



US008969530B2

(12) **United States Patent**
Wu et al.

(10) **Patent No.:** **US 8,969,530 B2**
 (45) **Date of Patent:** **Mar. 3, 2015**

(54) **ANTI-CLATHRIN HEAVY CHAIN
 MONOCLONAL ANTIBODY FOR
 INHIBITION OF TUMOR ANGIOGENESIS
 AND GROWTH AND APPLICATION
 THEREOF**

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(*) Notice: Subject to any disclaimer, the term of this
 patent is extended or adjusted under 35
 U.S.C. 154(b) by 229 days.

(21) Appl. No.: **13/644,311**

(22) Filed: **Oct. 4, 2012**

(65) **Prior Publication Data**

US 2013/0084298 A1 Apr. 4, 2013

Related U.S. Application Data

(60) Provisional application No. 61/543,118, filed on Oct.
 4, 2011.

(51) **Int. Cl.**

C07K 16/00 (2006.01)
C12P 21/08 (2006.01)
G01N 33/53 (2006.01)
A61K 39/395 (2006.01)
A61K 39/00 (2006.01)
C07K 16/18 (2006.01)
C07K 16/30 (2006.01)

(52) **U.S. Cl.**

CPC **C07K 16/18** (2013.01); **C07K 16/303**
 (2013.01); **C07K 2317/73** (2013.01)
 USPC **530/388.1**; 530/387.3; 530/387.7;
 435/7.1; 424/130.1; 424/133.1; 424/138.1;
 424/141.1; 424/155.1

(58) **Field of Classification Search**

None
 See application file for complete search history.

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(57)

ABSTRACT

A purified monoclonal antibody, or an. antigen-binding por-
 tion thereof, which specifically binds to human clathrin heavy
 chain (CHC) is disclosed. The antibody, or antigen-binding
 portion, thereof, exhibits at least one, two, three, four, five,
 six, seven, or all eight of the following properties: (a) specifi-
 cally binds to pancreatic adenocarcinoma cells; (b) binding to
 the cell surface and cytosol of cancer cells and tumor blood
 vessels; (c) internalized by CHC-expressing cells; (d) inhib-
 iting tumor growth, invasion ability, migration, and angiogen-
 esis; (e) inducing apoptosis in cancer cells and human umbili-
 cal vein endothelial cells; (f) inhibiting tumor growth and
 tumor blood vessels in pancreatic cancer in vivo; (g) sup-
 pressing epidermal growth factor, transferrin, and VEGF
 internalizations by cancer cells; and (h) suppressing hypoxia-
 inducible factor-1 α expression and vascular endothelial
 growth factor secretion. Methods for inhibiting tumor cell
 growth and/or angiogenesis, and detecting cancer in a subject
 is also disclosed.

