JAK/STAT3 signaling pathway mediates endothelial-like differentiation of immature dendritic cells

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Abstract. Endothelial-like differentiation (ELD) of dendritic cells (DCs) is a poorly understood phenomenon. The present study evaluated the effect on the ELD of DCs by using human esophageal squamous cell carcinoma (ESCC) cells with high or poor differentiation. The results demonstrated that KYSE450 (highly differentiated) and KYSE70 (poorly differentiated) cell supernatants induce the differentiation of immature DCs (iDCs), derived from healthy adult volunteers, away from the DC pathway and towards an endothelial cell (EC) fate. This effect was strongest in the cells treated with the KYSE70 supernatant. During the ELD of iDCs, sustained activation of JAK (janus tyrosine kinase)/STAT3 (signal transducer and activator of transcription 3) signaling was detected. Incubation of iDCs with the JAK inhibitor, AG490 blocked JAK/STAT3 phosphorylation and iDC differentiation. These results suggested that the JAK/STAT3 signaling pathway mediates ELD of iDCs. Furthermore, the poorly differentiated ESCC cells may have a greater effect on the ELD of iDCs than highly differentiated ESCC cells.

Introduction

Dendritic cells (DCs) are antigen-presenting cells that are important in the initiation and regulation of immune responses (1-3). DCs present antigenic peptides to initiate primary T-cell responses, and additionally, DCs express

costimulatory molecules that drive quiescent T cells into the cell cycle, promoting their differentiation (3,4). Previous studies have demonstrated the expression levels of endothelial (CD31, vWF and CD144) and dendritic precursor (CD205) cell surface markers and the antigen-presenting ability of DCs decrease significantly following their infiltration of tumors (5-8). However, the mechanisms behind these observations remain to be elucidated.

It has been reported previously that conditioned medium from murine Lewis lung carcinoma cells redirects the differentiation of CD34^{+} progenitor cells away from a DC pathway to an endothelial cell (ECs) fate (9). In addition, DC precursors can transdifferentiate into endothelial-like cells (ELCs) in mouse and human ovarian carcinomas following the addition of vascular endothelial growth factor-A (VEGF-A) and β-defensins (10). Furthermore, tumor-associated DCs incubated with the pro-angiogenic factors VEGF-A and oncostain M can transdifferentiate into ELCs, and this is suggested as an alternative pathway of tumor angiogenesis (11). Additional reports have demonstrated that DC progenitors or immature DCs (iDCs) have the ability to transdifferentiate into ELCs, potentially contributing to vasculogenesis in adult tissues. Therefore, DCs may be crucial to the neovascularization process in a number of physiopathological conditions (12,13).

STAT3 (signal transducer and activator of transcription 3) is activated by JAK (janus tyrosine kinase)-mediated tyrosine phosphorylation following receptor-ligand binding. The JAK/STAT3 signaling pathway regulates cell growth, proliferation, differentiation and apoptosis, and is important in the signal transduction of cytokines and growth factors (14,15). However, the function of the JAK/STAT3 signaling pathway on endothelial-like differentiation (ELD) remains to be elucidated.

Esophageal cancer (EC) is the sixth leading cause of cancer-associated mortality and the eighth most frequently diagnosed cancer worldwide (16). China has one of the highest incidences of esophageal cancer, with an estimate of >220,000 new detected cases and 200,000 mortalities every year (17). The predominant form of esophageal cancer is esophageal squamous cell carcinoma (ESCC), characterized by a poor prognosis and high invasiveness (18). It has been reported previously that tumor-associated factors derived from homogenates of EC9706 human ESCC cells may induce iDCs to differentiate into ELCs (19). However, the impact of

