate.

3. Results

3.1. Identification of UCMSC autophagy

To induce autophagy of UCMSCs, we treated them with different dosages (0, 100 nM, 1 μM and 10 μM) of rapamycin and identified utilizing immunofluorescent LC3 staining. As shown in Fig. 1, rapamycin enhanced autophagy (LC-3 fluorescence) in UCMSCs in a dose-dependent manner.

3.2. In vitro tube formation and screen of the optimal conditioned medium

UCMSCs were cultured in fresh DMEM with different dosages of rapamycin for 6 h, the medium was replaced with fresh one after washing with PBS for 3 times and incubated for an additional 24 h, and then the conditioned medium was collected. HUVECs were cultured in the different conditioned media in matrigel-coated plates for 3 h. As shown in Fig. 2, tube branching length, tube segment length, tube node

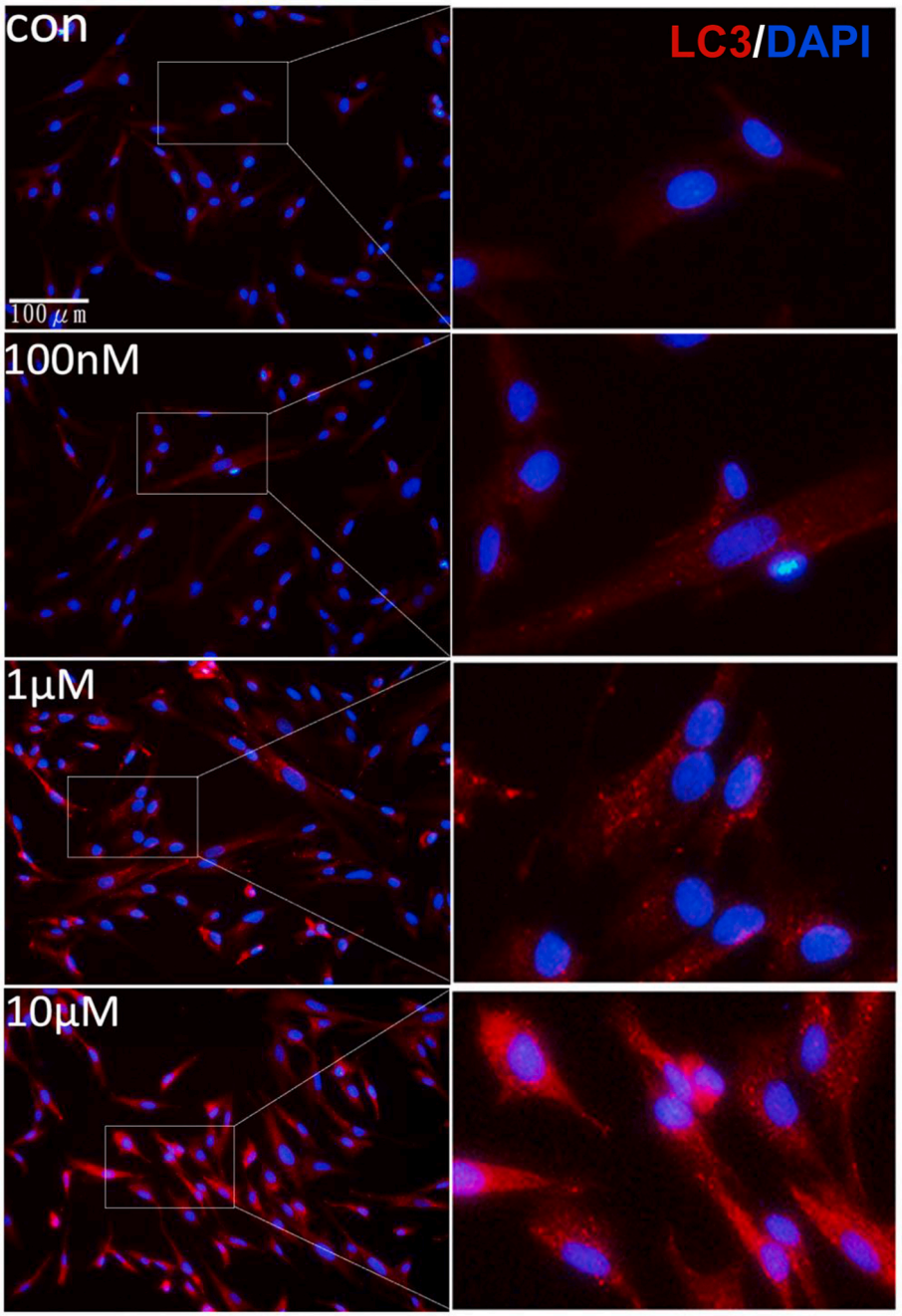

Fig. 1. Autophagy (LC3) levels in UCMSCs following exposure to 0, 100 nM, 1 μM and 10 μM rapamycin for 6 h. Red positive: LC-3 staining; Blue positive: DAPI staining (nuclei); n = 5/group; scale bar = 100 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

