Endothelial transdifferentiation of human HGC-27 gastric cancer cells *in vitro*

CHANGXIN CHEN[1], ZHIXIN HUNAG[2], MUCHENG WANG[1], ZICHENG HUANG[1], XIANGBO CHEN[1], ANYE HUANG[1], BINBIN ZHENG[1], LISHAN WU[1], YI LIU[1], XINWEN WANG[3*] and WEIFENG XU[4*]

[1]Department of Gastroenterology, Quanzhou First Hospital Affiliated to Fujian Medical University, Quanzhou, Fujian 362000; [2]Department of Thoracic Surgery, Fujian Medical University Union Hospital, Fuzhou, Fujian 350005; [3]Department of Orthopaedics, The Third Affiliated Hospital of Southern Medical University, Guangzhou, Guangdong 510630; [4]Department of Medical Oncology, The Affiliated Cancer Hospital of Zhengzhou University, Zhengzhou, Henan 450008, P.R. China

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Abstract. Malignant tumor cells are able to transdifferentiate into other cell types in various tissues or organs. Recent studies have demonstrated the ability of cancer cells to transdifferentiate into functional endothelial cells (ECs). However, whether human gastric cancer (GC) cells are able to transdifferentiate into other cell types has remained largely elusive. Furthermore, whether HGC-27 cells are able to participate in GC angiogenesis remains to be clarified. In the present study, the HGC-27 cell line grown under hypoxic conditions for 4 days exhibited the typical 'flagstone' appearance, which is typical for cultured ECs. HGC-27 cells cultured on Matrigel under hypoxic conditions gradually formed net-like structures. Furthermore, the cultured HGC-27 cells expressed CD31, CD34 and von Willebrand factor, the molecular markers for ECs, under hypoxic conditions. These results indicated that HGC-27 cells, cultured under hypoxic conditions, are able to transdifferentiate into EC-like cells *in vitro*.

Introduction

Gastric cancer (GC) is one of the most common, lethal neoplasms of the human digestive system and is the second most frequent cause of cancer-associated death worldwide (1). The highly aggressive advanced GC promotes distant metastasis, with typical metastatic sites being the lungs, liver and bones (2). Angiogenesis mostly be essential for nourishing the primary tumor (3). Therefore, it is imperative to explore the possible mechanisms of angiogenesis in GC to expand the current understanding of the disease etiology and prognosis and offer novel treatment strategies.

Tumor angiogenesis is a complex process involving activation of endothelial cells (ECs); degradation of the extracellular matrix; migration of ECs; as well as proliferation, tube formation and formation of adventitial membranes (4). Thus, ECs, usually generated from mesenchymal stem cells, bone marrow-derived endothelial progenitor cells and pre-existing ECs, have an essential role in tumor angiogenesis (5-8). Numerous studies have demonstrated the involvement of tumor cell-derived vascular ECs (VECs) in neoplasms such as myeloma, osteosarcoma, glioblastoma, ovarian cancer and neuroblastoma (9-13). Osteosarcoma cells transdifferentiate into endothelial cells under hypoxic conditions (14). First osteosarcoma cells differentiate into tumor stem cells under hypoxic conditions and then the tumor stem cells further differentiate into endothelial cells (14). Therefore, tumor cell-derived VECs are a typical source of ECs in tumor angiogenesis, although it remains elusive whether GC cells are able to differentiate into ECs. The present study aimed to determine whether human GC cells are able to differentiate into ECs to take on their morphological and functional properties.

Tumor cells are usually deficient in nutrients and oxygen due to the failure of blood supply to meet the metabolic requirements of these rapidly growing cells. Therefore, hypoxia is a common phenomenon in the development of most solid tumor types (15). Hypoxia-inducible factor (HIF), a transcription factor produced by tumor cells, mediates the growth, proliferation and metastasis of cancer cells under hypoxic conditions (16). HIF is a heterodimeric transcription factor containing a hypoxically inducible HIF-α subunit and a constitutively expressed HIF-β subunit (17). Therefore, the activity of HIF is determined primarily by the expression level of the α subunit (18). Hypoxia-induced tumor angiogenesis and

