Autophagy of umbilical cord mesenchymal stem cells conduces to pro-angiogenic function of conditioned medium

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Research

Keywords: Autophagy, umbilical cord mesenchymal stem cells, conditioned medium, angiogenesis, wound healing

Posted Date: February 16th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-227201/v1

Abstract

Background

Angiogenesis is a key prerequisite for wound healing. The conditioned medium following culture of umbilical cord mesenchymal stem cells (UCMSCs) has a potential to promote angiogenesis, but the efficacy is very low. Autophagy is an important process in protein recycling and a contributor for cell exocrine, which maybe stimulate the release of cytokines from UCMSCs to the medium and enhance the pro-angiogenic efficacy of the conditioned medium.

Methods

Autophagy in UCMSCs was induced by 100 nM, 1 μM and 10 μM rapamycin for 6-hour and then detected by LC-3 immunofluorescence staining. After induction, the cells were washed with PBS for 3 times and cultured in fresh medium without rapamycin for additional 24-hour. And then, the conditioned medium was collected for the following experiments. The angiogenic effects of different groups of conditioned medium were verified by *in vitro* and *in vivo* tube formation assays in the matrigel-coated plates and matrigel plaques injected in mouse inguinal areas. Finally, the expressions of angiogenic factors including VEGF, FGF-1, FGF-2, TGF-α, MMP-3, MMP-9, PDGF-α, PDGF-β, HIF-1α and Ang II in the autophagic and control UCMSCs were measured by q-PCR assay.

Results

Rapamycin induced autophagy of UCMSCs in a dose dependent manner, but the conditioned medium in 100 nM rapamycin-induced group was with the best pro-angiogenic efficacy. Thus, this group of medium was viewed as the optimal conditioned medium. The *in vivo* tube formation assay showed that angiogenesis in matrigel plaques injected daily with the optimal conditioned medium was more obvious than that injected with the control conditioned medium. Further, the expressions of VEGF, FGF-2, PDGF-α, MMP-9 and HIF-1α were markedly increased in UCMSCs following treatment with 100 nM rapamycin.

Conclusion

Appropriate autophagy improves the pro-angiogenic efficacy of the conditioned medium, which might be utilized to optimize the applications of UCMSCs-derived conditioned medium in wound healing and tissue repair.

Trial registration

Not applicable.

Background

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 wide range of sources, easy to obtain, low immunogenicity, 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 the actions of MSCs to re pair the damaged tissues or organs mainly results from their strong exocrine functions [5]. 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-a), 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]. Most of above exocrine factors participate in regulation of 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 following culture of normal UCMSCs contained a certain amount of FGF, VEGF and other angiogenic factors, and had a weak function to promote proliferation of vascular endothelial cells, but failed to affect agiogenesis [11]. Thus, the angiogenic function of conditioned medium from the untreated UCMSCs is very limited. To improve pro-angiogenic efficacy of UCMSCs-derived conditional medium, some groups recently attempted to use gene manipulation techniques to enhance the exocrine functions of UCMSCs [12, 13]. 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 pro-angiogenic efficacy of UCMSCs-derived conditioned medium in repair of 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]. However, there are some problems such as high cost, difficult operation and ethical restriction of using gene manipulation to improve the therapeutic efficacy of UCMSCs.

Autophagy is a critical physiological process to maintain cell homeostasis, and plays an important role in a series of cell functions [14, 15]. A recent study showed that autophagy is required to maintain the stemness and regenerative potential of hematopoietic stem cells (HSCs) [14]. 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 [14]. Thus, we hypothesized that strengthening autophagy in UCMSCs maybe increase their exocrine ability and improve pro-angiogenic efficacy of their conditioned medium. This study was designed to prove this hypothesis through *in vitro* and *in vivo* experiments.

Materials And Methods

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 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).

Cell culture

The 2nd generation UCMSCs were cultured in DMEM with 10% FBS, 100 U/mL penicinllin 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-hour culture, and then the medium was replaced every three days. When the cells grew to 80% confluence, they were seeded into 24-well plates and cultured in DMEM with different concentrations (0, 100 nM, 1 μM and 10 μM) of rapamycin for 6-hour to induce autophagy. After that, the medium was replaced following washing with PBS for 3 times, incubated with UCMSCs for additional 24-hour, and collected for the following experiments. The autophagy levels of different groups of UCMSCs were detected by immunofluorescence staining of LC-3.