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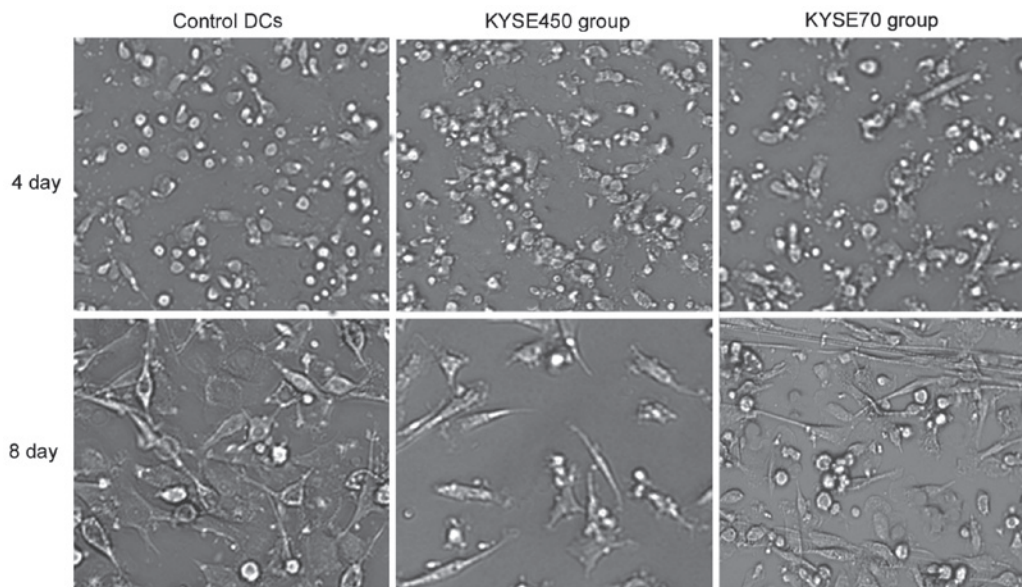Email: dongzm@zzu.edu.cn

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Figure 1. Cell morphology of the endothelial-like differentiation of iDCs induced by KYSE450 and KYSE70 supernatant. PBMCs were incubated in rhGM-CSF/rhIL-4 and 40% supernatant of KYSE450 (highly differentiated ESCC cells) or KYSE70 (poorly differentiated ESCC cells) was added at the end of day 2. The cells of the KYSE450 and KYSE70 groups had no obvious morphological change, similar to control DCs, following 4 days of induction. The cells of the KYSE450 group exhibited a fusiform shape following 8 days of induction. The KYSE70 group of cells was arranged as strip-shaped and funicular structures, distinctly different to the control dendritic cells, following 8 days of induction with the KYSE70 supernatant (magnification, x200). DCs; dendritic cells; PBMCs, peripheral blood mononuclear cells.

different tumor-differentiated degree ESCC on the ELD of iDCs is unclear, and the function of JAK/STAT3 signal in this process is unknown. In the present study, we investigated the effect on ELD of iDCs using cell culture supernatant obtained from the KYSE450 (high differentiation) and KYSE70 (poor differentiation) ESCC cell line, and demonstrated the role of JAK/STAT3 signal pathway therein.

Materials and methods

Preparation of KYSE450 and KYSE70 cell line supernatant. The KYSE450 and KYSE70 ESCC cell lines (Nanjing KeyGEN Biotech. Co., Ltd. (Nanjing, China) were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum. The cells were replenished with fresh medium at 60-80% confluency. The cells were used at passage 6-9. The supernatant was collected and filtered following 24 h of incubation, and stored at -20˚C.

Induction of ELCs from immature DCs. Peripheral blood mononuclear cells (PBMCs) were harvested from healthy adult volunteers (who provided written informed consent) and isolated using density gradient centrifugation with Ficoll-Paque. The purified cells were seeded in 12-well plates (11). Adherent cells (monocytes) were induced towards a DC fate using rhGM-CSF (100 ng/ml; Amoytop Biotech, Xiamen, China) and rhIL-4, (5 ng/ml; PeproTech China, Suzhou, China). 40% KYSE450 supernatant or 40% KYSE70 supernatant and 60% DCs medium was added at the end of day 2. Following 7 days of induction (day 9), the cells were harvested for use in experiments. As for controls, adherent cells (monocytes) were similarly induced towards DC fate using rhGM-CSF and rhIL-4. They were mature DCs when harvested for use in experiments.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. The total RNA 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 oligo-nucleotide primer pairs are as follows: GAPDH, F 5'-GAAGGCTGGGGCTCATTT-3' and R 5'-GAGGAGGCATTGCTGATGAT-3'; VEGF, F 5'-CCT CCGAAACCATGAACTTT-3' and R 5'-CCACCTCGA TGATTCTGC-3'; IL6, F 5'-TAGTGAGGAACAAGCCAG AGC-3' and R 5'-TGGCATTTGTGGTTGGGTCA-3'; JAK2, F 5'-AGAATGTCTTGGGGATGGCAG-3' and R 5'-TGATAG TCTTGGATCTTTGCTCG-3'; STAT3, F 5'-TGCTTCCCT GATTGTGACTG-3' and R 5'-CTGACAGATGTTGGA GATCACC-3'; CD144, F 5'-AAACACCTCACTTCCCCA TC-3' and R 5'-ACCTTGCCCACATATTCTCC-3'; and vWF, F 5'-ATGAGTATGAGTGTGCCTGC-3' and R 5'-GTAGAT GGTGCTTCGGTGG-3'. The PCR conditions used were: 95˚C for 10 min, 40 x (95˚C for 10 sec + 60˚C for 30 sec). Each sample was run in triplicate. Analysis of the RT-qPCR data was performed using Applied Biosystems 7500 Fast Real-Time PCR System, v2.0.5 (Applied Biosystems Life Technologies, Foster City, CA, USA).