18 Claims, 14 Drawing Sheets

FIG. 1

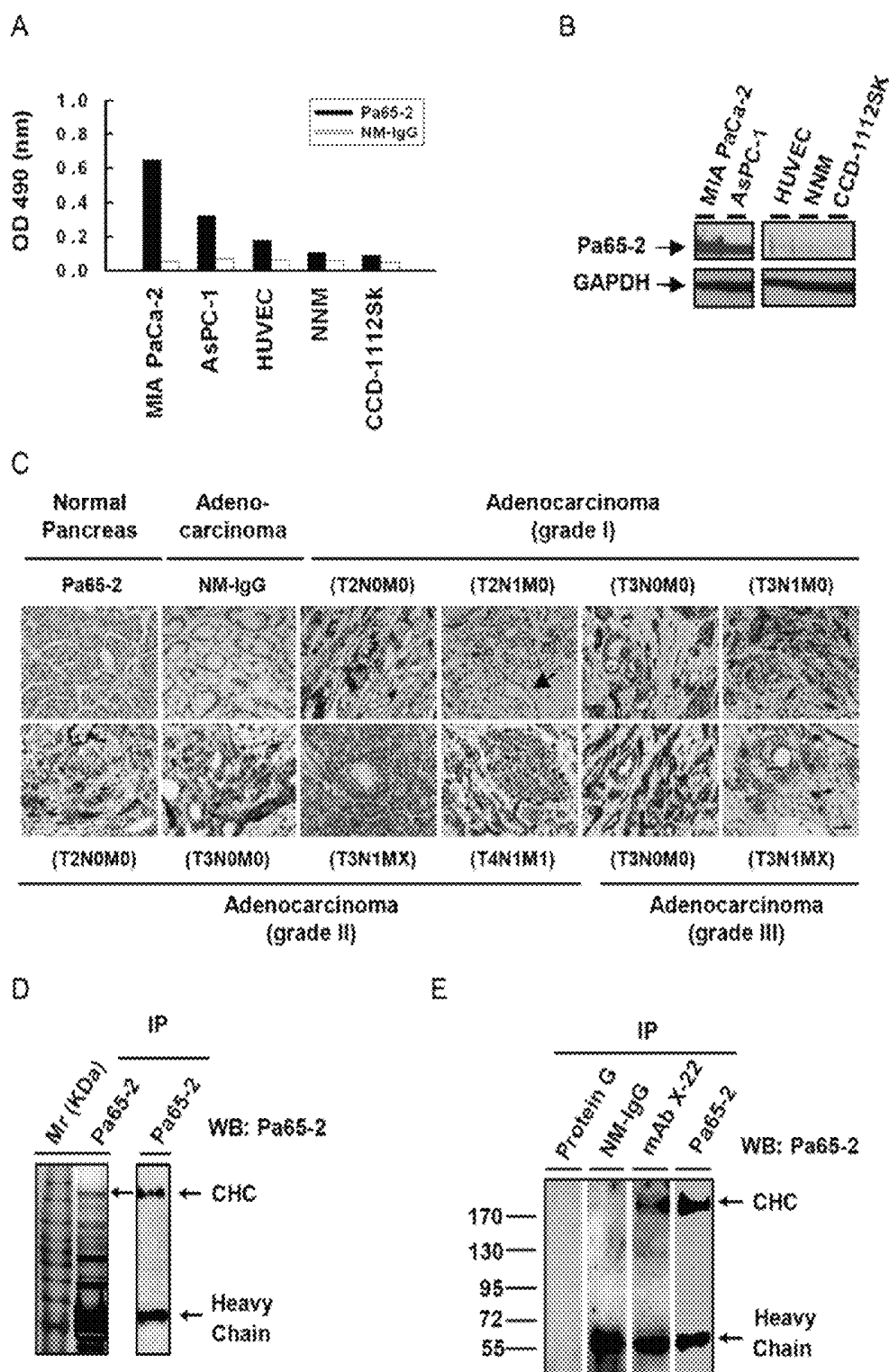


FIG. 2

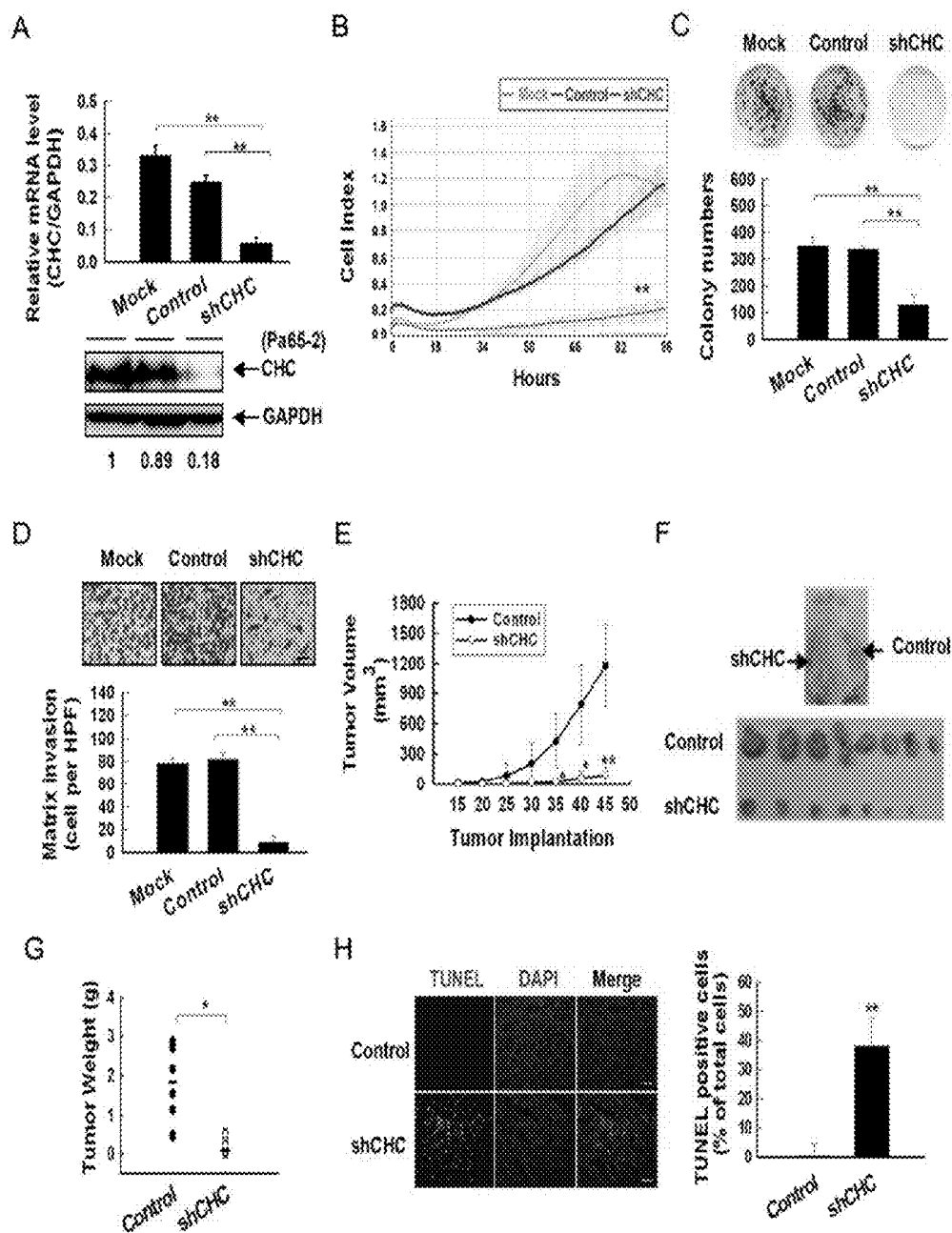


FIG. 3

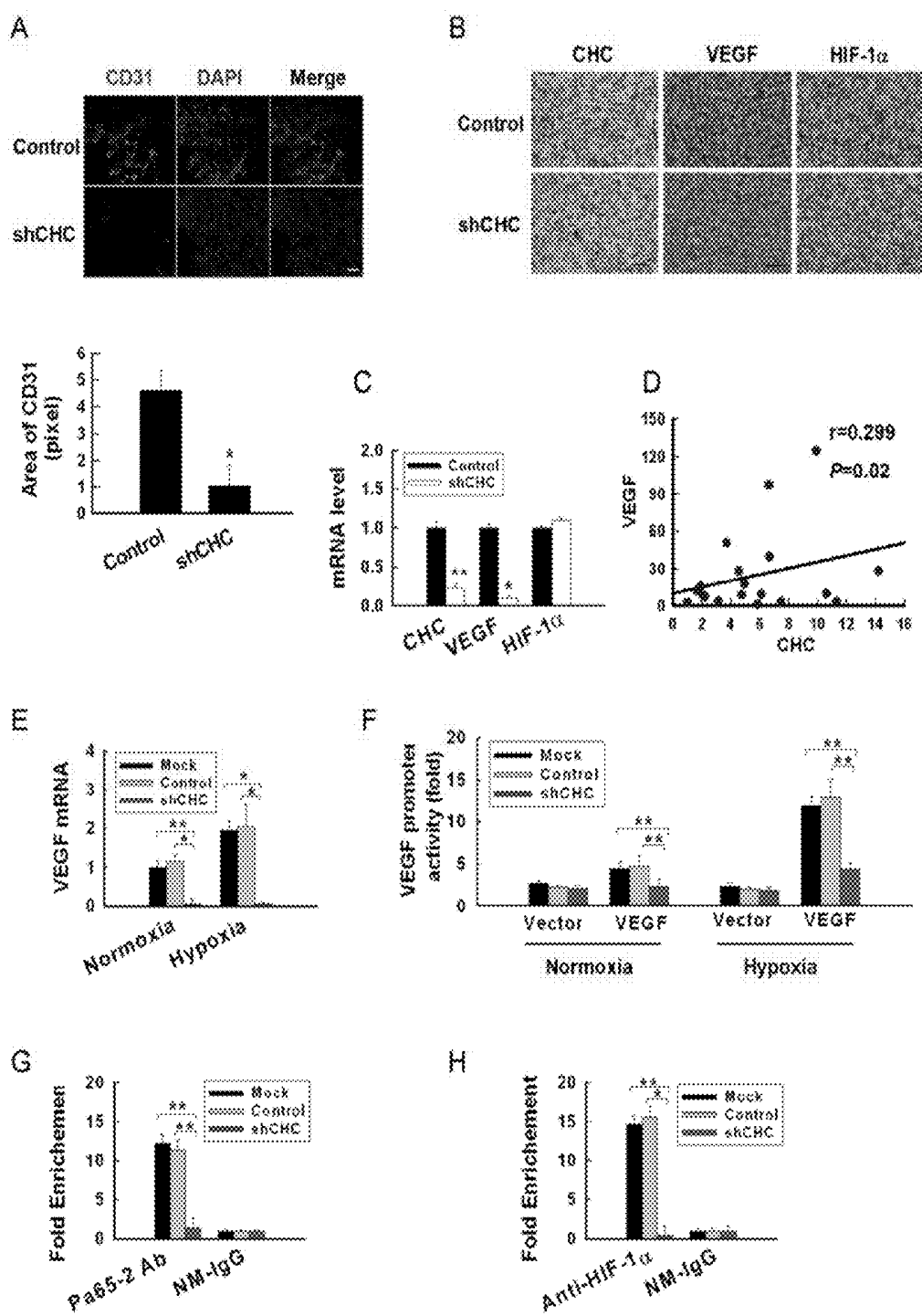


FIG. 4

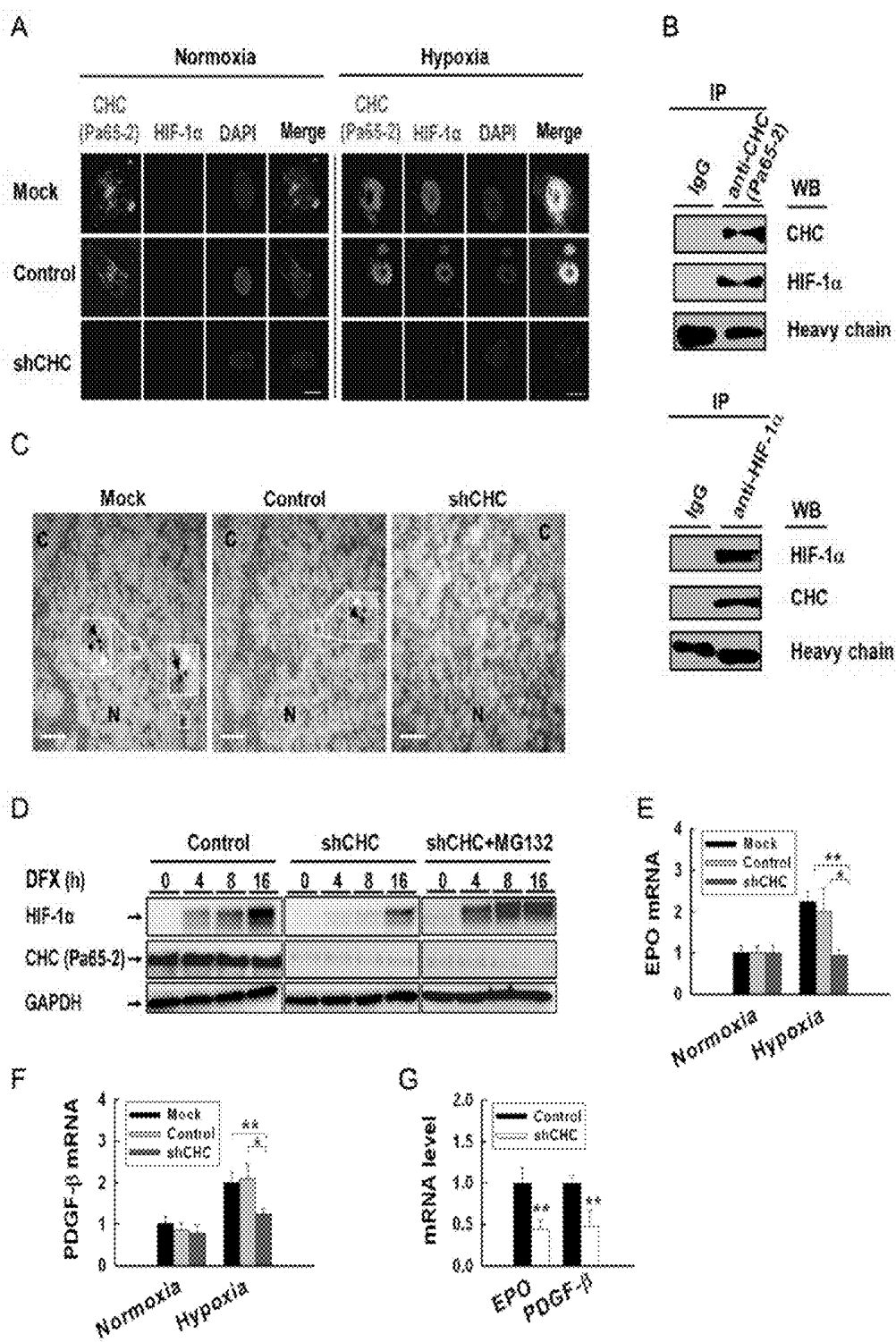


FIG. 5

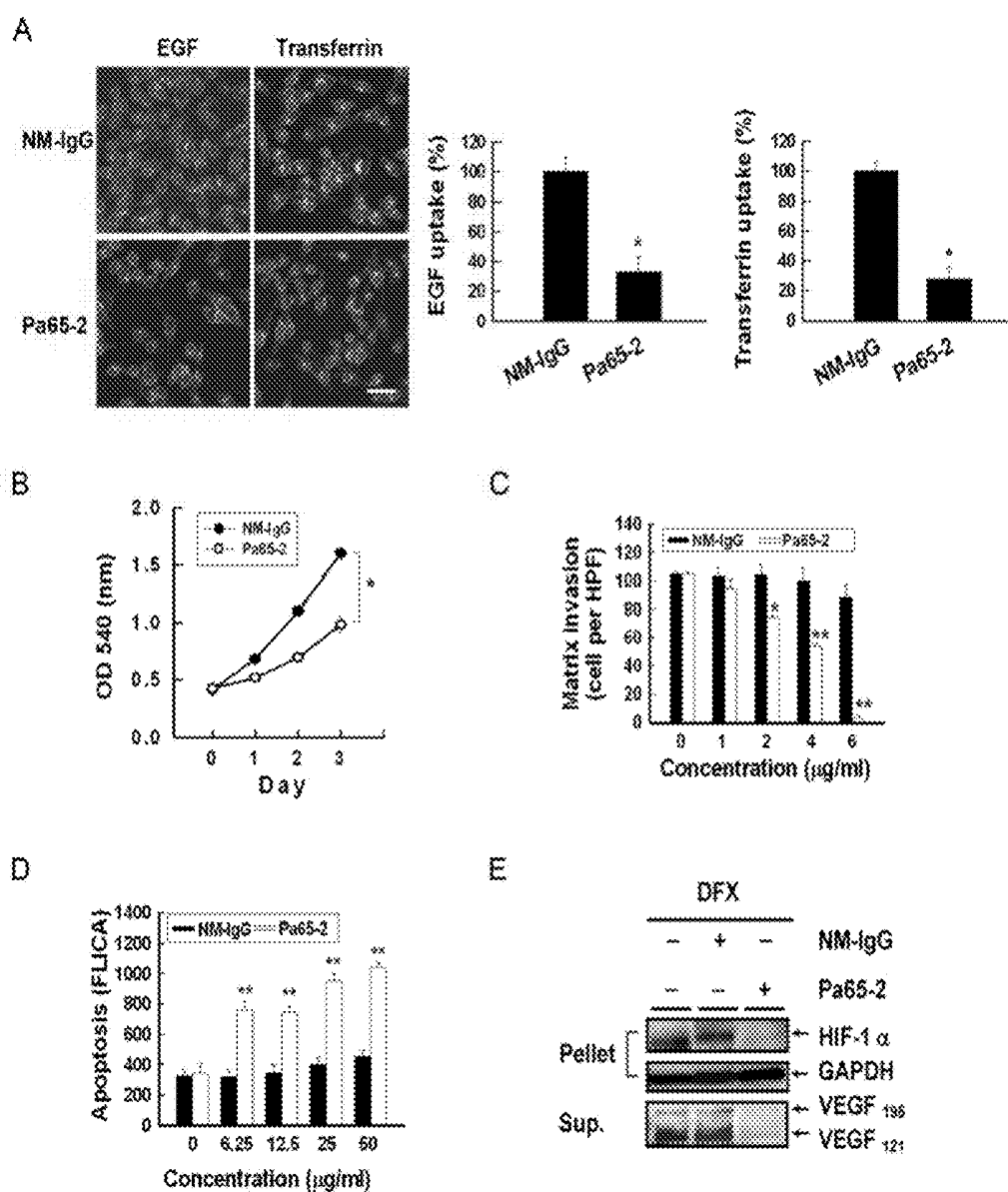


FIG. 6

