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NGF increases FGF2 expression and promotes endothelial cell migration and tube formation through PI3K/Akt and ERK/MAPK pathways in human chondrocytes

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SUMMARY

Objective: Vascular invasion is observed at the osteochondral junction in osteoarthritis (OA). Nerve growth factor (NGF) as an angiogenic factor is expressed in OA. This study is to investigate the effects of NGF on angiogenesis *in vitro* in human chondrocytes.
Design: Articular cartilages of knee joints were harvested from healthy and OA patients. Expressions of NGF and tropomyosin-related kinase A (TrkA) were detected by western blot, Safranin-O and fast green staining and immunohistochemistry in cartilage. Expression of fibroblast growth factor 2 (FGF2) was detected by western blot in cultured chondrocytes. Chondrocytes were transfected by lentiviral vectors to knock down TrkA. Migration and tube formation of human microvascular endothelial cell (HMVEC) were assessed by using transwell co-culture with chondrocyte after treatment of NGF.
Results: We confirmed expressions of NGF and TrkA were significantly up-regulated in OA. NGF induced expression of FGF2 in a time- and dose-dependent manner. Angiogenic activities of endothelial cells were greatly enhanced after co-cultured with NGF pre-treated chondrocytes, while knock-down of TrkA significantly abolished the above effects. We further found that NGF-induced expression of FGF2 promoted angiogenic activities of endothelial cells through PI3K/Akt and ERK/MAPK signaling pathways.
Conclusions: NGF promotes expression of FGF2 *in vitro* via PI3K/Akt and ERK/MAPK signaling pathways in human chondrocytes and it increases angiogenesis, which is mediated by TrkA. NGF could be responsible for vascular up-growth from subchondral bone in OA.

Introduction

Knee osteoarthritis (OA) is a degenerative disease mainly characterized by loss of articular cartilage, which is a source of pain and disability in elderly population[1]. Patients also suffer from swelling and stiffness of knee joint. Several factors including aging, obesity and trauma are associated with OA. But so far, the real underlying mechanisms of OA remain elusive.

Nerve growth factor (NGF), a classic member of the family of neurotrophic factors, was first isolated in nerve tissue[2]. It is a mediator regulating nerve growth as well as regulation of proliferation, differentiation, repair and survival[3]. However, recent studies showed that NGF is also potential in inducing angiogenesis in several physiological and pathological conditions[4–6]. Normal articular cartilage is avascular and aneural, but angiogenesis at the osteochondral junction and in non-calcified cartilage was observed in OA[7,8]. Previous studies further showed that expressions of NGF and its high affinity receptor, p140 tropomyosin receptor kinase A (TrkA), were both up-regulated in blood, synovial fluid and chondrocytes in OA[9,10]. Walsh *et al.* observed increased endothelial cell proliferation and vascular density in non-calcified articular cartilage with

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increased NGF expression within vascular channels in OA[11]. Cantarella *et al.* unraveled the NGF and its receptor TrkA dependent angiogenesis *in vitro* and *in vivo*[12]. These findings indicated that NGF is a pro-angiogenic factor mediated by TrkA and angiogenesis is a key process in OA. Research efforts have identified several promising inhibitors targeting NGF and the related pain mechanisms as well as their therapeutic effects[13–15], among which Tanezumab provided significant improvement in pain in many clinical OA studies[16]. However, so far few investigations have been carried out to elucidate the regulatory mechanism of NGF-induced angiogenesis in OA.

Fibroblast growth factor 2 (FGF2) is a member of the FGF family with mitogenic properties as well as an inducer of angiogenesis[17]. It is also involved in the regulation of articular cartilage homeostasis. Signaling molecules containing Src homology 2 or phosphotyrosine-binding domains, such as phosphatidylinositol 3-kinase (PI3K), are necessary to NGF signals[18]. NGF also transmits its signal to endothelial cells through the TrkA receptor. Stimulation of TrkA activity by NGF leads to activation of ERK1/2, PI3K, and Akt in endothelial cells, which is possibly responsible for their proliferation and migration in vitro[19].

In this study, we focus on the roles of NGF in FGF2 expression and migration of endothelial cell mediated by TrkA, with involvement of PI3K/Akt and ERK/MAPK signaling pathways in human chondrocytes.

Materials and methods

Patients selection and informed consent

Cartilage specimens were collected from patients with knee OA undergoing total knee arthroplasty ($n = 20$) and patients without knee arthropathy undergoing amputation of lower limbs due to trauma ($n = 14$) after written informed consent at The Second Affiliated Hospital of Zhejiang University School of Medicine. The stage of OA was assessed using X-ray and Kellgren–Lawrence Grading Scale. All methods were carried out in accordance with the relevant guideline and regulation of the 2nd Affiliated Hospital, School of Medicine, Zhejiang University, and all experimental protocols were approved by the 2nd Affiliated Hospital, School of Medicine, Zhejiang University Ethics Committee.

Isolation and culture of articular chondrocytes

The cartilage samples were washed under sterile conditions with PBS (Shanghai Long Island antibody diagnostic reagents company (FL-2004), Shanghai, China) after adjacent tissues were stripped off. 0.25% collagenase II (Sigma–Aldrich, St. Louis, MO, USA) was added for digestion at 37°C, 5% CO_2 for 5 h. Cells were harvested by centrifugation after 200 μm mesh strainer was used to filter the solution. The cells were cultured in DMEM (Gibco) with 15% FBS (Gibco) and 1% penicillin/streptomycin. Incubation condition is 5% CO_2 at 37°C. The medium was changed every 2–3 days. When cultured primary cells reached 80% confluence, they were detached by treatment with 0.25% (W/V) trypsin and 0.1% (W/V) ethylenediaminetetraacetic acid (Gibco) and subcultured at a density of 1×10^4 cells/cm^2. Cultured cells before passage 2 were used for experiments. To establish OA chondrocyte model, healthy chondrocytes were treated with 5, 10 and 20 ng/ml IL-1β for 24 h. In control group, the chondrocytes were non-treated with IL-1β. The concentration of NGF was 5, 10 and 20 ng/ml and chondrocytes were treated for 0, 12, 24 and 36 h.