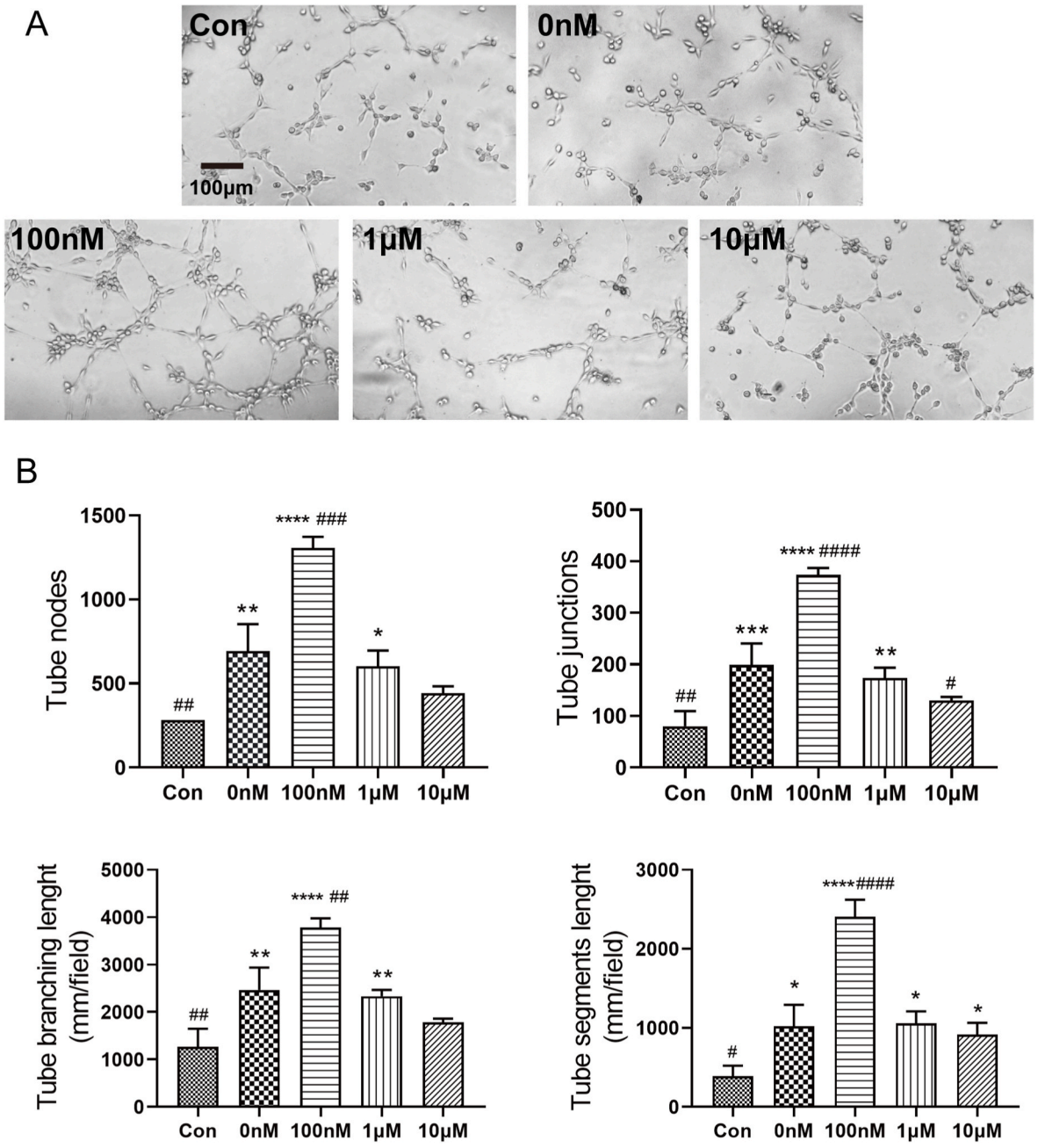

Fig. 2. Effects of different conditioned media on tube formation of HUVECs. A. The represent images show tube formation of HUVECs following culture with different conditioned media for 3 h. **B.** Quantification of tube branching length, tube segment length, tube node and tube junction of tube formation images. n = 4/group; scale bar = 100 μm $^{*}P < 0.05$, vs. control group; $^{**}P < 0.01$, vs. control group; $^{****}P < 0.0001$, vs. control group; $^{\#}P < 0.05$, vs. 0 nM group; $^{\#\#}P < 0.01$, vs. 0 nM group; $^{\#\#\#}P < 0.001$, vs. 0 nM group; $^{\#\#\#\#}P < 0.0001$, vs. 0 nM group.

and tube junction of HUVECs cultured with the 4 kinds of conditioned media were all markedly higher than that cultured with common medium (control). Of note, among the 4 kinds of conditioned media, the one induced with 100 nM rapamycin had the best pro-angiogenic efficacy. Thus, this medium was viewed as the optimal conditioned medium and used in the following experiments.

3.3. *In vivo* pro-angiogenic effect of the optimal conditioned medium

To further evaluate the pro-angiogenetic effect of the optimal conditioned medium, we performed *in vivo* experiments to investigate tube formation of HUVECs in matrigel matrix that was subcutaneously planted in mouse inguinal areas. H&E staining (Fig. 3A and B) showed that the number of newly formed vessels in the matrigel plaques treated with the optimal conditioned medium was significantly higher than that the control conditioned medium (0 nM group). This data was further confirmed by CD31 immunostaining, which also showed more newly formed vessels in matrigel plaques treated with the optimal conditioned medium than that treated with the control conditioned medium (Fig. 3C and D).

3.4. *Expressions of angiogenesis-related genes in autophagic UCMSCs*

Previous studies have shown that UCMSCs can secrete growth factors such as FGF, VEGF, EGF, TGF-β, IGF, NGF and HGF, and most of them are related to angiogenesis [9,10,23–25]. To further elucidate the

