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RESEARCH ARTICLE

MAPK/ERK1/2 signaling mediates endothelial-like differentiation of immature DCs in the microenvironment of esophageal squamous cell carcinoma

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Abstract Endothelial-like differentiation of dendritic cells (DCs) is a new phenomenon, and the mechanism is still elusive. Here, we show that the tumor microenvironment derived from the human esophageal squamous cell carcinoma (ESCC) cell line EC9706 can induce immature DCs (iDCs) differentiate toward endothelial cells, and become endothelial-like cells, but it has no obvious influence on mature DCs. During the course of endothelial-like differentiation of iDCs, a sustained activation of mitogen-activated protein kinase/extracelluar signal-regulated kinase1/2 (MAPK/ERK1/2) and cAMP response element-binding protein (CREB) was detected. Incubation of iDCs with MEK phosphorylation inhibitor PD98059 blocked the MAPK/ERK1/2 and CREB phosphorylation as well as the endothelial-like differentiation of iDCs. Inhibition of vascular endothelial growth factor-A (VEGF-A) in the microenvironment with its antibody blocked the endothelial-like differentiation and the phosphorylation of MAPK/ERK1/2 and CREB. These data suggest that MAPK/ERK1/2 signaling pathway activated by VEGF-A could mediate endothelial-like differentiation of iDCs in the ESCC microenvironment.

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Introduction

Dendritic cells (DCs) are antigen-presenting cells that play a key role in the initiation and regulation of immune responses. DCs not only provide antigenic peptides to initiate primary T cell responses but they also produce potent costimulatory molecules that drive quiescent T cells into the cell cycle and promote their differentiation [1, 2]. Many studies have shown that both the surface markers and presenting function of tumor-infiltrating DCs decreased significantly [3, 4]. However, little is known about the differentiation of DCs and the relationship between DCs and endothelial cells (ECs) in the presence of the tumor microenvironment.

DC precursors can be recruited by β-defensins and trans-differentiated into endothelial-like cells (ELCs) by vascular endothelial growth factor-A (VEGF-A) [5]. Furthermore, tumor-associated DCs incubated with pro-angiogenic factors, such as VEGF and oncostatin M, could trans-differentiate into ELCs, which may be an alternative pathway of tumor angiogenesis [6].

Many growth factors/cytokines and vasoactive substances can induce cell differentiation through different cellular signal transduction. Mitogen-activated protein kinase/extracelluar signal-regulated kinase1/2 (MAPK/ERK1/2) pathway has been shown to be an important signaling pathway that leads to cell differentiation by various of extracellular signals. ERK1/2 signaling is required for neural

specification in mouse embryonic stem cells and in the chick embryo [7]. Bone morphogenetic protein 4 induces neuronal differentiation of neuronal stem cells through Ras-ERK pathway [8]. ERK1/2 signaling pathway plays a key role in the process of mouse multipotent adult progenitor cell (MAPC) differentiation into EC in vitro [9]. One of the downstream signal molecules of ERK1/2 is the cAMP response element-binding protein (CREB). The phosphorylation of CREB mediated by ERK signaling responds to a variety of external signals to regulate cell differentiation and neurite outgrowth [10–12]. So, it is worthwhile to research further whether the MAPK/ERK1/2 signaling and CREB are involved in the endothelial-like differentiation of DCs.

Esophageal cancer, one of the most frequently diagnosed cancers in Asia, has a high incidence and mortality rate. Esophageal squamous cell carcinoma (ESCC) is the main type of esophageal cancer, which has strong invasiveness and a poor prognosis. In the present study, we examined the possibility of the endothelial-like differentiation of immature DCs (iDCs) and mature DCs (mDCs) in the microenvironment derived from ESCC cell line EC9706 culture supernatant, and investigated the role of p44/42 MAPK/ERK1/2 signaling and CREB in endothelial-like differentiation from iDCs. We show that EC9706 cell supernatant induces iDCs to differentiate from the DC pathway toward ECs, and form ELCs, but it has no obvious influence on mDCs. In addition, MAPK/ERK1/2 signaling activated by VEGF-A mediates endothelial-like differentiation of iDCs in the ESCC microenvironment, and CREB involves in this process.

Materials and methods

Preparation of the EC9706 cell culture supernatant [13]

EC9706 cells were cultured in RPMI 1640 medium with 10% fetal calf serum. The cells were replenished with fresh medium after reaching 60–80% confluence. The supernatant was collected and filtered after 24 h of incubation.

Isolation and culture of positive-control primary human umbilical vein endothelial cells

The aseptic cords were contributed by healthy parturient donors from the Third Affiliated Hospital, Zhengzhou University School of Medicine. An umbilical vein was digested with 2.5 g/l trypsin at 37°C for 10–15 min. The digested solution was collected and centrifuged (1,200 rpm, 8 min). The cells were suspended with endothelial cell medium (ECM) (ScienCell) and cultured on a 0.1% gelatin-coated 25-cm^2 flask in an atmosphere containing 5% CO_2 at 37°C. When the cells reached 80–90% confluence, von Willebrand factor (vWF) (Santa Cruz) and CD144 (Cell Signaling) were detected by immunocytochemistry, RT-PCR, and Western blot. DiI-Ac-LDL uptake was assayed to identify the cells.

Induction of ELCs from iDCs and mDCs

Briefly, peripheral blood mononuclear cells (PBMCs) were harvested from healthy adult volunteers by density-gradient centrifugation over Ficoll [6] and seeded in 24-well plates at 2×10^9/l for 3 h. The adherent cells (monocytes) were induced toward DCs with rhGM-CSF 100 μg/l (Amoytop), rhIL-4 5 μg/l (PeproTech), and 5 μg/l LPS (Sigma) on day 5. Then, 40% EC9706 supernatant was added at the end of days 2 and 7, respectively (divided into *iDCs-induced group* and *mDCs-induced group*), after 7 days of induction with EC9706 supernatant, and induced cells were harvested on day 9 (i.e., 2 + 7) and day 14 (i.e., 7 + 7), respectively, for study. Parallel culturing of control DCs (induction with rhGM-CSF 100 μg/l, rhIL-4 5 μg/l, and LPS 5 μg/l) were also harvested on days 9 and 14, respectively. VEGF-A in EC9706 cell supernatant was measured by human VEGF-A ELISA kit (Bender MedSystems). To detect the effect of VEGF-A blocked on the endothelial-like differentiation of iDCs, VEGF-A antibody (1 μg/ml) and isotype control IgG (Santa Cruz) were first admixed with the EC9706 supernatant.

Flow-cytometry analysis of DC surface markers

Monocytes, iDCs, and mDCs were harvested, respectively, on days 0, 2, 7. Cells of the iDCs-induced group, iDCs-induced group with PD98059, iDCs-induced group with VEGF-A Ab and its control DCs were harvested on day 9, and cells of mDCs-induced group and its control DCs were harvested on day 14. The cells were resuspended in PBS with a concentration of 1×10^6/ml. Monoclonal antibodies against human CD80, CD86, CD1a (FITC, BD), CD83, CD11c, and HLA-DR (PE, BD) (20 μl/10^6 cells) were incubated with the cells for 30 min in dark at 4°C. The cells were washed with PBS, resuspended in PBS and analyzed with flow-cytometer.

Immunofluorescence analysis

Induced cells, control DCs, and the human umbilical vein endothelial cells (HUVECs) were seeded in 96-well plates and incubated for 24 h, then fixed with 4% paraformaldehyde for 30 min. Rabbit anti-human vWF (Santa Cruz) was diluted 200 times as the primary antibody and FITC-conjugated goat anti-rabbit IgG was used as the secondary antibody. For detection, nuclear translocation of phospho-p44/42 MAPK/ERK Ab (Thr202/Tyr204; Cell Signaling) was diluted 100 times as the primary antibody and FITC-conjugated goat anti-rabbit IgG was used as the

secondary antibody, and the nuclear stain PI (Sigma) 200 μg/ml was used. The procedure was followed by the immunocyto-chemical protocol. Cells with green fluorescent particles in the cytoplasm were counted as having positive expression.

RNA isolation and RT-PCR

The total RNA of the PBMCs, the induced cells (iDCs-induced group, mDCs-induced group, control DCs) and the HUVECs was extracted with Trizol (Invitrogen), and converted to cDNA according to the protocol of the one-step RT-PCR kit (TaKaRa). The sequences for the oligonucleotide primer pairs are found in Table 1.