Correspondence to: Professor Weifeng Xu, Department of Medical Oncology, The Affiliated Cancer Hospital of Zhengzhou University, 127 Dongming Road, Zhengzhou, Henan 450008, P.R. China
E-mail: xwflz123@126.com

Professor Xinwen Wang, Department of Orthopaedics, The Third Affiliated Hospital of Southern Medical University, 183 West Zhongshan Avenue, Guangzhou, Guangdong 510630, P.R. China
E-mail: andrewbread@163.com

*Contributed equally

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the important role of HIF-α in the process have been widely reported (19-21). In the present study, it was hypothesized that GC cells are able to transdifferentiate into vascular EC-like cells under hypoxic conditions.

To investigate morphological and molecular changes, GC cells were cultured under hypoxic conditions, as well as on Matrigel (tube formation assay, a property of ECs). The results demonstrated that GC cells were able to transdifferentiate into EC-like cells *in vitro*.

Materials and methods

Cell lines and culture. The human GC (HGC) cell line HGC-27 was obtained from the Cell Bank of the Chinese Academy of Sciences. The cells were cultured in RPMI-1640 medium (GIBCO; Thermo Fisher Scientific, Inc.) supplemented with 20% fetal bovine serum (FBS; HyClone; Cytiva) and 100 units/ml penicillin/streptomycin.

Induced transdifferentiation of HGC cells. For the normoxia group, HGC cells were cultivated under normoxia conditions (5% CO_2, 95% air) in endothelial differentiation medium, i.e. RPMI-1640 medium supplemented with 20% FBS, 10 ng/ml vascular endothelial growth factor (VEGF; Invitrogen; Thermo Fisher Scientific, Inc.), 1% N2 supplement, 10 ng/ml epidermal growth factor, 5 ng/ml bone-derived fibroblast growth factor and 50 μg/ml heparin *in vitro*. For the control group, HGC cells were cultivated under normoxia conditions in RPMI-1640 medium only containing 10% FBS. For the hypoxia group, HGC cells were cultivated under hypoxia conditions in endothelial differentiation medium. For the HC group, HGC cells were cultivated under hypoxia conditions in RPMI-1640 medium only containing 10% FBS. The hypoxia group and GC group, HGC cells were placed in an incubator (Precision Scientific) with 1% oxygen, 5% CO_2 and 94% nitrogen. The cells of each group were cultured using their own specific aforementioned culture conditions for 4 days, their appearance was observed.

Three-dimensional culture. Matrigel (BD Biosciences) was thawed at 4˚C overnight. 96-well plates were incubated at 4˚C for 60 min. Matrigel (BD Biosciences) was poured onto the 96-well dish at 30 μl/well and then the dish was placed in a CO_2 incubator (humidified atmosphere with 5% CO_2/95% air) at 37˚C for 30 min. Subsequently, the HGC-27 cell suspensions ($1x10^5$ cells/200 μl) in endothelial differentiation medium or basic medium were added to the designated wells. In the hypoxia and GC groups, the cells were cultivated under hypoxia conditions (1% O_2, 5% CO_2, 94% nitrogen) and in the control and normoxia groups, the cells were cultivated under normoxia conditions (5% CO_2, 95% air). The cells were periodically observed by inverted phase-contrast microscopy and images were acquired. The number of tubes and the length of the branches were calculated using ImageJ software version 1.47 (National Institutes of Health).