Construction, production of lentivirus

The lentiviral short-hairpin RNA (shRNA) expressing constructs were purchased from Invitrogen (ThermoFisher Scientific, US). The target sequence for TrK-A shRNA is the following: 5′–CCAGTGACCTCAACAGGAAGAttcaagagaTCTTCCTGTTGAGGTCA-CTGG-3′. The constructs were transfected into 293T packaging cells along with the packaging plasmids using LipofectaminTM 2000 (Invitrogen, US) and the lentivirus containing supernatants were used to transduce chondrocytes.

Cell transfection

The chondrocytes were cultured in complete DMEM medium (Gibco, US) supplemented with 10% FBS at 37 °C with 5% CO_2. Prior to viral infection, cells were seeded in 6-wells plate at the density of 0.5×10^6 cells per well and grown until the cells at high confluence (>50%). Then, the cells were added into viral stock at a multiplicity of infection (MOI) of 100. 48 h after incubation, the RNA interference effect of Trk-A-shRNA lentivirus was determined by western blotting.

Western blot analysis

Total proteins from human cartilages or chondrocytes were extracted. Equal amounts of protein were separated by SDS-PAGE and then transferred onto a poly-vinylidene (PVDF) membranes (Millipore, USA). The membranes were incubated overnight at 4 °C with anti-NGF, TrkA, pAkt, Akt, pERK1/2, ERK1/2 and FGF2 antibodies. The membranes were then incubated with secondary antibody for 1 h. Finally, the quantification of western blot was determined using Quantity One software (Bio-Rad, Munich, Germany).

Migration and tube formation of HMVEC

The human microvascular endothelial cell (HMVEC) migration assay was performed using Transwell inserts (8.0 μm pore size; Costar) in 24-multiwell plates. HMVECs (10^5 cells in 200 μl of medium with 10% FBS) were then seeded into the upper chamber, and 300 μl of chondrocyte CM (culture medium) was placed in the lower chamber. Cells on the upper side of the Transwell membrane were examined and counted under a microscope.

Matrigel (BD Biosciences) was melted at 4 °C, added to 48 multiwell plates (Corning) at 100 μl/well, and then incubated at 37 °C for 30 min. HMVECs (1×10^5 cells) were resuspended in a 1:1 mixture of MV2 serum-free medium and chondrocytes (total 200 μl), and added to the wells. After 12 h of incubation at 37 °C, HMVECs tube formation was assessed by microscopy, and each well was photographed under a light microscope. The numbers of branches were calculated and quantified using Mac Biophotonics ImageJ software (NIH).

Statistical analysis

All numerical data were expressed as the mean value ± 95% confidence intervals (CI), with n equals to the number of samples analyzed. Statistical analysis was performed with Student's t-test and one-way analysis of variance (one-way ANOVA), and $p < 0.05$ was considered to be significant. The Tukey HSD/Dunnett T3 post hoc test was used for all pairwise comparisons, and significance was attained at $p < 0.05$. All statistical analyses were performed using the SPSS 20.0 software (SPSS, Chicago, IL, USA). For all experiments, $p < 0.05$ was considered to be significant and is

indicated by *. $p < 0.01$ is indicated by **. $p < 0.01$ vs NGF group is indicated by ##.

Results

Expressions of NGF and TrkA are up-regulated in OA

Expressions of NGF and TrkA in human cartilages and chondrocytes were evaluated by western blot and immunohistochemistry. In OA samples from patients undergoing total knee arthroplasty, expressions of NGF and its high affinity receptor, TrkA, were both up-regulated with significant difference detected by western blot compared with healthy group (Fig. 1(A), CI: (0.11, 0.28), $p < 0.0001$; CI: (0.08, 0.27), $p = 0.001$). The main clinical features of patients were shown in Table I. In Fig. 1(B), safranin-O and fast green staining showed a significant reduction of Safranin O-positive proteoglycan and chondrocytes and absence of tide mark in OA cartilages compared with healthy ones. According to immunohistochemistry, an increase in expressions of NGF as well as TrkA was observed in OA cartilages. After healthy human chondrocytes were stimulated by IL-1β, expressions of NGF and TrkA were significantly up-regulated according to western blot (Fig. 1(C), CI: (0.21, 0.70), $p = 0.002$; CI: (0.19, 0.63), $p = 0.004$), which is in accordance with the result that expressions of NGF and TrkA are up-regulated in OA clinical samples.

Table I
Clinical characteristics compared between healthy ($n = 14$) and OA ($n = 20$) patients

	Healthy (n = 14)	OA (n = 20)
Age, years	70.9 ± 18.2	67.2 ± 5.2
Gender		
Male	6	6
Female	8	14
Height, m	1.62 ± 0.094	1.60 ± 0.068
Weight, kg	65.5 ± 8.0	67.1 ± 7.7
BMI, kg^*m^{-2}	23.9 ± 3.2	27.3 ± 2.3
Side		
Right	6	10
Left	8	10
Kellgren–Lawrence	0 (65%) I (35%)	III (25%) IV (75%)

Effects of dose and treatment time of NGF on expression of FGF2 are in a time- and dose-dependent manner

We observed that after healthy human chondrocytes were treated by NGF, expression of FGF2 was up-regulated in a time- and