HUVECs were cultured in DMEM with 10% FBS, 100 U/mL penicinllin 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.

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 medium for 24-hour, 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 additional 3-hour. 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.

The In vivo tube formation assay

C57BL/6 mice were anesthetized with sodium pentobarbital (80 mg/kg, i.p.), and 200 μL matrigel mixed with HUVECs (5×10^4/100 μL) were subcutaneously injected into mouse inguinal areas. The conditioned medium (200 μL) was injected into mouse groins around matrigel plaques every day until sacrifice. One week later, the mice were sacrificed under anaesthesia, 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 [16].

Quantitative reverse-transcriptase polymerase chain reaction

UCMSCs were cultured in DMEM with or without rapamycin (100 nM) for 6-hour. 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 was 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 comparative threshold cycle method. The PCR primers were designed and synthesized by GeneCreate Biological Engineering Co., Ltd (Shanghai, China). The sequences of primers are listed in Table.1.

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 *t* tests or one-way ANOVA with Tukey's post-hoc adjustment for multiple comparisons when appropriate.

Results

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 staining of LC-3. As shown in Figure.1, rapamycin enhanced autophagy (LC-3 fluorescence) in UCMSCs in a dose-dependent manner.

In vitro **tube formation and screen of optimal conditional medium**

UCMSCs were cultured in fresh DMEM with different dosages of rapamycin for 24 hours, and then the conditioned medium was collected. HUVECs were cultured in different conditioned medium in matrigel-coated plates for 3-hour. As shown in Figure.2, tube branching length, tube segment length, tube node and tube junction of HUVECs cultured with 4 kinds of conditioned mediums were all markedly higher than that cultured with fresh medium (control). Of note, among 4 conditioned medium groups, the medium from UCMSCs cultured with 100 nM rapamycin had the best pro-angiogenic efficacy and the tube formation was most obvious in this group. Thus, this group of medium was viewed as the optimal conditioned medium and used in the following experiments.

In vivo **pro-angiogenic effect of 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 (Figure.3A and B) showed that the numbers of newly formed vessels in the matrigel plaques treated with the optimal conditioned medium were significantly higher than that treated with control conditioned medium. This data was further confirmed by immunostaining of CD31, which also showed more newly formed vessels in the matrigel plaques treated with optimal conditioned medium than that treated with control conditioned medium (Figure.3C and D).

Expressions of angiogenesis-related genes in autophagic UCMSCs

Previous studies had shown that UCMSCs could secrete growth factors such as FGF, VEGF, EGF, TGF-β, IGF, NGF and HGF, and most of them were related to angiogenesis [9,10,17 ~ 19]. To further elucidate the 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 untreated UCMSCs and autophagic UCMSCs (treated with 100nM rapamycin). As shown in Figure.4, the expressions of VEGF, FGF-2, PDGF-α, MMP-9 and HIF-1α were markedly increased in UCMSCs treated with 100nM rapamycin as compared with the untreated cells (0 nM group).

Discussion

MSCs are viewed as exocrine cells and 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 MSCs is one of the common and effective ways to utilize their exocrine functions [20]. UCMSCs are a kind of most commonly used MSCs, which are from the Wharton's Jelly of human umbilical cords. The conditioned medium following UCMSCs is rich in growth factors and has the potential to promote angiogenesis. However, in practical application, the effect this kind of unmodified conditioned medium on angiogenesis is very weak but this unmodified conditioned medium is very 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.