RNA isolation and reverse transcription-quantitative (RT-q) PCR. Total RNA was isolated from each group using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.), and quantified spectrophotometrically (at 260 nm). RNAase-Free DNase I (Invitrogen; Thermo Fisher Scientific, Inc.) was used to remove genomic DNA contamination. First-strand complementary (c) DNA was synthesized from total RNA (500 ng), the ReverTra Ace kit (Toyobo) and oligo (dT) 20 primers (performed according to the manufacturer's protocol). PCR amplification of cDNA template was performed using the LightCycler 480 real-time PCR system (Roche). The reaction was performed in a 384-well plate using the LightCycler 480 SYBR Green I Master kit (Roche). The PCR reaction system consisted of 0.75 μl of template cDNA, 0.6 μl of 2.5 μM primer mix, 2.5 μl of 2X SYBR Green I Master Mix and 1.15 μl RNAse-free water in a final volume of 5 μl. The primer sequences were as follows: CD31 forward, 5'-ACATGGCAACAAGGCTGT GTA-3' and reverse, 5'-CCTCAAACTGGGCATCATAAG-3' (GenBank accession no. NM_000442); CD34 forward, 5'-CCA CTCGGTGCGTCTCTCTAGGAGC-3' and reverse, 5'-TTG TCTCTGGAGTTGAAACGTTGGC-3' (GenBank accession no. NM_001025109); von Willebrand factor (vWF) forward, 5'-CTGAAGAGTCATCGGGTCAACTGT-3' and reverse, 5'-AGCATGAAGTCATTGGCTCCGTTCT-3' (GenBank accession no. NM_000552); β-actin forward, 5'-TTCTGTGGC ATCCACGAAACT-3' and reverse, 5'-GAAGCATTTGCG GTGGACGAT-3' (GenBank accession no. NM_001101). The optimal conditions for PCR amplification of the cDNA were as follows: Initiation at 95˚C for 30 sec, followed by 40 cycles of 95˚C for 15 sec and 60˚C for 60 sec. The fluorescence threshold value was calculated using Lightcycler 480 series software. The calculation of relative changes in mRNA levels was performed using melting curve analysis with normalization to the housekeeping gene β-actin. Data analysis of the relative real time PCR was based on the $2^{-\Delta\Delta Cq}$ method (22). PCR amplification was repeated three times.

Immunofluorescence. HGC-27 cells were plated on sterile glass coverslips with Lysin in a 6-well culture plate and cultivated under different conditions according to the group. After 48 h of incubation, the cells plated on glass coverslips were fixed with 4% paraformaldehyde on ice for 30 min and treated with 0.3% TritonX-100 solution (Sigma-Aldrich; Merck KGaA) for 10 min at room temperature. Subsequently, the cells were blocked with 1% bovine serum albumin (BSA) (Sigma-Aldrich; Merck KGaA) for 20 min at room temperature. Subsequently, they were incubated overnight at 4˚C with antibodies against CD31 (1:2,000; mouse anti-human; cat. no. ab218; Abcam), CD34 (1:2,000 rabbit anti-human; cat. no. ab81289; Abcam) and vWF (1:2,000 mouse anti-human; cat. no. ab194405; Abcam). The cells were incubated in the dark at room temperature for 1 h with secondary antibodies: PE-conjugated anti-rabbit antibodies (1:200; cat. no. P-2771MP; Invitrogen; Thermo Fisher Scientific Inc.) or FITC-conjugated anti-mouse antibodies (1:200; cat. no. A0568; Beyotime Institute of Biotechnology). Nuclei were counterstained with DAPI (Sigma-Aldrich; Merck KGaA). Subsequently, images were captured using an Olympus BX51 epifluorescent microscope (Olympus BX51; Olympus Corp.).

Statistical analysis. Statistical analysis was performed using SPSS 20.0 (IBM Corp.) and GraphPad Prism software version 5.0 (GraphPad Software, Inc.). Data were expressed as the mean ± standard deviation. Groups were compared by one-way ANOVA followed by the Newman-Keuls or Dunnett

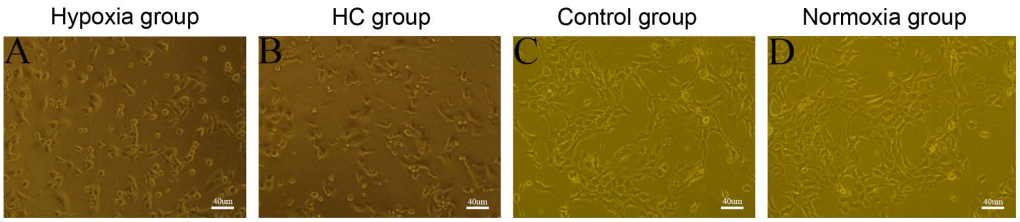

Figure 1. Morphological changes of HGC-27 cells following culture under hypoxia. Photomicrographs of HGC-27 cells following culture in (A) endothelial differentiation medium under hypoxia; (B) essential medium under hypoxia conditions; (C) endothelial differentiation medium under normoxia; (D) essential medium under normoxia (magnification, x100; scale bar, 40 μm). The 'flagstone' appearance of HGC-27 cells was observed after culture under hypoxia conditions for 4 days. Under normoxia, the cells adhered to the culture dish rather than developing a 'flagstone' appearance. HC, HGC-27 cells in essential medium under hypoxia.