Immunofluorescence. Control DCs, the KYSE450 cell group (induced by KYSE450 cell supernatant) and the KYSE70 cell group (induced by KYSE70 cell supernatant) were seeded in 12-well plates and incubated for 24 h, then fixed with 4% para-formaldehyde for 30 min. Antibodies were diluted in BSA-PBS (cat no. A8010; Solarbio, Beijing, China). A rabbit anti-human von Willebrand factor (vWF) antibody (1:100; cat no. sc-73268; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and a Cy3-conjugated goat anti-rabbit IgG secondary antibody were used to detect vWF expression and localization in the three cell groups. To visualize cluster of differentiation 144 (CD144),
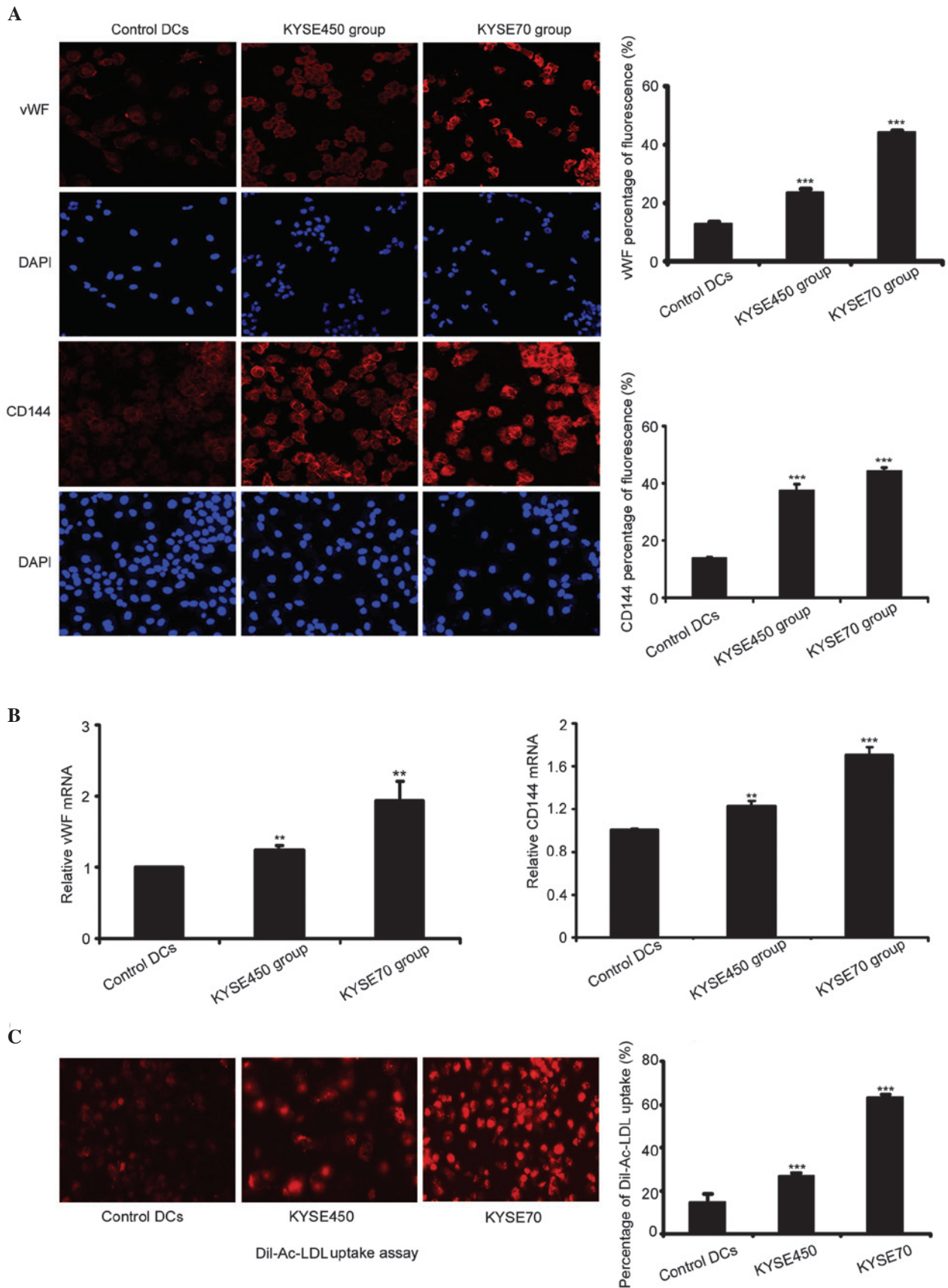
Figure 2. Expression of endothelial cell markers vWF and CD144 increased following induction by KYSE450 and KYSE70 supernatant. (A) Immunofluoresence was used to detect the expression of vWF and CD144 in control DCs and the KYSE450 and KYSE70 groups (magnification, x200). Data are the mean ± standard deviation (SD), n=4, ***P<0.001 vs. control DCs. (B) RT-qPCR detection of mRNA levels of vWF and CD144 in control DCs and the KYSE450 and KYSE70 groups. Data are the mean ± SD, n=3, **P<0.01, ***P<0.001 vs. control DCs. (C) Dil-Ac-LDL uptake was enhanced following induction by KYSE450 and KYSE70 supernatant (magnification, x200). Data are the mean ± SD, n=4, ***P<0.001 vs. control DCs. DCs, dendritic cells; vWF, von Willebrand factor; CD144, cluster of differentiation 144; DiI-Ac-LDL, DiI-labeled acetylated low-density lipoprotein; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