In this study, we tried to improve angiogenic effect of the conditioned medium through enhancing authophagy of UCMSCs. We used 0, 100 nM, 1 μM and 10 μM rapamycin (6 hours) to induce authophagy of UCMSCs, and then utilized *in vitro* tube formation experiment to compare the pro-angiogenic effect of 4 kinds of conditioned medium. Our data showed that all 4 kinds of conditioned medium from UCMSCs could markedly promote the tube formation of HUVECs cultured in the matrigel-coated plates (Figure.2). Of note, among 4 kinds of conditioned medium, the conditioned medium from 100 nM rapamycin-induced UCMSCs had much greater efficiency to enhance tube formation, instead of the conditioned medium from 1 μM and 10 μM rapamycin-induced UCMSCs (Figure.2). However, the autophagy in UCMSCs induced by rapamycin was increased in a dose-dependent manner (Figure.1). This indicates that only appropriate autophagy has 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 [21]. 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 control conditioned medium.

It has been known that VEGF and FGF-2 are the strongest promoters for angiogenesis [22]. 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 PDG-α and -β) has also the function to enhance angiogenesis in the presence of other growth factors such as FGF2 [23]. Our data showed that the expression of PDG-α, but not PDG-β was significantly increased in the autophagic UCMSCs. HIF-1α is a transcription factor in mammalian cells, which plays an essential role in cellular and systemic homeostatic responses to hypoxia. It has been reported that HIF-1α has the potential to enhance VEGF expression and promote angiogenesis [24]. A recent study also showed that overexpression of HIF-1α in bone marrow MSCs (BMMSCs) could induce MSCs to form tubes by themselves [25]. Our data showed that autophagy markedly increased HIF-1α expressions in 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 expressions of FGF-1, FGF-1, TGF-α and Ang II were also increased in the autophagic UCMSCs, but not significant.

Conclusions

This study shows that appropriate autophagy of UCMSCs effectively improves pro-angiogenic efficiency of the conditioned medium and increases the expressions of VEGF, FGF-2, PDGF-α, MMP-9 and HIF-1α in UCMSCs. This finding offers a new insight for improving therapeutic efficiency the conditioned medium from MSCs in wound healing, as well as tissue injury and repair.

Declarations

Ethical Approval and Consent to participate

The animal use in this study was reviewed and approved by the Experimental Animal Ethics Committee of Xinxiang Medical University.

Consent for publication

All authors had read and approved to publish this manuscript.

Availability of data and materials

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

Competing interests

The authors declare that they have no conflict of interest.

Funding

This study was funded by the National Key Research and Development Program of China (No: 2018YFC1105800); the Supporting Plan for Scientific and Technological Innovative Talents in Universities of Henan Province (No: 19HASTIT004); and Henan Outstanding Young Scholars Fund (No: 212300410012).

Authors' contributions

XW designed and supervised this study. WW, XL, CC and DL performed the experiments. GY analyzed the data. XW wrote the manuscript and ZG revised the manuscript.

Acknowledgements

We sincerely thank the Stem Cell and Biotherapy Engineering Research Center of Henan Province for a gift of UCMSCs.

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Tables

Table.1 Primers for real-time PCR.

Primers	Forward	Reverse
VEGF-A	AGGGCAGAATCATCACGAAGT	AGGGTCTCGATTGGATGGCA
FGF-1	GCCCTGACCGAGAAGTTTAATC	CCCCGTTGCTACAGTAGAGG
FGF-2	AGAAGAGCGACCCTCACATCA	CGGTTAGCACACACTCCTTTG
TGF-α	AGGTCCGAAAACACTGTGAGT	AGCAAGCGGTTCTTCCCTTC
MMP-3	CTGGACTCCGACACTCTGGA	CAGGAAAGGTTCTGAAGTGACC
MMP-9	TGTACCGCTATGGTTACACTCG	GGCAGGGACAGTTGCTTCT
PDGF-α	GCAAGACCAGGACGGTCATTT	GGCACTTGACACTGCTCGT
PDGF-b	CTCGATCCGCTCCTTTGATGA	CGTTGGTGCGGTCTATGAG
HIF-1α	CACCACAGGACAGTACAGGAT	CGTGCTGAATAATACCACTCACA
Ang II	CTCGAATACGATGACTCGGTG	TCATTAGCCACTGAGTGTTGTTT
GAPDH	GGCTGTTGTCATACTTCTCATGG	GGAGCGAGATCCCTCCAAAAT