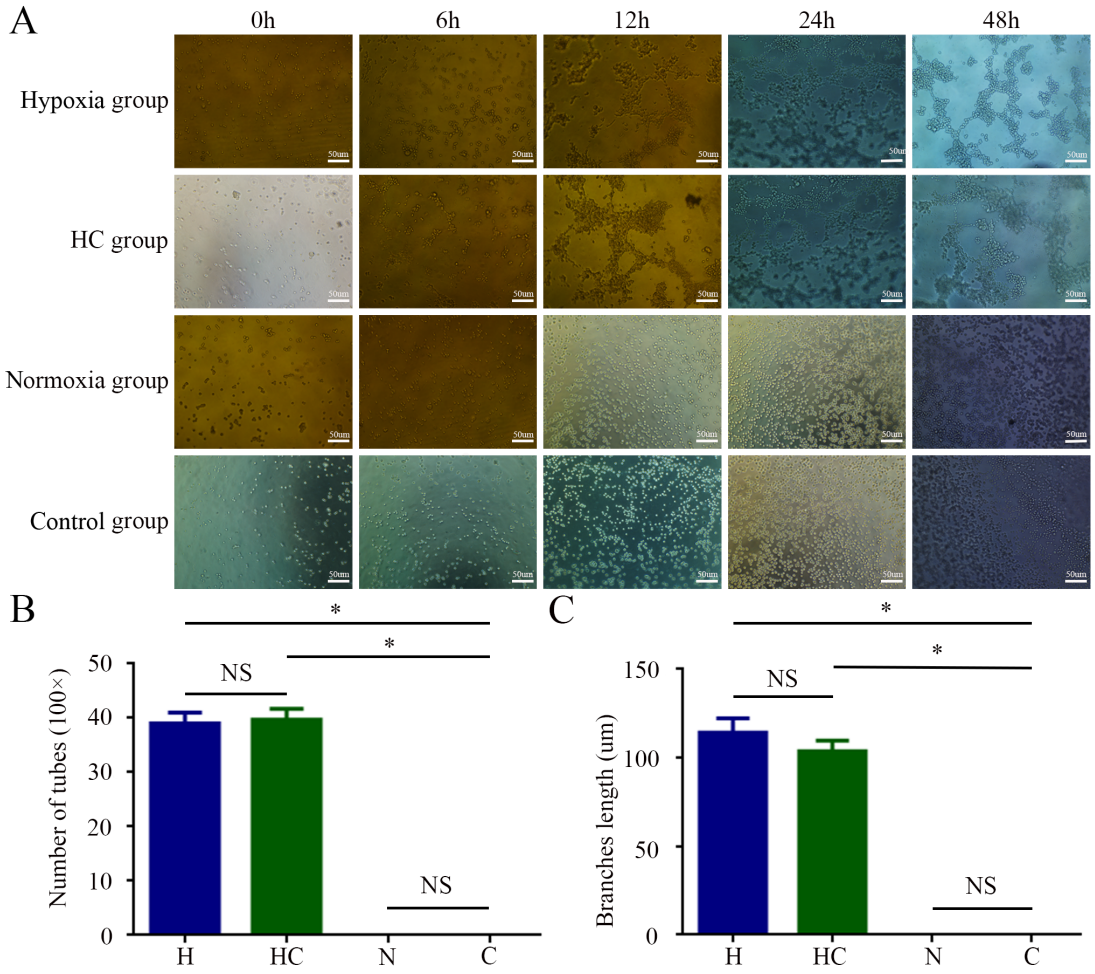

Figure 2. (A) HGC-27 cells were cultivated on Matrigel under different conditions. Representative images indicated that HGC-27 cells cultured for 0, 6, 12, 24 and 48 h formed a network. The cells were cultured under hypoxia conditions, H group and HC group (magnification, x100; scale bar, 50 μm). (B) Number of tubes in the photomicrographs of HGC-27 cells cultured under different conditions. (C) Branch length measurements from photomicrographs of tube formation. *P<0.05. Groups: H, HGC-27 cells in endothelial differentiation medium under hypoxia; HC, HGC-27 cells in essential medium under hypoxia; N, HGC-27 cells in endothelial differentiation medium under normoxia; C, HGC-27 cells in essential medium under normoxia. NS, no significance.