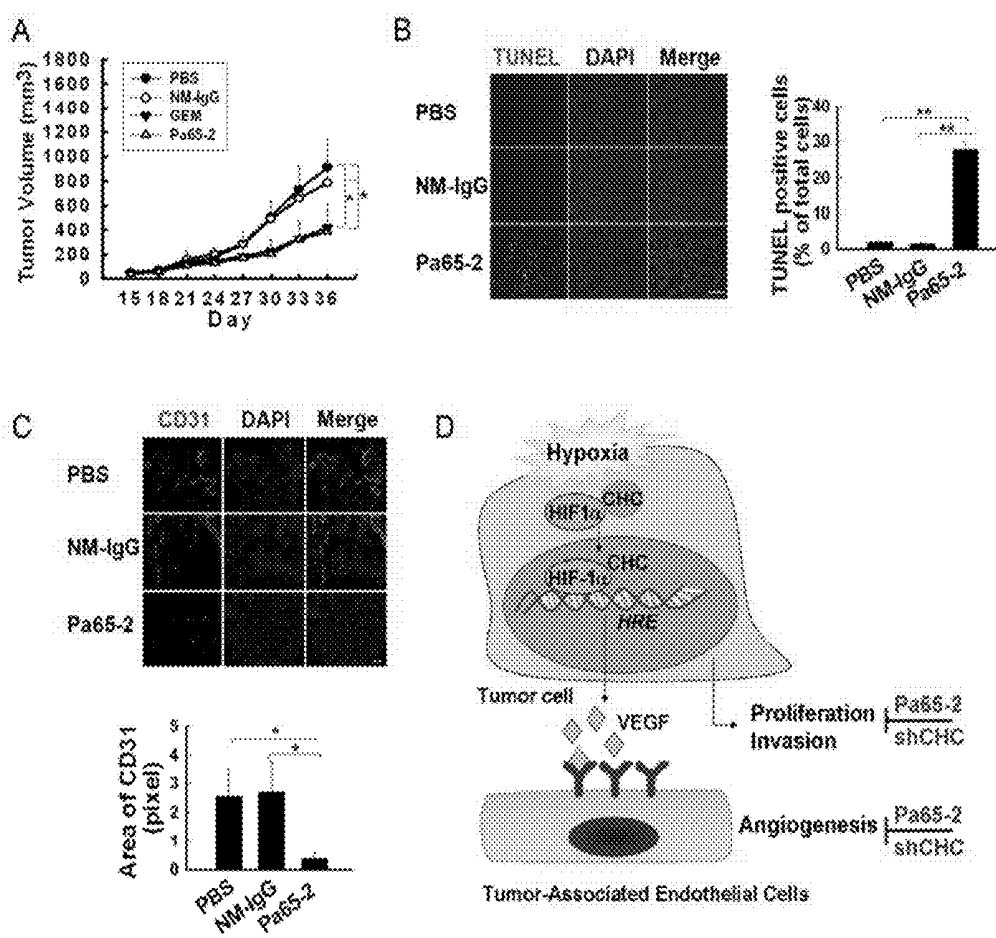


FIG. 7

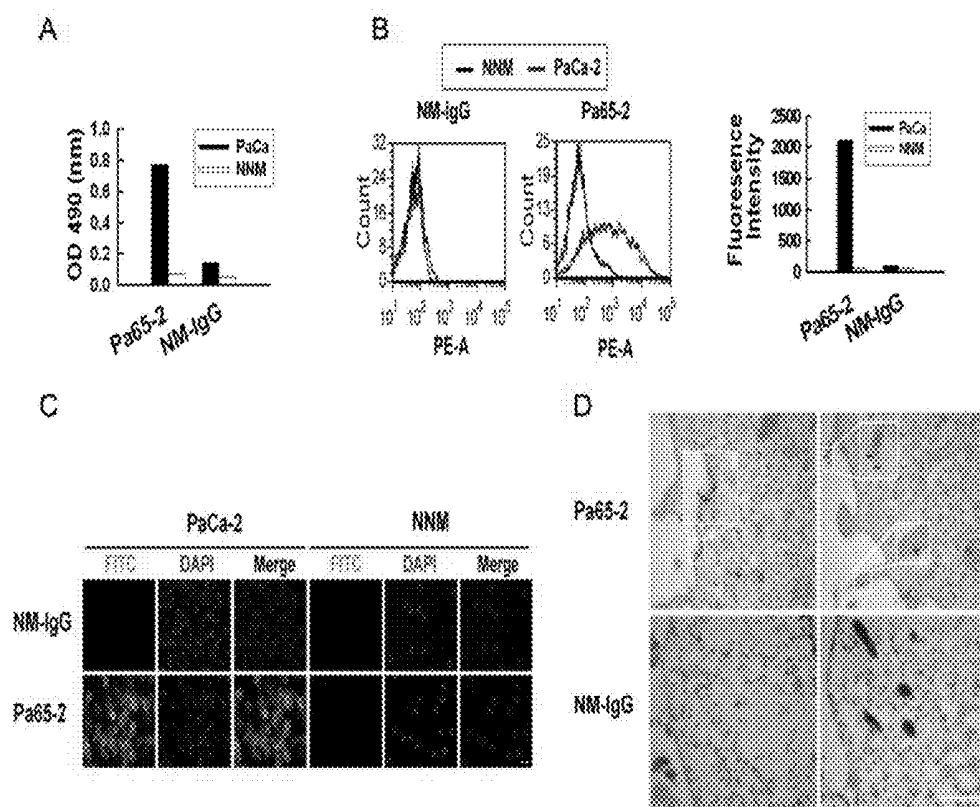


FIG. 8

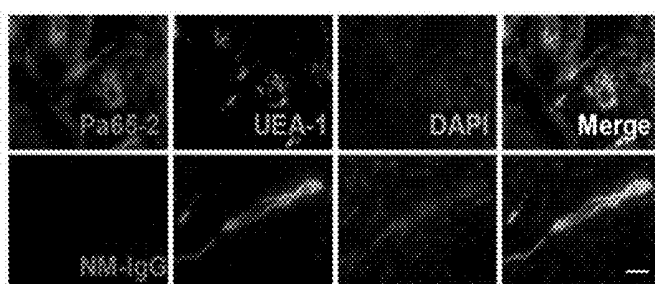
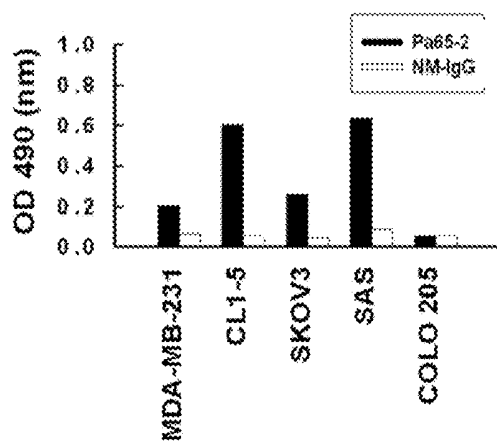


FIG. 9

A



B

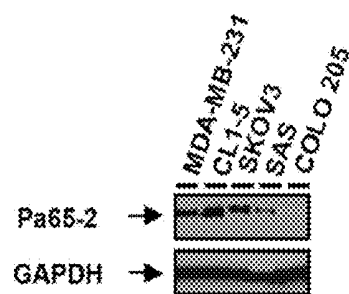
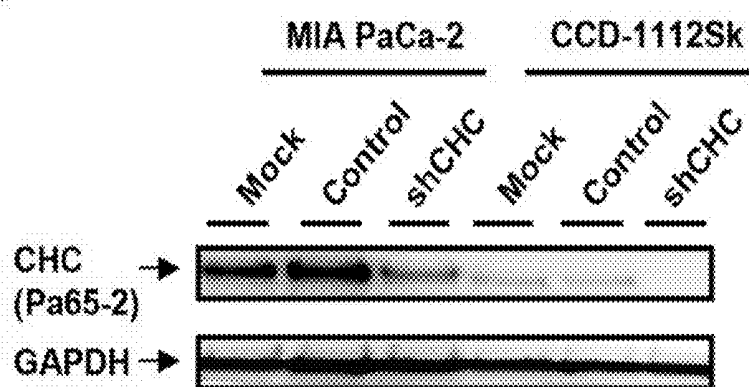