Figures

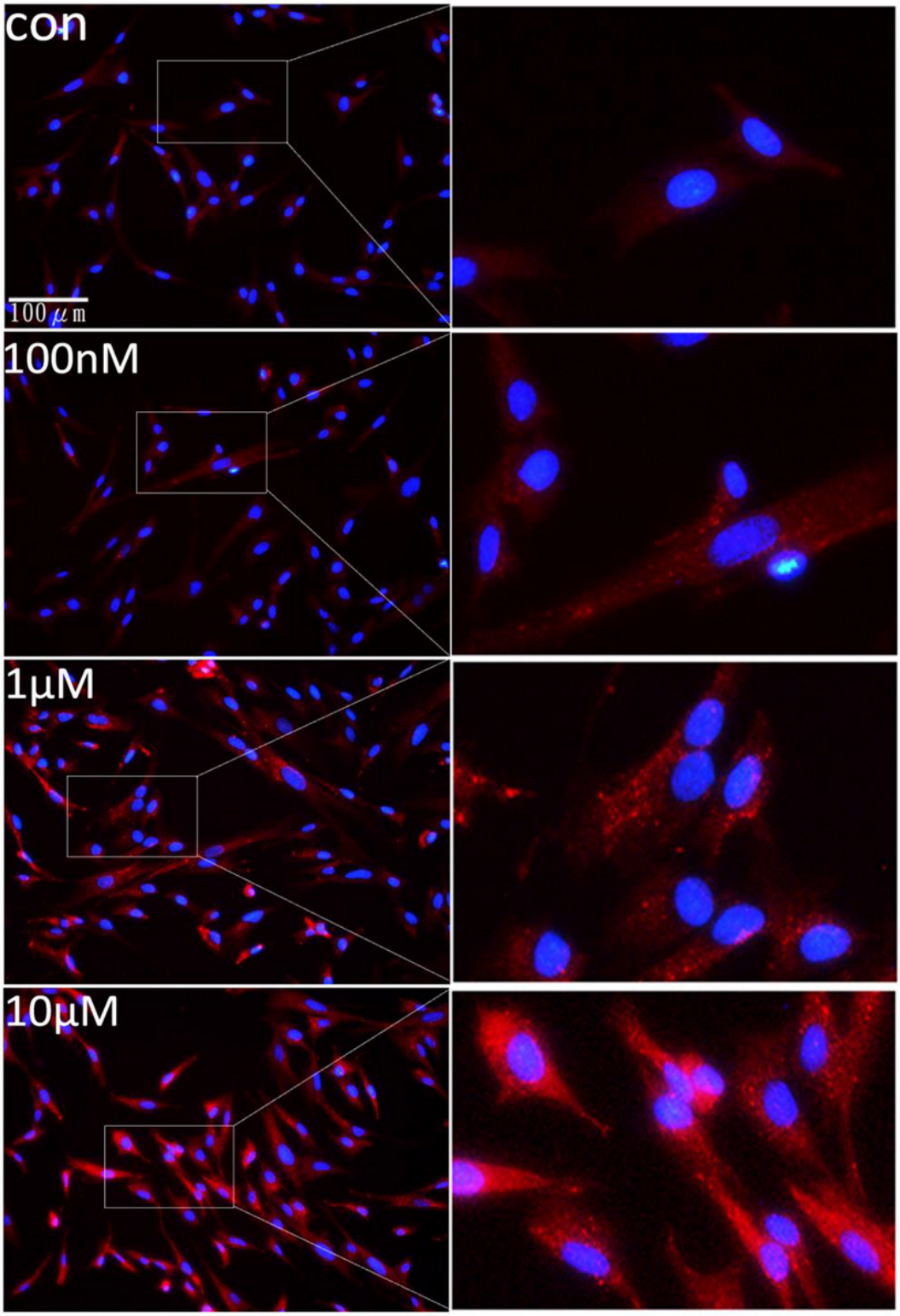

Figure 1

Autophagy (LC3) levels in UCMSCs following exposure to 0, 100 nM, 1 μM and 10 μM rapamycin for 6-hour. n=5/group; scale bar=100μm.