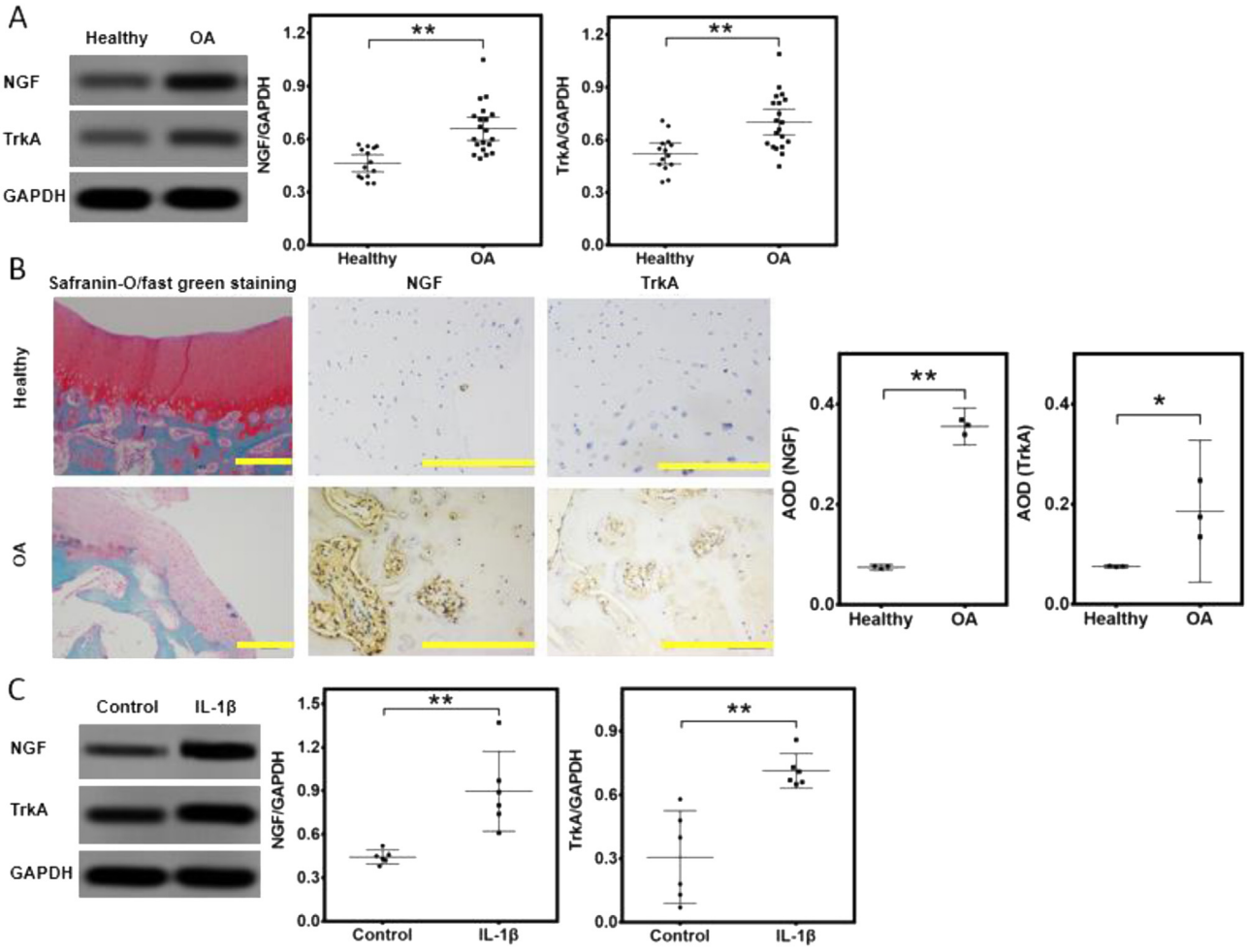

Fig. 1. Expressions of NGF and its receptor TrkA are up-regulated in OA. (A) Western blot and quantitative analysis showed expressions of NGF and TrkA are up-regulated in human OA cartilages ($n = 20$) compared with healthy ones ($n = 14$). (B) Safranin-O and fast green staining showed loss of proteoglycan and chondrocytes, as well as absence of tide mark in human OA cartilages compared with healthy ones. Immunohistochemistry of human cartilages and quantitative analysis showed increased expressions of NGF and TrkA. (C) Western blot and quantitative analysis showed up-regulated expressions of NGF and TrkA in IL-1β stimulated human healthy chondrocytes compared with healthy ones ($n = 6$). $^{**}p < 0.01$. $^{*}p < 0.05$. Scale bar = 300 μm. Data are represented as individual data points with 95% CI.

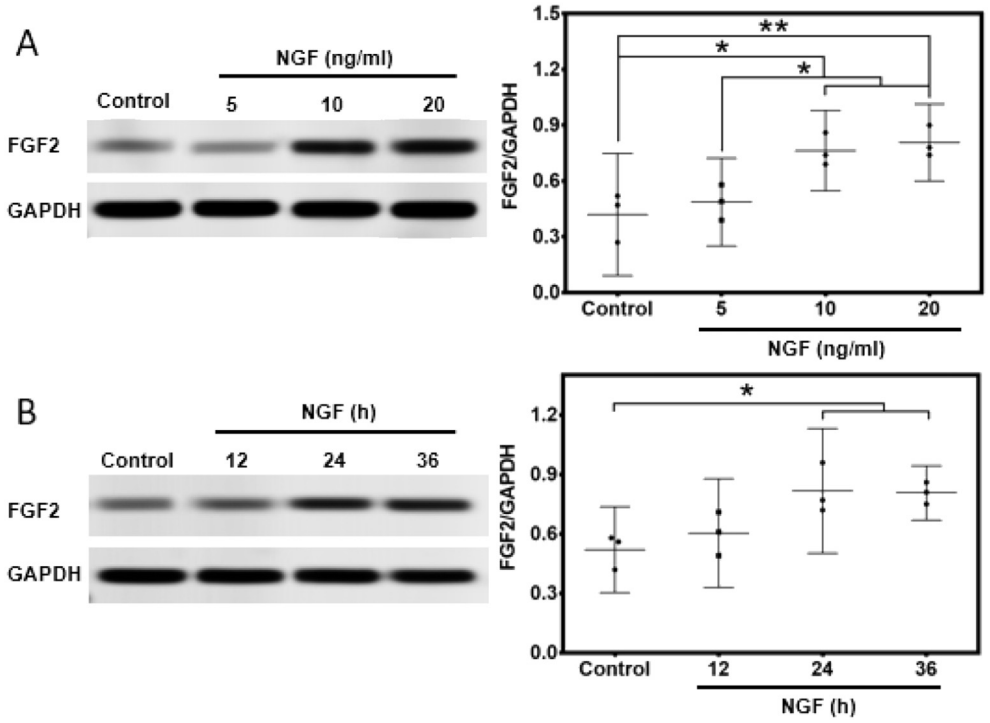

Fig. 2. Effect of dose (A) and treatment time (B) of NGF on expression of FGF2. Healthy human chondrocytes were treated by NGF with different doses. Western blot and quantitative analysis were performed to examine expression of FGF2 at different time points. Expression of FGF2 is generally dependent on dose and treatment time of NGF. Moreover, with review of previous studies, 10 ng/ml and 24 h were chosen for subsequent experiments. $n = 3$. $^{*}p < 0.05$, $^{**}p < 0.01$. Data are represented as individual data points with 95% CI.

dose-dependent manner according to western blot. Expression of FGF2 in 5 ng/ml group was significantly lower than that of 10 ng/ml group (Fig. 2(A), CI: (−0.54, −0.01), $p = 0.041$). Expression of FGF2 in control group was significantly lower than that of 24 h group (Fig. 2(B), CI: (−0.55, −0.04), $p = 0.025$). Moreover, with review of previous studies, treatment of NGF for 24 h with a dose of 10 ng/ml was decided as the treatment time and dose of NGF in our study.

