CHC promotes tumor growth and angiogenesis through regulation of HIF-1α and VEGF signaling

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1. Introduction

About 95% of pancreatic cancer cases are adenocarcinomas. The overall five-year survival rate of pancreatic adenocarcinoma is about 5%. It is the fourth leading cause of cancer death in the United States [1]. Pancreatic cancer often recurs after initial treatment despite the use of chemotherapy or radiation therapy [2]. At present, there is no effective treatment for pancreatic cancer. The most commonly used medicine to treat pancreatic cancer is gemcitabine (GEM), a pyrimidine nucleoside drug, but it is only moderately effective [3].

Two monoclonal antibody (mAbs) drugs currently in clinical trial for targeted therapy against pancreatic cancers are cetuximab [4] and bevacizumab [5,6], targeting epidermal growth factor receptor (EGFR) and vascular endothelial growth factor (VEGF), respectively. However, clinical trial data showed that using either cetuximab or bevacizumab, in combination with small molecule drugs had no significant improvement in the overall survival of patients [4–7]. Since EGFR and VEGF may not be a suitable target for pancreatic cancer treatment, it is important to identify a suitable target for targeted therapy of pancreatic cancer.

We have generated several mAbs recognizing pancreatic cancer cells. One of these mAbs, Pa65-2, can recognize clathrin heavy chain (CHC). Clathrin, encoded by the CLTC gene at 17q23.2, is a trimer of heavy chains (CHC). In vitro and in vivo study showed that suppression of CHC either by shRNA or by Pa65-2 inhibited tumor growth and angiogenesis. One of the key functions of CHC was to bind with the hypoxia-inducing factor (HIF)-1α protein, increasing the stability of this protein and facilitating its nuclear translocation, thereby regulating the expression of VEGF. Taken together, our findings indicate that CHC plays a role in the processes of tumorigenesis and angiogenesis. Pa65-2 antibody or CHC shRNA can potentially be used for pancreatic cancer therapy.
in many types of solid tumors [13–15], and the results from several studies suggest that agents acting directly or indirectly against the expression of HIF-1α have anticancer effects [16,17].

In the present study, we found that CHC associates with the HIF-1α, increases the stability of this protein, and facilitates its nuclear translocation, thereby regulating VEGF gene expression in cancer cells. Our newly generated Pa65-2 inhibited tumor growth and angiogenesis, suggesting that this mAb can potentially be used to inhibit tumor angiogenesis and tumorigenesis in pancreatic cancer.

2. Materials and methods

2.1. Cell lines and culture

Human pancreatic adenocarcinoma cell lines (MIA PaCa-2 and AsPC-1), and a human skin fibroblast cell line (CCD-1112Sk), were purchased from American Type Culture Collection (ATCC). These cells were cultured in accordance with cell bank protocols and had been passaged for less than 6 months after resuscitation. Normal human nasal mucosal (NNM) epithelia was a primary culture derived from a nasal polyp [18]. Human umbilical vein endothelial cells (HUVECs) were purchased (Lonza) and grown in EBM-2 medium (Lonza).

2.2. Generation of monoclonal antibodies

Monoclonal antibodies against MIA PaCa-2 were generated following a standard procedure [19] with slight modifications [20]. Briefly, female BALB/c mice were immunized intraperitoneally with MIA PaCa-2 four times at 3-week intervals. On day 4 after the final boost, splenocytes were harvested from the immunized mouse spleen and fused with NSI/1-Ag4-1 myeloma cells by 50% polyethylene glycol (GIBCO). Those hybridomas, positive for MIA PaCa-2 but negative for NNM, were then subcloned by limited dilution and preserved in liquid nitrogen. Ascites were produced in pristane-primed BALB/c mice and mAbs purified with protein G Sepharose 4G gel (GE).