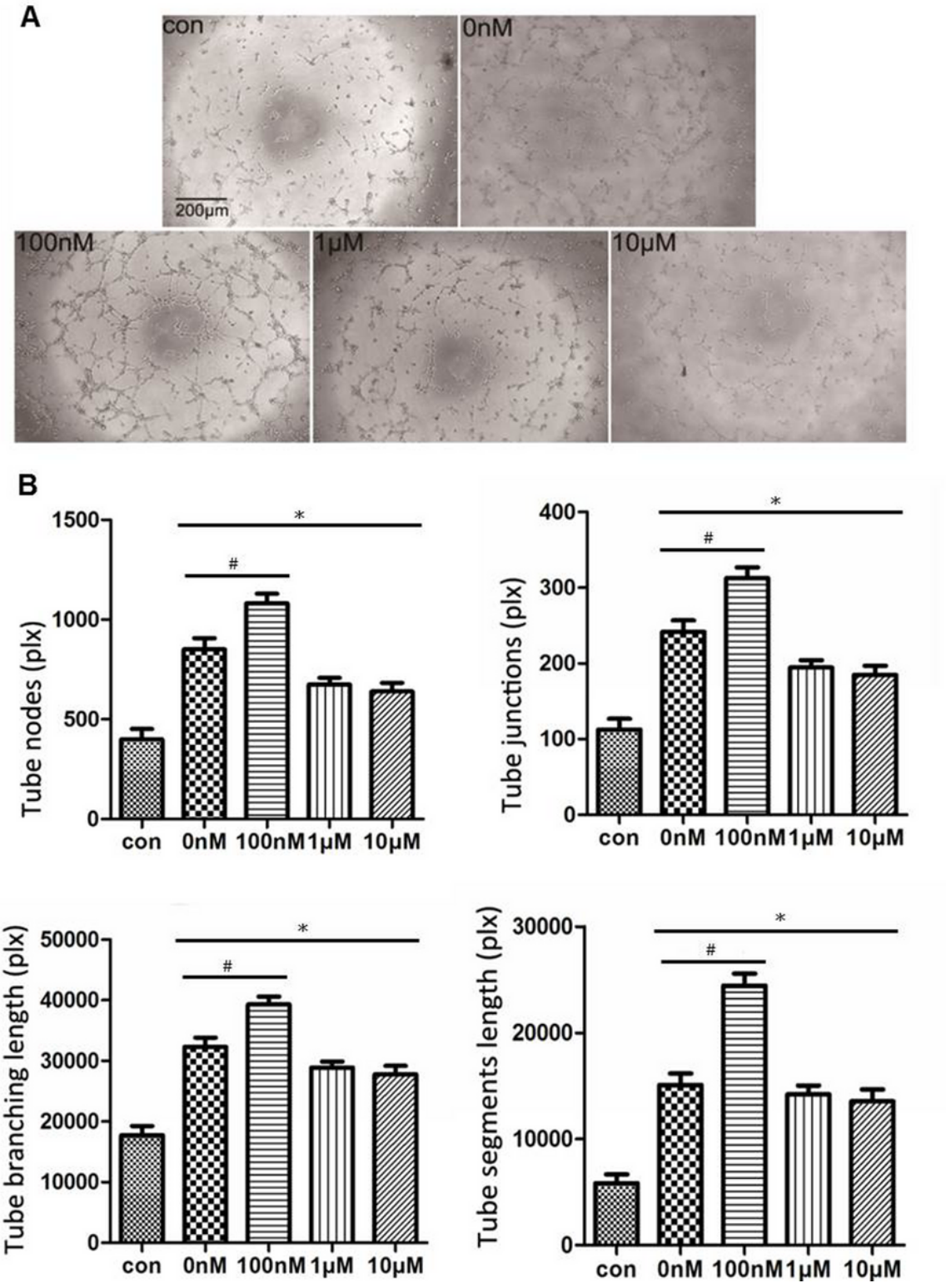

Figure 2

Effects of different conditioned mediums on tube formation of HUVECs. A. The represent images show tube formation of HUVECs following culture with different conditional mediums for 3 hours. B. Quantification of tube branching length, tube segment length, tube node and tube junction of tube formation image. n=4/group; scale bar=200μm. *P<0.01, vs. control group; #P<0.01, vs. 0 nM group.