Western-blot analysis

Total protein concentration of each group cells was measured by Bradford method. Cell lysates of 50 μg were resolved to 10% SDS-PAGE gel and transferred to PVDF membrane. vWF Ab diluted 300 times, CD144 Ab (Cell Signaling) diluted 1,000 times, phospho-p44/42 MAPK/ERK Ab (Thr202/Tyr204; Cell Signaling) diluted 1,000 times, anti-p44/42 MAPK/ERK Ab (Thr202/Tyr204; Cell Signaling) diluted 1,000 times, phospho-CREB Ab (Cell Signaling) diluted 1,000 times, and CREB Ab (Cell Signaling) diluted 1,000 times were added, respectively, overnight at 4°C. HRP-IgG secondary antibody was incubated for 2 h at room temperature. The membranes were visualized with ECL. The Gel Doc Imaging System was used for detecting the gray value of the protein bands.

DNA-binding assay for CREB

Nuclear extracts were prepared according to the manufacturer's protocol of nucleoprotein extraction kit (Beyotime). The single-stranded oligonucleotide CRE probes for the CRE binding site (5′-AGTTGAGGGGACTTTCCCAG GC-3′), mutational CRE probe (5′-TGTCGAATGCAA ATCACTAGAA-3′) were synthesized (Sangon). To label DNA probes, the Biotin 3′ End DNA Labeling Kit (Beyotime) was used according to the manufacturer's protocol. The single-stranded oligonucleotide probes were annealed to be double-strand. Electrophoretic mobility shift assay (EMSA) was conducted using the LightShift Chemiluminescent EMSA Kit (Beyotime). For each sample, 20 μg of nuclear protein was used. The DNA-protein complexes were separated from the unbound DNA probes by electrophoresis on 6% polyacrylamide gels. A transferal of the protein from the polyacrylamide gel onto the nylon membrane was made using the semi-dry transfer method. The membranes were visualized with ECL.

Detection of Weibel–Palade bodies by transmission electron microscopy

The cells of the iDCs-induced group, the control DCs, and the HUVECs were digested by 0.25% trypsin and centrifuged with 2,000 rpm for 10 min, and pro-fixed with 2.5% glutaraldehyde overnight. The samples were post-fixed by 1% osmic acid, dehydrated with alcohol gradient, dehydrated with 100% acetone, embedded in epoxy resin 812, 815, sliced by Leica ultramicrotomy, and double electron stained with uranyl acetate and citric acid. The photographs were taken by transmission electron microscopy (TEM) (Hitachi H-7500).

In vitro angiogenesis assay

The cells were digested and washed twice and resuspended with PBS. The cells were seeded in the 96-well plates (coated with fibronectin for 45 min) with 1×10^5 cells/well, in quadruplicate, and were cultured with ECM at 37°C with 5% CO_2. The cells were observed with inverted microscope.

DiI-Ac-LDL and India ink uptake assay [6, 14]

The cells were seeded in 96-well plates and incubated for 36 h, then, DiI-Ac-LDL (10 μg/ml) (Biomedical Technologies, Inc.) was added and the cells were further incubated at 37°C for 4 h. The medium containing DiI-Ac-LDL was removed, and washed three times with PBS, then India ink (10 μl/ml) was added into the medium and the cells were incubated at 37°C for another 4 h. The India ink medium was then removed and the cells were washed three times with PBS. The cells were then observed with inverted fluorescence microscopy.

Table 1 Primer sequences of related genes for RT-PCR analysis

Genes	Accession no.	Forward primer	Reverse primer
CD1a	M28825	GCTGCACTCTGGAAAGGTCT	CCAAAGCGCAAGACCTAT CA
vWF	M25865	CACCGTTTGCCCACCCTTCG	GCCCACTGGGAGCCGACACT
CD144	X79981	GATGCAGAGGCTCATGATGC	TTGGTACATGACAGAGGCGT
GAPDH	M33197	AAGGTCGGAGTCAACGGATTTG	CTTGACAAAGTGGTCGTTGAGG

Statistical analysis

Data were expressed as mean ± standard deviation (SD) with at least three separate experiments and analyzed by one-way ANOVA and q-test. Significance was defined as $p < 0.05$.

Results

The changes of morphology and associated antigen of endothelial-like differentiation of iDCs and mDCs in the ESCC microenvironment

The control DCs extended obvious protrusions on day 4, but in the iDCs-induced group, few cell protrusions were extended. On day 9, the control DCs gradually became larger, round, and had burr-like protuberances, and some of them were suspended (Fig. 1Ad). However, the cells of the iDCs-induced group were slender and tended to form tube-like structures (Fig. 1Ab), similar to the appearance of HUVECs (Fig. 1Aa). Some cells were arrayed into cord-like structures (Fig. 1Ac), a typical appearance of ECs. Any differences in morphology between the mDCs-induced group (Fig. 1Ae) and the control DCs (Fig. 1Af) were not obvious.

To identify the effect of EC9706 supernatant on the expression of DC surface markers in induced cells, CD86, CD1a and CD11c were detected by flow-cytometry (FCM). The expression levels of CD86, CD1a and CD11c were decreased in iDCs-induced group cells (37.54 ± 9.64, 23.37 ± 10.71, 32.25 ± 11.36) compared with the control DCs (65.27 ± 3.10, 53.17 ± 9.16, 51.45 ± 7.27) ($n = 5$, $p < 0.05$), but the antigens expressed in mDCs-induced group (58.21 ± 1.38, 45.64 ± 2.65, 34.21 ± 2.81) and the control group (60.13 ± 1.98, 50.83 ± 2.03, 38.88 ± 3.28) ($n = 5$, $p > 0.05$) had no difference (Fig. 1Cb).

Next, we identified the effect of EC9706 supernatant on the expression of EC surface markers in induced cells by RT-PCR, Western blot and Immunofluorescence analysis. The expression of CD144 (582 bp) and vWF (434 bp) could not be detected and CD1a (601 bp) was very weak in these cells (Fig. 1Da). After the induction with the EC9706 supernatant for 7 days, the CD1a expression decreased and the CD144 and vWF expression increased in iDCs-induced group cells as compared with the control. Interestingly, the control DCs also expressed CD144 and vWF, just at low levels (Fig. 1Db). There was no CD144 or vWF expression in mDCs-induced group cells and its control DCs, and only CD1a could be detected (Fig. 1Dc). The expression of CD144 and vWF in HUVECs was strong (Fig. 1Dd). Western blot analysis demonstrated that the expression of CD144 (135KD) and vWF (210KD) was higher in the iDCs-induced group cells than its control DCs ($n = 3$, $p < 0.01$). In both mDCs-induced group cells and the control, CD144 and vWF cannot be detected (Fig. 1E). Immunofluorescence analysis also revealed the enhanced expression of vWF in iDCs-induced group cells (Fig. 1B).

The functional changes of the endothelial-like differentiation of iDCs and mDCs in the ESCC microenvironment

To demonstrate that the different stages of DCs further induced by EC9706-conditioned medium had the endothelial ability to form into tube-like structures, the cells of iDCs-induced group and mDCs-induced group were seeded on fibronectin-coated plates. Surprisingly, the results showed that the cells of iDCs-induced group appeared in fusiform shape and had the tendency of vortex distribution in the first 24 h (Fig. 2Aa), which was very similar to the positive control HUVECs (Fig. 2Ac). They tended to form tube-like structure (Fig. 2Ab), which was similar to the tube-like structure formed by HUVECs (Fig. 2Ad). However, the cells were distributed evenly in control DCs of iDCs-induced group (Fig. 2Ae, f), mDCs-induced group and its control DCs (Fig. 2Ag, h).

Although shared by other cells as macrophages and monocytes, the uptake of DiI-Ac-LDL is considered to be one of the typical functions of ECs [15, 16]. However, macrophages and monocytes not only uptake DiI-Ac-LDL but also uptake of India ink [14]. In the present study, the results showed that PBMCs had weak uptake of DiI-Ac-LDL, and strong uptake of India ink (Fig. 2B). The cells of the iDCs and mDCs-induced group did not uptake the India ink, which excluded the possibility that monocytes and macrophages were mixed in with the induced cells [14]. The increased uptake of DiI-Ac-LDL in cells of iDCs-induced group showed that the iDCs tended to differentiate toward ECs. However, the cells of the mDCs-induced group still had weak uptake of DiI-Ac-LDL (Fig. 2C).