FIG. 10

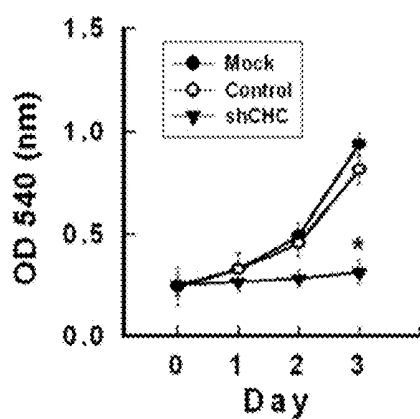
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101 AHTMTDDVTF WKWISLNTVA LVTDNNAVYHW SMEGESQPVE MFDNRSSLAG
151 CQIINYRTDA KOKWLLLTGI SAQQSRVYGA MQLYSVDRKY SQPIEGHAAS
201 FAQPKMEGNA EESTLFCFAV RGGAGGKLHI LEVGTPTTGN QPFPPKAVDV
251 FFPPEAQNDP PVAMQISEKH DVVFLITKYG VHLVDLETG TCIVMNRISG
301 ETIFVYAPHE ATAGIIGYNR KGQVLSVCVE GENIIPYITN YLONPDIALR
351 MAVRNNLACA EELFARKFNA LEAQGNVSEA AKVAANAPKG IIRTPDTIRR
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451 WLKEDKLECS EELQDLVKS V DPTLALS VYL HANVPNKVIQ CFAETGQVQK
501 IYLYAKKVG Y IPDWIFLLRN VMRISPDQGG QFAQMLVQDE EPLADITQIV
551 DVFMEYNLIQ OCTAFLLDAU KNNRPSEGPI QTRLLEMNLM HAPQVADAIL
601 GNQMFTBYDR AHIAQLCEKA GLIQRALEHF IDLYDIKRAV VHTHLNPEW
651 LVNVFGSLSY EDSLECCRAM LSANIRQNLO ICVQVASNYH EQLSTQSLIE
701 LFSFYSFPEG LFFYFLOSVN FSQDPDVHFK YIQAACTGQ IKEVERICRE
751 SNCYDPERVK NFKLKAALTD QLPILIVCDR FDFVHDLVLY LYRNNIQKVI
801 ELYVQKVNFS RLPVVI GGLL DVDCSEDEVK NLILVVRGQF STDELVAEVE
851 KRNRLEKLLLP WLEARI HEGC EEPATBNALA KLYIDSNNP ERFIRENPPY
901 DSEYVGKVCU KRDPHLACVA YENGQCDEL INVCNENSLF KSLSRVLRK
951 KDPFLWGSVL LESNPPYRRL IDQYVQTALS ETQDPSEVS VTKAFMTADL
1001 FNELIELLEK IYLDNSVFSR HRNLQNLIL TAIKADRIRV MEYINRLUNY
1051 DAPDIANI AI SNELFEKFAF IFRKFDVNTS AVQVLEHIG NIDRAYEPAE
1101 RCNEPAVWSQ LAKAQLQEGM VKEAIDSYIK ADDPSSYMEV YQAANTSGNW
1151 EELVKYLQMA RKKARGSYVE TELIFALAKT NRLAELEFFI NGPNNAHIQQ
1201 YGDRCYDEKM YDAAKLLYNN VSNFGRLAST LYBLGEYQAA YDGRKANST
1251 RTWKREYCFAC YDGKEFRLAQ MCGLNIVVHA DELEELINYY QDRGYFEELI
1301 TMLVAAALQE RANMGMFTEL AILYSKFKPQ KMRENLELFW SRVNI PKVLR
1351 AAEEQAHLWAE LVFLYDKYEE YDNALIIYMMN HPTDAWKEG FKDIIITKYAN
1401 YELYVRAIQF YLEFKPLLLN DULMVLSPLR DHTRAVNYFS KVKQLPLVKP
1451 YLRSYQNNNN KSYNCSLNNL FITKEEDYQAL RTSIDAYDNF DNISLAQRIE
1501 KHELICTFRI AAYLFKGNR WRQSVELCKE DSYKGDAMQV ASESKDTECA
1551 EELLQWFLQE EKRECFGACL FTCYDILLRPD VVLETAWEHN IMDTAMPYFI
1601 QYMKREYLT KY DKLDASES LR KEESQATETQ PIVYGGPQLM LTAGPSVAVP
1651 FQAPFGVGYI APPYGGPQPG FGYSM

FIG. 11

A



B



C

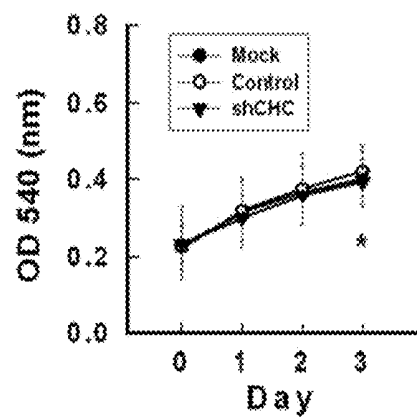


FIG. 12

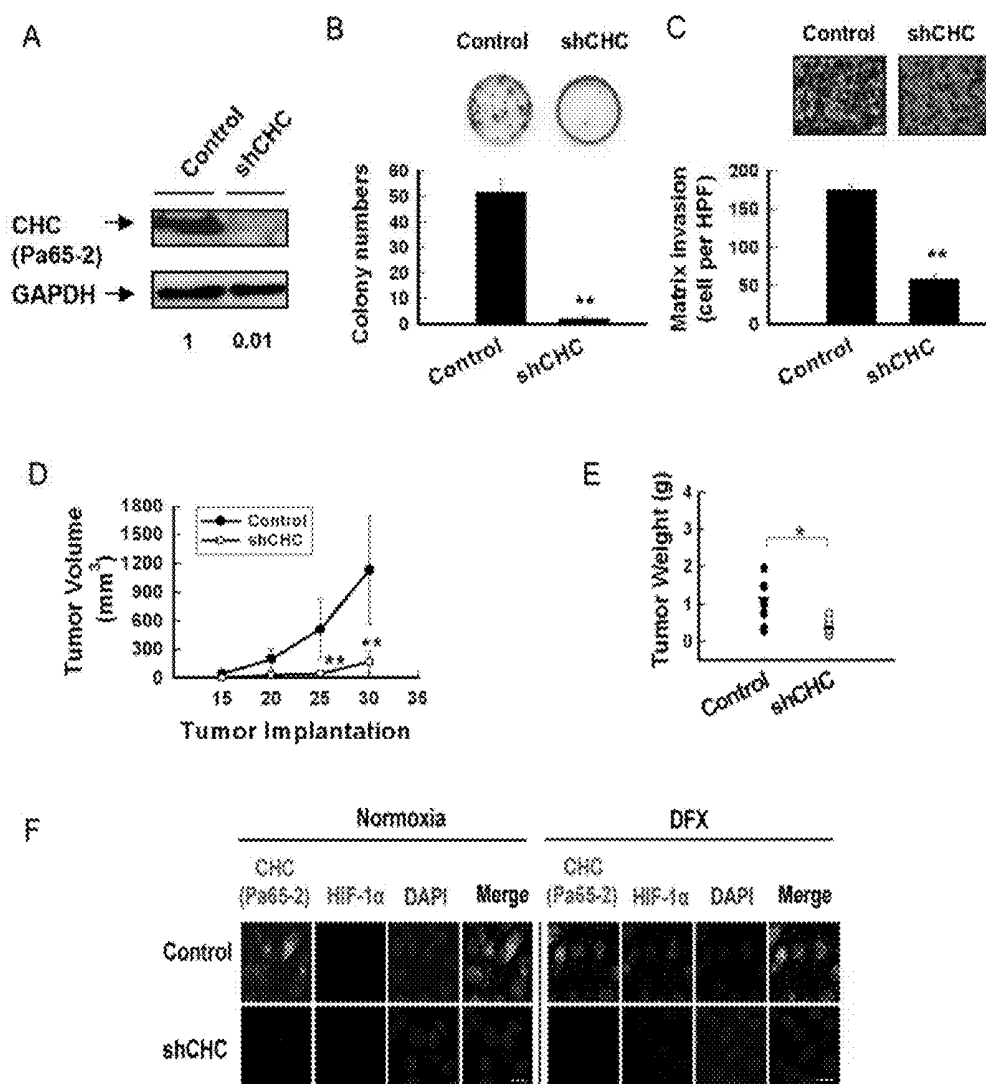


FIG. 13

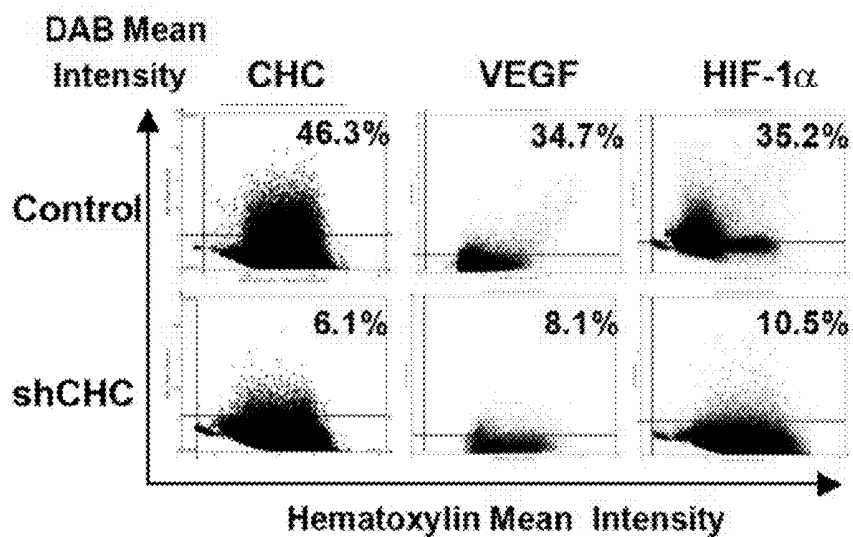
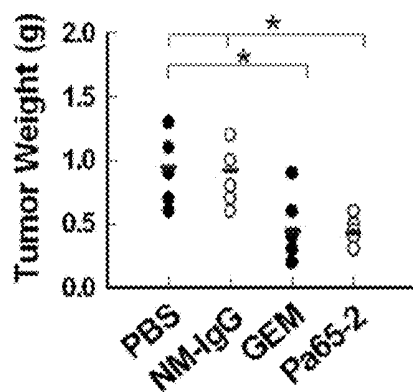