Effects of NGF on expression of FGF2, migration and tube formation of endothelial cells are mediated by TrkA in chondrocytes

The RNA interference (RNAi) lentiviral vector particles targeting TrkA gene was constructed and evaluated. As is shown in Fig. 3(A), relative protein expression of TrkA in shRNA-TrkA group was significantly down-regulated comparing with control according to

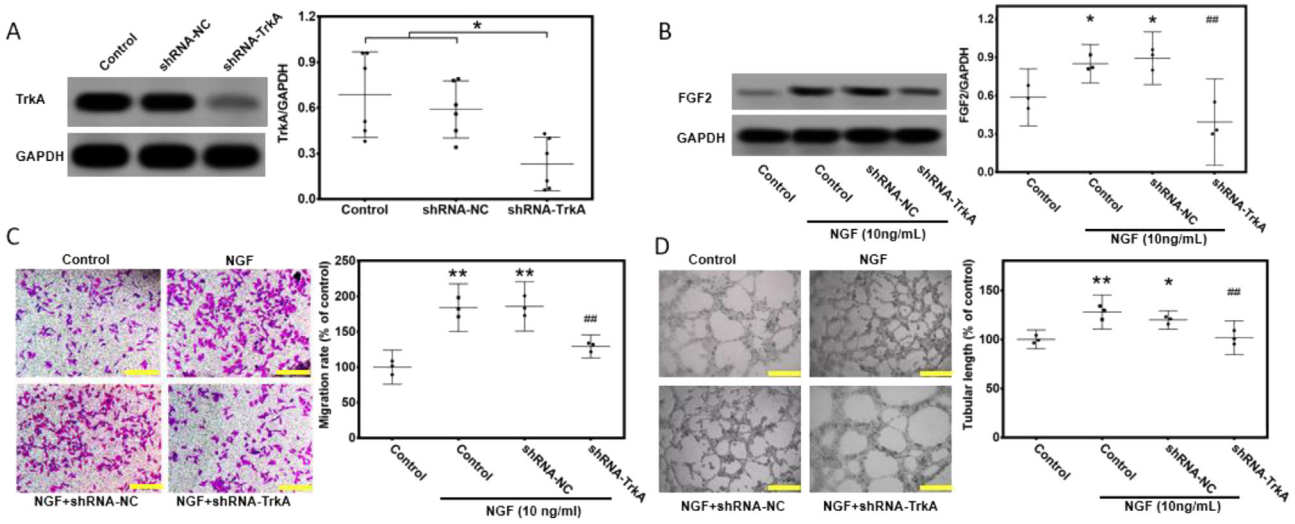

Fig. 3. Effects of NGF on expression of FGF2, migration and tube formation of endothelial cells are mediated by TrkA in human healthy chondrocytes. (A) Western blot and quantitative analysis showed expression of TrkA in human chondrocytes was down-regulated after knock-down of TrkA by transfection of lentiviral vector ($n = 6$). (B) Western blot and quantitative analysis showed up-regulated expression of FGF2 in human chondrocytes in NGF group compared with control group and down-regulated expression of FGF2 in NGF + shRNA-TrkA group compared with NGF group ($n = 3$). (C) Migration and quantitative analysis showed an increase after endothelial cells were co-cultured with NGF-treated human chondrocytes. Migration was decreased in shRNA-TrkA + NGF group compared with NGF group ($n = 3$). (D) Tube formation and quantitative analysis showed an increase in NGF group. Tube formation was decreased in shRNA-TrkA + NGF group compared with NGF group ($n = 3$).$^{*}p < 0.05$ vs control, $^{**}p < 0.01$ vs control, $^{\#\#}p < 0.01$ vs NGF group. Scale bar = 150 μm. Data are represented as individual data points with 95% CI.

western blot (CI: (−0.84, −0.08), $p = 0.02$) and negative control (CI: (−0.64, −0.07), $p = 0.014$) groups. As expected, no difference between control group and negative control group was statistically insignificant. These results confirmed that TrkA was successfully knocked down.

To further investigate the role of NGF on chondrocytes, western blot, migration and tube formation assays were used. As is shown in Fig. 3(B), expression of FGF2 was evidently up-regulated in NGF and NGF + shRNA-NC groups compared with the control group (CI: (0.01, 0.52), $p = 0.044$; CI: (0.05, 0.56), $p = 0.021$), while NGF + shRNA-TrkA group showed down-regulated expression of FGF2 (CI: (−0.71, −0.20), $p = 0.002$) comparing with NGF group.

Angioneic activities of endothelial cells (migration and tube formation) as shown in Fig. 3(C) and (D) were enhanced after co-culture with human chondrocytes in NGF and NGF + shRNA-NC groups comparing with control group (migration: CI: (54.3, 113.8), $p < 0.0001$; CI: (56.0, 115.6), $p < 0.0001$; tube formation: CI: (13.3, 42.7), $p = 0.001$; CI: (5.2, 34.6), $p = 0.011$). Migration rate and tubular length were both abolished in NGF + shRNA-TrkA group comparing with NGF group (CI: (−84.5, −25.0), $p = 0.002$; CI: (−41.0, −11.6), $p = 0.002$). These results showed that NGF induces angiogenesis *in vitro* via its receptor TrkA in human chondrocytes.