In addition, EC-specific functional structure of Weibel–Palade (WP) bodies were detected by TEM. The results showed that WP body and plenty of mitochondria were present in the cells of the iDCs-induced group (Fig. 2D). No WP body was found in the control DCs.

Thus, the above results showed that iDCs differentiated into ELCs in the ESCC microenvironment, both phenotypically and functionally, but this microenvironment had no obvious influence on mDCs.

MAPK/ERK1/2 signaling and CREB was activated during endothelial-like differentiation of iDCs in the ESCC microenvironment

To determine the function of MAPK/ERK1/2 signaling and CREB in the process of endothelial-like differentiation of

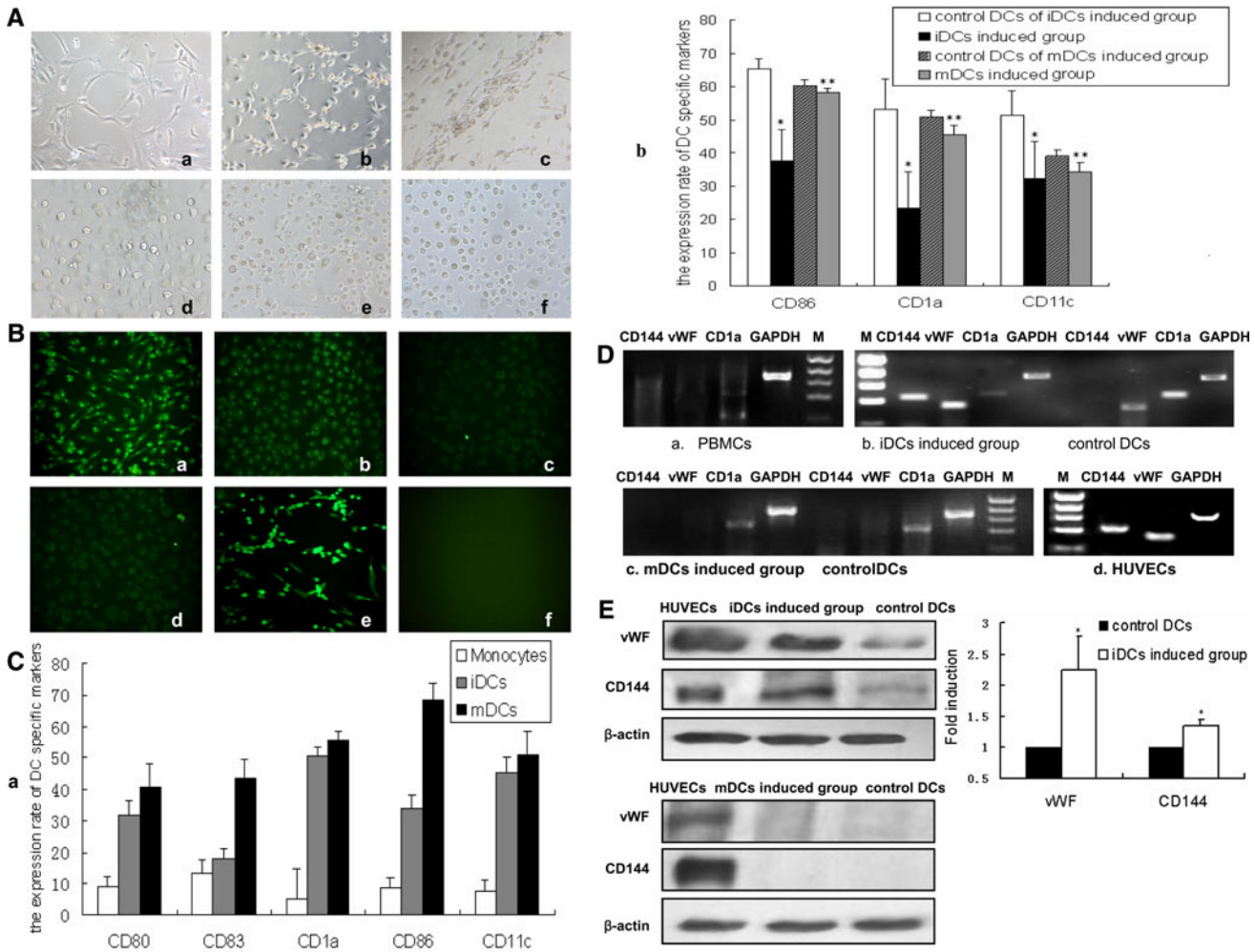
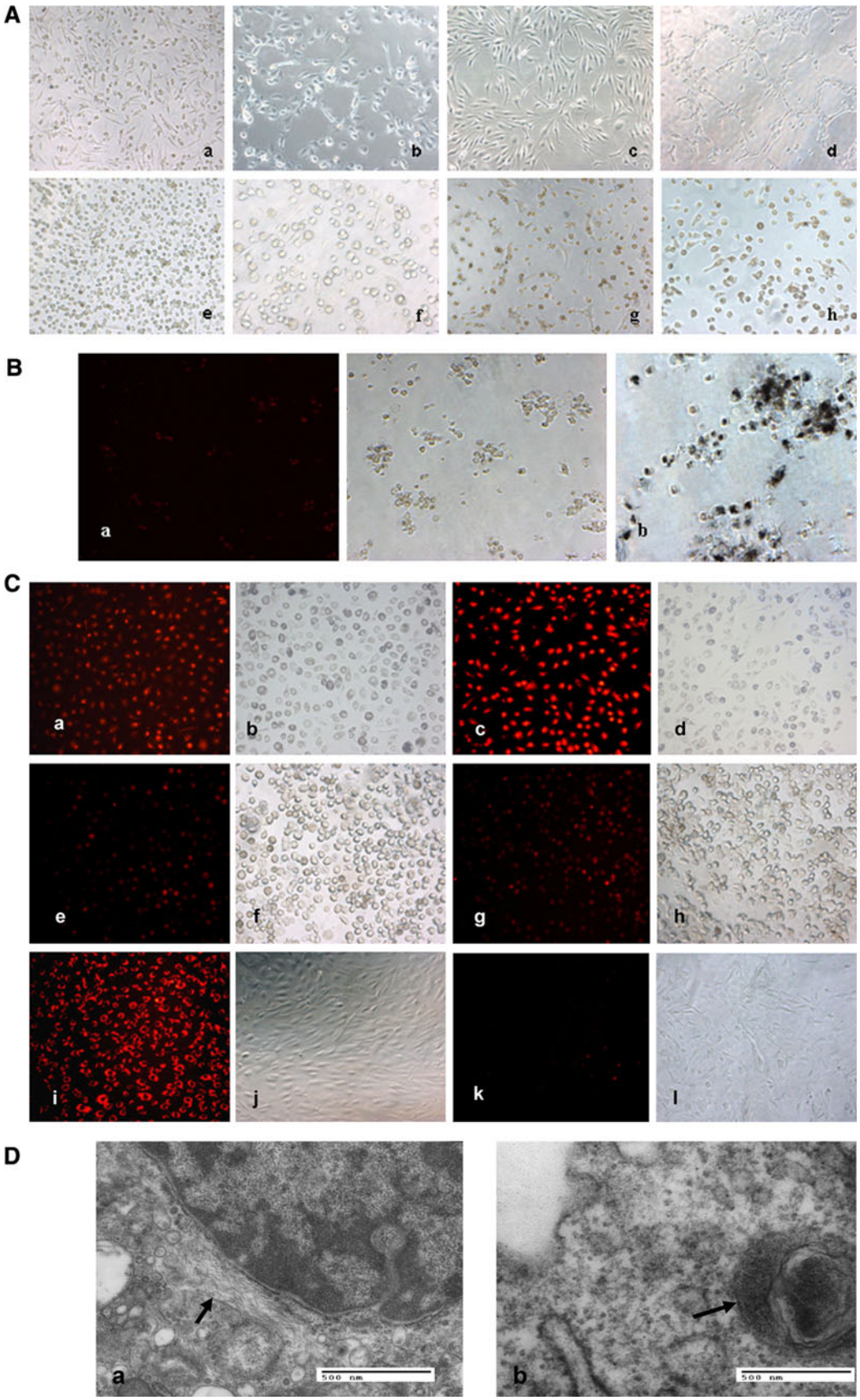