FIG. 16

A



B

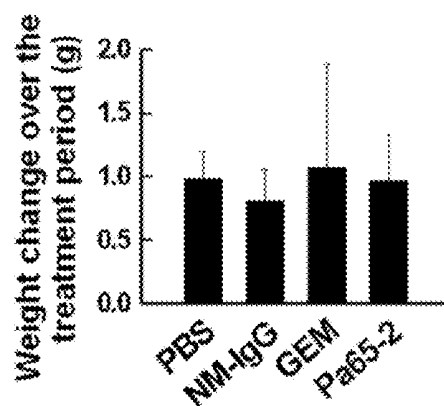


FIG. 14

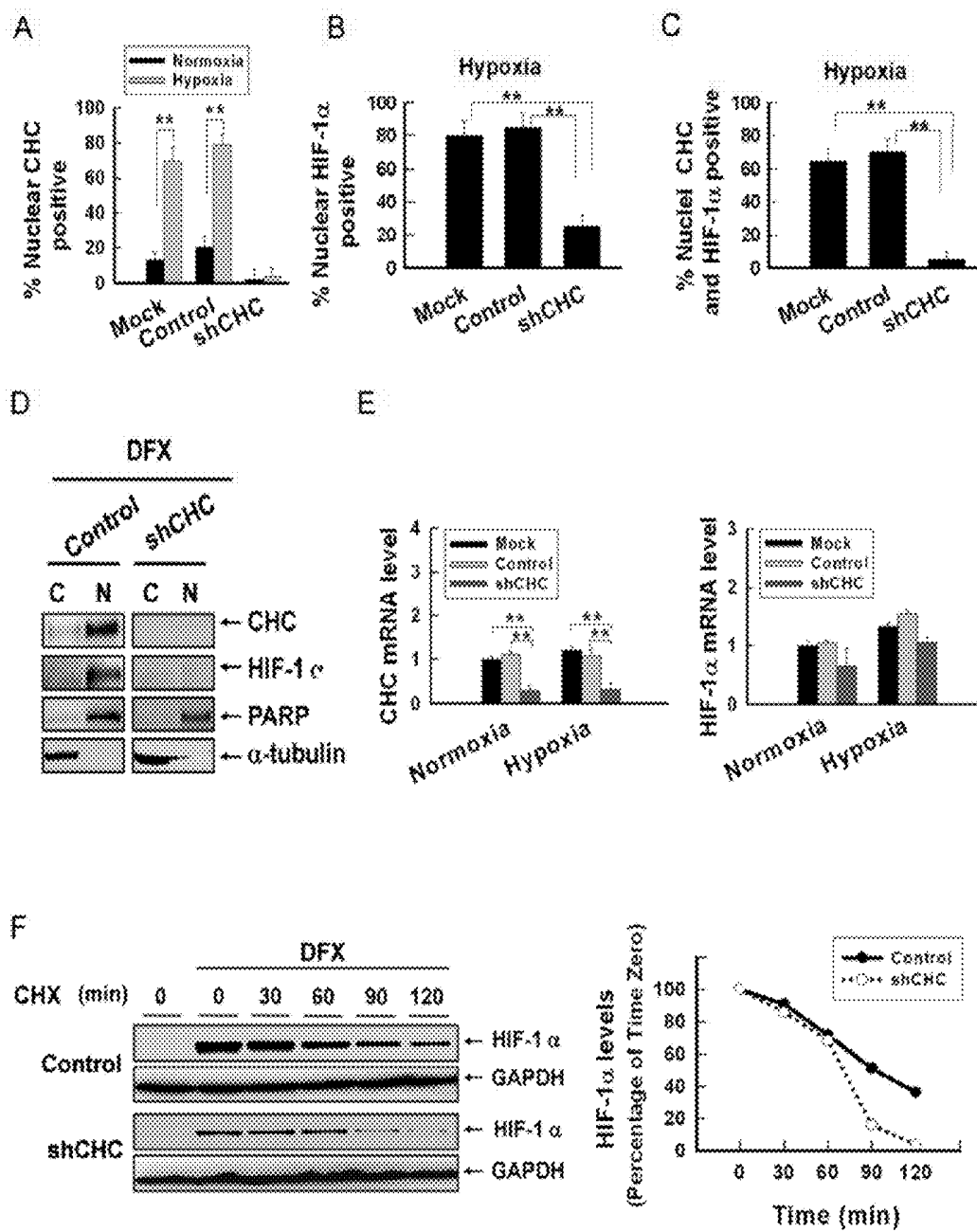
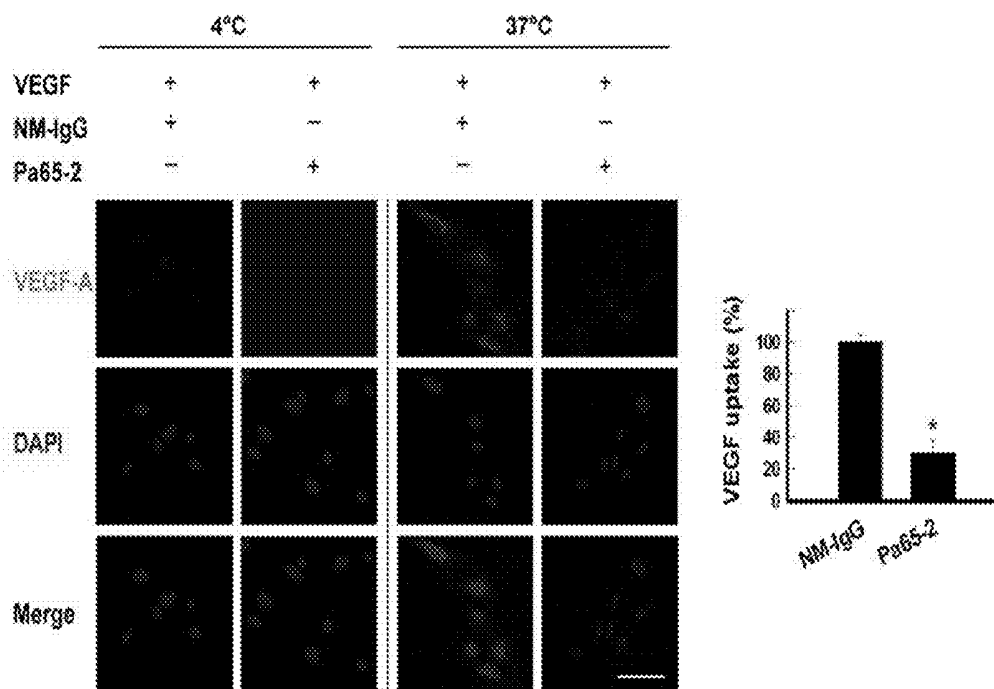
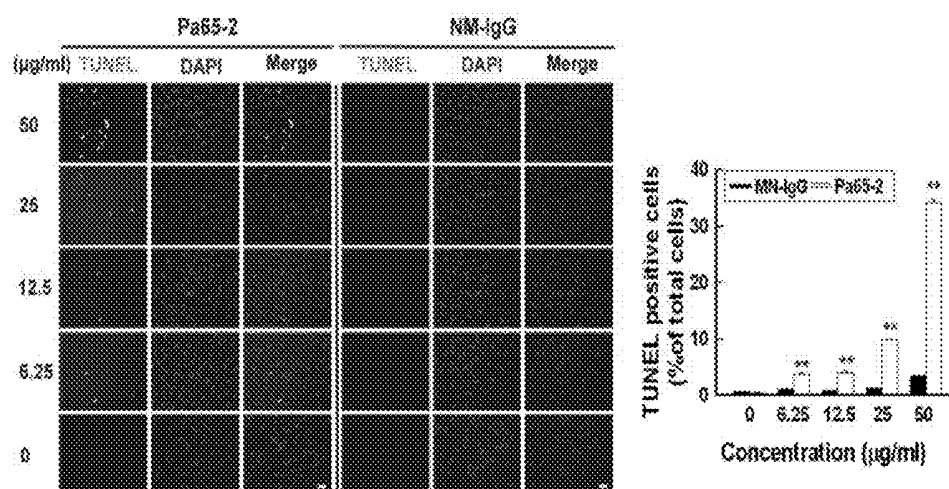


FIG. 15

A



B



1

ANTI-CLATHRIN HEAVY CHAIN MONOCLONAL ANTIBODY FOR INHIBITION OF TUMOR ANGIOGENESIS AND GROWTH AND APPLICATION THEREOF

REFERENCE TO RELATED APPLICATION

The present application claims the priority to U.S. Provisional Application Ser. No. 61/543,118, filed Oct. 4, 2011, which is herein incorporated by reference in its entirety.

FIELD OF THE INVENTION

The present invention relates generally to anti-cancer agent, and more specifically to antibodies for treating cancers.

BACKGROUND OF THE INVENTION

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. Pancreatic cancer often recurs after initial treatment despite the use of chemotherapy or radiation therapy. 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.

Two monoclonal antibody (mAbs) drugs currently in clinical trial for targeted therapy against pancreatic cancers are cetuximab and bevacizumab, 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 pancreatic cancer patients.