2.3. Identification of the target protein of Pa65-2

MIA PaCa-2 cell lysates were purified by protein G Sepharose (GE), coupled with Pa65-2 and eluted with elution buffer [21]. The eluates were separated by SDS–PAGE. The band of interest was cut from the gel, reduced with dithioerythritol (DTE) alkylated with iodoacetamide (IAA) and digested with trypsin for 16 h at 37 °C [21]. The digested peptides were analyzed by LC–MS/MS sequencing in the Core Facility for Proteomics and Structural Biology Research at Academia Sinica (Taipei).

2.4. Immunoprecipitation and immunoblotting assay

Cells were extracted with RIPA buffer and the supernatants were immunoprecipitated using either anti-CHC antibody, Pa65-2, mAb X-22 (Affinity), or anti-HIF-1α antibody (BD), and then analyzed by immunoblotting [22]. The signals were developed using enhanced chemiluminescence reagents (ECL) (Thermo).

2.5. Cell proliferation analysis and invasion assays

RT-CEL (ACEA), a microelectronic cell sensor system, was used to count the number of living cells. Cells (5 × 10^4) were seeded into each sensor-containing well in microtiter plates. The electronic sensors provided a continuous (every 6 h), quantitative measurement of the cell index in each well. Cell growth was measured for 72 h, and cell indices for each well were recorded at all time points. Cell invasion was assayed in 24-well Biocoat Matrigel invasion chambers (8 μm; Millipore) according to the manufacturer’s directions. Cells were counted under a microscope in five predetermined fields. Assays were performed in triplicate.

2.6. shRNA transfection and luciferase reporter gene assays

Lentiviruses (pLKO.1) containing the CHC shRNA ID TRCN0000007984 (Academia Sinica, Taipei) and pLKO.1 empty vector controls were generated and used to infect MIA PaCa-2 cells. For plasmid transfection, 293T cells were seeded at a density of 60% in a six-well plate and incubated for 24 h. The cells were co-transfected with 1.5 μg of CHC shRNA or pLKO.1 empty vector controls with pCMV-AB8.91 and pMD.G, using Lipofectamine2000 (Life Technologies), according to the recommended protocol. Conditioned medium was exchanged the following day and lentivirus-containing supernatant was harvested 48 h later. For lentiviral infection, cells were seeded at a density of 70% confluence and incubated for 24 h. Lentiviral infection was added 10% (v/v) of lentivirus-containing medium to the cell culture and incubated for 48 h. Transduced cells were selected using puromycin (2 μg/ml, Sigma) for 4 days [23,24]. Transfection efficacy achieved 80%. The VEGF reporter plasmids contains nucleotides −2274 to +379 of the VEGF gene inserted into luciferase reporter pGL2-Basic (Promega) as previously described [25]. VEGF promoter primers sequences are presented in Supplementary Table 1. Luciferase reporter gene assays were conducted using the Renilla Luciferase Assay System (Promega) according to the manufacturer’s directions. The Renilla luciferase was constructed for normalization of transfection efficiency. Relative light units were calculated as the ratio of Firefly luciferase to Renilla luciferase activity (normalized luciferase activity).

2.7. Quantitative reverse transcription polymerase chain reaction (RT-PCR)

RNA extractions were performed using the RNeasy Mini kit (Qiagen) according to the manufacturer’s instructions. First strand cDNA was synthesized from 1.0 μg of total RNA with SuperScript III reverse transcriptase (Life Technologies). CLTC, VEGF, HIF-1α, erythropoietin (EPO), platelet-derived growth factor-β (PDGF-β), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers sequences are presented in Supplementary Table 1. Real-time PCR was performed using a LightCycler 480 System (Roche). Cycling temperatures were as follows: denaturing 94 °C, annealing 60 °C, and extension 70 °C. Data were normalized by the expression level of GAPDH in each sample.

2.8. Hypoxia assay

For hypoxia experiments, MIA PaCa-2 cells were grown in a Ruskin Hypoxic Chamber (APM-50D, 18 Astec, Japan) and treated with either 1% O2; 5% CO2 for 18 h, or given DFX, deferoxamine (100 μM, Sigma) treatment for 5 or 16 h. For proteasome inhibitor treatment, MG132 (10 μM, Sigma) was added to culture medium then incubated 5–17 h.

