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Paracrine signaling by VEGF-C promotes non-small cell lung cancer cell metastasis via recruitment of tumor-associated macrophages

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ABSTRACT

High expression of tumoral vascular endothelial growth factor C (VEGF-C) is correlated with clinical non-small cell lung cancer (NSCLC) metastasis and patient survival. Nevertheless, the comprehensive mechanisms accounting for VEGF-C-mediated cancer progression remain largely unclear. The present study found that VEGF-C expression was upregulated in various NSCLC cell lines. By utilizing transwell migration assay, we found that both recombinant VEGF-C protein and overexpression of VEGF-C in NSCLC cells (A549 and H441 cell lines) could efficiently enhance RAW264.7 cell (murine macrophages) migration. However, recombinant VEGF-C treatment had no effects on both CD206 (an M2 macrophage marker) expression and M1/M2 cytokine profiles of macrophages. Furthermore, additional treatment of recombinant Flt-4/Fc, the specific VEGFR-3 inhibitor or the specific VEGFR-2 inhibitor significantly suppressed macrophage migration compared with A549-CM (conditioned medium) or H441-CM alone group, confirming that NSCLC cells-derived VEGF-C is sufficient to promote macrophage migration. Interestingly, VEGF-C could stimulate the Src/p38 signaling via VEGFR-2/3 axis in macrophages, and inhibition of Src/p38 signaling obviously reversed the enhancement effect of VEGF-C on macrophage migration. Finally, the functional importance of macrophage infiltration induced by tumoral VEGF-C in promoting metastasis was established in a mouse model. In conclusion, our results highlight a novel function of tumoral VEGF-C that paracrinely induces macrophage recruitment, and resultantly promotes NSCLC cell metastasis. Therefore, VEGF-C/VEGFR-2/3 axis may be a promising microenvironmental target against progression of NSCLC.

1. Introduction

Lung cancer is the leading cause of cancer–related deaths worldwide, and non-small cell lung cancer (NSCLC) accounts for up to 80% of all lung cancer cases [1]. Tumor-associated macrophages (TAMs) are a major component of the leukocyte infiltration of many solid tumors, and the infiltration of TAMs correlates with cancer metastasis process and poor prognosis in most types of human cancer including NSCLC [2,3]. However, the mechanism of cancer-mediated macrophage infiltration remains unclear.

Vascular endothelial growth factors (VEGFs) are a family of secreted polypeptides with a highly conserved receptor-binding cystine-knot structure, comprising several different proteins, including VEGF-A, VEGF-B, VEGF-C, VEGF-D and placental growth factor [4]. Expression of VEGFs is commonly observed in most aggressive tumors, and profoundly associates with the poor prognosis of cancer patients [5]. VEGF-C is the relatively newly defined VEGFs family member, and compared with the well-characterised VEGF-A, many functions and molecular mechanisms involved in the cancer progression mediated by the VEGF-C remain undefined.

VEGF-C functions through interacting with its receptors, VEGFR-3 (also called Flt-4) and VEGFR-2 [6]. In NSCLC, a previous report indicated that high expression levels of both VEGF-C and its receptor Flt-4 show the poor prognosis of cancer patients [7]. Besides, another work indicated that VEGF-C is frequently upregulated in cancer tissues, and interestingly, high tumoral but not stromal VEGF-C status shows poor prognosis in NSCLC patients [8]. Specifically, recent studies showed that the specific VEGFR-3-TK inhibitor (SAR131675) could significantly reduce TAMs infiltration and aggregation in 4T1 (a murine breast cancer cell line) tumor-beard mice [9,10]. As VEGFR-3 was confirmed

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to be abundantly expressed in monocytes and TAMs [11,12], these findings strongly suggest that VEGF-C/VEGFR-3 axis mediates TAMs infiltration of cancer tissues. Nevertheless, the definite role of tumoral VEGF-C in NSCLC-induced TAMs infiltration and the resultant impact on cancer metastasis remain still unexplored.

In the present study, we demonstrate that paracrine VEGF-C from NSCLC cells can increase macrophage migration in vitro and promote macrophages infiltration in vivo, mainly via VEGFR2/3/Src/p38 pathway in macrophages, thus facilitating cancer cell metastasis. Therefore, the VEGF-C/VEGFR-2/3 axis may be a promising micro-environmental intervention target in NSCLC therapy.

2. Materials and methods

2.1. Reagents

Recombinant VEGF-C and VEGFR-3 Fc chimera recombinant protein (Flt-4/Fc) were purchased from R&D. SAR131675 (VEGFR-3 inhibitor), SB203580 (p38 inhibitor), saracatinib (Src inhibitor) and SKLB1002 (VEGFR-2 inhibitor) were from Selleck Chemicals. Cell culture reagents were obtained from Invitrogen. All other reagents were from Sigma unless stated otherwise.

2.2. Cell lines, conditioned medium preparation and transfection

Murine macrophage cell line RAW264.7, normal lung epithelial cell line BEAS-2B cells and NSCLC cell lines (A549, H460, H358, H441 and HCC827) were originally from ATCC. NSCLC cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco-BRL, Gaithersburg, MD, USA). BEAS-2B cells were cultured in epithelia cell medium (Gibco-BRL, Gaithersburg, MD, USA). All of them were supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin. Cells were cultured in a standard humidified incubator at 37 °C in a 5% CO2 atmosphere. To obtain the conditioned medium (CM) , NSCLC cell lines (A549 and H441) were seeded at 2×10^6 cells/75 cm^2. Then, the supernatants were harvested at 24 h of further incubation.

VEGF-C in pcDNA3.1 (+) vector (Invitrogen, The Netherlands) or empty vector were transfected into A549 and H441 cells using liposomes according to a previous study [13]. The VEGF-C-overexpressing cells were selected for stable transfection over 14 days using normal culture medium with 600 μg/mL G418 sulphate (Calbiochem, Germany).