a rabbit anti-human CD144 antibody (1:200; cat no. 2500; CST Biological Reagents Company Ltd., Shanghai, China) and a 555-conjugated goat anti-rabbit secondary antibody were used. The anti-p-JAK2 (Tyr 1007/Tyr 1008) primary antibody (1:100; cat no. cc-16566-R; Santa Cruz Biotechnology, Inc.) and a 555-conjugated goat anti-rabbit secondary antibody (cat no. P0178-1; Beyotime Institute of Biotechnology, Shanghai, China) were used to detect phosphorylated JAK2. The p-STAT3 (B-7) primary antibody (1:100; cat no. sc-8059; Santa Cruz Biotechnology, Inc.) and a 488-conjugated goat anti-mouse IgG antibody (cat no. P0188-1; Beyotime Institute of Biotechnology) were used to detect phosphorylated STAT3. Cells with fluorescent particles in the cytoplasm were counted as positive expression.

Di1-labeled acetylated low-density lipoprotein (LDL) uptake assay. Control DCs and the KYSE450 and KYSE70 cell groups were seeded in 96-well plates. Following incubation for 36 h, Di1-labeled acetylated-LDL (10 μg/ml; Biomedical Technologies, Stoughton, MA, USA) was added to each well, and all the groups were further incubated at 37˚C for another 4 h. The medium containing Dil-Ac-LDL was removed and all the cell samples were washed three times with phosphate-buffered saline and then observed using a IX2-IL100 fluorescence microscope (Olympus Corporation).

Statistical analysis. Data were presented as the mean ± standard deviation (SD), taken from at least three separate experiments and analyzed using one-way analysis of variance. A statistically significant difference was defined as $P<0.05$.

Results

Changes in iDC morphology during the ELD of iDCs, induced by KYSE450 and KYSE70 supernatant. Immature DCs induced by KYSE450 or KYSE70 supernatant for 4 days appeared round, with a similar form to control DCs. However, following induction of 8 days, the KYSE450 group cells exhibited a fusiform shape, and a number of cells from the KYSE70 group was arranged in cord-like structures, a typical appearance of ECs (Fig. 1).