PI3K/Akt signaling pathway is partially involved in NGF-induced expression of FGF2, migration and tube formation of endothelial cells

To detect whether PI3K/Akt signaling is involved in NGF-induced expression of FGF2, migration and tube formation of endothelial cells, the human chondrocytes were treated with NGF in the presence or absence of LY294002, the PI3K inhibitor. As is shown in Fig. 4(A), the results showed that NGF promoted the phosphorylation of Akt compared with the control group (CI: (0.14, 0.35), $p < 0.0001$). According to western blot, NGF increased the expression of FGF2 in chondrocytes significantly (CI: (0.16, 0.48), $p < 0.0001$). However, the LY294002 + NGF combined treatment

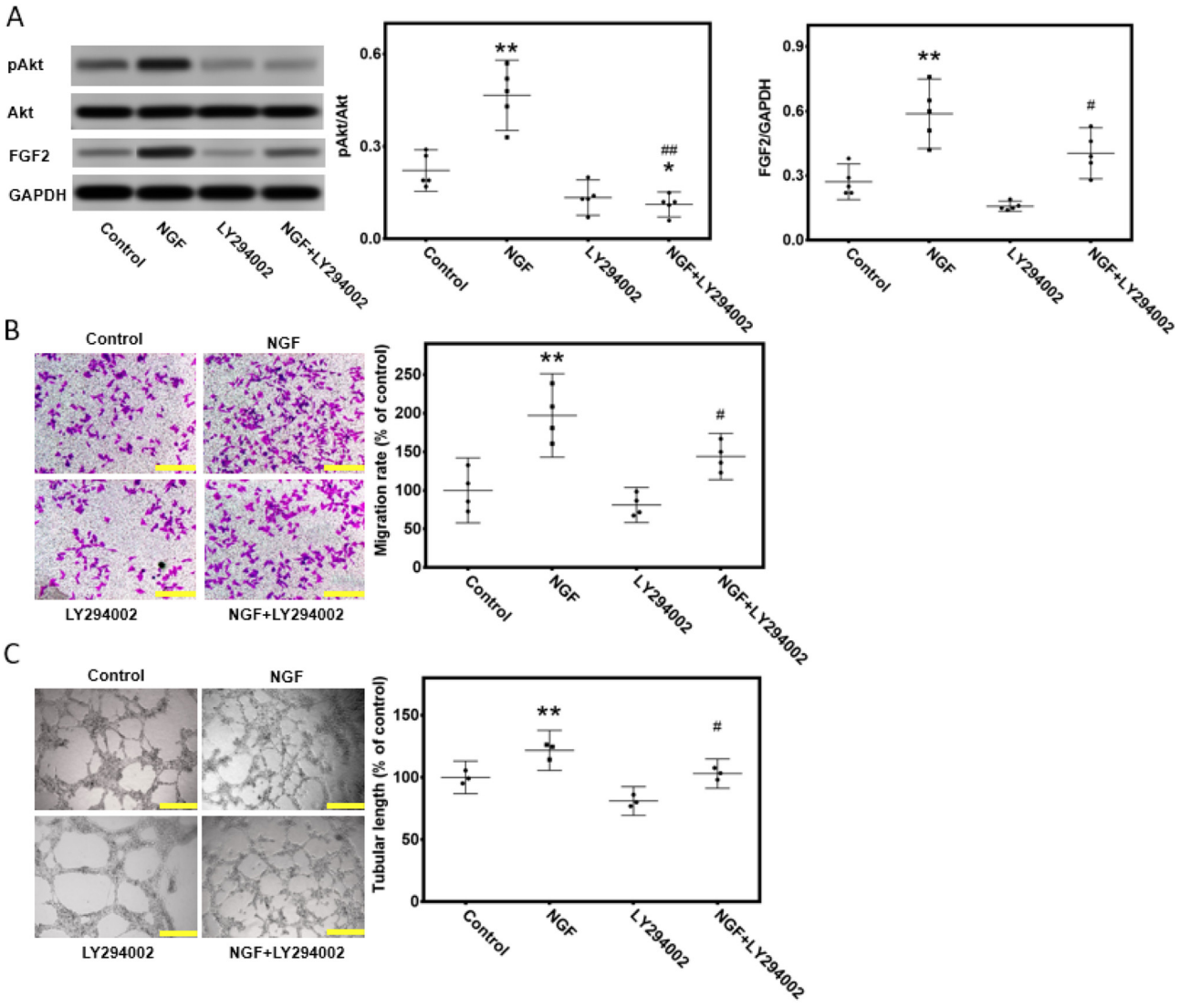

Fig. 4. PI3K/Akt signaling pathway is partially involved in NGF-induced increased expression of FGF2, migration and tube formation of endothelial cells. (A) Western blot and quantitative analysis showed up-regulated expressions of pAkt/Akt and FGF2 in human chondrocytes in NGF group compared with control group and down-regulated expressions of pAkt/Akt and FGF2 in NGF + LY294002 (a PI3K inhibitor) group compared with NGF group ($n = 5$). (B) Migration and quantitative analysis showed an increase after endothelial cells were co-cultured with NGF-treated human chondrocytes. Migration was decreased in NGF + LY294002 group compared with NGF group ($n = 4$). (C) Tube formation and quantitative analysis showed an increase in NGF group. Tube formation was decreased in NGF + LY294002 group compared with NGF group ($n = 3$). $^{*}p < 0.05$ vs control, $^{**}p < 0.01$ vs control; $^{\#}p < 0.05$ vs NGF group, $^{\#\#}p < 0.01$ vs NGF group. Scale bar = 150 μm. Data are represented as individual data points with 95% CI.

partially decreased the NGF-induced FGF2 expression (CI: (−0.34, −0.02), $p = 0.021$) and markedly decreased the phosphorylation of Akt (CI: (−0.46, −0.25), $p < 0.0001$).

Similarly, NGF enhanced angiogenic activities of endothelial cells and it was blocked with the addition of LY294002 as shown in Fig. 4(B) and (C). Migration rate and tubular length were both reduced in NGF + LY294002 group comparing with NGF group (CI: (−104.7, −1.7), $p = 0.042$; CI: (−32.6, −4.7), $p = 0.011$). These results showed that NGF induces angiogenesis and expression of FGF2 *in vitro* via PI3K/Akt signaling pathway in human chondrocytes.