Fig. 1 Cell morphology and associated antigen expression of the endothelial-like differentiation of iDCs and mDCs. **A** Morphology change of the induced cells. Positive control HUVECs (*a*). PBMCs were incubated in rhGM-CSF/rhIL-4, and the 40% EC9706 supernatant was added at the end of day 2 (cells of the iDCs-induced group). The cells of the iDCs-induced group presented tendency of tube-like structures (*b*) and strip-shaped and funicular structures (*c*) after adding EC9706 supernatant for 7 days. The control DCs of iDCs-induced group were parallel cultured with rhGM-CSF/rhIL-4 on day 9 (*d*). The cells of mDCs-induced group (*e*) and its control DCs (*f*) on day 14. Magnification ×200. **B** The expression of the EC marker vWF in induced cells by immunofluorescence. vWF expression in the cytoplasm of iDCs-induced group (*a*), control DCs of iDCs-induced group (*b*), mDCs-induced group (*c*), control DCs of mDCs-induced group (*d*), the positive control HUVECs (*e*), and the negative-control group (PBS) (*f*). Magnification ×200. **C** FCM analysis of the expression rates of DC-specific markers in monocytes, iDCs, and mDCs (*a*) and in induced cells (*b*). Results shown are mean ± SD ($n = 3$) (*a*). Results shown are mean ± SD ($n = 5$) (*b*), * $p < 0.05$ vs. control DCs of iDCs-induced group. ** $p > 0.05$ vs. control DCs of mDCs-induced group (*b*). **D** RT-PCR detection the CD144, vWF, and CD1a expression in PBMCs, iDCs-induced group, mDCs-induced group, control DCs, and HUVECs. *M* marker: 1,500, 1,000, 750, 500, and 250 bp from *top* to *bottom*. **E** Western blotting detection the CD144 and vWF in the induced cells. Results shown are mean ± SD ($n = 3$). * $p < 0.01$ vs. control DCs

iDCs, we examined if MAPK/ERK1/2 signaling and CREB was activated by the EC9706 supernatant. Results showed that the phosphorylation of MAPK/ERK1/2 increased in a time-dependent manner in the differentiating iDCs (Fig. 3A). EC9706 supernatant stimulates a strong phosphorylation of p44/42 MAPK/ERK1/2 after 15 min of incubation, and the activation sustained during the observation period of 60 min in iDCs. PD98059 is a selective inhibitor of MEK1/2, the upstream regulator of phosphorylation of MAPK/ERK1/2. Therefore, we used PD98059 50 μM to block MEK1/2 to determine the phosphorylation of MAPK/ERK and its downstream CREB. Results showed that phosphorylation of MAPK/ERK1/2 and CREB was obviously inhibited in the presence of PD98059 ($n = 3$, $p < 0.05$) (Fig. 3A). To further demonstrate the involvement of CREB in this process, we showed CREB DNA-binding activity by EMSA. Increased CRE DNA-binding activity was detected in the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◀ **Fig. 2** Analysis of the functional change of the endothelial-like differentiation of iDCs and mDCs. **A** The formation of tube-like structures on fibronectin-coated plates. The cells of iDCs-induced group appeared with fusiform shapes and a vortex distribution (*a*). The cells of iDCs-induced group had a tendency to form tube-like structures (*b*). The HUVECs appeared with fusiform shapes, a vortex distribution (*c*) and tube-like structures (*d*). The control DCs of iDCs-induced group (*e*, *f*), the cells of mDCs-induced group (*g*) and its control DCs (*h*) appeared round and evenly distributed. Magnifications for *a*, *c* and *e* are ×100. Magnifications for *b*, *d*, *f*, *g* and *h* are ×200. **B** DiI-Ac-LDL and Indian ink uptake assay of the PBMCs. *a* DiI-Ac-LDL uptake assay of the PBMCs (*left panel*) is phase contrast photographs that is related to (*right panel*). Magnification ×200. *b* Indian ink uptake assay of the PBMCs. Magnification ×400. **C** DiI-Ac-LDL and Indian ink uptake assay of the induced cells. DiI-Ac-LDL uptake of the control DCs of iDCs-induced group (*a*), the iDCs-induced group (*c*), the control DCs of mDCs-induced group (*e*), the mDCs-induced group (*g*), the positive control HUVECs (*i*), the negative control 3T3 cells (*k*). *b*, *d*, *f*, *h*, *j* and *l* are phase contrast photographs that are related to *a*, *c*, *e*, *g*, *i* and *k*, respectively, which show no India ink uptake. Magnification ×200. **D** Acquisition of the endothelial feature, WP body, as seen by TEM. *a* Organized structure of the cells of iDCs-induced group. *Arrow* shows microfilaments. Magnification ×50,000. *b* The presence of WP body in the cells of iDCs-induced group. *Arrow* shows WP body. Magnification ×50,000

nucleus extract of iDCs-induced group by EMSA (Fig. 3B).

Inhibition of the activation of MAPK/ERK1/2 suppressed the endothelial-like differentiation of iDCs

After 7 day induction of EC9706 supernatant, the expression of phospho-p44/42 MAPK/ERK1/2 and CREB were still strong in the differentiating iDCs (Fig. 4A), and the total p44/42 MAPK/ERK1/2 remained unchanged, which suggests that phospho-p44/42 MAPK/ERK1/2 and CREB may be critical in the process of endothelial-like differentiation of iDCs. In order to verify that MAPK/ERK1/2 signaling pathway is essential for the differentiation, the effect of PD98059 10 μM on the endothelial-like differentiation of iDCs was investigated. Results showed that MAPK/ERK1/2 and CREB phosphorylation was obviously inhibited after a 7-day induction of EC9706 supernatant in the presence of PD98059 ($n = 3$, $p < 0.01$) (Fig. 4A). Inhibition of MAPK/ERK1/2 and CREB phosphorylation by PD98059 was accompanied by a significantly decreased expression of EC-specific antigen of CD144 and vWF in induced cells ($n = 3$, $p < 0.01$) (Fig. 4A, B). Furthermore, DiI-Ac-LDL uptake was reduced in the cells of the iDCs-induced group in the presence of PD98059 (Fig. 4C). Thus, inhibition of MAPK/ERK1/2 phosphorylation by PD98059 results in the inhibition of endothelial-like differentiation of iDCs, both morphologically and functionally.

The nuclear translocation of phosphorylation of MAPK/ERK1/2 is required to activate some transcription factors to lead to cell proliferation and differentiation [17, 18]. Before induction of the EC9706 supernatant, the level of phospho-MAPK/ERK1/2 (green) in iDCs was low (Fig. 4Da). iDCs incubated with EC9706 supernatant for 7 days showed the presence of phospho-MAPK/ERK1/2 (green) in the nuclei (red) of differentiating iDCs (Fig. 4Db). In the presence of PD98059 for 7 days, MAPK/ERK1/2 phosphorylation as well as nuclear translocation were inhibited (Fig. 4Dc). Thus, the phosphorylation and translocation of MAPK/ERK1/2 induced by EC9706 supernatant is required for the endothelial-like differentiation of iDCs.

Blocking VEGF-A in EC9706 supernatant inhibited the activation of MAPK/ERK1/2 and CREB

VEGF is a multifunction cytokine that promotes EC differentiation, proliferation, and angiogenesis [19, 20]. In this study, to determine the role of VEGF-A during the process of the endothelial-like differentiation of iDCs, first of all, we check the content of VEGF-A in the EC9706 supernatant by ELISA kit. Results showed that the content of VEGF-A in the EC9706 supernatant was 0.5827 ± 0.0627 ng/ml. Next, we used VEGF-A Ab (1 μg/ml) to block VEGF-A in the EC9706 supernatant to investigate its effect on MAPK/ERK1/2 signaling and CREB. Western blot showed that the level of phospho-ERK1/2 and CREB drastically decreased in the VEGF-A blocking group compared to the iDCs-induced group ($n = 3$, $p < 0.01$) (Fig. 5), which showed that VEGF-A played a starter role during the process of MAPK/ERK1/2 and CREB was activated by EC9706 supernatant.