2.3. Western blotting and ELISA

Western blotting protocol was according to our previous report [14]. Primary antibodies were anti-VEGF-C (1: 1000; CST #2445), Src (1: 1000; CST #2109), p-Src (1:1000; CST #12432), p38 (1: 1000; CST #8690), p-p38 (1:1000; CST #4511), and β-tubulin (1:5000; ab6046). The secondary antibody was Goat anti-rabbit IgG (1:7500; Proteintech, USA, SA00001-2). The bands were detected by ECL detection reagent. The supernatants of macrophage culture were centrifuged before ELISA. IL-10, IL-12 and IL-23 were measured by commercial ELISA kits (R&D, Minneapolis, MN) according to the manufacturer's instructions.

2.4. Real-time RT-PCR

Total RNA was extracted from cells using Trizol Reagent (Invitrogen), and then complementary DNA (cDNA) was synthesized using ReverTra Ace reverse transcriptase (TOYOBO, Japan, FSQ-301) according to the manufacturer's instructions. Real-time RT-PCR was performed with the SYBR Green Realtime PCR Master Mix (TOYOBO, Japan, QPK-201) on an iCycler (Bio-Rad) following the manufacturer's instructions. The primer sequences were as follows: VEGF-C forward primer: GCAGTTACGGTCTGTGTCCA; VEGF-C reverse primer: TTGAG GTTGGCCTGTTCTCT; GAPDH forward primer: GGAGTCAACGGATTT GGT; GAPDH reverse primer: GTGATGGGATTTCCATTGAT. The gene expression levels for each amplicon were calculated by the ΔΔCT method [15] and normalized against GAPDH mRNA.

2.5. In vitro migration assay

The RAW264.7 cell migration was assayed using the Falcon TM Cell Culture Inserts containing polycarbonate membranes with pore sizes of 8 μm. Briefly, Macrophages were seeded (1×10^5 cells/well) in the upper chamber of a transwell and placed it on the 24-well plate. The conditioned medium of A549 or H441 cells were added into the lower chamber. In the coculture experiments, A549 or H441 cells (5×10^4 cells/ insert) were seeded into the lower chamber of 24-well plates. After 10 h, the cell suspension in the upper chamber was aspirated, and the upper surface of the filter was carefully cleaned with cotton plugs. After migration through the polycarbonate membrane, macrophages were stained with crystal violet and images from five representative fields of each membrane were taken. The migratory cells within the lower chamber were counted.

2.6. In vivo metastasis model

BALB/C nude mice (6–8 weeks old) were housed under standard conditions according to the institutional guidelines for animal care. For experimental metastasis , A549 or VEGF-C-overexpressing A549 cells (5×10^6 per mouse) were injected into the tail vein of nude mice. For therapeutic experiments, mice were oral administration of SAR131675 (100 mg/Kg/day) after 5 days post injection of A549 cells. In another therapeutic experiment group, mice were received clodronate encapsulated in liposome nanoparticles (5 mg/mL) to deplete macrophages in mice according to another report [16] before tail-injection of VEGF-C-overexpressing A549 cells. Mice were euthanized after 4 weeks and the livers were harvested, and the number - of visible surface metastases was counted. The liver tissues were either processed for immunohistochemistry.

2.7. Immunohistochemistry

The liver tissue sections were deparaffinized with xylene and rehydrated with graded ethanol. Antigen retrieval was performed by boiling the sections in low-pH citrate buffer for 15 min. The sections were stained and visualized by BBI Science Life ABC kit. Primary antibody was rat anti-mouse F4/80 at a 1:200 dilution (Novus). Images were captured using the AxioVision Rel.4.6 computerized image analysis system (Carl Zeiss). The F4/80 expression levels in the IHC slices were determined by measuring the cumulated integrated optical density (IOD) using IPP software (Media Cybernetics, Inc., USA).

2.8. Statistical analysis

Statistical analysis was evaluated by Student's test for simple comparisons between two groups and one-way ANOVA for comparisons among multiple groups using GraphPad Prism 5. Data are expressed as mean ± S.D. P value of < 0.05 was considered statistically significant.

3. Results

3.1. VEGF-C induces migration of macrophages but not macrophage polarization

To determine the role of tumoral VEGF-C in macrophage recruitment, we first detected the mRNA expression levels of VEGF-C in both NSCLC cell lines and normal lung epithelium cells by qRT-PCR. The VEGF-C mRNA levels in NSCLC cell lines (A549, H460, H358, H441 and HCC827) were significantly higher than those in BEAS-2B cells (Fig. 1A).