ERK/MAPK signaling pathway is involved in NGF-induced expression of FGF2, migration and tube formation of endothelial cell

To detect whether ERK/MAPK signaling is involved in NGF-induced expression of FGF2, migration and tube formation of endothelial cells, the human chondrocytes were treated with NGF in the presence or absence of U1026, the ERK inhibitor. As is shown in Fig. 5(A), the results showed that NGF promoted the phosphorylation of ERK compared with the control group (CI: (0.37, 0.70), $p < 0.0001$). The U1026 + NGF combined treatment decreased the NGF-induced FGF2 expression (CI: (−0.48, −0.21), $p < 0.0001$) and markedly decreased the phosphorylation of ERK (CI: (−0.62, −0.29), $p < 0.0001$).

Similarly, NGF enhanced angiogenic activities of endothelial cells and it was blocked with the addition of U1026. Migration rate and tubular length were both reduced in NGF + U1026 group comparing with NGF group (CI: (−419.3, −356.9), $p < 0.0001$; CI: (−37.1, −18.9), $p < 0.0001$). These results showed that NGF induces angiogenesis and expression of FGF2 *in vitro* via ERK/MAPK signaling pathway in human chondrocytes.

NGF-induced FGF2 expression promotes migration and tube formation of endothelial cells

To detect whether NGF-induced expression of FGF2 is related to migration and tube formation of endothelial cells, the human chondrocytes were treated with NGF in the presence of antibody to FGF2 and BGJ398 (an FGFR inhibitor).

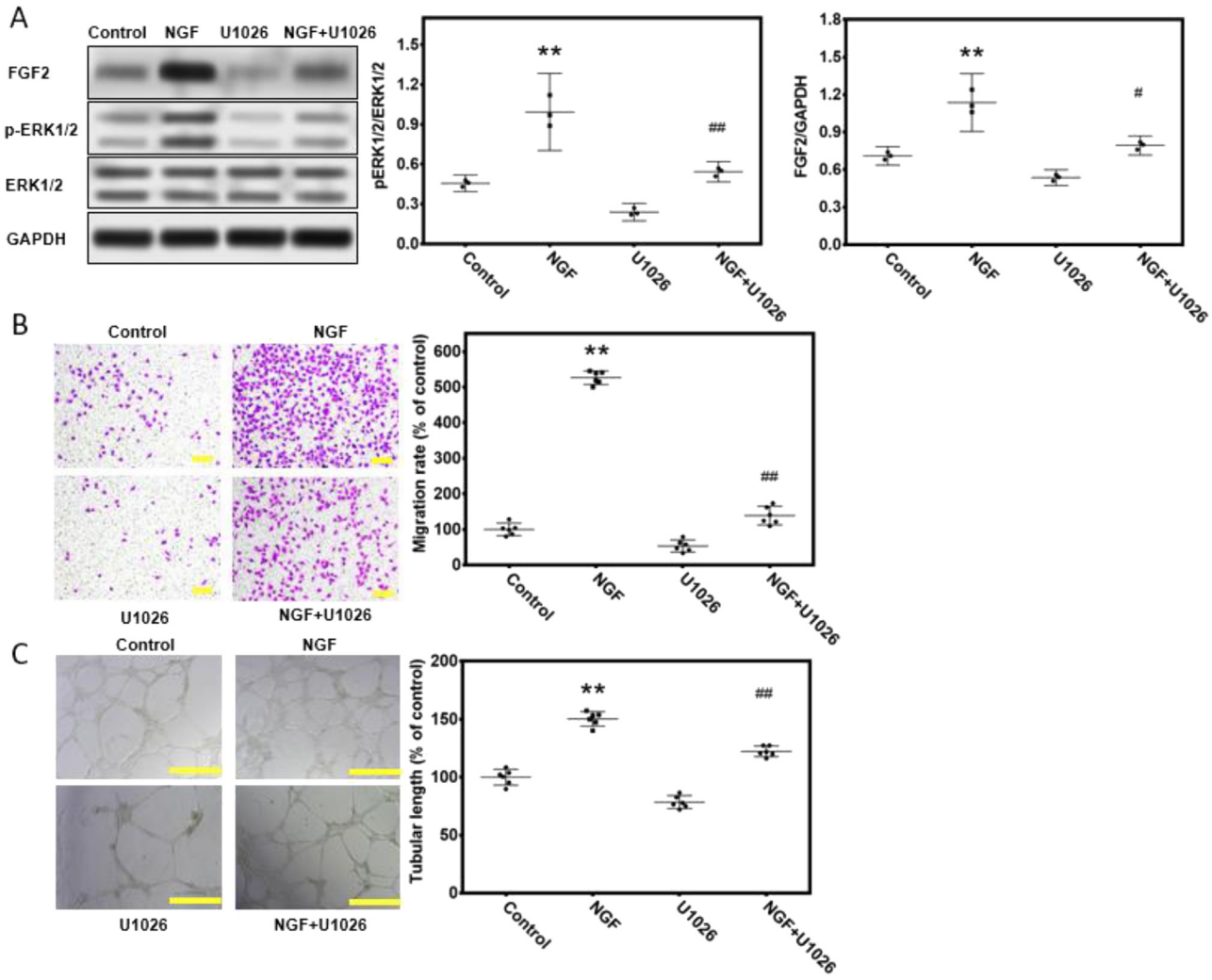

Fig. 5. ERK/MAPK signaling pathway is partially involved in NGF-induced increased expression of FGF2, migration and tube formation of endothelial cells. (A) Western blot and quantitative analysis showed up-regulated expressions of pERK1/2/ERK and FGF2 in human chondrocytes in NGF group compared with control group and down-regulated expressions of pERK1/2/ERK and FGF2 in NGF + U1026 (an ERK inhibitor, 10 μM) group compared with NGF group ($n = 3$). (B) Migration and quantitative analysis showed an increase after endothelial cells were co-cultured with NGF-treated human chondrocytes. Migration was decreased in NGF + U1026 group compared with NGF group ($n = 6$). (C) Tube formation and quantitative analysis showed an increase in NGF group. Tube formation was decreased in NGF + U1026 group compared with NGF group ($n = 6$). $^{*}p < 0.05$ vs control, $^{**}p < 0.01$ vs control; $^{\#}p < 0.05$ vs NGF group, $^{\#\#}p < 0.01$ vs NGF group. Scale bar = 150 μm. Data are represented as individual data points with 95% CI.