Blocking VEGF-A in EC9706 supernatant results in the inhibition of the endothelial-like differentiation of iDCs

To determine the role of VEGF-A in the endothelial-like differentiation of iDCs, VEGF-A Ab (1 μg/ml) was used to investigate its effect on cell morphology, the expression of EC-specific marker vWF and CD144, and the function of DiI-Ac-LDL uptake. The cells of the iDCs-induced group showed slender spindle (Fig. 6Ab), which was similar to HUVECs (Fig. 6Ad). There was almost no slender branch in cells of blocking VEGF-A group (Fig. 6Ac), which was similar to control DCs (Fig. 6Aa). Immunofluorescence showed that vWF expression decreased in the cells of blocking VEGF-A group compared to the cells of the iDCs-induced group (Fig. 6B). Western blotting showed that compared to the cells of the iDCs-induced group, CD144 and vWF expression decreased in the cells of blocking VEGF-A group ($n = 3$, $p < 0.01$) (Fig. 6C). Uptake assay showed that the uptake of DiI-Ac-LDL

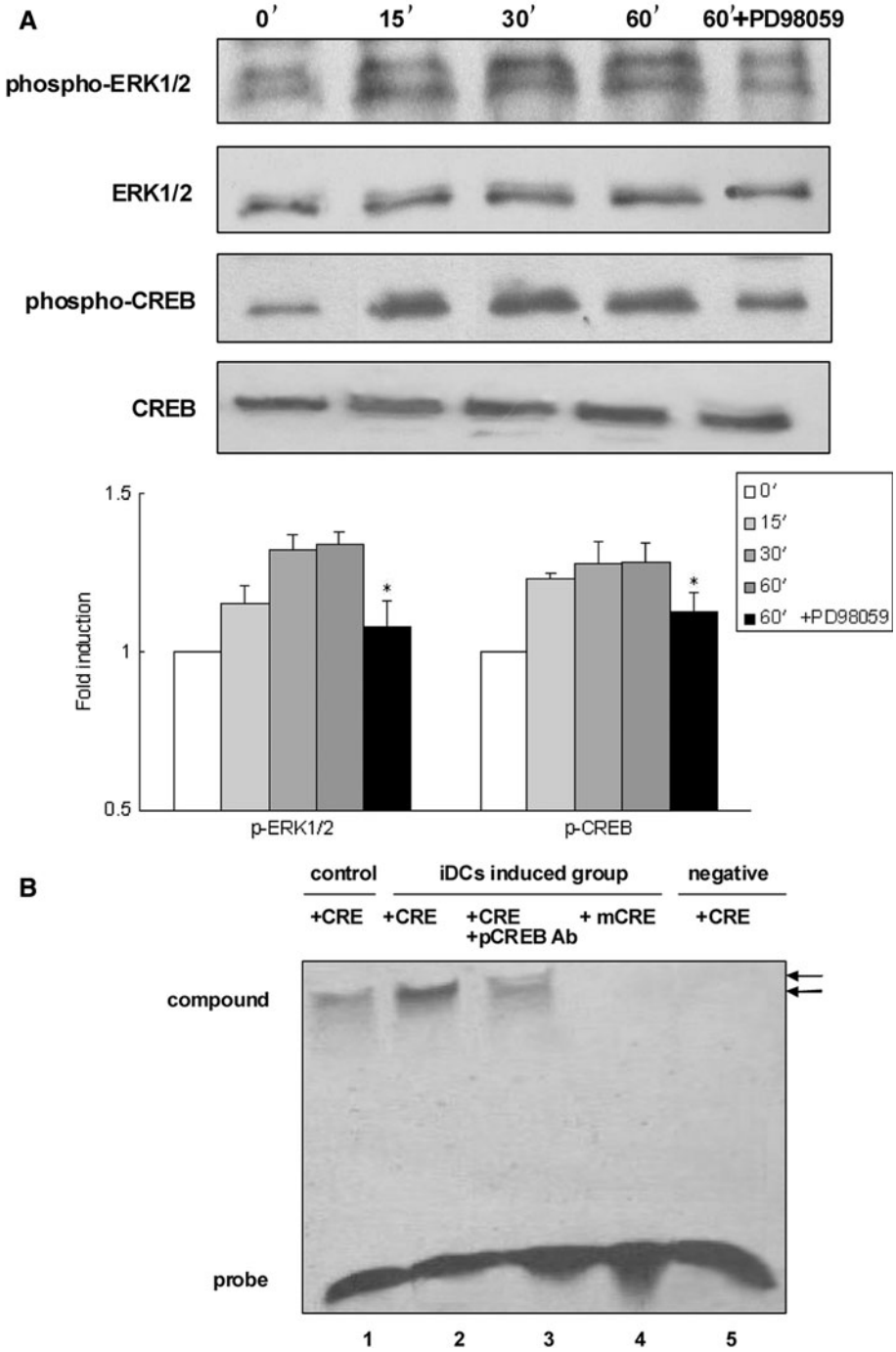


Fig. 3 The activation of MAPK/ERK1/2 and CREB by EC9706 supernatant and the inhibition of the activation of MAPK/ERK1/2 and CREB with PD98059 (**A**). ERK1/2 and CREB was activated after 15 min of incubation with EC9706 supernatant which sustained throughout the period of 60 min in iDCs, and the activation of ERK1/2 and CREB was inhibited by PD98059. Results are mean ± SD ($n = 3$).* $p < 0.05$ vs. 30′, 60′ group. Increased CRE DNA-binding activity was detected in the nucleus extract of iDCs-induced group by EMSA (**B**). *Lane 1* control containing CRE probes (before induced by EC9706 cell supernatant). *Lane 2* iDCs-induced group containing CRE probes. *Lane 3* iDCs-induced group containing CRE probes and p-CREB antibodies. *Lane 4* iDCs-induced group containing mutational CRE probes. *Lane 5* negative control containing only CRE probes without nuclear extract

decreased in the cells of the VEGF-A blocking group. There was no difference between the blocking VEGF-A group and control DCs (Fig. 6D).

The nature of the cells of differentiation-inhibited by PD98059 or the VEGF-A Ab

In this study, the differentiation-inhibited cells had the change of on-cell morphology and cell markers (Figs. 4, 6). The expression of vWF and CD144 also decreased, and the DiI-Ac-LDL uptake decreased in cells of the iDCs-induced group with PD98059 or with VEGF-A Ab. The cell morphology of differentiation-inhibited cells became similar to the control DCs. The expression of DC markers in differentiation-inhibited cells were checked by FCM (Fig. 7), and the result showed that the expression of DC markers decreased when differentiation was inhibited by PD98059, on the contrary, the expression of DC markers increased when differentiation was inhibited by VEGF-A Ab, which suggests it is a good way to