Protein and mRNA expression of the EC markers vWF and CD144 were detected by immunofluorescence and RT-qPCR. The protein expression levels of EC markers were significantly increased in cells induced by KYSE450 or KYSE70 supernatant, compared with the control DCs (n=4, $P<0.001$). The immunostaining intensity of the two EC markers was greater in the poorly differentiated ESCC group (KYSE70) compared with the highly differentiated group (KYSE450; Fig. 2A). RT-qPCR analysis demonstrated enhanced expression of vWF and CD144 mRNA in the KYSE450 and KYSE70 groups compared to the control DCs (n=3, $P<0.01$, $P<0.001$). Furthermore, the mRNA expression levels of vWF and CD144 in the KYSE70 group were enhanced significantly in comparison to the KYSE450 group (Fig. 2B; n=3, $P<0.001$). The uptake of Dil-Ac-LDL is considered to be a characteristic function of ECs, although this is performed by other cell types, such as macrophages and monocytes (20,21). Previously, we demonstrated that PBMCs had a weak uptake of Dil-Ac-LDL (19). By contrast, in the current study, the cells induced by KYSE450 or KYSE70 supernatant exhibited a strong uptake of Dil-Ac-LDL compared to the control DCs (n=4, $P<0.001$). Additionally, the KYSE70 group cells demonstrated a stronger uptake compared to the KYSE450 group (Fig. 2C; n=4, $P<0.001$). The above results demonstrated that iDCs induced by KYSE450 and KYSE70 supernatant differentiate towards an EC phenotype, and the KYSE70 supernatant induced a greater effect during this transition.

KYSE450 and KYSE70 supernatants induce ELD and activate the JAK/STAT3 signaling pathway in iDCs. During the process of the ELD of iDC, JAK/STAT3 upregulation at the mRNA level was detected by RT-qPCR. The results showed that the mRNA expression levels of JAK and STAT3 increased in the KYSE450 and KYSE70 groups in comparison to the control DCs (Fig. 3A; $P<0.05$, $P<0.01$, $P<0.001$). Furthermore, the KYSE70 group exhibited slightly increased mRNA levels of JAK and STAT3 compared with the KYSE450 group, however this difference was not statistically significant (n=3, P=0.054, P=0.617, respectively). The mRNA expression levels of VEGF-A and IL-6, cytokines downstream of JAK/STAT3 signaling were determined. VEGF-A and IL-6 mRNA levels increased significantly in the KYSE450 and KYSE70 groups compared to the control DCs ($P<0.01$ for VEGF-A; $P<0.001$ for IL-6), with a significantly higher level of expression in the KYSE70 group than KYSE450 (Fig. 3A; $P<0.01$ for VEGF-A; $P<0.001$ for IL-6). Immunocytochemical analysis demonstrated that JAK and STAT3 were phosphorylated at higher levels in the KYSE450 and KYSE70 groups compared with the control DCs, and the fluorescence intensity in the KYSE70 group was stronger than that in the KYSE450 group (Fig.3B; n=3; $P<0.001$).

Blocking JAK signaling in iDCs inhibits ELD induced by KYSE450 and KYSE70 supernatants. AG490 (AG) is an inhibitor of JAK. To confirm whether the JAK/STAT3 signaling pathway is involved in the ELD of iDCs, we added 10 μmol AG490 to the KYSE450 and KYSE70 supernatant-treated cells at the end of day 2. Following 8 days of incubation, the mRNA expression levels of vWF and CD144 had significantly decreased in the KYSE450 + AG and KYSE70 + AG groups (Fig. 4A; n=3, $P<0.01$, $P<0.001$). Furthermore, the levels of these proteins were significantly decreased, as demonstrated by a reduction in the fluorescence intensity of CD144 and vWF in the KYSE450 + AG and KYSE70+AG groups compared with the control KYSE450 group or KYSE70 group (Fig. 4B; n=3, $P<0.001$). Additionally, Dil-Ac-LDL uptake was significantly reduced in the KYSE450 and KYSE70 groups incubated with AG490 (Fig. 4C; n=4, $P<0.001$).

In summary, the results demonstrated the JAK/STAT3 signaling pathway promotes the ELD of iDCs induced by KYSE450 and KYSE70 supernatant, and blocking JAK/STAT3 signaling inhibits the ELD of iDCs.