As is shown in Fig. 6(A), migration of endothelial cells was reduced in NGF + anti-FGF2 and NGF + BGJ398 groups compared with control group (CI: (−377.7, −321.2), $p < 0.0001$; CI: (−387.4, −314.0), $p < 0.0001$). In Fig. 6(B), tube formation assay showed significantly reduction in NGF + anti-FGF2 and NGF + BGJ398 groups compared with control group (CI: (−97.2, −64.7), $p < 0.0001$; CI: (−76.3, −43.8), $p < 0.0001$).

Discussion

In this study, we confirmed that OA resulted in up-regulated expressions of NGF and its high affinity receptor, TrkA, in human cartilages and chondrocytes. Furthermore, we demonstrated that NGF-induced expression of FGF2 in chondrocytes was mediated by TrkA recognition through activating PI3K/Akt and ERK/MAPK signaling pathways and promoted angiogenesis *in vitro*, which may lead to vascular up-growth from subchondral bone in OA.

OA is a complex disease, in which angiogenesis plays an important role. Angiogenic effect of NGF has been previously found in different *in vitro* and *in vivo* models, such as chick embryo chorioallantoic membrane (CAM)[20], mice[21] and rats[22]. Angiogenesis is well regulated and organized and is a process induced by angiogenic factor and receptors. In fact, the role that NGF plays in cartilage repair during OA was not much studied before researchers paid attention to insulin-like growth factor (IGF)-1, FGF2, transforming growth factor (TGF)-β and bone morphogenetic protein (BMP) family members[23,24]. NGF binds to TrkA and it is important for the formation of nociceptive sensory neurons during development[25]. *In vitro*, β-NGF increases the proliferation of human dermal, umbilical vein and choroidal cells, and rat brain endothelial cells. β-NGF also increases migration of human choroidal and pig aortic endothelial cells[19,26], and promotes capillary tube formation by human umbilical vein endothelial cells. Park *et al.* found that invasion and cord formation were strongly enhanced via PI3K/Akt signaling pathway by adding NGF, while migration of endothelial cells was not as enhanced[21]. It is reported that distinct steps stimulated by FGF2 are all the steps in angiogenesis, including proliferation of endothelial cell, cord formation, invasion and migration[27]. According to our data, significant increase in migration and tube formation of endothelial cells were observed after co-culture with NGF-treated chondrocytes. This difference may be attributed to the chondrocytes involved in our study. Also, chondrocytes in NGF + shRNA-TrkA group were shown to suppress angiogenic activities of endothelial cells and expression of FGF2. Previous studies have shown that the effects of NGF are mainly attributed to TrkA[18,28]. Upon NGF binding, TrkA dimerizes and autophosphorylates multiple tyrosines within its cytoplasmic domain[18]. Accordingly, we believe that NGF receptor TrkA