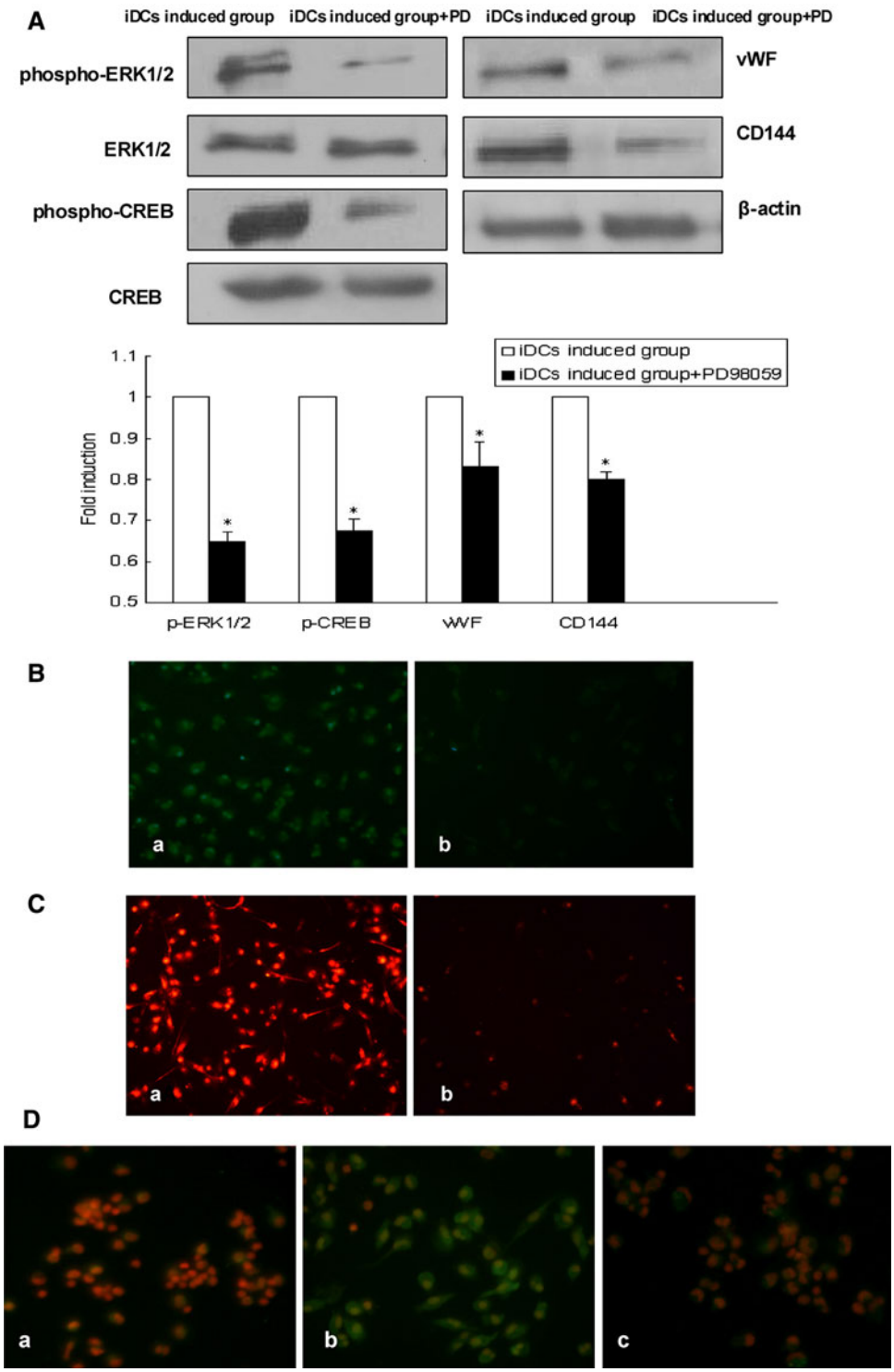


Fig. 4 PD98059 blocking the activation of MAPK/ERK1/2 and CREB, and inhibiting endothelial-like differentiation of iDCs. **A** 10-μM PD98059 obviously blocked the activation of ERK1/2 and CREB, and significantly decreased the expression of EC-specific antigen vWF and CD144 in the cells of the iDCs-induced group. Western blotting results shown are mean ± SD ($n = 3$). * $p < 0.01$ vs. iDCs-induced group. **B** 10-μM PD98059 decreasing the expression of vWF in cells of iDCs-induced group + PD (*b*) vs. cells of iDCs-induced group (*a*) as seen by immunofluorescence (200×). **C** 10-μM PD98059 decreasing the DiI-Ac-LDL uptake in cells of iDCs-induced group + PD (*b*) vs. cells of iDCs-induced group (*a*) (200×). **D** PD98059 inhibited nucleus translocation of phospho-ERK1/2 in iDCs treated with EC9706 supernatant for 7 days. Undifferentiated iDCs before induction by EC9706 supernatant (*a*). iDCs incubated with EC9706 supernatant showed the presence of phospho-ERK1/2 (*green*) in the nuclei (*red*) of endothelial-like differentiation of iDCs (*b*). Phospho-ERK1/2 as well as nuclear translocation were inhibited in the presence of PD98059 for 7 days (*c*) (200×)

enhance the ability of DC antigen presentation by blocking VEGF-A.

In brief, our results showed that VEGF-A in EC9706 supernatant played a starter role to active MAPK/ERK1/2 signaling pathway-mediated endothelial-like differentiation of iDCs in the ESCC microenvironment.

Discussion

It is widely accepted that DCs are terminally differentiated cells. However, recent studies have shown that stromal cell-derived transforming growth factor-beta can induce mDCs to differentiate into a new regulatory DC subset [21,

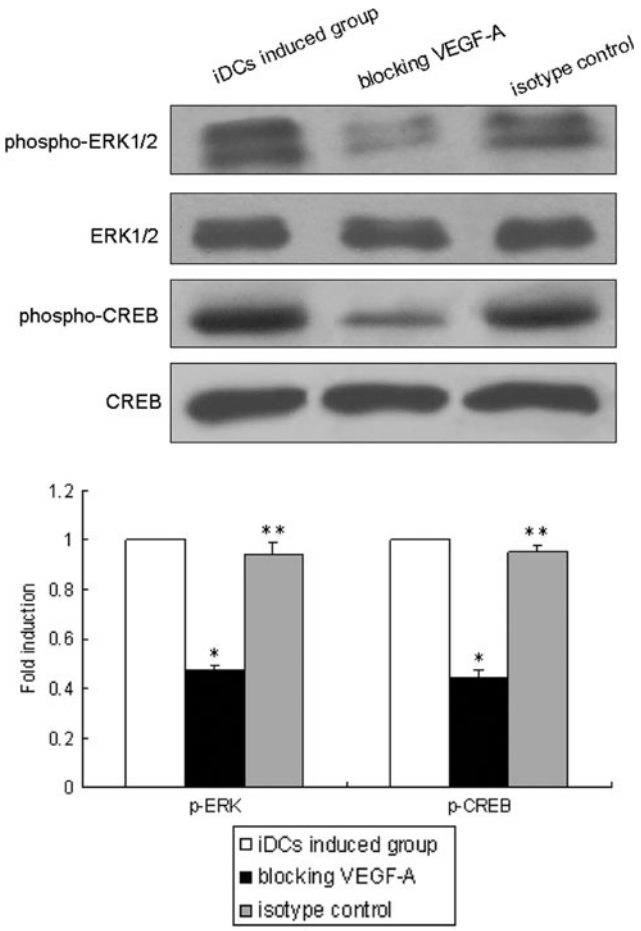


Fig. 5 Western-blot analysis of the expression of phospho-ERK1/2 and phospho-CREB in cells of blocking VEGF-A. The expression of p-ERK1/2, p-CREB was still high in differentiating iDCs after 7-day induction of EC9706 supernatant. The activation of ERK1/2 and CREB was inhibited through blocking the VEGF-A in EC9706 supernatant with VEGF-A Ab 1 μg/ml. Results are mean ± SD ($n = 3$). * $p < 0.01$ vs. the iDCs-induced group and isotype control; * $p > 0.05$ vs. the iDCs-induced group

22]. In this study, we investigated the effect of the microenvironment produced by the ESCC cell line EC9706 supernatant on the differentiation of different stages of DCs derived from PBMCs and found that iDCs differentiate from the DC pathway toward ECs in the microenvironment. However, this microenvironment has no obvious influence on mDCs. PBMCs behave as pluripotent stem cells which can be induced to acquire lymphocyte, endothelial, macrophage, neuronal, epithelial, and hepatocyte phenotypes in the absence of a fusion with preexisting mature tissue cells [23]. In this study, iDCs still keep some property of PBMCs, so it is possible to differentiate toward ECs in a specific microenvironment.