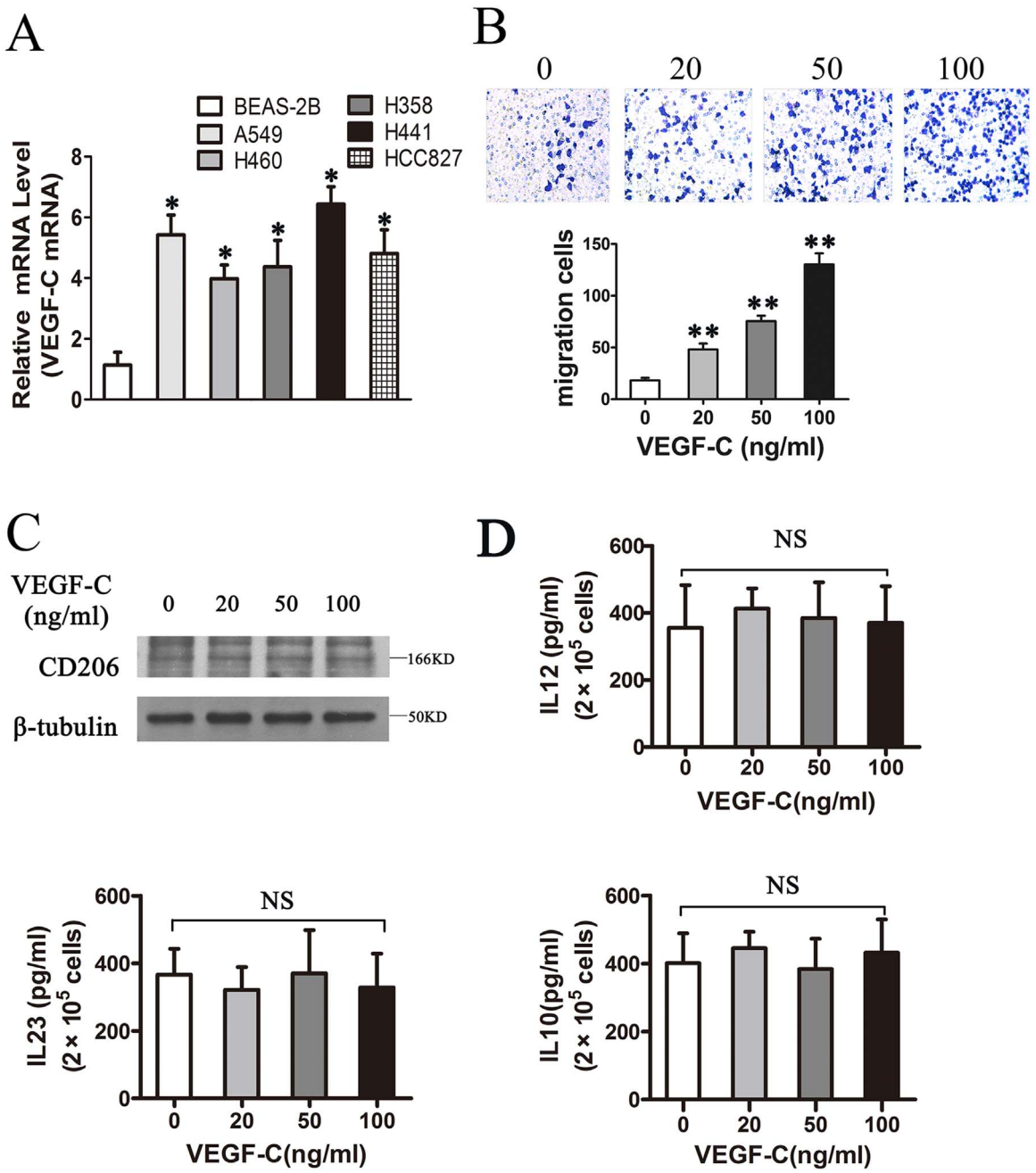

Fig. 1. Recombinant VEGF-C protein induces migration of macrophages but not macrophage polarization. (A) Real-time RT-PCR of VEGF-C mRNA expression in NSCLC cell lines and BEAS-2B cells. Gene expression data were presented as the fold increase in gene expression relative to the BEAS-2B cells (set arbitrarily to 1). *Significantly different from BEAS-2B, $p < 0.05$. (B) Macrophage migration increased after treatment with VEGF-C (20–100 ng/mL). * *Significantly different from control group, $p < 0.001$. (C) The effects of VEGF-C on CD206 expression in macrophage cells analyzed by western blotting after 48-h treatment. (D) M1/M2 cytokine profiles were detected by enzyme-linked immunosorbent assay (ELISA). The data represent at least three independent experiments. NS, not significant.

In order to define the effect of VEGF-C on macrophage migration, we used murine macrophage cell line RAW264.7 according to another study [17]. Fig. 1B showed that treatment with recombinant VEGF-C could substantially enhance macrophage migration in a concentration-dependent manner (with the concentrations ranging from 20–100 ng/mL) after 10-h incubation determined by transwell assay.

As TAMs can be sub-classified into classically (M1) and alternatively (M2) activated phenotypes [18], we next to determine whether there is a relationship between VEGF-C and macrophage polarization. The detection methods of M1 or M2 polarization of RAW264.7 cells were according to another report [19], as mannose receptor CD206 (an M2 macrophage marker) was detected by western blotting and levels of M1 cytokines (interleukin (IL) 12 and IL23), and M2 cytokine (IL10) were determined by ELISA. We found that VEGF-C had none effect on CD206 expression (Fig. 1C). Additionally, we collected macrophages after treatment with VEGF-C (100 ng/mL) for 48 h to detect M1/M2 cytokine profiles by ELISA. The results showed that the levels of both M1 cytokines (IL12 and IL23), and M2 cytokine (IL10) showed no significant differences between the VEGF-C treatment group and the control group (Fig. 1D).

Therefore, the above results indicate that recombinant VEGF-C promotes the migration of macrophages, while exerts no effects on macrophage polarization.