2.9. Chromatin immunoprecipitation (ChIP)

The protocol for chromatin immunoprecipitation (ChIP) has been described previously [26]. Briefly, control and shCHC-expressing MIA PaCa-2 cells were fixed with 1% formaldehyde, lysed in lysis buffer, sonicated, and clarified by centrifugation. The supernatant was immunoprecipitated with anti-CHC, anti-HIF-1α (Abcam) or NM-IGC (Sigma) antibodies. The precipitates were then amplified by the LightCycler 480 System. The relative abundance of specific sequences in immunoprecipitated DNA was determined using the ΔΔCt method with Ct obtained for total extracted DNA (Input DNA) as a reference value. The amount of immunoprecipitated target was quantified by real-time PCR, and the value of immunoprecipitated target was calculated as the ratio of IP DNA to the total amount of input DNA used for the immunoprecipitation (IP/input) to obtain relative-fold enrichment value. ChIP primers sequences are presented in Supplementary Table 1.

2.10. Immuno-electron microscopy

Cells were gently scraped out of the flasks using a cell scraper (Costar) and fixed in paraformaldehyde and glutaraldehyde. Following fixation, cell pellets were washed with buffer and 30% glycerol and gently agitated overnight at room temperature. Cells were subjected to freeze substitution in an AFIs (Leica), in which they were dehydrated by methanol at −91 °C for 4–5 days. Cells were then warmed to −50 °C, embedded in Lowicryl HM20, and polymerized at −50 °C by UV. Ultrathin sections of 90–200 nm thickness were obtained using an Ultracut UC7 (Leica). The sections were incubated with the Pa65-2 mouse IgG or anti-HIF-1α rabbit IgG. The secondary antibodies (Jackson), goat anti-mouse IgG (Fab’2 fragment) conjugated with 18 nm gold particles, or goat anti-rabbit IgG (Fab’2 fragment) conjugated with 12 nm gold particles, were then applied to their respective sections. Finally, the sections were stained with uranyl acetate and lead citrate, and examined by TEM (Hitachi).

2.11. Inhibition of cell internalization by Pa65-2

EGF uptake assays were carried out using a fluorescence-based approach, as previously described [27]. Cells were washed with serum-free medium, incubated in 1% BSA in serum-free medium at 37 °C, and pretreated with Pa65-2 or NM-IGC (50 μg/ml) at 37 °C. They were then incubated with or without Alexa 555-EGF (1 μg/ml, Life Technologies) at 37 °C or 4 °C. The cells were imaged using a Leica TCS SP confocal microscope (Leica).