test. Comparison between groups H and GC was performed by the Newman-Keuls test, while groups H, HC or N and group C were compared by Dunnett's test. P<0.05 was considered to indicate statistical significance.

Results

HGC-27 cells exhibit the morphological features of ECs after transdifferentiation. To investigate the transdifferentiating capability of the tumor cells, HGC-27 cells were cultured under different conditions to determine the presence of the characteristic 'flagstone' appearance, as well as microtube formation on Matrigel. The tube-formation capacity of the cells was evaluated by determining the branch length and number of tubes. On day 4 of HGC-27 cell cultivation under hypoxic conditions with or without endothelial differentiation medium, a typical flagstone morphology was noted (Fig. 1A and B). However, HGC-27 cells cultured under normoxic conditions, with or

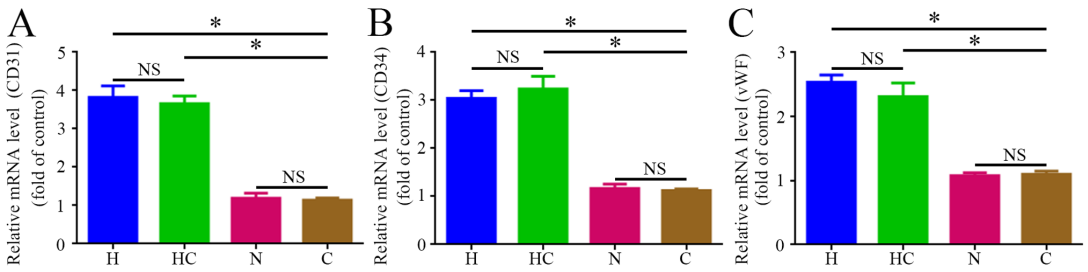

Figure 3. Changes in (A) CD31, (B) CD34 and (C) vWF mRNA levels in HGC-27 cells after exposure to different culture conditions as detected by reverse transcription-quantitative PCR. *P<0.05. Groups: H, HGC-27 cells in endothelial differentiation medium under hypoxia; HC, HGC-27 cells in essential medium under hypoxia; N, HGC-27 cells in endothelial differentiation medium under normoxia; C, HGC-27 cells in essential medium under normoxia. NS, no significance; vWF, von Willebrand factor.