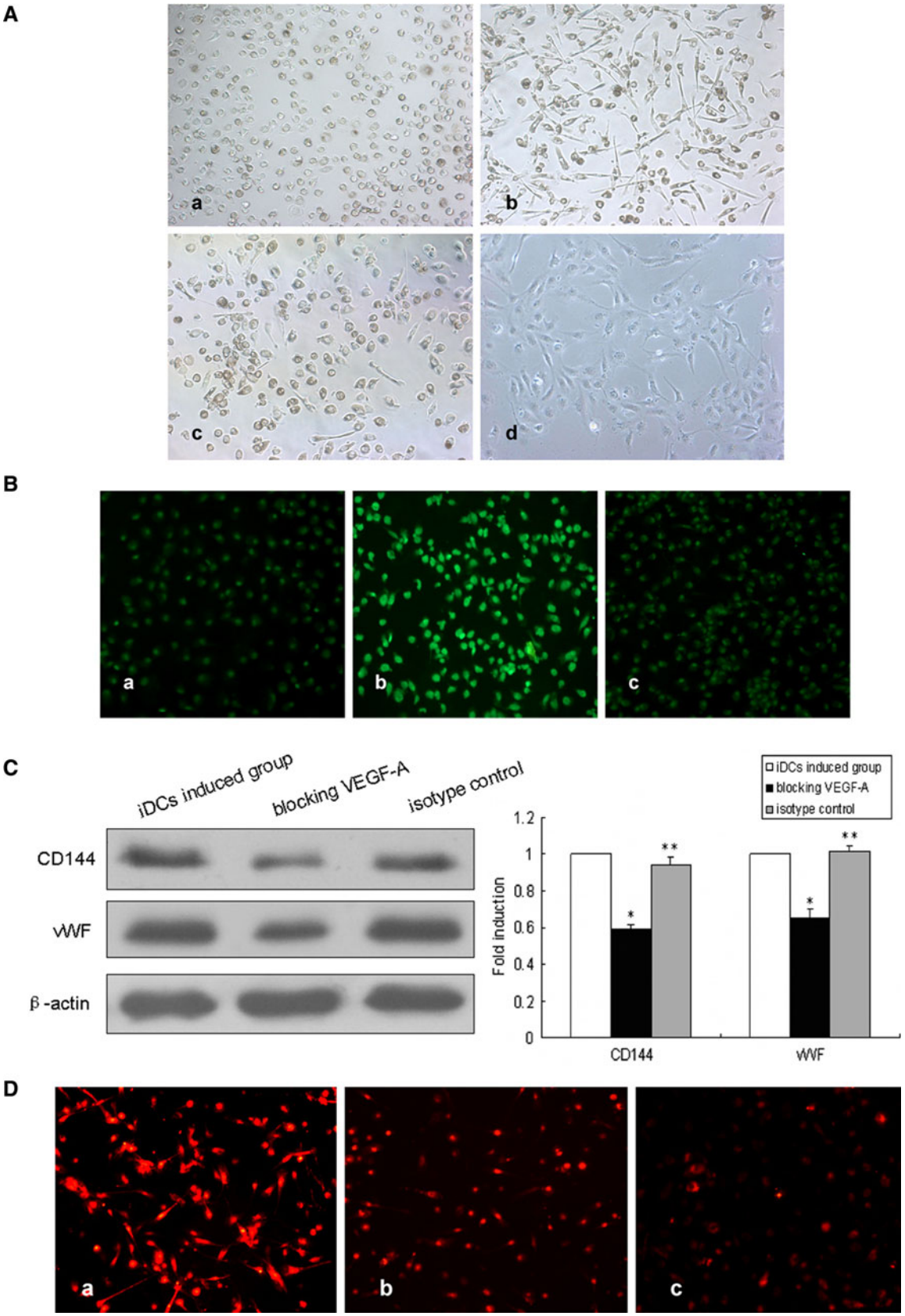
With the detection by RT-PCR and Western-blot analysis, we found that monocyte-derived DCs expressed the EC-specific markers vWF and CD144 at low levels. This

Fig. 6 Blocking VEGF-A in EC9706 supernatant results in the inhibition of the endothelial-like differentiation of iDCs. **A** Morphological changes after blocking VEGF-A in EC9706 cell supernatant by VEGF-A Ab. control DCs (*a*), iDCs-induced group (*b*), blocking VEGF-A group (*c*), HUVECs (*d*) (magnification ×200). **B** Immunofluorescence analysis of the decreasing expression of vWF after blocking VEGF-A in EC9706 cell supernatant, control DCs (*a*), iDCs-induced group (*b*), blocking VEGF-A group (*c*), (magnification ×200). **C** Western-blot analysis of the decreasing expression of CD144 and vWF after blocking VEGF-A. Results shown are mean ± SD ($n = 3$). * $p < 0.01$ vs. iDCs-induced group and isotype control; * $p > 0.05$ vs. iDCs-induced group. **D** Blocking VEGF-A decreasing the DiI-Ac-LDL uptake in cells of blocking VEGF-A (*b*) vs. cells of the iDCs-induced group (*a*). Control DCs (*c*). (magnification ×200) ▶

indicated that a phenotypic overlap exists between DCs and the microvascular endothelium. This finding was also confirmed by other researchers in mRNA level or by immunocytochemistry [6, 24]. In fact, DC and EC lineages are close relatives and exert a reciprocal effect on their differentiation [25–27]. Monocytes can differentiate into macrophages, DCs, or ECs in different microenvironments [28, 29], and ECs can also act as antigen-presenting cells [30].

In the presence of this tumor microenvironment, iDCs could up-regulate the expression of the EC markers vWF and CD144, while decreasing the DC markers CD1a, CD86, and CD11c. Additionally, WP bodies and tube-like structures formation, and DiI-Ac-LDL uptake was enhanced in these induced cells. All the above evidence has shown that iDCs have differentiated into ELCs. No expression of vWF, CD144 in PBMCs excluded the possibility of differentiation directly from PBMCs to ELCs. Additionally, no cell proliferation was observed during the entire culture period of the PBMCs. So, the expression of EC markers in tumor media-treated cells is a result of cell differentiation exclusive of the outgrowth of a contaminating subpopulation of endothelial or hematopoietic progenitor cells. However, in contrast, the results showed that this microenvironment had no obvious influence on mDCs. This perhaps brings forward evidence that these mDCs could be used relatively safely in an anti-ESCC vaccine.

The function of these ELCs was further verified by two generally accepted criteria. The first criterion was the uptake of DiI-Ac-LDL. Most induced cells exhibited uptake of DiI-Ac-LDL, but not India ink, distinguishing them from mononuclear cells and macrophages [14]. The other criterion is tube-like formation on fibronectin-coated plates. Interestingly, fusiform shapes and a tendency toward vortex distribution appeared, and tube-like structures formed, in these differentiated cells, which were similar to the characteristics of the positive-control HUVECs. In addition, electron microscopy was used to


A
a
b
c
d
B
a
b
c
C
iDCs induced group
blocking VEGF-A
isotype control
CD144
vWF
β-actin
iDCs induced group
blocking VEGF-A
isotype control
Fold induction
1.2
1
0.8
0.6
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0.2
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CD144
vWF
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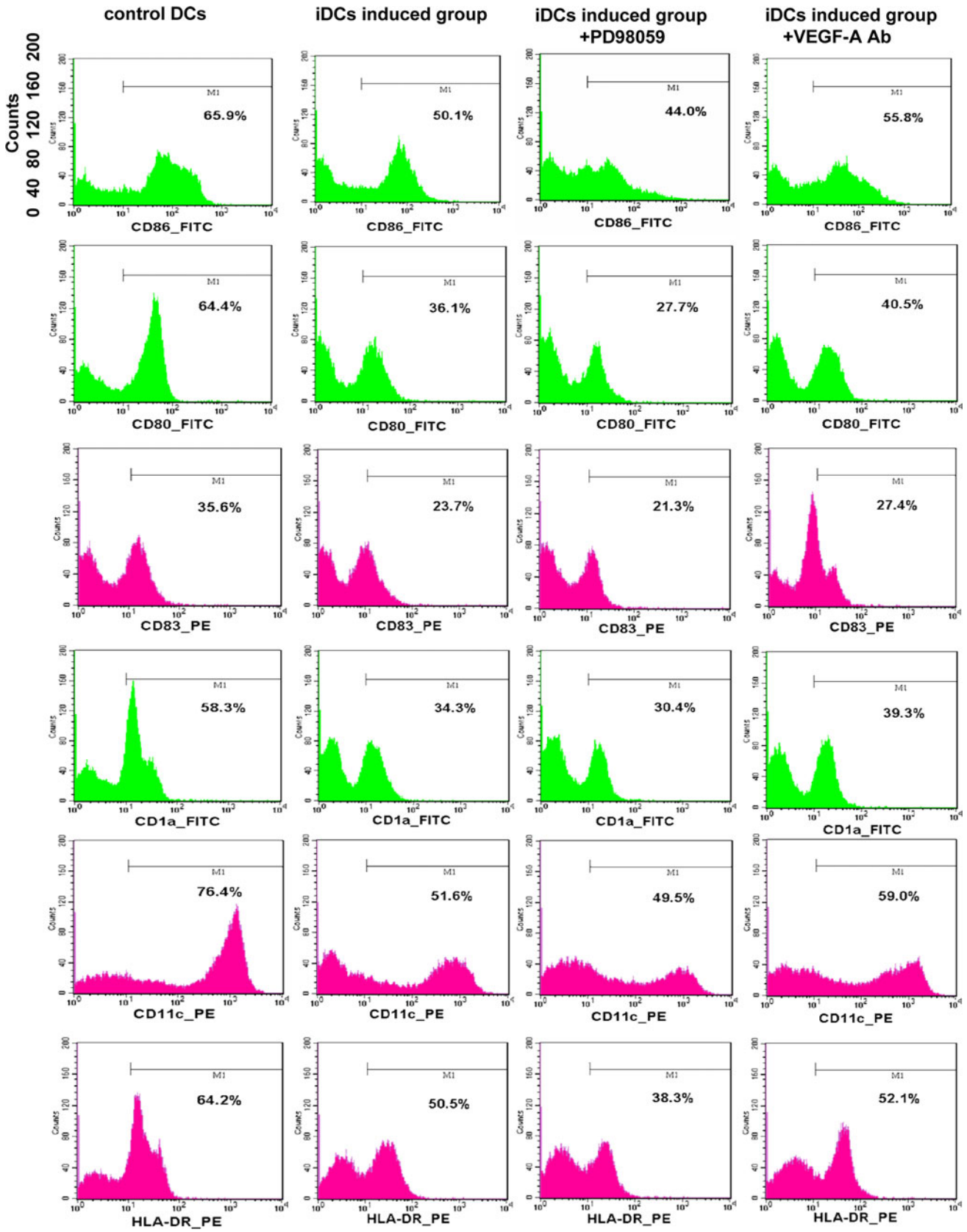
control DCs
iDCs induced group
iDCs induced group +PD98059
iDCs induced group +VEGF-A Ab
Counts
0 40 80 120 160 200
M1
65.9%
50.1%
44.0%
55.8%
CD86_FITC
64.4%
36.1%
27.7%
40.5%
CD80_FITC
35.6%
23.7%
21.3%
27.4%
CD83_PE
58.3%
34.3%
30.4%
39.3%
CD1a_FITC
76.4%
51.6%
49.5%
59.0%
CD11c_PE
64.2%
50.5%
38.3%
52.1%
HLA-DR_PE