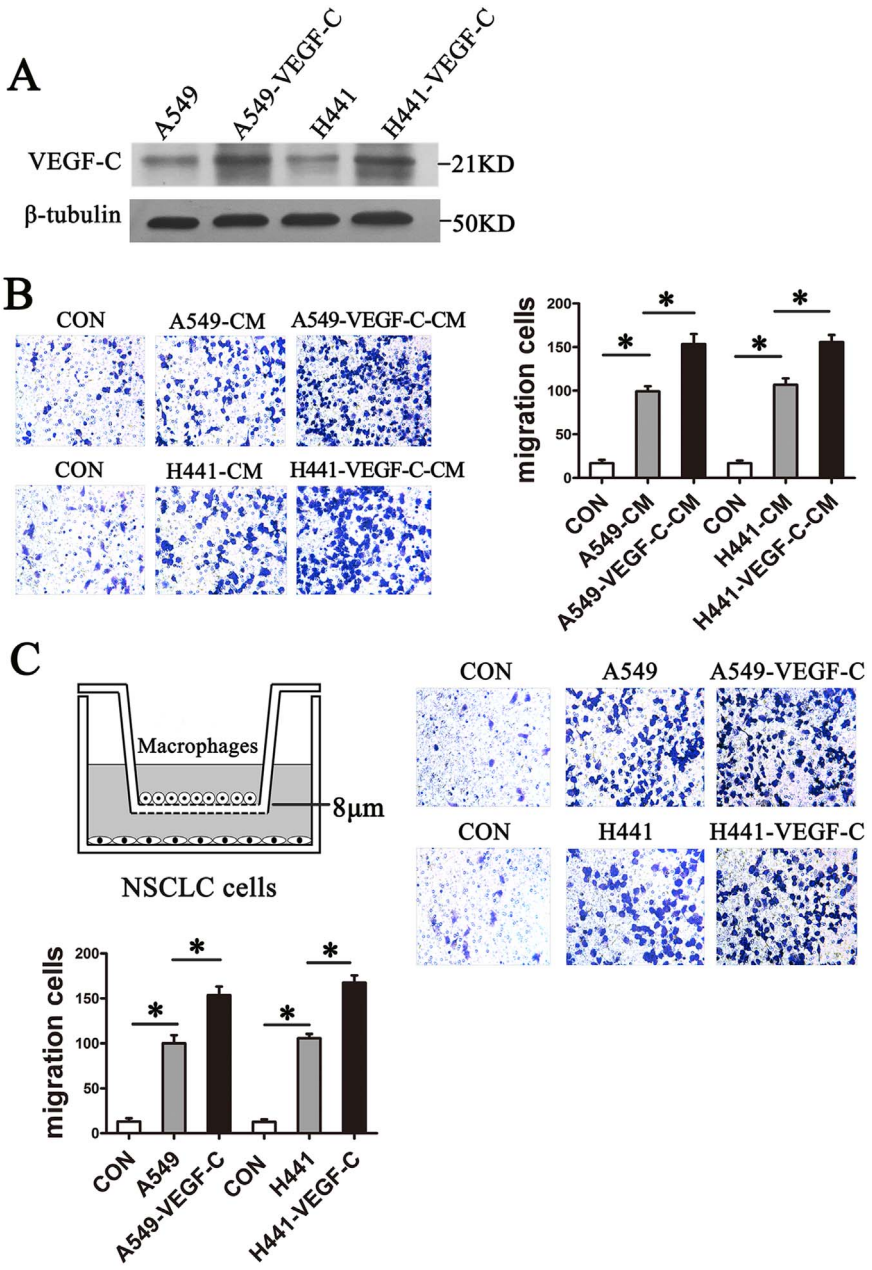

Fig. 2. VEGF-C overexpression increases macrophage migration. (A) Western blotting analyses of VEGF-C expression in transfected NSCLC cell lines. (B) The conditioned medium (CM) of tumor cells promoted the macrophage migration. The CM of VEGF-C-overexpressing A549 and H441 cells increased macrophage cell migration compared with CM of parental A549 and H441 cells. (C) Macrophage cell migration experimental system. The Falcon TM Cell Culture Inserts containing polyethylene terephthalate (PET) membranes with pore sizes of 8 μm. VEGF-C overexpression in A549 and H441 cells enhanced macrophage migration. The data represent at least three independent experiments. *$p < 0.05$.

3.2. VEGF-C overexpression increases macrophage migration

To further confirm whether VEGF-C can promote macrophage migration, we constructed the stable VEGF-C-overexpressing A549 and H441 cells by transfection with a plasmid coding for VEGF-C. The transfection efficiency was assayed by western blotting (Fig. 2A).

We collected conditioned medium (CM) from A549, VEGF-C-overexpressing A549 (A549-VEGF-C), H441 and H441-VEGF-C cells. In the experiments, A549-CM, A549-VEGFC-CM, H441-CM and H441-VEGF-C-CM were used to incubate macrophage for 10 h. The migration of macrophages treated by A549-CM and H441-CM was strikingly increased compared with the control group. Furthermore, the CM of VEGF-C-overexpressing A549 and H441 cells enormously increased macrophage migration compared with CM of parental A549 and H441 cells (Fig. 2B).

Next, we used a transwell cell coculture system (transwell chambers, 8-μm pore size, Falcon, Oxnard, CA) to coculture the RAW264.7 (1×10^5) and NSCLC cells (5×10^4) without direct contact (Fig. 2C, upper panel) for 10 h. Fig. 2C (lower panel) showed that macrophages co-cultured with A549 and H441 cells exhibited higher migration ability than the cells cultured alone. Besides, VEGF-C overexpression in A549 and H441 cells could further enhance macrophage migration.

3.3. Tumoral VEGF-C promotes macrophage migration via VEGFR-2/3 axis

The above results cannot necessarily indicate the role of tumoral VEGF-C in enhancement of macrophage migration. VEGF-C has been shown to signal through the receptors VEGFR-3 (also called Flt-4) and VEGFR-2 [20], however, VEGFR-3 uniquely responds to its specific ligand, VEGF-C [6]. As VEGFR-3 is abundantly expressed in macrophages