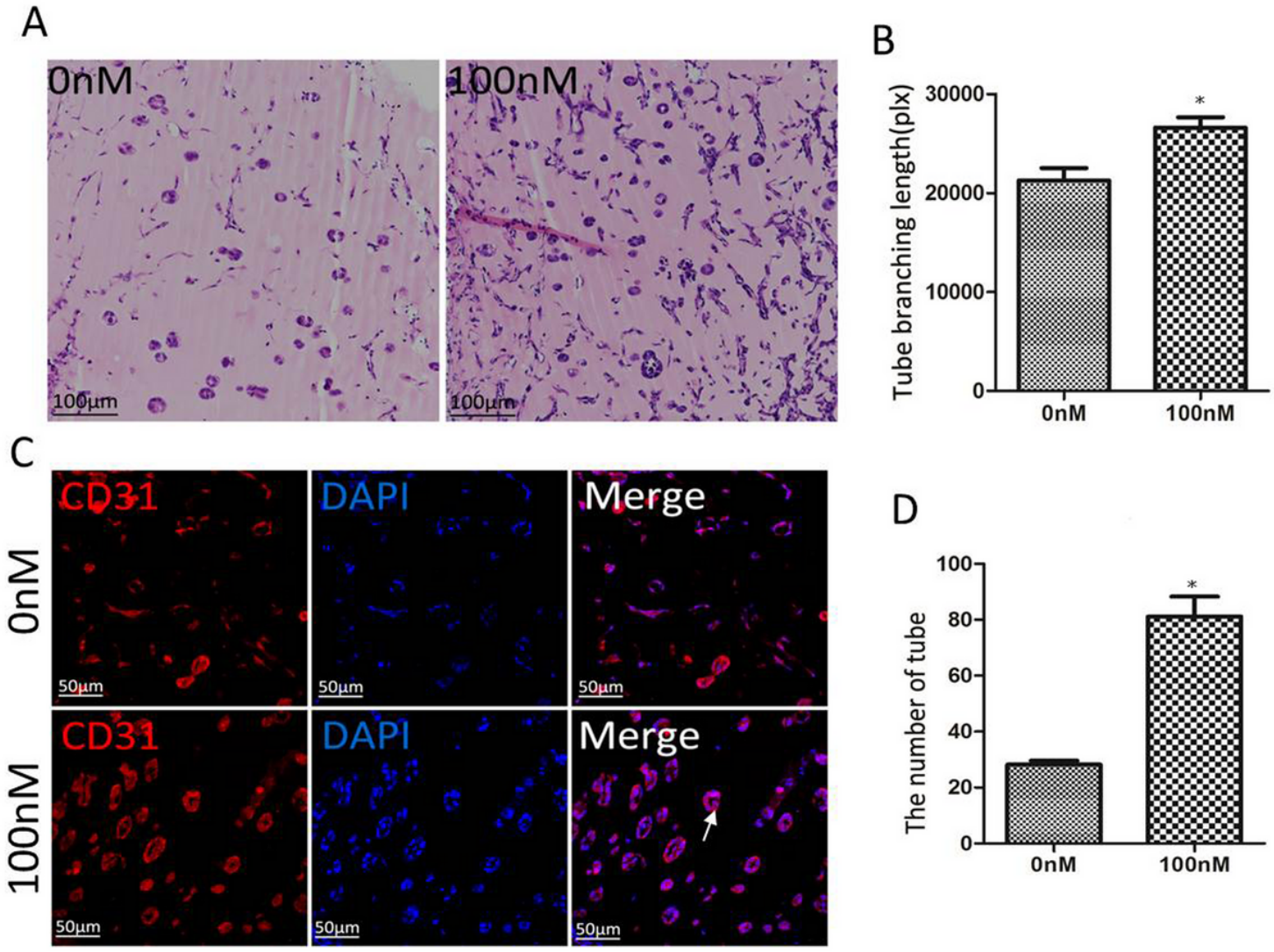

Figure 3

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 in the matrigel plaques treated with control or optimal 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 in the matrigel plaques treated with control or optimal conditioned medium. D. Quantification of the numbers of newly formed blood vessels in CD31 staining images. n=4/group; scale bar=100μm. *P<0.01, vs. control conditioned medium (0 nM) group.