Discussion

It has previously been reported that incubation of tumor-associated DCs with VEGF and oncostatin M can direct progenitor
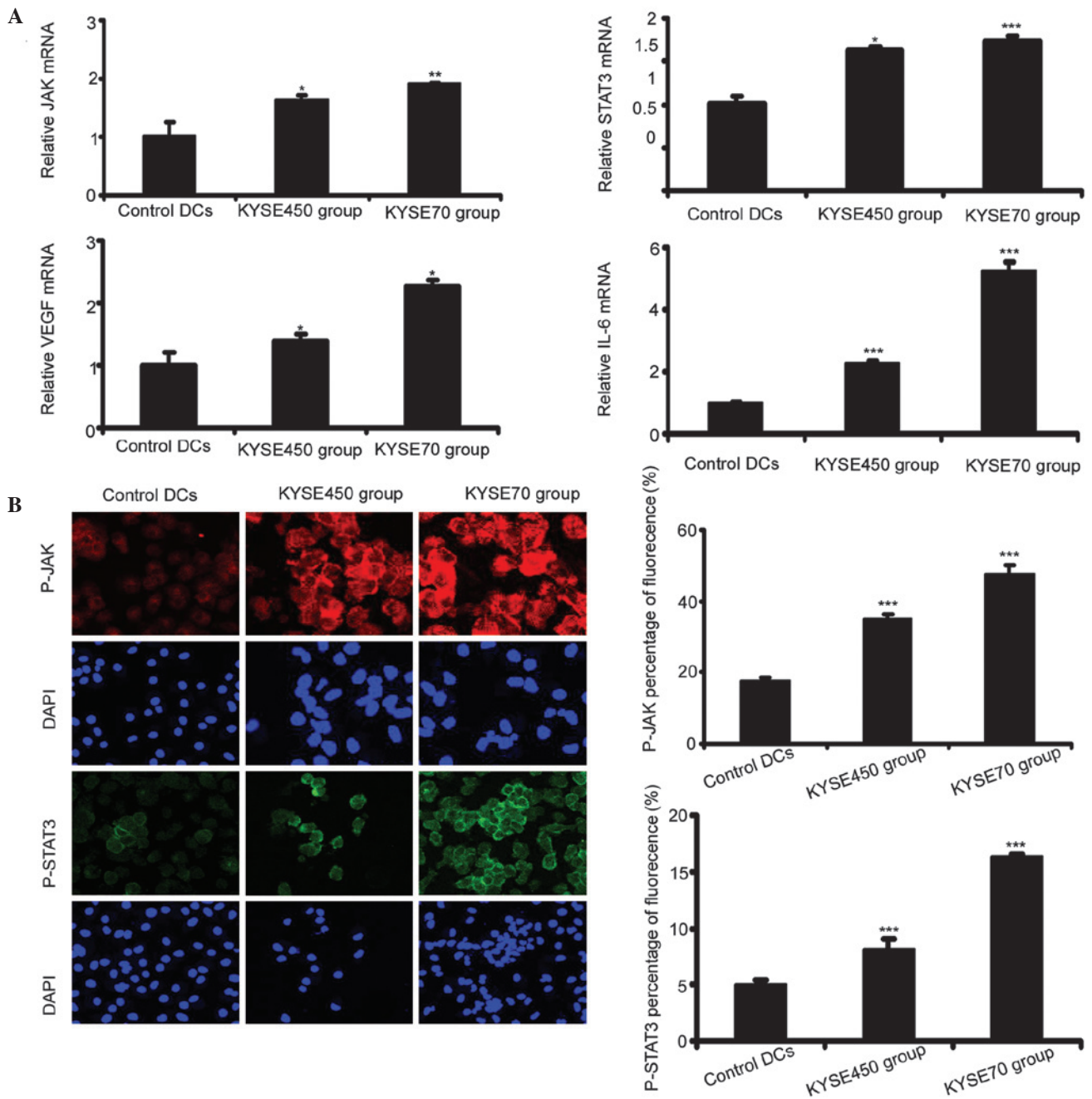
Figure 3. Activation of JAK/STAT3 signaling by KYSE 450 and KYSE70 supernatant. (A) RT-qPCR analysis revealed that JAK and STAT3 mRNA expression levels increased significantly following incubation with KYSE450 and KYSE70 supernatant. Additionally, the known targets of JAK/STAT3 signaling, VEGF and IL-6 had significantly increased mRNA expression. Data are the mean ± SD (n=3). *P<0.05, **P<0.01, ***P<0.001 vs. control dendtritic cells (DCs). (B) Immunofluorescence detection of the activation of JAK and STAT3 in control DCs and the KYSE450 and KYSE70 groups (magnification, x200). Data are the mean ± SD (n=3). ***P<0.001 vs. control DCs. JAK, janus tyrosine kinase; VEGF, vascular endothelial growth factor; STAT3, signal transducer and activator of transcription 3.