Therefore, it is important to identify a suitable target for developing targeted therapy against pancreatic cancer.

SUMMARY OF THE INVENTION

In one aspect, the invention relates to a purified monoclonal antibody, or an antigen-binding portion thereof, which specifically binds to human clathrin heavy chain (CHC) comprising the amino acid sequence of SEQ ID NO: 1.

In another aspect, the invention relates to an isolated monoclonal antibody, or a binding fragment thereof. The isolated monoclonal antibody, or a binding fragment thereof comprises a heavy chain variable region and a light chain variable region, in which the heavy chain variable region comprises: (i) complementarity determining region 1 (CDR1) comprising SEQ ID NO: 4; (ii) complementarity determining region 2 (CDR2) comprising SEQ ID NO: 5; and (iii) complementarity determining region 3 (CDR3) comprising SEQ ID NO: 6; and the light chain variable region comprises: (i) CDR1 comprising SEQ ID NO: 7; (ii) CDR2 comprising SEQ ID NO: 8; and (iii) CDR3 comprising SEQ ID NO: 9.

In another aspect, the invention relates to a method for inhibiting tumor cell growth and/or tumor angiogenesis, which comprises administering to a subject, in need thereof a composition comprising the aforementioned purified monoclonal antibody, or antigen-binding portion thereof, and a pharmaceutically acceptable carrier.

Further in another aspect, the invention relate to a method for inhibiting tumor growth and/or tumor angiogenesis, which comprises: administering to a subject in need thereof a

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composition comprising the aforementioned isolated monoclonal antibody, or binding fragment thereof, and a pharmaceutically acceptable carrier.

Further in another aspect, the invention relate to an isolated single-chain variable fragment comprising: (a) the heavy chain variable region (SEQ ID NO: 2) and the light chain, variable region (SEQ ID NO: 3) of the isolated antibody or binding fragment as aforementioned; and (b) a linker peptide connecting the heavy chain variable region (SEQ ID NO: 2) and the light chain variable region (SEQ ID NO: 3).

Further in another aspect, the invention relate to a method for detecting cancer in a subject, which comprises: (a) applying the isolated monoclonal antibody, or binding fragment thereof, of claim 4 to a cell or tissue sample obtained from the subject; and (b) assaying the binding of the isolated monoclonal antibody, or binding fragment thereof to the cell or the tissue sample; and (c) comparing the binding with a normal control to determine the presence of the cancer in the subject, wherein the cancer expresses human clathrin heavy chain.

Yet in another aspect, the invention relates to a composition comprising: (a) the aforementioned isolated monoclonal antibody or binding fragment thereof; and (b) a pharmaceutically acceptable carrier.

These and other aspects will become apparent from the following description of the preferred embodiment taken in conjunction with the following drawings, although variations and modifications therein may be affected without departing from the spirit and scope of the novel concepts of the disclosure.

The accompanying drawings illustrate one or more embodiments of the invention and, together with the written description, serve to explain the principles of the invention. Wherever possible, the same reference numbers are used throughout the drawings to refer to the same or like elements of an embodiment.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows generation and characterization of mAbs against pancreatic cancer. (A) ELISA and (B) Western blot analysis of the binding activity of Pa65-2 in pancreatic adenocarcinoma cell lines and various normal cell lines. NM-IgG was used as a control. (C) Immunohistochemistry analysis of Pa65-2 in human pancreatic cancer tissue array. The arrow indicates a blood vessel-like structure. Scale bar, 50 μ m. (D) Purification of Pa65-2-targeted protein by immunoaffinity chromatography. Lane 1, molecular weight marker; lane 2, purified proteins from Pa65-2-conjugated affinity column; and lane 3, Western blot analysis of purified proteins from Pa65-2-conjugated affinity column. (E) MIA PaCa-2 whole-cell lysates were immunoprecipitated with Pa65-2 and anti-CHC and control antibodies followed by Western blot analysis.

FIG. 2 shows inhibition of pancreatic cancer growth by suppressing CHC expression, (A) The results of CHC knock-down were determined by quantitative RT-PCR (upper panel) and Western blot analysis (lower panel). The band signal of CHC was normalized to GAPDH. Error bars denote $\pm$ standard deviation (SD). **P<0.01. (B) A microelectronic cell sensor system analysis of cell proliferation in mock, control vector and shCHC-transfected MIA PaCa-2 cells. Error bars denote $\pm$ standard deviation (SD). **P<0.01. (C) Colony formation potential of MIA PaCa-2 cells. Representative images are shown in the upper panel. Error bars denote $\pm$ SD. **P<0.01. (D) Knockdown of CHC expression reduced invasion. Scale bar, 40 μ m; Error bars denote $\pm$ SD. **P<0.01. (E) Eight NOD/SOD mice were injected s.c. (1×10^7 cells) in the lateral

portion of the hind limb, right side with, the control vector-transduced MSA PaCa-2, and left side with the CHC shRNA-transduced MIA PaCa-2. The tumors were measured with calipers every five days, and tumor volume was calculated as $\text{length} \times (\text{width})^2 \times 0.52$. Error bars denote $\pm$ SD. * $P < 0.05$; ** $P < 0.01$. (F) Representative images of tumor-bearing mice (upper panel) and tumor burden (lower panel) are shown. (G) Tumor weight was measured. Error bars denote $\pm$ SD. * $P < 0.05$. (H) Detection of apoptosis induced by CHC shRNA in MIA PaCa-2 cells by TUNEL staining. Scale bar, 200 μ m; Error bars denote $\pm$ SD. ** $P < 0.01$.

FIG. 3 shows CHC regulation of VEGF expression in pancreatic cancer. (A) Expression of endothelial cell marker CD31 in CHC knockdown tumor sections by immunofluorescent staining (upper panel). Quantification of fluorescence intensity was performed using MetaMorph software (lower panel). Scale bar, 200 μ m; Error bars denote $\pm$ SD. * $P < 0.05$. (B) Immunohistochemical localization of CHC, VEGF and HIF1- α in tumor sections. Scale bar, 40 μ m. (C) Quantitative RT-PCR analyses of CHC, VEGF and HIF-1 α gene expressions in xenograft tumors. Gene expression levels were normalized to GAPDH signal. Error bars denote $\pm$ SD. * $P < 0.05$; ** $P < 0.01$. (D) CHC and VEGF mRNA levels in samples obtained from 19 human pancreatic cancer patients were measured by quantitative RT-PCR analysis. Correlation between CHC and VEGF expression was evaluated by Spearman's analysis, ($r = 0.299$; $P = 0.02$). (E) Quantitative RT-PCR analysis of VEGF mRNA in CHC knockdown MIA PaCa-2 cells. Expression of VEGF was normalized to GAPDH. Error bars denote $\pm$ SD. * $P < 0.05$; ** $P < 0.01$. (F) Effect of CHC on activation of the VEGF promoter in MIA PaCa-2 cells after hypoxia. The cells were transfected with reporter plasmids and subjected to luciferase assay. Error bars denote $\pm$ SD. ** $P < 0.01$. (G) and (H) Analysis of binding of CHC and HIF-1 α on the VEGF promoter by chromatin immunoprecipitation (ChIP) analyses after hypoxia for 16 h. Specific primer sets for the VEGF promoter region containing two HIF-1 α binding sites (-534 to -158) were used. A ChIP assay was performed using antibodies against (G) CHC (Pa65-2) and (H) HIF-1 α . Normal mouse IgG was used as a negative control. Error bars denote $\pm$ SD. * $P < 0.05$; ** $P < 0.01$.

FIG. 4 shows CHC interacts with and stabilizes HIF-1 α in MIA PaCa-2 cells. (A) Co-localization of CHC and HIF-1 α after double immunofluorescent staining with antibodies against CHC (FITC staining would show green color) or HIF-1 α (Rhodamine staining would show red color) was analyzed by confocal microscopy. Scale bar, 10 μ m. (B) Co-immunoprecipitation of cell lysates with antibodies against CHC and HIF-1 α and subsequent immunoblotting with Pa65-2 and anti-HIF-1 α antibodies. (C) Co-localization of CHC and HIF-1 α in the nuclei of MIA PaCa-2 cells by double labeling immuno-electron microscopy. Mock (left panel) and control (middle panel) cells both showed co-localization of CHC, labeled by 18 nm colloidal gold conjugated antibody (arrows, insert), and HIF-1 α , labeled by 12 nm colloidal gold conjugated antibody (white arrows, insert), in the nuclear regions. N. shCHC-transduced cells (right panel) showed marked reduction of CHC signaling and negligible labeling of HIF-1 α in the nucleus (C, cytosol, N, nucleus). Scale bar, 200 nm. (D) MIA PaCa-2 cells were incubated with DFX (100 μ M) for 0 to 16 h in the absence (left, and middle panel) or presence of the proteasome inhibitor MG-132 (10 μ M) (right panel). Whole-cell lysates from different time intervals were analyzed by Western blot analysis. (E) Quantitative RT-PCR analyses of EPO and (F) PDGF- β mRNA in CHC knockdown MIA PaCa-2 cells. Error bars denote $\pm$ SD. * $P < 0.05$; ** $P < 0.01$. (G) Quantitative RT-PCR analyses of EPO and

PDGF- β gene expressions in xenograft tumors. Gene expression levels were normalized to GAPDH signal. Error bars denote $\pm$ SD. ** $P < 0.01$.