2.12. Immunofluorescent staining

Cells were incubated with Pa65-2 and anti-HIF-1α antibodies, and then with FITC- or Rhodamine-conjugated secondary antibodies (Jackson). Images were captured by confocal microscopy (Leica). For cell surface staining, MIA PaCa-2 cells were seeded on coverslips and grown to 80% confluence. The live cells were incubated with Pa65-2 for 30 min at 4 °C. After being washed twice with PBS, the cells were fixed by 4% paraformaldehyde and blocked by adding 3% BSA. The cells were
analyses were done using one-way ANOVA with Tukey’s test to compare the three
ined by light microscopy. Tissue sections were stained with antibodies specific
(DAB). The preparations were lightly counterstained with hematoxylin and exam-
cluded antigen retrieval in 10 mM sodium citrate buffer (pH 6.0) in a pressure coo-
was treated with Pa65-2 or NM-IgG for 24 h. After incubating with FLICA, cells was read at ex/em of 550/595 nm with fluo-
alyzed with confocal microscopy. To identify the target of Pa65-2, MIA PaCa-2 total cell lysates were prepared and purified by Pa65-2-conjugated immunoaffinity chromatography. Silver stain and Western blotting demonstrated that Pa65-2 recognized a target protein with a molecular weight of 190 kDa (Fig. 1D). According to LC–MS/MS analysis and Swiss-Prot database searching, the target protein of Pa65-2 is human clathrin heavy chain (CHC) (Supplementary Fig. 4). The specificity of Pa65-2 to CHC was confirmed by conducting immunoprecipitation and Western blot analysis in parallel using a commercial CHC antibody, mAb X-22 (Fig. 1E). Western blot analysis using Pa65-2 showed a dramatic decrease in signal after CHC knockdown (Fig. 2A). These data further confirmed that Pa65-2 specifically rec-
3.2. Suppressing CHC expression inhibits pancreatic cancer growth
To evaluate the functional role of CHC in tumorigenesis, CHC expression was knocked down by shRNA in MIA PaCa-2 cells. When CHC were knocked down in MIA PaCa-2 cells (Fig. 2A), the growth rate (Fig. 2B), colony formation (Fig. 2C) and invasion ability (Fig. 2D) of the cancer cells were significantly reduced. However, suppression of CHC expression in human skin fibroblast had no effect on proliferation rate of the cells (Supplementary Fig. 5). To examine the effect of down-regulating CHC expression on tumor growth in vivo, a xenograft model was generated by injecting MIA PaCa-2 cells with CHC gene knockdown on one side of the NOD/SCID mouse, and control on the other side. We found that knockdown of CHC markedly reduced xenograft tumor growth (Fig. 2E–G). On average, the suppression of CHC resulted in a 92.6% reduction in tumor growth compared with the controls at day 45 (Fig. 2E). On day 50, mice were sacrificed and tumors were dissected (Fig. 2F) to measure the tumor weight (Fig. 2G). While one mouse showed no tumor on the CHC knockdown side, the other seven mice showed tumors with miniscule sizes on their CHC knockdown sides (Fig. 2F). The same results were observed in CHC knockdown CL1-5 lung cancer cells (Supplementary Fig. 6A–E). Furthermore, TUNEL staining showed that CHC knockdown increased apoptosis of the tumor cells (Fig. 2H). These results

stained by FITC-labeled goat anti-mouse IgG (Jackson ImmunoResearch) and DAPI (Life Technologies). All fluorescence images were obtained by confocal microscopy (Leica).

2.13. Apoptosis assay
Apoptosis of cultured cells was verified through the detection of caspase activ-
ity using sulfurhodamine FLICA apoptosis detection kit (Immunochemistry Tech-
nologies). Cells in 96-well culture plates were treated with Pa65-2 or NM-IgG for 24 h. After incubating with FLICA, cells was read at ex/em of 550/595 nm with fluo-

2.14. Animal models
All animal experiments were performed as per the guidelines of the National Laboratory Animal Center. The protocol was approved by the Committee on the Eth-
ics of Animal Experiments of Academia Sinica. (Taipei). (Permit Number: MMI-
ZOOWH2009102). A xenograft model was generated by injecting NOD/SCID mice with MIA PaCa-2 cells transduced with either CHC shRNA or control vector. The two kinds of transduced cells were injected into different lateral sides of the hind limbs of eight animals at the same time. For analysis of antitumor efficacy of Pa65-2, NOD/SCID mice bearing MIA PaCa-2-derived pancreatic cancer xenografts (≈50 mm^3) were intravenously injected in the tail vein with Pa65-2, or gemicitabine, or NM-IgG, or equivalent volumes of PBS. Tumors were measured by calipers every three days, and mice were observed routinely for weight loss as a symptom of drug toxicity. The tumor volumes were calculated as length × (width)^2 × 0.52. Ani-
mals were treated following the guidelines established by Academia Sinica (Taipe-

2.15. CD31 staining and terminal deoxynucleotidyl transferase-mediated dUTP nick end Labeling (TUNEL) assay
CD31 staining and TUNEL assays were carried out as described previously [28]. The slides were then visualized under a fluorescent microscope and analyzed with MetaMorph software.