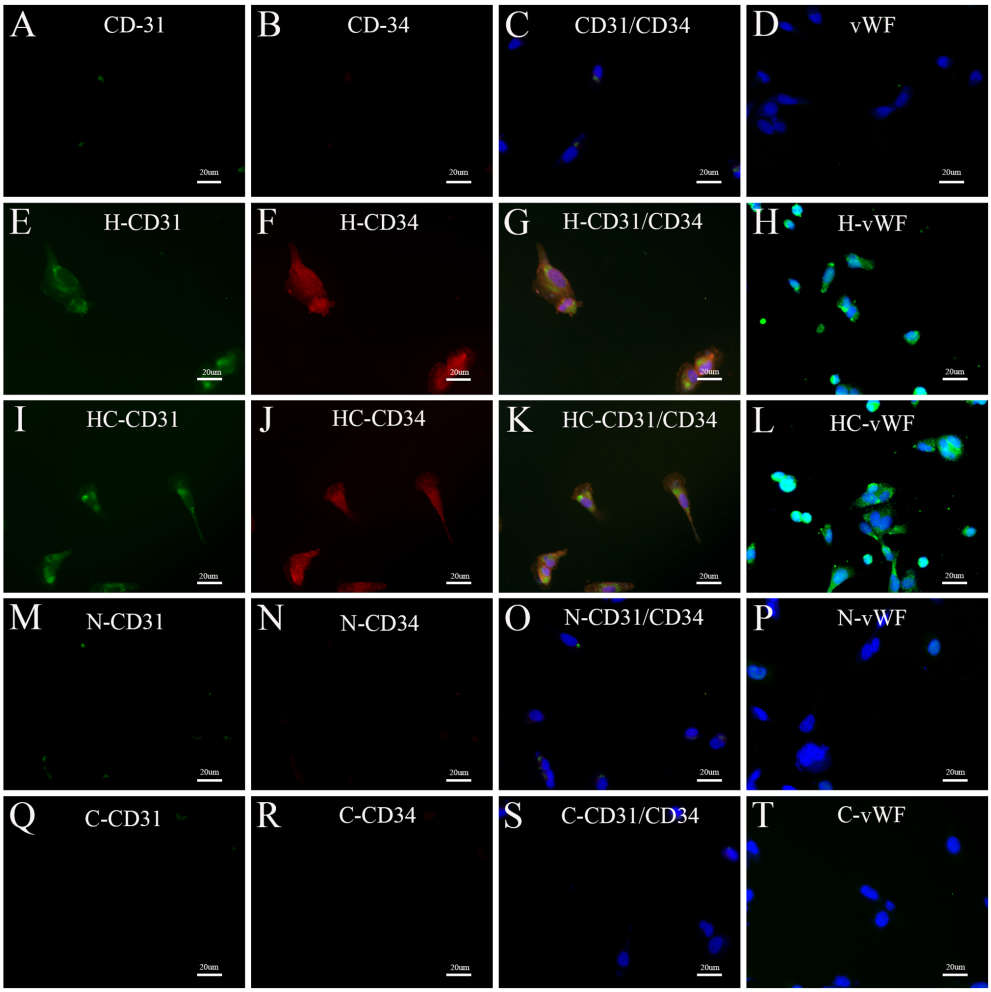

Figure 4. Epifluorescent microscopy of HGC-27 cells prior to or after culture under different conditions. (A-D) Immunofluorescent staining performed on HGC-27 cells prior to culture under different conditions (A) CD-31, (B) CD-34, (C) CD31 + CD34 merge and (D) vWF. (E-H) Immunofluorescent staining of cells in the H group; (E) CD-31, (F) CD-34, (G) CD31 + CD34 merge and (H) vWF. (I-L) immunofluorescent staining of cells in the GC group; (I) CD-31, (J) CD-34, (K) CD31 + CD34 merge and (L) vWF. (M-P) Immunofluorescent staining of cells in the N group; (M) CD-31, (N) CD-34, (O) CD31 + CD34 merge and (P) vWF. (Q-T) Immunofluorescent staining of cells in the C group (Q) CD-31, (R) CD-34, (S) CD31 + CD34 merge and (T) vWF. CD31 is displayed in green, CD34 in red and vWF in green (the nuclei stained with DAPI, blue) (magnification, x400; scale bar, 20 μm). Groups: H, HGC-27 cells in endothelial differentiation medium under hypoxia; HC, HGC-27 cells in essential medium under hypoxia; N, HGC-27 cells in endothelial differentiation medium under normoxia; C, HGC-27 cells in essential medium under normoxia. vWF, von Willebrand factor.

without endothelial differentiation medium, adhered to the culture dish rather than developing the flagstone appearance (Fig. 1C and D). HGC-27 cells cultivated on Matrigel under hypoxic conditions, with or without endothelial differentiation medium, underwent a series of morphological changes: From a single cell or a cluster of cells (0 h) to discontinuous net-like structures (6 h), to continuous net-like structures (12 h) and to a significant increase in the number of the net-like structures (24 h), and the net-like structures continued to exist at 48 h after seeding (Fig. 2A). Furthermore, the branch lengths and

the number of tubes at 24 h did not demonstrate any obvious differences between H and HC groups (Fig. 2B and C). However, HGC-27 cells grown on Matrigel under normoxic conditions, with or without endothelial differentiation medium, did not form any obvious net-like structures even at 48 h after seeding (Fig. 2A).