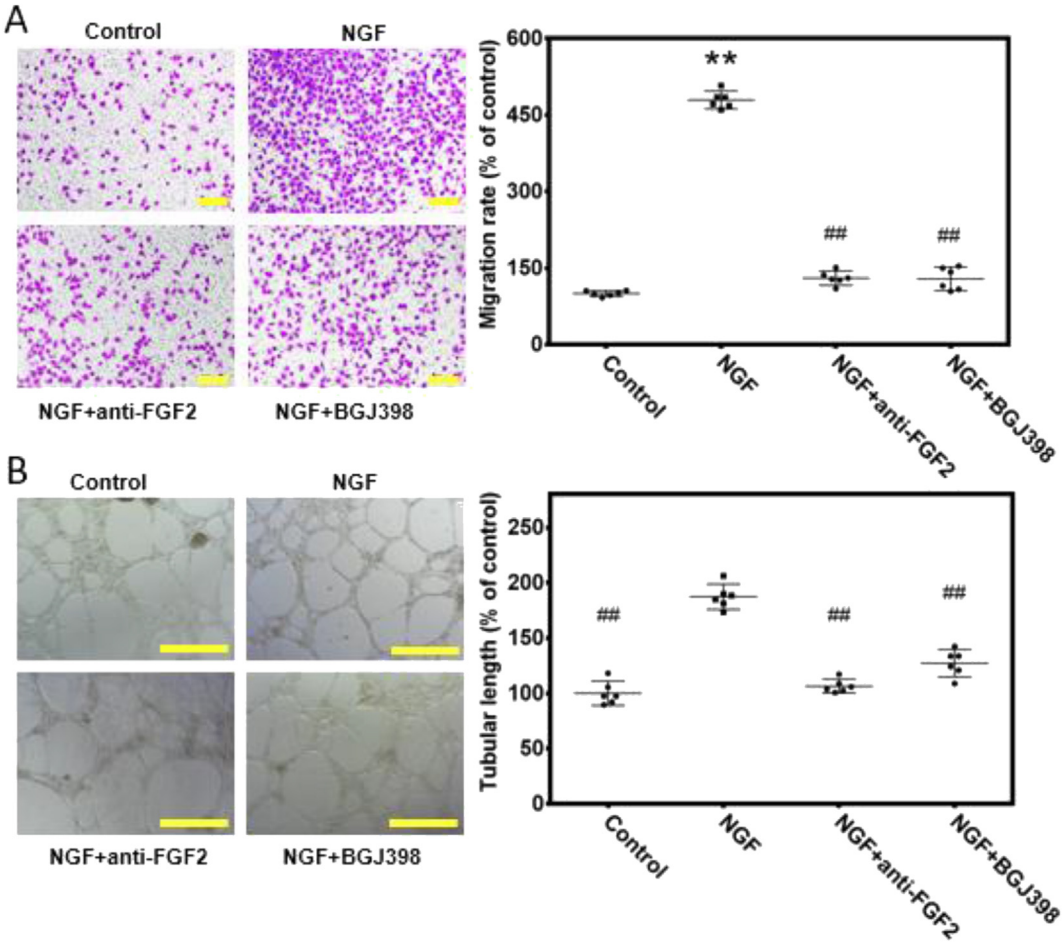

Fig. 6. NGF-induced FGF2 expression promoted migration and tube formation of endothelial cells. (A) Migration and quantitative analysis showed an increase after endothelial cells were co-cultured with NGF-treated human chondrocytes. Migration was decreased in NGF + anti-FGF2 (an antibody to FGF2, 1:500) and NGF + BGJ398 (an FGFR inhibitor, 1 μM) groups compared with NGF group ($n = 6$). (B) Tube formation and quantitative analysis showed an increase in NGF group. Tube formation was decreased in control, NGF + anti-FGF2 and NGF + BGJ398 groups compared with NGF group ($n = 6$). $^{*}p < 0.05$ vs control, $^{**}p < 0.01$ vs control; $^{\#}p < 0.05$ vs NGF group, $^{\#\#}p < 0.01$ vs NGF group. Scale bar = 150 μm. Data are represented as individual data points with 95% CI.

participates in functions of NGF in chondrocytes. Furthermore, this is probably a reason for up-growth of vessels from subchondral bone impairing cartilage repair.

The PI3K/Akt signaling pathway is one of the most important downstream targets of receptor tyrosine kinases, integrin receptors, and the Ras pathway[29]. It plays an important role in the physiological effect of NGF, especially in the angiogenesis process[30]. Previous studies have not only reported PI3K/Akt as a critical signaling pathway for cell proliferation, anti-apoptosis and migration, but also as a pathway for promotion of angiogenesis through specific stimulations[31,32]. Activating PI3K could induce the assembly of receptor-PI3K complexes, after which Akt is activated. Through phosphorylation, activated Akt mediated the activation and inhibition of several targets, resulting in cellular growth, survival and proliferation through various mechanisms[29]. Our results showed that PI3K/Akt pathway was activated by NGF and moreover, activation of PI3K/Akt, up-regulated expression of FGF2 and angiogenic activities of endothelial cells were blocked by inhibitor LY294002. These results indicated that PI3K/Akt activation plays an important role in NGF-induced angiogenesis *in vitro* in chondrocytes.

NGF and FGF2 are both important neurotrophic factors. Several studies have demonstrated that FGF2 can promote mitosis in the early phase of neural stem cells proliferation, while NGF supports their survival. NGF accelerates the proliferation of neural progenitor cells in conjunction with FGF2[33]. Noureddini *et al.* reported that NGF and FGF2 could induce cholinergic neuron differentiation[34]. Davidson *et al.* found that NGF colocalized with FGF2 protein and mRNA in effusions and solid tumors. In that study, Davidson *et al.* further concluded that coexpression of NGF with molecules involved in angiogenesis suggest that the proangiogenic role attributed to NGF *in vitro* and *in vivo* may be relevant in clinical cancer[35]. However, the relationship between NGF and FGF2 in chondrocytes is scarcely documented. In our study, data showed that NGF leads to up-regulation of FGF2, which is mediated by TrkA and is via PI3K/Akt and ERK/MAPK. Accordingly, it is suggested that NGF-induced angiogenesis in chondrocytes is related to FGF2 expression. Vascular endothelial growth factor (VEGF) as another well known angiogenic factor in OA was also examined in our study. However, we found that PI3K/Akt signaling pathway was not involved in NGF-induced expression of VEGF. According to Walsh et al., chondrocyte expression of VEGF is a particular feature in OA and is associated with vascular growth at osteochondral junction[11]. Further investigation is needed to explore the underlying mechanism of VEGF.

Monoclonal antibodies to NGF including tanezumab and fulranumab were used as treatments for pain, for example, osteoarthritis[36]. However, US FDA put anti-NGF trials on hold because of reports of osteonecrosis and rapid progression of OA which needed arthroplasty. The trials for tanezumab and fulranumab resumed after FDA lifted its hold in 2015. To date, the mechanism remains unknown. One possible explanation for progressive OA is nerve damage leading to loss of ability to feel pain in joint with reduced joint proprioception[37]. Another possible explanation for the apparent role of NSAIDs is that inhibited bone healing capacity caused by this class of drugs[38–40]. The most consistent and convincing results were seen in patients with symptomatic OA of knee or hip and low-dose antibody may have a favourable risk–benefit profile in OA patients without related predisposing factors.

There are limitations in this study that should be noted. First, the major steps of angiogenesis *in vitro* include endothelial cell proliferation, migration, invasion, and tube formation, while in our study, only migration and tube formation were evaluated. Second, this study focused on only knee joint, whether the mechanism applies to other osteoarthritic joint needs more investigations. Finally, according to Vincent et al. perlecan and FGF2 co-localised within pericellular matrix of chondrocytes and pericellular FGF2 mediates activation of ERK pathway upon loading of cartilage[41]. Our data only showed that FGF2 participated in promotion of angiogenesis *in vitro* through co-culture of chondrocytes and endothelial cells. However, the real process *in vivo* is different from that of our experiment. Therefore, whether FGF2 secreted by chondrocytes has the same effect in cartilage still needs confirmation of further experiments. Our future work will further investigate the angiogenic effect of NGF *in vivo* via PI3K/Akt and ERK/MAPK signaling pathways in chondrocytes.

Conclusion

In summary, our results confirmed that expressions of NGF and TrkA are up-regulated in OA. NGF induces expression of FGF2 and results in angiogenesis *in vitro* in human chondrocytes. Further mechanistic analyses disclosed that PI3K/Akt and ERK/MAPK signaling pathways are involved. Collectively, these findings provided a better understanding of the mechanism of NGF induced angiogenesis *in vitro* underlying OA.

Author contributions statement

X.D, X.Y, Y.Q and T.Z contributed to the conception and design of the study. X.Y, Y.Q, T.Z, J.F, X.L, T.X, X.D and Q.Y performed the experiments and contributed to the analysis and interpretation of data. X.Y and Y.Q contributed to draft manuscript. All approved the submitted manuscript.

Competing interests statement

The authors declare no competing interests.

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Supplementary data

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