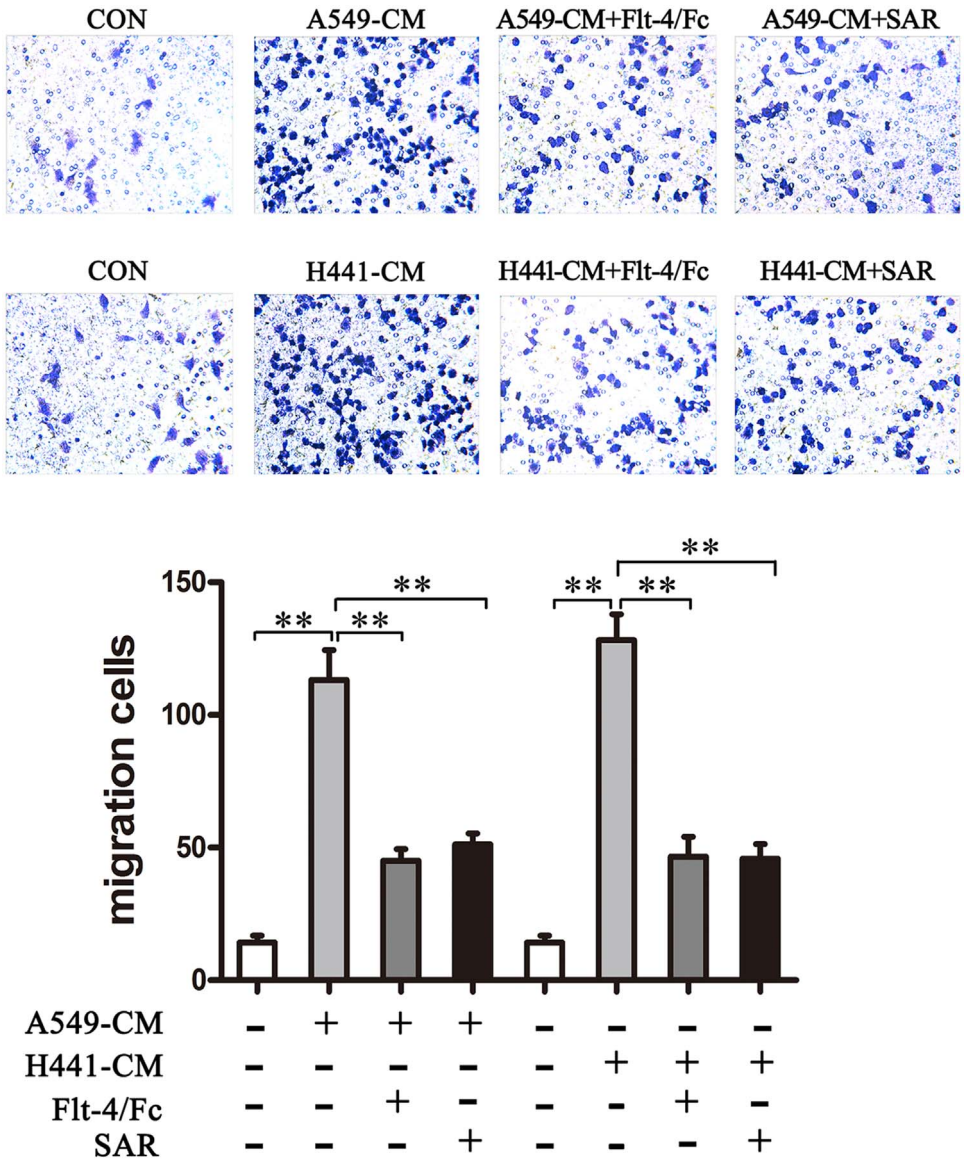

Fig. 3. Tumoral VEGF-C promotes macrophage migration via VEGFR-3 axis. Macrophage cell (1×10^5 cells/well) were treated with Flt-4/Fc (100 ng/mL) or SAR131675 (1 μM) in the CM of NSCLC cells. Macrophage migration was detected by migration assay. The data represent at least three independent experiments. * *$p < 0.001$.

[21], we next sought to block VEGFC/VEGFR-3 axis to determine the role of tumoral VEGF-C in macrophage recruitment.

Recombinant Flt-4/Fc can specifically neutralize the secreted form of VEGF-C [7], and was therefore utilized to interrupt VEGF-C signaling. Flt-4/Fc (100 ng/mL) was added into the CM of NSCLC cells followed by incubation of macrophages for 10 h. Fig. 3 showed that both A549-CM and H441-CM could increase macrophage migration, however, additional treatment with Flt-4/Fc could substantially suppress macrophage migration compared with A549-CM and H441-CM groups. Next, we used SAR131675, a potent and selective VEGFR-3-TK inhibitor, to selectively block VEGFR-3 in macrophages. As shown in Fig. 3, similar with Flt-4/Fc, additional administration of SAR131675 (1 μM) in A549-CM or H441-CM could efficiently reduce macrophage migration compared with A549-CM or H441-CM treatment alone. As VEGF-C is also known as the ligand of VEGFR2, to further confirm the role of tumoral VEGF-C in macrophage recruitment, we sought to determine the effect of VEGFR2 blockade by SKLB1002 (a selective VEGFR2 inhibitor) on VEGF-C-induced macrophage migration. The results showed that administration of SKLB1002 (32 nM) in A549-CM or H441-CM could also substantially reduce macrophage migration compared with A549-CM or H441-CM treatment alone. These data suggest that NSCLC cells-derived VEGF-C promotes the migration of macrophages via VEGFR-2/3 axis in macrophages.

3.4. The Src/p38 MAPK signaling pathway is required for VEGF-C-mediated macrophage migration

As the Src/p38 MAPK signaling has been confirmed to play a critical role in VEGF-C-mediated cancer cell migration [7], we speculate whether tumoral VEGF-C promotes macrophage migration also via the Src/p38 MAPK axis. We performed experiments using A549 cells as a cell model. Fig. 4A showed that A549-CM activated the Src/p38 signaling in macrophages after 4-h treatment as indicated by enhanced expression of phosphorylated Src (p-Src) and p-p38 without effects on total Src and p38 expression, and addition of either Flt-4/Fc or SAR131675 in A549-CM could significantly reverse such effect. Besides, addition of SKLB1002 in A549-CM also efficiently reversed A549-CM-induced activation of Src/p38 signaling in macrophages after 4-h treatment (Supplementary Fig. 2). Furthermore, recombinant VEGF-C (100 ng/mL) significantly stimulated the Src/p38 signaling in macrophages after 4-h incubation (Fig. 4B).