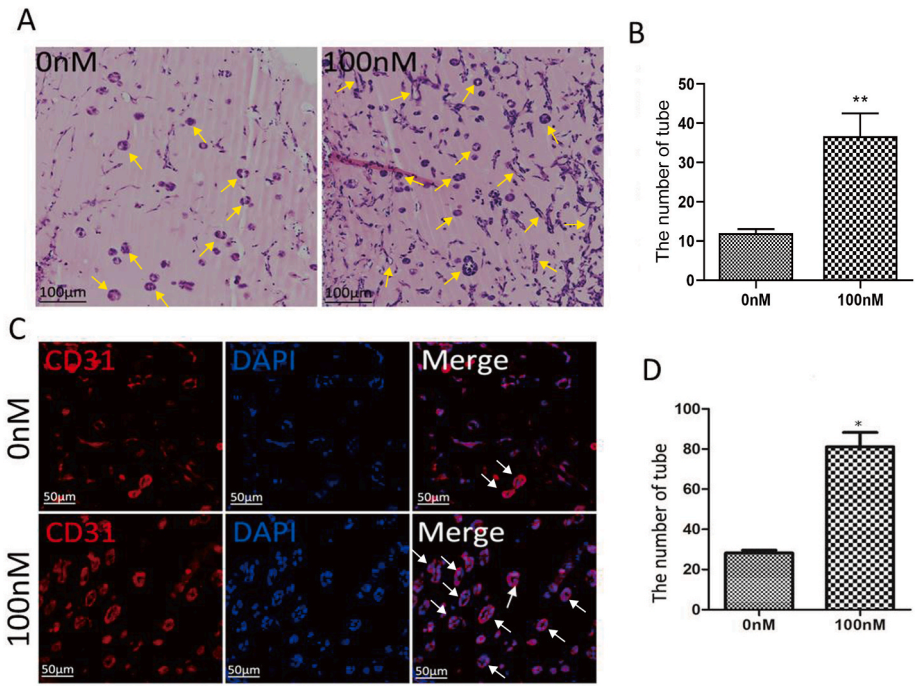

Fig. 3. Effects of conditioned medium on the formation of blood vessels in the planted matrigel plaques. A. H&E staining shows the newly formed blood vessels (yellow arrows indicated) in the matrigel plaques treated with 0 nM or the optimal (100 nM) conditioned medium. **B.** Quantification of the numbers of newly formed blood vessels in H&E staining images. **C.** Immunostaining of CD31 shows the newly formed blood vessels (white arrows indicated) in the matrigel plaques treated with 0 nM or the optimal (100 nM) conditioned medium. **D.** Quantification of the numbers of newly formed blood vessels in CD31 staining images. n = 4/group; scale bar = 50 μm $^{*}P < 0.05$, vs. 0 nM group; $^{**}P < 0.01$, vs. 0 nM group. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

mechanisms of pro-angiogenic effect of the conditioned medium, we compared VEGF, FGF-1, FGF-2, TGF-α, matrix metalloproteinase (MMP)-3, MMP-9, PDGF-α, PDGF-β, hypoxia inducible factor-1α (HIF-1α) and angiotensin II (Ang II) expressions at the transcriptional level in the groups treated with the optimal conditioned medium and control conditioned medium (0 nM group). As shown in Fig. 4, the expressions of VEGF, FGF-2, MMP-9, PDGF-α and PDGF-β were markedly increased in UCMSCs induced with 100 nM rapamycin compared to the 0 nM group.