2.16. Analysis of tissue samples
Human pancreatic cancer tissue and normal pancreatic tissue arrays were pur-
chased from Pantomics Inc. The immunohistochemistry assay was performed on 5 μm sections cut from paraffin blocks and float-mounted on plus-coated glass slides (Fisher Scientific). The essential steps of immunohistochemistry assay in-
cluded antigen retrieval in 10 mM sodium citrate buffer (pH 6.0) in a pressure coo-
ker for 10 min, blocking endogenous peroxidase with 3% hydrogen peroxide, blocking nonspecific protein binding with an avidin–biotin blocking kit (Vector), and incubating with primary mouse monoclonal antibody at a dilution of 1:200 for 30 min, then incubating with chromosomes 3', 3'aminobenzidine (DAB). The preparations were lightly counterstained with hematoxylin and exam-
ined by light microscopy. Tissue sections were stained with antibodies specific for CHC (Pa65-2), VEGF (GeneTex), HIF-1α (Millipore), NM-IgG, and UEA-1–FITC (Vector). Quantification of DAB intensity was by HistoQuest software (TissueGnos-
tics). The protocol was approved by the Institutional Review Board of Human Sub-
jects Research Ethics Committee of Academia Sinica (Taipei). (Permit Number: AS-
IRB01-11030).

2.17. Statistical analyses
All experiments were performed in triplicates. Statistical analyses were done using unpaired Student’s t-tests to compare the two treatment groups. Statistical analyses were done using one-way ANOVA with Tukey’s test to compare the three treat-
ment groups and in vivo experiments. *, p < 0.05, **, p < 0.01 were considered significant.

3. Results
3.1. Generation and characterization of mAbs against pancreatic cancer
To obtain a potential target for pancreatic adenocarcinoma therapy, we immunized BALB/cJ mice with MIA PaCa-2 cells (Supplementary Fig. 1A). More than 6000 hybridoma clones were obtained. Supernatants from each fusion well were then tested for the production of specific antibodies against MIA PaCa-2 antigens by ELISA assay. Sixteen monoclonal antibodies that exhibited higher reactivities against MIA PaCa-2 cells were generated (data not shown). One of these monoclonal antibodies, Pa65-2, was found to specifically recognize MIA PaCa-2 cells but not normal nasal mucosa (NNM) cells, as confirmed by ELISA, flow cytometry, and immunofluorescent analyses (Supplementary Fig. 1C–E). Pa65-2 antibody recognized a single band of Mr. 190 kDa protein on the pancreatic cancer cell, MIA PaCa-2 by Western blotting (Supple-
mental Fig. 1B). The immunoglobulin (Ig) isotype determination showed that the Pa65-2 is an IgG1. Immuno-electron microscopy showed cellular localization of Pa65-2 on the plasma membrane of MIA PaCa-2 cells (Supplementary Fig. 1F). Confocal microscopy also showed that the Pa65-2 bound to cell membranes of live MIA PaCa-2 cells at 4 °C (Supplementary Fig. 1G). Pancreatic ade-
nocarcinoma cell lines, MIA PaCa-2 and AsPC-1, and normal cell lines, HUVEC, NNM, and CCD-1112Sk, were screened by ELISA and Western blot analyses to characterize the binding property of Pa65-2. In general, Pa65-2 had strong binding affinity to pancreatic cancers cell lines (Fig. 1A and B).

All 40 specimens of pancreatic adenocarcinoma tissues were positively stained by Pa65-2, while six normal pancreatic tissues were not, as shown by the pancreatic cancer tissue arrays (Fig. 1C and Supplementary Table 2). Notably, the target antigen of Pa65-2 is present not only in tumor tissues but also in tumor blood vessels (Fig. 1C, arrow and Supplementary Fig. 2). Pa65-2 can also bind to other types of cancer cell lines, including MDA MB-231 (breast cancer), CL1-5 (lung cancer), SKOV3 (ovary cancer) and SAS (oral cancer), as shown by ELISA and Western blot analysis (Supplementary Fig. 3).