cell differentiation away from the DC pathway to an ELC fate (11). Furthermore, the tumor microenvironment can induce the ELD of iDCs, but not of mature DCs (22-24). While these previous studies have examined the effect of DC differentiation stage on the transition to ELCs, to the best of our knowledge, there is no previous study that addresses whether the degree of differentiation of tumor cells can influence the ELD of iDCs. In the present study, the impact of the microenvironment produced by ESCC cell line KYSE450 (highly differentiated) and KYSE70 (poorly differentiated) supernatant on the differentiation of iDCs derived from PBMCs, was

investigated. The results demonstrate that iDCs induced by the KYSE70 supernatant appeared with fusiform shapes and were arranged into cord-like structures in the KYSE70 and KYSE450 groups. Additionally, the KYSE70- induced group expressed higher levels of the EC markers vWF and CD144, and exhibited stronger Dil-Ac-LDL uptake in comparison with the KYSE450-induced group. These results demonstrate that iDCs can transdifferentiate into ELCs in an ESCC microenvironment, and the supernatant from the poorly differentiated KYSE70 cells had a greater effect on ELD than the supernatant from the highly differentiated KYSE450 cells.
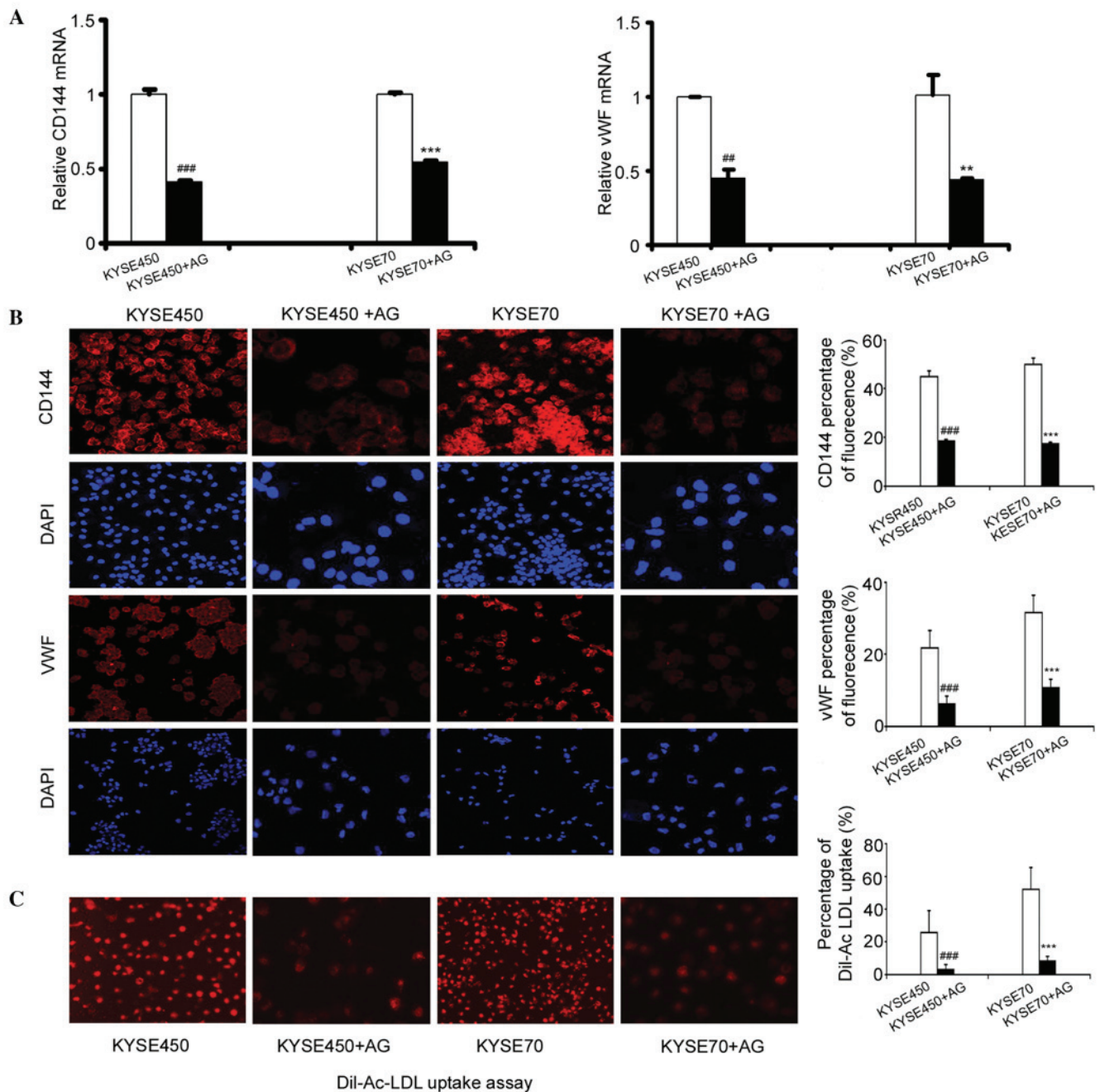
Figure 4. Blocking the activation of JAK and STAT3 inhibited endothelial-like differentiation of iDCs. (A) Addition of 10 µmol AG490 at the end of day 2, decreased the expression of vWF and CD144 in iDCs induced by KYSE450 and KYSE70 supernatant. RT-qPCR results shown are the mean ± SD (n=3). ##P<0.01, ###P<0.001 vs. KYSE450 group. **P<0.01, ***P<0.001 vs.KYSE70 group. (B) Immunofluorescence detection of the protein expression of the EC markers CD144 and vWF in the KYSE450, KYSE450 + AG, KYSE70, and KYSE70 + AG groups (magnification, x200). Data are the mean ± SD (n=3). ***P<0.001 vs. KYSE450 group. ***P<0.001 vs. KYSE70 group. (C) Dil-Ac-LDL uptake decreased after induction by KYSE450 + AG490 and KYSE70 + AG490. Original magnification, x200. Results shown are the mean ± SD (n=4). ***P<0.001 vs. KYSE450 group. ***P<0.001 vs. KYSE70 group. AG, AG490; iDCs, immature dendritic cells; vWF, von Willebrand factor; CD144, cluster of differentiation 144; DiI-Ac-LDL, DiI-labeled acetylated low-density lipoprotein; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