Increased expression of the EC markers CD31, CD34 and vWF after transdifferentiation. To confirm whether HGC-27 cells transdifferentiated to ECs under hypoxic conditions, the mRNA levels of the EC markers CD31, CD34 and vWF were examined. Compared with the control group, the transcription levels of these markers in HGC-27 cells were significantly increased following exposure to hypoxic conditions, H and HC groups. However, the transcription levels of these markers were not significantly different between the N and C groups after the cells were exposed to normoxic conditions (Fig. 3). In addition, immunofluorescent staining was performed to assess the expression of these markers in HGC-27 cells. Prior to treatment, HGC-27 cells were rarely positive for CD31, CD34 and vWF. Of note, after exposure to hypoxic conditions, most HGC-27 cells were positive for CD31, CD34 and vWF. However, after culture under normoxic conditions, N (in endothelial differentiation medium) and C groups (in RPMI-1640 medium only containing 10% FBS), only a small percentage of HGC-27 cells were positive for CD31, CD34 and vWF (Fig. 4). These results were consistent with those of the RT-qPCR analysis.

Discussion

Transdifferentiation occurs when a fully differentiated cell loses its original phenotype under the stimulation of certain factors and acquires a different cell phenotype (23). In the present study, HGC-27 cells were demonstrated to be able to transdifferentiate into EC-like cells with characteristic morphological and functional properties under hypoxic conditions. GC cells originate from the endoderm (24), but the sole source of ECs appears to be the mesoderm (25), implying that the transformation of HGC-27 cells into EC-like cells was certainly transdifferentiation.

Blood supply has a crucial role in tumor survival, proliferation and metastasis (26). Rapid growth of tumors causes a hypoxic microenvironment that stimulates the formation of new blood vessels (27). An important part of the tumor neovascularization process is VEC formation (28). In the present study, it was demonstrated that HGC-27 cells transdifferentiated into EC-like cells under hypoxic conditions. HGC-27 cells also expressed the EC biomarkers CD31, CD34 and vWF. HGC-27 cells cultured under hypoxic conditions had the characteristic flagstone appearance, the typical morphological feature of ECs. Furthermore, HGC-27 cells cultured on Matrigel gradually formed net-like structures.

Hypoxia is able to stimulate the formation of new blood vessels (29). This mechanism was thought to be hypoxia activating VEGF, which in turn stimulated the proliferation and migration of endothelial cells and promoted the production of new blood vessels (30). The present study provided evidence for the transdifferentiation capability of HGC-27 cells into EC-like cells under hypoxic conditions independent of exogenous VEGF. These results suggested that hypoxia-induced transdifferentiation of HGC-27 cells into EC-like cells is exogenous VEGF-independent.

Several studies have demonstrated the pivotal role of transcription factors in regulating a cell's fate (31). Cell fate is related to not only the type of the transcription factor but also the proportion of different transcription factors (32). These observations indicate that cell type-specific programming may be revocable and the programming may be re-regulated by modifying the activities of certain key transcription factors (33). Several types of malignant tumor cell may be transdifferentiated into EC-like cells to acquire the phenotype of ECs, a process in which transcription factors are involved (34,35). However, the mechanisms of HGC-27 cell transdifferentiation into EC-like cells and the transcription factors involved in the process remain elusive, warranting further research.

There were certain shortcomings to the present study. First, regarding the mechanism of HGC-27-cell transdifferentiation into EC-like cells, the transcription factors and potential other influencing factors involved in this process remain elusive. Furthermore, the signaling pathways involved in the mechanism of HGC-27-cell transdifferentiation under hypoxic conditions requires further investigation. Finally, whether human HGC-27 gastric cancer cells are able to transdifferentiate into endothelial cells *in vivo* remains elusive and may be assessed in a future study.

In conclusion, HGC-27 cells cultured in hypoxic conditions demonstrated the morphological characteristics of ECs and expressed EC markers CD31, CD34 and vWF. The present study demonstrated that HGC-27 cells can transdifferentiate into endothelial cells under hypoxic conditions *in vitro*.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

CXC conducted the experiments and drafted the manuscript. ZXH, MCW, ZCH, XBC and AYH contributed to statistical analysis and manuscript writing. BBZ, LSW and YL participated in performing the cell experiments. XWW and WFX conceived the present study and helped revise the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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