To identify the target of Pa65-2, MIA PaCa-2 total cell lysates were prepared and purified by Pa65-2-conjugated immunoaffinity chromatography. Silver stain and Western blotting demonstrated that Pa65-2 recognized a target protein with a molecular weight of 190 kDa (Fig. 1D). According to LC–MS/MS analysis and Swiss-Prot database searching, the target protein of Pa65-2 is human clathrin heavy chain (CHC) (Supplementary Fig. 4). The specificity of Pa65-2 to CHC was confirmed by conducting immunoprecipitation and Western blot analysis in parallel using a commercial CHC antibody, mAb X-22 (Fig. 1E). Western blot analysis using Pa65-2 showed a dramatic decrease in signal after CHC knockdown (Fig. 2A). These data further confirmed that Pa65-2 specifically rec-

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indicate that CHC may play a role in the regulation of tumorigenesis.

3.3. CHC regulates VEGF expression in pancreatic cancer

Knockdown of CHC markedly reduced tumor growth (Fig. 2). To investigate whether loss of CHC expression could inhibit tumor angiogenesis, immunofluorescent staining with CD31 was performed. Knockdown of CHC resulted in a 78% reduction in tumor blood vessels in mouse xenografts (Fig. 3A). Since it is well known that HIF-1α-mediated VEGF axis plays an important role in tumor angiogenesis, we further investigated whether knockdown of CHC had an impact on HIF-1α and VEGF expressions. Results showed that the protein expression levels of CHC, VEGF, and HIF-1α in the tumor xenografts were significantly reduced in the CHC knockdown group (Fig. 3B and Supplementary Fig. 7). In addition, mRNA level of CHC and VEGF were lowered in CHC knockdown tumor tissues, compared to the control tumor tissues (Fig. 3C). However, the decreased expression of HIF-1α in CHC knockdown xenograft tumor was observed only in protein level (Fig. 3B and Supplementary Fig. 7) but not in the mRNA level (Fig. 3C).

We further investigated the molecular mechanism of CHC on the regulation of VEGF gene expression. VEGF mRNA was increased in mock cells under hypoxia treatment, whereas it was decreased markedly under both hypoxic and normoxic conditions when CHC was knocked down (Fig. 3D). Luciferase assay also showed...
that CHC can participate in the regulation of VEGF-A promoter activity (Fig. 3E). To directly determine whether CHC binds to the hypoxia-response element (HRE) of VEGF-A promoter, we performed chromatin immunoprecipitation (ChIP) assays in CHC-expressing and knockdown cells. We found that CHC bound to the VEGF-A promoter region (Fig. 3F) and that there was a significant decrease in the binding of CHC and HIF-1α to the VEGF-A promoter region in CHC knockdown cells (Fig. 3F and G). These observations suggest that CHC may interact with HIF-1α and co-localize onto the HRE of the VEGF-A promoter, which can then induce VEGF-A expression.

3.4. CHC interacts with and stabilizes HIF-1α in MIA PaCa-2 cells

To investigate whether CHC could interact with HIF-1α, we assessed the co-localization of CHC and HIF-1α by confocal microscopy. Interestingly, CHC was found in 80% of the nuclei of the MIA PaCa-2 cells, while HIF-1α was found in 83% of nuclei of MIA PaCa-2 cells during hypoxia. Together, 70% of the nuclei of the MIA PaCa-2 cells contained both CHC and HIF-1α during hypoxia (Fig. 4A and Supplementary Fig. 8A–C). However, when CHC was knocked down, a low level of HIF-1α was detected in either cytoplasm or nuclei (Fig. 4A and Supplementary Fig. 8A–C). The same results were observed in CL1-5 lung cancer cells (Supplementary Fig. 6F). Subsequently, immunoprecipitation and immuno-electron microscopy were applied to verify protein interaction between CHC and HIF-1α. As shown in Fig. 4B, HIF-1α and CHC were co-immunoprecipitated with each other. The double labeling immuno-electron microscopy data further showed that CHC and HIF-1α were present in the nuclei as well as in the cytoplasm of MIA PaCa-2 cells. The co-localization of CHC and HIF-1α was particularly noticeable inside the nuclei in both mock and control cells (Fig. 4C), suggesting an interaction between the two proteins.