4. Discussion

MSCs are viewed as exocrine cells and are widely used in regenerative medicine. It is recently recognized that the exocrine functions of MSCs are responsible for their main actions in tissue and organ repair. The conditioned medium derived from MSC culture is one of the common and effective ways to utilize their exocrine functions [26]. UCMSCs are a kind of most commonly used MSCs, which are from the Wharton's Jelly of human umbilical cords. The conditioned medium following culture of UCMSCs is rich in growth factors and has the potential to promote angiogenesis. However, in the practical application, the effect of this kind of unmodified conditioned medium on angiogenesis is very

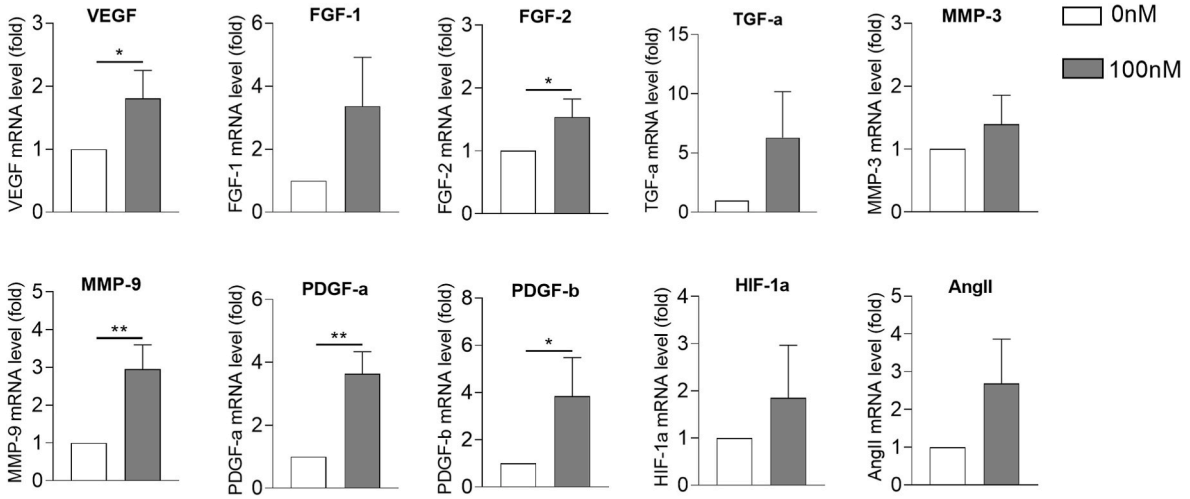

Fig. 4. Effect of autophagy on expressions of VEGF, FGF-1, FGF-2, TNF-α, MMP-3, MMP-9, PDGF-α, PDGF-β, HIF-1α and Ang II in UCMSCs. n = 3/group; $^{*}P < 0.05$, vs. 0 nM group; $^{**}P < 0.01$, vs. 0 nM group.

weak, and its effect on wound healing and tissue repair is also very limited [11]. Thus, how to improve pro-angiogenic efficiency of MSC-derived conditioned medium is a key topic in current and future studies.