◀ **Fig. 7** FCM analysis of the expression rate of DC-specific markers after blocking by PD98059 or VEGF-A. Cells of the iDCs-induced group, the iDCs-induced group with PD98059 (10 μM), the iDCs-induced group with VEGF-A Ab (1 μg/ml) and its control DCs were harvested on day 9. Monoclonal antibodies against human CD86_FITC, CD80_FITC, CD83_PE, CD1a_FITC, CD11c_PE and HLA-DR_PE were incubated with the cells

determine the nature of a cell. The cells of the iDCs-induced group exhibited WP bodies and plenty of microfilaments, which are typical morphological properties of ECs. Altogether, our data support the idea that the iDCs could transform into ELCs in the tumor microenvironment.

Endothelial-like differentiation of DCs is a new phenomenon, and related research is still limited. It is intriguing to hypothesize that DCs or DC precursors might turn into ELCs under the influence of VEGF-A [5, 31, 32]. Therefore, its mechanisms need further research. Many growth factors/cytokines and vasoactive substances can led to cell differentiation through cellular signal transduction [33]. The MAPK/ERK1/2 signaling pathway plays a key role in mediating signals from membrane receptors to the nucleus, and is involved in multiple physiological processes, including cell growth, cell differentiation, cell proliferation, and apoptosis [34, 35]. Some studies have shown that the MAPK/ERK pathway plays a central role in VEGF-induced bone marrow stem cells differentiation into ECs [9] and the activation of MAPK/ERK is required for the DC-like differentiation of U937 cells [36]. Basic fibroblast growth factor-induced neuronal differentiation of mouse bone marrow stromal cells also depend on MAPK/ERK pathway [33]. Whether MAPK/ERK is involved in the endothelial-like differentiation of iDCs needs to be clarified. In this study, we observed that a sustained activation of the ERK1/2 is required for endothelial-like differentiation of iDCs, which is consistent with the research reported that a sustained activity of ERK results in neuronal differentiation [7] and bone marrow stem cell differentiation [9].

Cell differentiation is a complex process including many molecules, some of them need to be transcribed and translated. Once activated, ERK1/2 can translocate into the nucleus to phosphorylate related transcription factors and regulate their activity. It has been well established that the nuclear translocation of activated MAPK/ERK is important for the differentiation of various cells [9, 37]. In this study, our results demonstrate that MAPK/ERK1/2 phosphorylation is required for endothelial-like differentiation of iDCs, and activated MAPK/ERK1/2 was present in the nucleus of cells incubated with EC9706 supernatant. PD98059, an inhibitor of MEK, not only inhibited the activation of ERK1/2 but also inhibited the nuclear translocation of phosphorylated ERK1/2.

CREB as a nuclear transcription factor is one of the downstream signal molecules of ERK1/2. The phosphorylation of CREB mediated by ERK signaling responds to a variety of external signals, which plays a key role in cell differentiation and neurite outgrowth [10–12]. It remains to be explored, however, whether CREB participates in the endothelial-like differentiation of iDCs. Here, we observed that the activation of ERK1/2 and CREB were triggered by the EC9706 supernatant. In addition, the necessity of ERK1/2 activation in the process of endothelial-like differentiation is supported by the experiment that the differentiation is impaired when the phosphorylation of ERK1/2 is inhibited by PD98059.

Various extracellular factors are involved in the process of cell development, among them, growth factors play a central role during the process of the differentiation of ECs. Recently, VEGF has been shown to be an important cytokine that induces a series of biological effects in ECs [38, 39]. VEGF is essential for the differentiation from stem cells toward hematopoietic cells [40]. VEGF signaling is required for endothelial differentiation during vasculogenesis [19]. Transduction with an adenoviral vector expressing the VEGF(165) gene led to significantly enhanced endothelial differentiation of human embryonic stem cells [41]. High expression of VEGF can be detected in a variety of human tumor tissues [42, 43]. However, little is known about the role of VEGF-A in the endothelial-like differentiation of iDCs. VEGF, as an important angiogenesis regulatory factor, can significantly stimulate tumor angiogenesis [44, 45]. Its characterized receptors are the tyrosine kinases, VEGF receptor 1 (VEGFR1), and VEGF receptor 2 (VEGFR2). VEGFR2 signaling activates a variety of downstream mediators in EC, including Src and Ras/Raf/MEK/ERK pathways [46]. Antibody and small-molecule kinase inhibitors of VEGFR2 have been shown to inhibit tumor angiogenesis [47]. SU5416, a VEGF receptor tyrosine kinase inhibitor, partially rescued the mDC phenotype in the presence of VEGF, implying the involvement of both tyrosine kinase-dependent and -independent inhibitory mechanisms [48]. The effects of VEGFR-2 blockade were examined in mice in vivo and the result showed that SU5416 abolished the vascular migration of CD11c + cells, confirming the importance of tyrosine kinase signaling in DC endothelialization [5]. In this study, we observed that blocking VEGF-A in EC9706 supernatant by VEGF-A antibody inhibited ERK1/2 and CREB phosphorylation and the endothelial-like differentiation of iDCs, which shows that VEGF-A in EC9706 supernatant is a trigger for activating the MAPK/ERK1/2 signaling pathway, and by blocking VEGF-A, the expression of DC markers increased. Therefore, this study provides a new strategy for inhibiting the endothelial-like

differentiation of iDCs and enhancing the ability of DCs' antigen presentation.

Altogether, this study showed that iDCs could differentiate into ELCs in a specific microenvironment, and the MAPK/ERK1/2 signaling pathway activated by VEGF-A-mediated endothelial-like differentiation of iDCs in the ESCC microenvironment, which provided a molecular explanation for the endothelial-like differentiation of iDCs. It has traditionally been believed that tumor vascularization is ascribed to the sprouting of ECs from existing vessels, however recent studies indicate that the recruitment of endothelial progenitors that differentiate into ECs plays an important role in the formation of tumor neovessels [49–51]. DCs and their precursors might contribute to new vessel formation by two possible mechanisms: by stimulating angiogenesis from existing vessels through the release of pro-angiogenic factors [52, 53], or by contributing to vasculogenesis by trans-differentiation into ELCs. The redirection of differentiation from the DC pathway to ECs could restrict the potential anti-tumor reactivity of DCs, and thereby facilitate tumor growth and immune escape. However, to this point, research on the differentiation of DCs into ELCs is still very limited, and it is worthwhile to research this further.

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