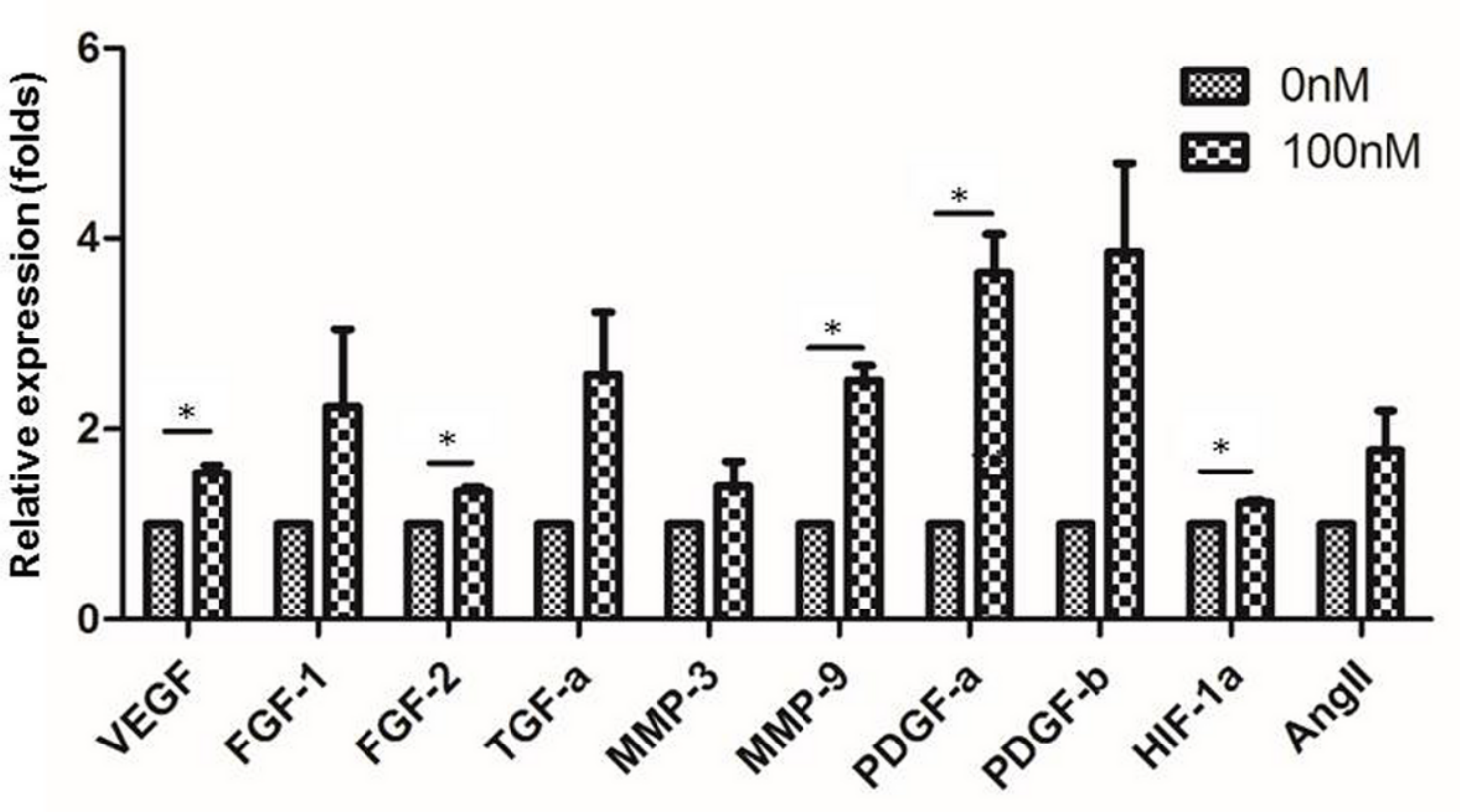

Figure 4

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. control group.