As shown in Fig. 4C, recombinant VEGF-C (100 ng/mL) treatment

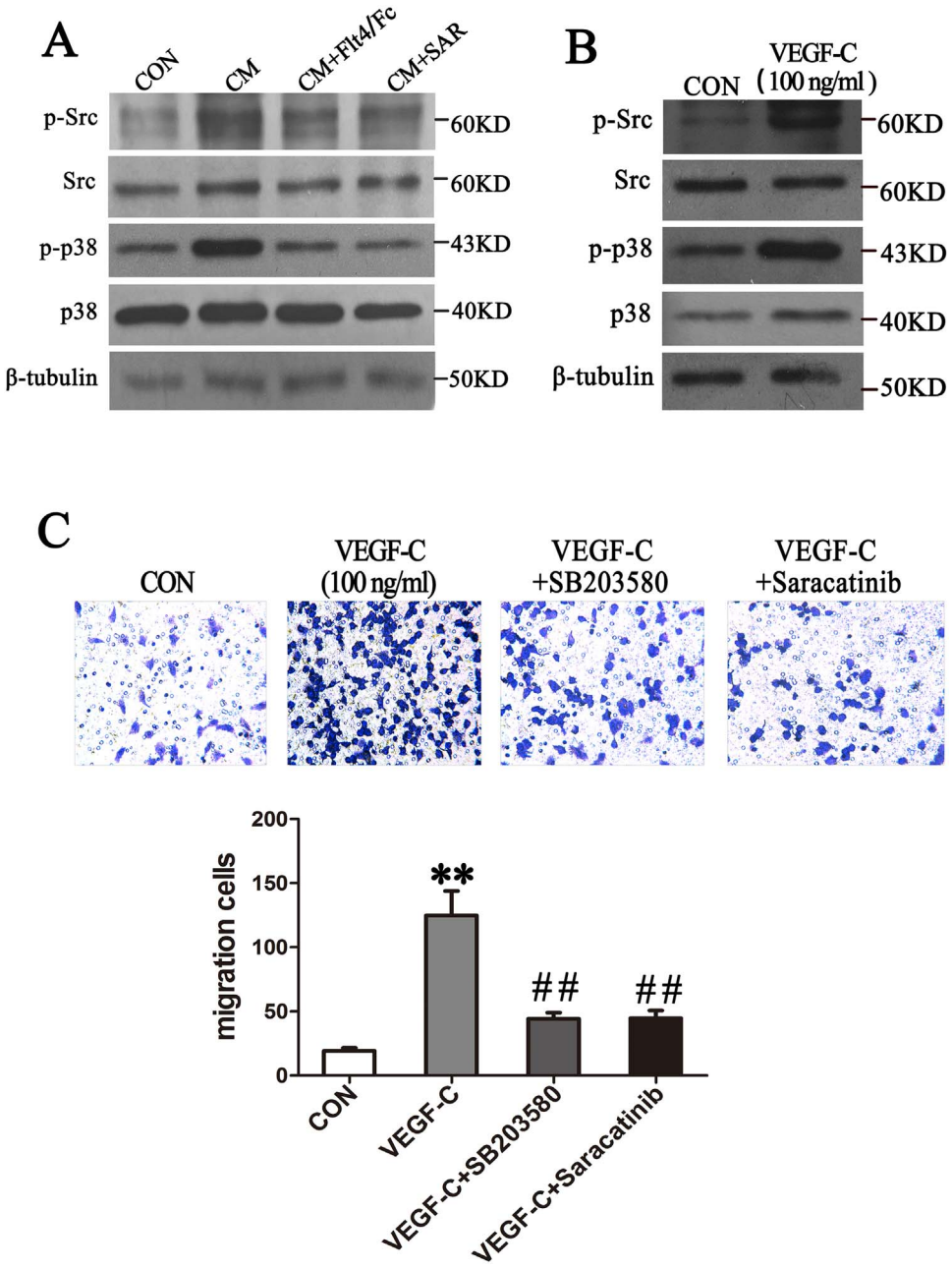

Fig. 4. The Src/p38 MAPK signaling pathway is required for VEGF-C-mediated macrophage migration. (A) The effects of Flt-4/Fc or SAR131675 in A549-CM on p-Src, Src, p-p38 and p38 levels in macrophage cells analyzed by western blotting. (B) The effect of VEGF-C on p-src, src, p-p38 and p38 levels in macrophage cells analyzed by western blotting. (C) Migration assay of macrophage. Macrophages were treated with VEGF-C (100 ng/mL), VEGF-C plus pretreatment with SB203580 (p38 inhibitor, 1 μM) or saracatinib (Src inhibitor, 1 μM) for 1 h. The data represent at least three independent experiments. * *Significantly different from control group, $p < 0.001$. $^{\#\#}$ Significantly different from the VEGF-C group, $p < 0.001$.

for 10 h substantially enhanced the migration ability of macrophages, and such effects could be obviously reversed by either pretreatment of macrophages with the Src inhibitor (Saracatinib, 1 μM) or the p38 inhibitor (SB203580, 1 μM) for 1 h.

The above results together strongly support that NSCLC cells-derived VEGF-C can sufficiently promote macrophage migration via activation of VEGFR-2/3-mediated Src/p38 signaling in macrophages.

3.5. Tumoral VEGF-C enhances macrophage infiltration and afterwards induces liver metastasis in vivo

Based on the above observations, we hypothesized that tumoral VEGF-C could promote NSCLC cell metastasis via recruitment of macrophages. To test this hypothesis, we injected A549 cells (5×10^6 per mouse) into the tail vein of nude mice to detect cell metastasis in vivo. Livers from these mice were harvested after 4 weeks and further tested for the presence of F4/80-positive cells by immunohistochemistry staining.