This may be due to increased IL-6 and VEGF protein expression in the KYSE70 group, a hypothesis that is supported by our previous findings showing VEGF can induce the ELD of iDCs (22,23). It is known that PBMCs are pluripotent stem cells and can be induced to acquire macrophage, lymphocyte, epithelial, endothelial, neuronal, and hepatocyte phenotypes in the absence of fusion with pre-existing mature tissue cells (25). In the present study, iDCs retained some features of

PBMCs, thus, iDCs may differentiate into ELCs in the ESCC microenvironment to varying degrees.

A large number of growth factors, cytokines and vasoactive substances can induce cell differentiation through cellular signal transduction. The JAK/STAT3 signaling pathway is important in cell growth, differentiation, proliferation and apoptosis, and involved in the abnormal differentiation of DCs (26). Previous studies have demonstrated that STAT3 is a key component

of diverse signal transduction pathways generated by a large number of cytokines and growth factors, such as IL-6, epidermal growth factor and platelet-derived growth factor. Furthermore, the receptors for these ligands are associated with JAK (27). The results of the current study revealed the JAK/STAT3 signaling pathway was activated during the ELD of iDCs, and the cells in the KYSE70 group exhibited higher levels of p-JAK and p-STAT3, compared with the cells in the KYSE450 group. AG490 is the inhibitor of the JAK/STAT3 signaling pathway, and not only inhibited the activation of JAK signaling, but also inhibited the differentiation of iDCs to ELCs.

In conclusion, the present study demonstrated that iDCs may differentiate into ELCs in response to an ESCC microenvironment, and the degree of differentiation of the ESCC cells may affect the phenotype of the resulting ELC. Poorly differentiated ESCCs had a greater effect on the ELD of iDCs than highly differentiated ESCCs. Additionally, JAK/STAT3 signaling is involved in this process.

Acknowledgements

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