FIG. 5 shows inhibition of ligand internalization and pancreatic cancer cells growth by Pa65-2. (A) Immunofluorescent analysis of EGF and transferrin internalization in MIA PaCa-2 cells after CHC inhibition by Pa65-2. MIA PaCa-2 cells, which were pretreated with Pa65-2 or NM-IgG at 37° C. and subsequently incubated with Alex 555-EGF (1 μ g/ml) or Alex 555-transferrin (50 μ g/ml) at 37° C. (red: Rhodamine staining), were subjected to plasma membrane staining using WGA-Alex 647 (green) and nucleus staining using DAPI (blue). Scale bar, 20 μ m. (left, panel). The percentage of EGF-Alex 555 (middle panel) or transferrin-Alex 555 (right panel) staining was calculated based on a total of 100 MIA PaCa-2 cells. Staining was analyzed by confocal microscopy. Error bars denote $\pm$ SD. * $P < 0.05$. (B) MIA PaCa-2 cells were treated with Pa65-2 or NM-IgG (50 μ g/ml) for 3 days. Cell proliferation was detected by MTT (3-(4,5-cimetlylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) analysis. Error bars denote $\pm$ SD. * $P < 0.05$. (C) Pa65-2 inhibited MIA PaCa-2 cell invasion. Error bars denote $\pm$ SD. * $P < 0.05$; ** $P < 0.01$. (D) Pa65-2 induced MIA PaCa-2 cell apoptosis. Error bars denote $\pm$ SD. ** $P < 0.01$. (E) MIA PaCa-2 cells were incubated with DFX (100 μ M) in the presence of Pa65-2 (50 μ g/ml) or NM-IgG (50 μ g/ml) for 48 h. The culture media and whole-cell lysates were analyzed by Western blot analysis.

FIG. 6 shows Pa65-2 suppression of pancreatic xenograft tumor growth. (A) Tumor-bearing mice (average tumor sizes ranging from 40 to 60 mm³), received i.v. injections with 10 mg/kg of Pa65-2 or 15 mg/kg of gemcitabine (GEM), or NM-IgG, or PBS. Tumor sizes were measured every three days ($n = 6$, each group). Error bars denote $\pm$ SD. * $P < 0.05$. (B) Detection of Pa65-2-induced apoptosis in tumor tissues by TUNEL staining (left panel). Quantification of fluorescence intensity was performed using MetaMorph software (right panel). Scale bar, 200 μ m; Error bars denote $\pm$ SD. ** $P < 0.01$. (C) Expression of endothelial marker (CD31) within tumor sections was analyzed by immunofluorescent staining (upper panel). Quantification of fluorescence intensity was performed using MetaMorph software (lower panel). Scale bar, 200 μ m; Error bars denote $\pm$ SD. * $P < 0.05$. (D) Schematic representation of the proposed mechanism of CMC mediation of tumors genesis. Under hypoxic conditions, CHC has an additional role of mediating hypoxia-induced angiogenesis. CHC interacts with and stabilizes HIF-1 α . CHC plays an assisting role in HIF-1 α nuclear localization and HRE promoter binding, which lead to an increased production of VEGF. Suppression of CHC expression can inhibit tumorigenesis and angiogenesis.

FIG. 7 shows the results of determination of the binding activities of Pa65-2 on MIA PaCa-2 cells. (A) Pa65-2 shows high affinity against MIA PaCa-2 cells by ELISA. Normal mouse IgG (NM-IgG) was used for negative control. (B) Flow cytometric analysis of Pa65-2 binding activity on MIA PaCa-2 and NNM (left panel) cells, fluorescent intensity indicates the binding activity of Pa65-2 on cells (right panel). (C) Immunofluorescent analysis of Pa65-2 localization in MIA PaCa-2 and NNM cells. Scale bar, 50 μ m. (D) Pa65-2 shows high affinity against MIA PaCa-2 cell membrane and cytosol by labeling immuno-electron microscopy. Normal mouse IgG (NM-IgG) was used, for negative control. Scale bar. 500 nm.

FIG. 8 shows the results of immunofluorescent staining of Pa65-2 and Ulex europeus agglutinin-1 (UEA-1), which indicates they are co-localized. These results indicate Pa65-2 is

also expressed in blood vessels of human pancreatic tumor mass (red, Pa65-2; green, UEA-1). Scale bar, 20 μ m.

FIG. 9 shows binding activity of Pa65-2 against various cancer cell lines. (A) ELISA analysis of the binding activity of Pa65-2 in various cancer cell lines. NM-IgG was used as a control. (B) Western blot analysis of Pa65-2 in various cancer cell lines.

FIG. 10 shows the alignment of peptide sequences obtained from LC-nanoESI-MS/MS analysis of the Pa65-2-target protein and the sequence of human CHC protein. The letters in bold and underlined represent the Pa65-2-target protein sequences that match the sequences of human CHC protein (SEQ ID NO: 1).

FIG. 11 shows suppression of CHC expression had no effect on human skin fibroblast cell growth. (A) The results, of CHC knockdown were determined by Western blot analysis. MIA PaCa cells were used, as a control. (B) MIA PaCa-2 cells and (C) CCD-112Sk cells proliferation was detected by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) analysis. Error bars denote $\pm$ SD. * P <0.05.

FIG. 12 shows suppression of CHC expression inhibits lung cancer cells growth and invasion. (A) CHC knockdown was determined by Western blot. (B) Knockdown of CHC expression reduced colony formation potential of CLI-5 cells. Error bars denote $\pm$ SD. ** P <0.01. (C) Knockdown of CHC expression reduced cell invasion. Scale bar, 40 μ m; Error bars denote $\pm$ SD. ** P <0.01. (D) Eight NOD/SCID mice were injected s.c. (1×10^7 cells) in the lateral portion of the hind limb, left side for the control vector-transduced CLI-5 cells, and right side for the CHC shRNA-transduced CLI-5 cells. The tumors were measured with calipers every five days, and tumor volume was calculated as $\text{length} \times (\text{width})^2 \times 0.52$. Error bars denote $\pm$ SD. ** P <0.01. (E) Tumor weight was measured. Error bars denote $\pm$ SD. * P <0.05. (F) Colocalization of CHC and HIF-1 α , shown by immunofluorescence, in CLI-5 cells incubated with DFX (100 μ M) for 5 h (right, panel) or with 20% O₂ for 5 h (left panel). Scale bar, 10 μ m.

FIG. 13 shows quantification of CHC, VEGF and HIF-1 α staining was performed on consecutive sections using HistoQuest analysis software.

FIG. 14 shows CHC knockdown facilitated HIF-1 α protein degradation in MIA PaCa cells. The percentage of (A) CHC, (B) HIF-1 α , (C) HIF-1 α and CHC positive nuclei is shown in FIG. 4A. Three independent fields of 1 mm² each were counted. The application of the quantitative methods described previously (3), Error bars denote $\pm$ SD. ** P <0.01. (D) Immunoblot analysis of cytoplasmic, C, and nuclear, N, fraction of MIA PaCa-2 cells treated with DFX (100 μ M) for 16 h (anti-PRAP-1 and anti- α -tubulin serving as internal control), (E) Quantitative RT-PCR analysis of CMC (left panel) and HIF-1 α (right panel) mRNA in CHC knockdown MIA PaCa-2 cells cultured in normoxic or hypoxic conditions. Gene expression levels were normalized to GAPDH signal. Error bars denote $\pm$ SD. ** P <0.01. (F) MIA PaCa cells were first incubated in the presence of DFX (100 μ M) for 16 h and then treated, with CHX (5 μ g/ml) in control and shCHC cells for indicated times. Cells were harvested and whole-cell lysates analyzed for HIF-1 α protein level by immunoblotting (left panel). The band intensity, as determined by densitometry, was normalized to the GAPDH signal and expressed as percent of the HIF-1 α abundance at time zero (right panel).

FIG. 15 shows inhibition of endothelial cells growth in vitro by Pa65-2. (A) Immunofluorescent analysis of VEGF internalization in HUVECs after CHC inhibition by Pa65-2. HUVECs were pretreated with Pa65-2 or NM-IgG at 37° C., and subsequently incubated with or without VEGF (40 ng/ml) at 37° C. or 4° C. The cells were stained to detect antibodies

against VEGF-A (red: Rhodamine staining) and for the nucleus using DAPI (blue). Scale bar, 50 μ m. (B) Detection of Pa65-2-induced apoptosis in HUVEC by TUNEL staining. Scale bar, 100 μ m; Error bars denote $\pm$ SD. ** P <0.01.

FIG. 16 shows the results of comparing antitumor efficacy of Pa65-2 and GEM in NOD/SCID bearing human pancreatic cancer xenografts. (A) Tumor weight was measured. Error bars denote $\pm$ SD. * P <0.05. (B) Different treatments have no effects on body weight change ($n=6$ in each group).

DETAILED DESCRIPTION OF THE INVENTION

Definitions

The terms used in this specification generally have their ordinary meanings in the art, within the context of the invention, and in the specific context where each term is used. Certain terms that are used to describe the invention are discussed below, or elsewhere in the specification, to provide additional guidance to the practitioner regarding the description of the invention. For convenience, certain terms may be highlighted, for example using italics and/or quotation marks. The use of highlighting has no influence on the scope and meaning of a term; the scope and meaning of a term is the same, in the same context, whether or not it is highlighted. It will be appreciated that same thing can be said in more than one way. Consequently, alternative language and synonyms may be used for any one or more of the terms discussed herein, nor is any special significance to be placed upon whether or not a term is elaborated or discussed herein. Synonyms for certain terms are provided. A recital of one or more synonyms does not exclude the use of other synonyms. The use of examples anywhere in this specification including examples of any terms discussed herein is illustrative only, and in no way limits the scope and meaning of the invention or of any exemplified term. Likewise, the invention is not limited to various embodiments given in this specification.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention pertains. In the case of conflict, the present document, including definitions will control.

As used herein, “around”, “about” or “approximately” shall generally mean within 20 percent, preferably within 10 percent, and more preferably within 5 percent of a given value or range. Numerical quantities given herein are approximate, meaning that the term “around”, “about” or “approximately” can be inferred if not expressly stated.