Fig. 5A showed that after 5 days post injection of cancer cells, oral administration of SAR131675 (100 mg/Kg/day) to block VEGF-C function resulted in a significant decrease in the incidence of metastasis and the number of visible liver metastases. To further explore the possible role of VEGF-C in tumor microenvironment, we stained the tissue sections of livers with anti-F4/80 antibody. The results clearly showed that SAR131675 treatment could reduce the recruitment of F4/80-positive macrophages in the liver tissues (Fig. 5B).

To further confirm the role of tumoral VEGF-C-mediated macrophage infiltration in cancer cell metastasis, we next injected VEGF-C-

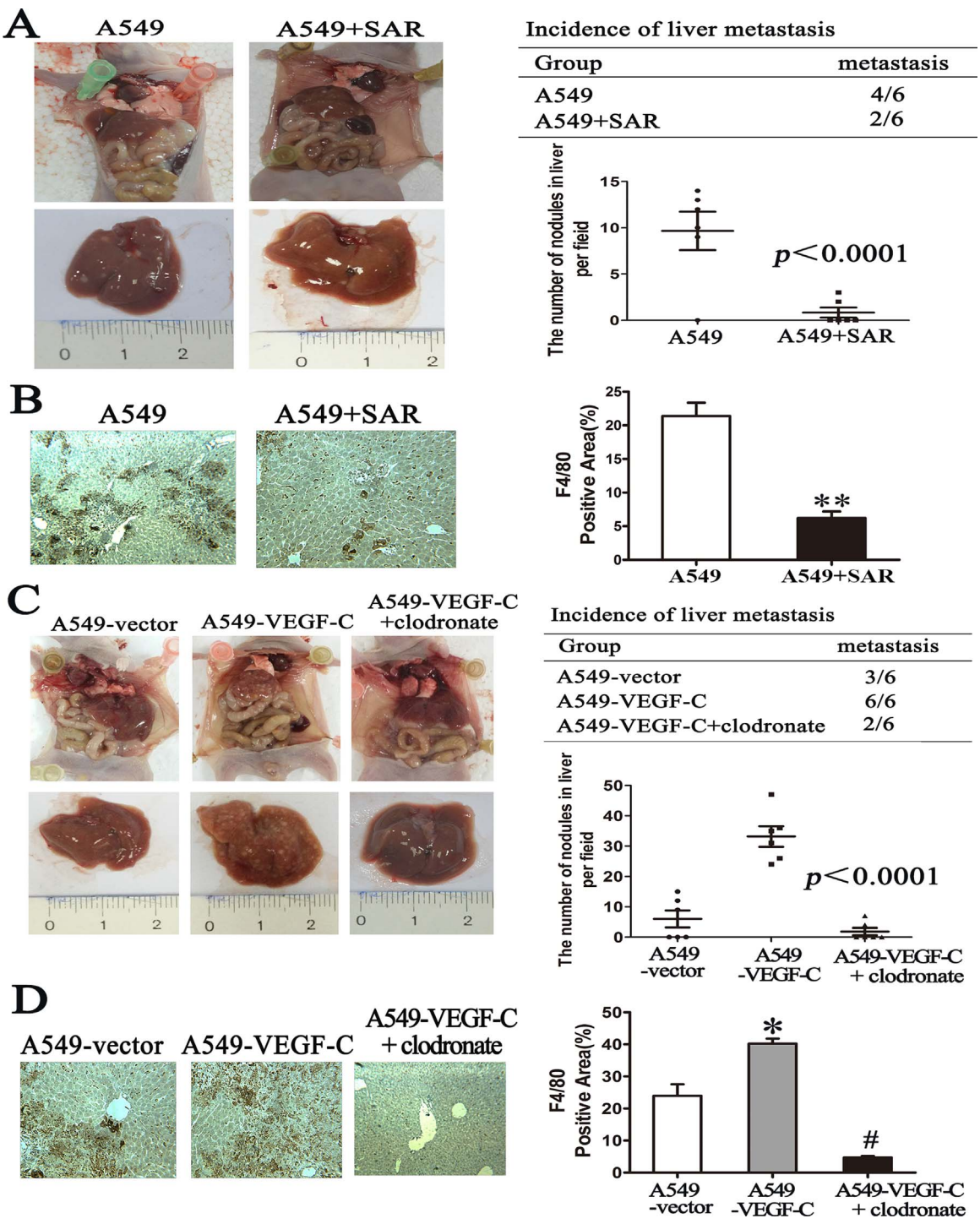

Fig. 5. Tumoral VEGF-C enhances macrophage infiltration and afterwards induces liver metastasis *in vivo*. (A) Photos of liver metastasis and scatter plots showing the incidence and number of visible liver metastases 4 weeks after tail-vein injection of A549 cells and treatment with SAR131675 (100 mg/Kg/day) (n = 6 per group). $p < .0001$. (B) The liver tissues were stained with antibody to F4/80, respectively. Magnification, 20 × . The positive staining of F4/80 expression per field from paraffin-embedded sections of A549 cells or those treated with SAR131675 was determined by immunohistochemistry and morphometric quantification. * *Significantly different from control group, $p < 0.001$. (C) VEGF-C-overexpressing A549 cells were injected into the tail vein of nude mice, and the nude mice were treated with clodronate before injection of VEGF-C-overexpressing A549 cells. Photos of liver metastasis and scatter plots showing the incidence and number of visible liver metastases 4 weeks after injection were established (n = 6 per group). $p < .0001$. (D) The liver tissues were stained with antibody to F4/80, respectively. Magnification, 20 × . The positive staining of F4/80 expression per field from paraffin-embedded sections of A549-VEGF-C cells or those treated with clodronate was determined by immunohistochemistry and morphometric quantification. *Significantly different from control group, $p < 0.005$. # Significantly different from the VEGF-C group, $p < 0.005$.