We noticed that the protein level of HIF-1α, which was induced by hypoxia, was decreased in CHC knockdown cells (Fig. 4C and Supplementary Fig. 8D), whereas the mRNA level of HIF-1α was not affected by CHC knockdown (Supplementary Fig. 8E),
suggesting that CHC may influence HIF-1α protein stability. To test whether knockdown of CHC decreased the stability of HIF-1α protein, cells were treated with cycloheximide (CHX) to block de novo protein synthesis. The results suggested that suppression of CHC decreased HIF-1α protein stability (Supplementary Fig. 8F). However, the decreased protein level of HIF-1α in CHC knockdown cells was rescued with the presence of MG132 (a proteasome inhibitor) (Fig. 4D). We further analyzed the expressions of HIF-1α-dependent genes in CHC knockdown cells. The results showed that knockdown of CHC inhibited EPO (Fig. 4E) and PDGF-β gene expressions (Fig. 4F). Similar data were also obtained in tumor xenograft (Fig. 4G). Together, these data suggest that the interaction between CHC and HIF-1α may protect HIF-1α protein stability.

3.5. Inhibition of pancreatic cancer growth and angiogenesis by Pa65-2

Clathrin-mediated endocytosis (CME) is a major mechanism for the internalizations of plasma-membrane receptors [29]. We evaluated the effect of Pa65-2 on the internalization of epidermal growth factor (EGF), known ligand for CME [30]. As shown in Fig. 5A, treatment with Pa65-2 blocked EGF uptake in MIA PaCa-2 cells. In addition, treatment of Pa65-2 not only inhibited cancer cell proliferation (Fig. 5B) and migration (Fig. 5C), but it also induced cell apoptosis in MIA PaCa-2 cells (Fig. 5D). Western blot analysis showed that treatment with Pa65-2 suppressed hypoxia-induced HIF-1α expression and VEGF secretion (Fig. 5E). However, treatment with normal mouse IgG (NM-IgG) showed no inhibitory activities (Fig. 5A–E). Moreover, we also found that Pa65-2 suppressed VEGF internalization and induced apoptosis in HUVECs (Supplementary Fig. 9).

Since CHC shRNA knockdown severely affected xenograft tumor growth in vivo, we investigated whether Pa65-2 could be used to directly inhibit tumor angiogenesis and growth in pancreatic cancer. NOD/SCID mice were inoculated with MIA PaCa-2 cells. When the tumors grew up to 50 mm³, mice were administered with Pa65-2 (10 mg/kg), NM-IgG, or PBS every three days.
Results showed that in the Pa65-2 treated groups, the average tumor volume were about 2-fold smaller than the control groups at day 36 ($n=6$, $p<0.05$; Fig. 6A and Supplementary Fig. 10A). When analyzing the tumor sections, we found that the apoptotic cells were increased ($n=6$, $p<0.01$; Fig. 6B) while the blood vessels were decreased ($p<0.05$; Fig. 6C) in Pa65-2-treated tumors. We further compared the inhibitory effect of Pa65-2 and gemcitabine, a widely accepted first-line treatment for pancreatic cancer, on tumor growth. Results showed that treatment with Pa65-2 had equivalent effects as treatment with gemcitabine (Fig. 6A and Supplementary Fig. 10B). These findings show that Pa65-2 possesses the ability to inhibit pancreatic tumor growth and angiogenesis.

### 4. Discussion

Pa65-2, generated by screening hybridomas against MIA PaCa-2, was found to specifically bind to the cell surface and cytosol of various cancer and tumor blood vessels through interaction with its target, CHC. This study demonstrated that CHC expression is upregulated in cancer cells as well as in tumor-associated endothelial cells. We found that inhibition of CHC by Pa65-2 or shRNA could inhibit tumor growth and angiogenesis. ChIP assays indicated that CHC interacted with HIF-1α and participated in the regulation of VEGF transcription. Knockdown of CHC expression also decreased the protein stability of HIF-1α. To our knowledge, this is the first study to demonstrate that CHC promotes angiogenesis.
and tumor growth by stabilizing HIF-1α, followed by upregulating the expression of VEGF.