Autophagy is a cell survival mechanism, which can be induced by several chemical inducers, such as rapamycin, spermidine, tacrolimus, and so on [27,28]. Among these inducers, rapamycin is a commonly used one that has been widely utilized by many studies [27,29]. In this study, we tried to improve angiogenic effect of the conditioned medium through enhancing autophagy of UCMSCs. We used 0, 100 nM, 1 μM and 10 μM rapamycin (6 h) to induce autophagy of UCMSCs, and then utilized *in vitro* tube formation experiment to compare the pro-angiogenic effects of 4 kinds of conditioned medium. Our data showed that all 4 kinds of conditioned medium derived from UCMSC culture could markedly promote the tube formation of HUVECs cultured in the matrigel-coated plates (Fig. 2). Of note, among the 4 kinds of conditioned media, the one induced with 100 nM rapamycin had much greater efficiency to enhance tube formation, instead of the conditioned medium in 1 μM and 10 μM rapamycin-induced groups (Fig. 2). However, the autophagy in UCMSCs induced by rapamycin was increased in a dose-dependent manner (Fig. 1). This indicates that only appropriate level of autophagy has the optimal efficiency to improve angiogenic effect of the conditioned medium. But, it is still not known why the angiogenic effect of the conditioned medium from higher autophagic MSCs is lower than that from lower autophagic MSCs. Maybe excess autophagy induces apoptosis of UCMSCs, and thus disturbances their exocrine function. In fact, it has been reported that excess autophagy causes autophagic cell death and apoptosis in other cell lineages. For instance, Li et al. reported that excessive activation of autophagy induced apoptosis of H9c2 cells [30]. We also tested the effect of conditioned medium on angiogenesis in matrigel plaques transplanted into the mice. The *in vivo* data showed that tube formation in the plaques treated daily with the optimal conditioned medium is more obvious than that treated with the control conditioned medium (0 nM group).

It has been known that VEGF and FGF-2 are the strongest promoters for angiogenesis [31]. Our data indicated that enhancing autophagy also markedly induced expressions of VEGF and FGF-2 in UCMSCs at the transcription level. MMP3 and MMP-9 are also essential factors for angiogenesis, which can cleave pre-VEGF into mature ones. In this study, we found that the expression of MMP-9, not MMP-3 was significantly increased in the autophagic UCMSCs (treated with 10 μM rapamycin). PDGF (including PDGF-α and -β) has also the function to enhance angiogenesis in the presence of other growth factors such as FGF2 [32]. Our data showed that both PDGF-α and PDGF-β was significantly increased in the autophagic UCMSCs. The upregulations of above-mentioned factors in the autophagic UCMSCs may be partially responsible for pro-angiogenic effect of the conditioned medium. In addition, the expression levels of FGF-1, TGF-α, MMP-3, HIF-1α and Ang II also increased in the autophagic UCMSCs, but not significant.

It is known that blood vessel formation is a key step in many biological processes including organ development, tissue regeneration and wound healing. The conditioned medium derived from stem cell culture is considered one of the options for promoting angiogenesis, but its efficiency has always been unsatisfactory. In this study, we obtained a highly efficient conditioned medium for promoting angiogenesis from UCMSCs with appropriate autophagy. This achievement can expand application of UCMSC-conditioned medium in tissue repair, tissue regeneration and tissue engineering. However, there are some weaknesses in this study. A weakness is not to investigate the substantial therapeutic effect of the optimal conditioned medium for wound healing and tissue repair, which may to some extent limit application of the screened conditioned medium. Another weakness is that lack of a rescue experiment to directly prove autophagy, rather than other effects of rapamycin on UCMSCs, results in functional changes of the conditioned medium, which may also limit application of the screened conditioned medium.

5. Conclusions

This study shows that appropriate levels of autophagy of UCMSCs effectively improves pro-angiogenic efficiency of the conditioned medium and increases the expressions of VEGF, FGF-2, MMP-9, PDGF-α and PDGF-β in UCMSCs. This finding offers a new insight for improving therapeutic efficiency the conditioned medium derived from MSC culture in wound healing, as well as tissue injury and repair.

Availability of data and materials

The datasets generated for this study are available on request to the corresponding author.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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