overexpressing A549 cells into the tail vein of nude mice. As Fig. 5C showed, compared with the vector control group, VEGF-C overexpression could obviously increase the metastatic incidence and the number of visible surface metastases on the mice liver, accompanied by enhanced infiltration of F4/80-positive macrophages in the liver tissues (Fig. 5D). Finally, clodronate encapsulated in liposome - nanoparticles (5 mg/mL) was used to deplete macrophages in mice before tail-injection of cancer cells. We found that clodronate treatment could efficiently reverse the enhancing effect of VEGF-C overexpression on cell metastasis in vivo (Fig. 5C), and concomitantly eliminated

macrophage infiltration in the liver tissues to the level rarely detected (Fig. 5D).

Taken together, these results intensely suggest that NSCLC cells-derived VEGF-C can promote macrophage recruitment, and resultantly leads to tumor metastasis.

4. Discussion

Production and secretion of VEGFs is commonly found in most aggressive cancer, and profoundly influences cancer progression and the prognosis of cancer patients [22]. VEGFs have an important role in the growth and metastasis of neoplasms because of its angiogenic and lymphangiogenic properties [23]. VEGF-C is the relatively newly defined VEGFs family member, and also characterised as an important lymphangiogenic and angiogenic growth factor involved in cancer metastasis [24]. Recently, growing evidences have shown that VEGF-C affects cancer progression and chemotherapy resistance by direct effects on tumor cells themselves [7,25–27]. In the present study, we found that VEGF-C was upregulated in various cancer cells and worked paracrinely to induce macrophage infiltration, and thus promoted NSCLC cell metastasis in vivo. Therefore, our study reveals a novel function of VEGF-C that affects cancer progression via direct action on macrophages.

A previous study showed that VEGF-C is commonly expressed in NSCLC cells and stromal macrophages, and its expression is frequently upregulated in tumor cells. More importantly, tumor showing high VEGF-C expression in tumor cells exhibits poor prognosis, while the status of VEGF-C in stromal macrophages is not correlated with the prognosis of NSCLC patients [8]. However, the potential mechanisms for these differences remain unclear. An interesting study indicates that in contrast to VEGF-A, VEGF-C does not exert direct influence on the growth of primary tumors, but instead induces lymph node lymphangiogenesis in the promotion of cancer metastasis [28]. Therefore, our data combined with the above report strongly support that tumoral VEGF-C contributes to cancer progression mainly via action on tumor microenvironment (including stromal macrophages) while not cancer cells themselves, and thus may explain, at least partly, the reason that stromal macrophages-derived VEGF-C had little effect on cancer progression.

Macrophages constitute a major component in cancer microenvironment. High density of CD68-positive tumor-associated macrophages in the tumor stroma is associated with poor overall survival of NSCLC patients, and in fact, a subgroup of the peritumoral M2 macrophages have undesirable effects on prognosis of NSCLC [18,29,30]. However, in this study, our results showed that VEGF-C had no effects on macrophage M2 polarization, thus excluded the possibility that tumoral VEGF-C exerted direct action on M2 macrophage-induced cancer metastasis. In other words, our results strongly suggest that tumoral VEGF-C induces macrophage infiltration and subsequently facilitates other factors to elicit macrophage M2 polarization. Therefore, further work is needed to explore the major ‘cofactors’ that ultimately initiate and promote M2 macrophage-mediated NSCLC metastasis.

As mentioned above, VEGFR-3 is abundantly expressed on the surface of macrophages. In the present study, we found that either Flt-4/Fc or the SAR131675 (a specific VEGFR-3 inhibitor) could efficiently reverse the enhancement effect of VEGF-C on macrophage migration. VEGF-C is also known as the ligand of VEGFR2. This study also found that VEGFR2 blockade by SKLB1002 could substantially reduce VEGF-C–induced macrophage migration. What are the mediators for VEGF-C/VEGFR-2/3 axis-mediated macrophage migration? Src kinase-dependent p38 activation has been confirmed to be an important couse for cell migration [28], and Su et al. showed that VEGF-C/VEGFR-3 axis promoted cancer cell migration via stimulating Src/p38 pathway [7]. Therefore, we inferred that whether Src/p38 signaling is also involved in tumoral VEGF-C-mediated macrophage migration. We found that A549-CM activated the Src/p38 signaling in macrophages, which could be significantly reversed by addition of Flt-4/Fc, SAR131675 or SKLB1002. Furthermore, this study showed that recombinant VEGF-C also significantly stimulated the Src/p38 signaling in macrophages, and inhibition of Src/p38 signaling could obviously reverse the enhancement effect of VEGF-C on macrophage migration. Taken together, these findings support that tumoral VEGF-C promotes macrophage migration probably via VEGFR-2/3/Src/p38 signaling axis in macrophages.

Our work has some limitations. We did not investigate the downstream target genes of Src/p38 signaling responsible for tumoral VEGF-C-mediated macrophage migration. Furthermore, many clinical data are needed to further confirm our findings.

Notwithstanding these limitations, our study does demonstrate that tumor-derived VEGF-C can induce macrophage infiltration via action on its receptors VEGFR-2/3 in macrophages, and resultantly promote NSCLC cells metastasis in vivo. Therefore, VEGF-C/VEGFR-2/3 axis may be a promising microenvironmental target against progression of NSCLC.

Acknowledgements

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Conflict of interest statements

None.

Statement of author contributions

DCY, YY, YB, ML, WQP and YZC carried out experiments. DCY, YY and YB analysed data. LB and ZLYconceived experiments and wrote the paper. All authors had final approval of the submitted and published versions.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.yexcr.2018.02.005.

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