CHC has been implicated with a role in cancer. Overexpression of CHC, which is associated with a malignant phenotype, has been reported in hepatocellular carcinoma (HCC) tissues and has been used for the early detection of the disease [31,32]. Clathrin-mediated endocytosis has been reported to enhance receptor tyrosine kinase signaling activity [33] and functions [34], which may play a role in tumor development. Recent research indicates that pharmacological inhibitors of c-Met endocytosis or clathrin knockdown are able to diminish tumor growth and metastasis [35], providing evidence that CHC may promote tumorigenesis. In addition, several studies have shown that endocytosis of VEGFR-2 in endothelial cells occurs mostly through clathrin-dependent endocytosis [36]. Our current study found higher expression levels of CHC in pancreatic cancer tissues as well as in other cancer types. In addition, we found that Pa65-2 binds to tumor cells as well as to tumor-associated endothelial cells. These findings suggest that the expression of CHC in tumor and tumor-associated endothelial cells may be responsible for increasing uptake of growth factors by endocytosis under pathological conditions. Several studies have shown that endocytosis is a dynamic process [37]. During the cellular uptake, receptors and their associated ligands cluster into clathrin-coated pits by association with clathrin adaptor proteins (APs) [38]. Adaptor proteins then recruit and interact with cytoplasmic clathrin to move the clathrin-coated pits to the membrane [29]. Clathrin adaptors were thought to bind to the clathrin, which provides the force required to deform the membrane into a curved bud [39], and then the large GDPase dynamin is involved in the detachment of clathrin-mediated endocytosis from the membrane [40]. However, in this study, we found that Pa65-2 bound to the cell surface of MIA PaCa-2 cells using FACS (Supplementary Fig. 1D), immuno-electron microscopy (Supplementary Fig. 1F) and confocal microscopy (Supplementary Fig. 1G) analyses. In pathological conditions such as cancer, cells might express CHC on plasma membrane that could be recognized by anti-CHC antibody.

Recently, it has been reported that CHC binds to p53 [41] and NF-κB [42] in the nuclei. Here, we also found CHC may interact with HIF-1α in the nucleus and the cytoplasm under hypoxic conditions. HIF-1α protein degradation has been reported to lead to a decrease in the transcription of HIF-1α downstream genes [43-45]. In our current study, diminishing CHC levels by shRNA resulted in decreased protein stability of HIF-1α and decreased expressions of HIF-1α downstream genes. CHC may serve as both a stabilizer and transport mediator [46] that translocate HIF-1α from the cytosol to the nucleus under hypoxic conditions. After CHC knockdown, HIF-1α may become unprotected from proteasomal degradation and hence, less stable, which results in less HIF-1α localization in nuclei. Previous reports used structure prediction to describe the interaction between Asn1288 of CHC, a crucial amino acid residue, and p53 [47]. CHC may stabilize HIF-1α and regulate HIF-1α-mediated transcription in a similar manner. However, further investigation is required to identify the binding interface between CHC and HIF-1α.

Base on our studies, we propose that the CHC promotes tumor growth and angiogenic process through two pathways. One is to increase the uptake of growth factors or receptors by clathrin-mediated endocytosis; the other is to increase the expression of VEGF, possibly by interacting and stabilizing HIF-1α under hypoxic condition (Fig. 6D). In conclusion, we have generated a monoclonal antibody, Pa65-2, which specifically binds to pancreatic cancer cells and tumor-associated endothelial cells through its recognition of CHC. Our results strongly implicate CHC in promoting tumor growth, invasion and angiogenesis in vitro and in vivo. Pa65-2 inhibited EGF and VEGF internalizations, and had similar anti-tumor activity as gemcitabine. Our results suggest that Pa65-2 mAb or CHC shRNA can potentially be used to reduce tumorigenesis and angiogenesis.
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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.canlet.2012.12.001.

References