Abbreviations: mAb, monoclonal antibodies; CHC, clathrin Heavy Chain; HIF-1 α , Hypoxia-inducible factor 1 α ; VEGF, vascular endothelial growth factor; NNM, Normal nasal mucosal; FACS, flow cytometric analysis; ELISA, Enzyme-linked immunosorbent assay; EMT, epithelial-mesenchymal transition; Quantitative Reverse Transcription Polymerase Chain Reaction (RT-PCR); ChIP, Chromatin Immunoprecipitation; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HUVEC, Human Umbilical Vein Endothelial Cells; IHC, immunohistochemistry; DFX, deferrioxamine; EKE, hypoxia responsive element; UEA-1, Ulex europaeus 1 agglutinin; CDR, complementarity-determining region; LC-nanoESI-MS/MS, liquid chromatography-nano-electrospray ionization tandem mass spectrometry.

As used herein, “preparation” shall generally mean something prepared, manufactured, a substance especially prepared.

As used herein, the term “antibody” means an immunoglobulin (Ig) molecule or a fragment of an immunoglobulin

molecule having the ability to specifically bind to a particular antigen. The Ig monomer is a "Y"-shaped molecule that consists of four polypeptide chains; two identical heavy chains and two identical light chains connected by disulfide bonds. The arms of the Y, for example, contain the site that bind antigen and, therefore, recognize specific foreign objects. This region of the antibody is called the Fab (fragment, antigen binding) region.

Antibodies are well known to those of ordinary skill in the science of immunology. As used herein, the term "antibody" means not only full-length antibody molecules but also fragments of antibody molecules retaining antigen binding ability. Such fragments are also well known, in the art and are regularly employed both in vitro and in vivo. In particular, as used herein, the term "antibody" means not only full-length immunoglobulin molecules but also antigen binding active fragments such as the well-known active fragments $F(ab')_2$, Fab, Fv, and Fd.

The fragment antigen-binding (Fab fragment) is a region on an antibody that binds to antigens. It is composed of one constant and one variable domain of each of the heavy and the light chain. The two variable domains bind the epitope on their specific antigens. Fc and Fab fragments can be generated in the laboratory. The enzyme papain can be used to cleave an immunoglobulin monomer into two Fab fragments and an Fc fragment. The enzyme pepsin cleaves below hinge region, so a $F(ab')_2$ fragment and a pFc' fragment is formed. The enzyme IdeS (Immunoglobulin degrading enzyme from *Streptococcus pyogenes*, trade name FabRICATOR™) cleaves IgG in a sequence specific manner at neutral pH. The $F(ab')_2$ fragment can be split into two Fab' fragments by mild reduction.

The variable domain of an antibody is referred to as the Fv region and is the most important region for binding to antigens.

The Fv fragment consists of the heavy chain variable domain (VH) and the light chain variable domain (VL) held together by strong noncovalent interaction. Thus, each Fv fragment contains one intact antigen-binding site and represents the minimal active fragment derivable from an antibody molecule.

The variable regions of the heavy and light chains can be fused together to form a single-chain variable fragment (scFv), which is only half the size of the Fab fragment, yet retains the original, specificity of the parent immunoglobulin.

It has been reported that "fully" human antibodies may avoid some of the side effects of humanized and chimeric antibodies. Two successful approaches were identified—phage display-generated antibodies and mice genetically engineered to produce more human-like antibodies. Phage display could be used such that variable antibody domains could be expressed on filamentous phage antibodies.

It is now well-established in the art that the non-CDR regions of a mammalian antibody may be replaced with similar regions of conspecific or heterospecific antibodies while retaining the epitopic specificity of the original antibody. This is most clearly manifested in the development and use of "humanized" antibodies in which non-human CDRs are covalently joined to human FR and/or Fc/pFc' regions to produce a functional antibody. Thus, for example, PCT International Publication Number WO 92/04381 teaches the production and use of humanized murine RSV antibodies in which at least a portion of the murine FR regions have been replaced by FR regions of human origin. Such antibodies, including fragments of full-length antibodies with antigen-binding ability, are often referred to as "chimeric" antibodies. Such chimeric antibodies may be produced in which some or

all of the FR regions of the antibody have been replaced by other homologous human PR regions.

Humanized forms of non-human (e.g., murine) antibodies are chimeric immunoglobulins, immunoglobulin chains or fragments thereof (such as Fv, Fab, Fab', $F(ab')_2$ or other antigen-binding subsequences of antibodies) which contain minimal sequence derived from non-human immunoglobulin. Humanized antibodies include human immunoglobulins (recipient antibody) in which residues from a complementary determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat or rabbit having the desired specificity, affinity and capacity. In some instances, Fv framework residues of the human immunoglobulin are replaced by corresponding non-human residues. Humanized antibodies may also comprise residues which are found neither in the recipient antibody nor in the imported CDR or framework sequences. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are those of a human immunoglobulin consensus sequence. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin [Jones et al., Nature, 321:522-525 (1986); Riechmann et al., Nature, 332:323-329 (1988); and Presta, Curr. Op. Steel Biol., 2:593-596 (1992)].

Methods for humanizing non-human antibodies are well known in the art. Generally, a humanized antibody has one or more amino acid residues introduced into it from a source which is non-human. These non-human amino acid residues are often referred to as "import" residues, which are typically taken from an "import" variable domain. Humanization can be essentially performed following the method of Winter and co-workers [Jones et al., Nature, 321:522-525 (1986); Riechmann et al., Nature, 332:323-327 (1988); Verhoeven et al., Science, 239:1534-1536 (1988)], by substituting rodent CDRs or CDR sequences for the corresponding sequences of a human antibody. Accordingly, such "humanized" antibodies are chimeric antibodies (U.S. Pat. No. 4,816,567), wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some CDR residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies.

Human antibodies can also be produced using various techniques known in the art, including phage display libraries. The techniques of Cole et al. and Boerner et al. are also available for the preparation of human monoclonal antibodies (Cole et al., Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, p. 77(1985) and Boerner et al., J. Immunol., 147(1) 86-95 (1991)]. Similarly, human antibodies can be made by introducing of human immunoglobulin loci into transgenic animals, e.g., mice in which the endogenous immunoglobulin genes have been partially or completely inactivated. Upon challenge, human antibody production is observed, which closely resembles that seen in humans in all respects, including gene rearrangement, assembly, and antibody repertoire. This approach is described, for example, in U.S. Pat. Nos. 5,545,807; 5,545,806; 5,569,825; 5,625,126; 5,633,425; 5,661,016. Such fully human or humanized monoclonal antibodies will have particular utility in that they will not evoke an immune response against the antibody itself. See U.S. Pat. No. 7,622,113, which is herein incorporated by reference in its entirety.

The antibody may be labeled and may be immobilized on a solid support. The word "label" when used herein refers to a detectable compound or composition which is conjugated directly or indirectly to the antibody so as to generate a "labeled" antibody. The label may be detectable by itself (e.g. radioisotope labels or fluorescent labels) or, in the case of an enzymatic label, may catalyze chemical alteration of a substrate compound or composition which is detectable.

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 (~190 kDa each), each paired with a light chain (25-27 kDa). The basic unit of its assembly is the triskelion, which has a flexible, three-armed polyhedral cage-like structure. Clathrin plays a crucial role in clathrin-mediated endocytosis (CME) pathway, a major route for membrane trafficking. It is involved in the ubiquitous uptake of ligand-receptor complexes, membrane transporters, and adhesion molecules. Clathrin also stabilizes the fibers of the spindle apparatus during mitosis. However, the main mechanism of CHC in tumorigenesis remains unknown.

We found that CHC may regulate the stability of the α subunit of hypoxia inducible factor-1 (HIF-1 α). When cells are exposed to hypoxic conditions, HIF-1 α is stabilized, which subsequently upregulates several downstream genes to promote cell survival in low-oxygen conditions. HIF-1 α accumulation mediates cellular and systemic adaptive responses to maintain oxygen homeostasis. It also upregulates hypoxia-inducible genes, which are involved, in angiogenesis, erythropoiesis, energy metabolism as well as cell survival decisions in all metazoan species. In cancerous conditions, cells within rapidly growing solid tumors are exposed to chronic or intermittent hypoxia. Therefore, tumor cells encounter powerful selective pressure from hypoxia during their progression, invasion, and metastasis. An elevated expression of hypoxia-responsive proteins is a poor prognostic sign in many types of solid tumors, and the results from several studies suggest that agents acting directly or indirectly against the expression of HIF-1 α have anticancer effects.

In the present study, it was found that CHC, which is associated with the HIF-1 α , increases the protein's stability and facilitates its nuclear translocation, thereby regulating VEGF gene expression in cancer cells. The 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.

In one aspect, the invention relates to a purified monoclonal antibody, or an antigen-binding portion thereof, which specifically binds to human clathrin heavy chain (CHC) comprising the amino acid sequence of SEQ ID NO: 1.

In one embodiment of the invention, the purified monoclonal antibody or antigen-binding portion thereof comprises: (a) a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 2; and (b) a light chain variable region comprising the amino acid sequence of SEQ ID NO: 3.

In another embodiment of the invention, the purified monoclonal antibody or antigen-binding portion thereof binds to cells selected from the group consisting of pancreatic cancer cells, breast cancer cells, lung cancer cells, ovary cancer cells, oral cancer cells, and tumor-associated endothelial cells.

In another aspect, the invention relates to an isolated monoclonal antibody, or a binding fragment thereof. The isolated monoclonal antibody, or a binding fragment thereof comprises a heavy chain variable region and a light chain variable region, in which the heavy chain variable region comprises: (i) complementarity determining region 1 (CDR1) comprising SEQ ID NO: 4; (ii) complementarity determining region

2 (CDR2) comprising SEQ ID NO: 5; and (iii) complementarity determining region 3 (CDR3) comprising SEQ ID NO: 6; and the light chain variable region comprises: (i) CDR1 comprising SEQ ID NO: 7; (is) CDR2 comprising SEQ ID NO: 8; and (iii) CDR3 comprising SEQ ID NO: 9.

In one embodiment of the invention, the isolated antibody or binding fragment comprises a heavy chain variable domain (V_H) SEQ ID NO: 2 and/or a light chain variable domain (V_L) SEQ ID NO: 3.

In another embodiment of the invention, the binding fragment comprises an Fv fragment of the antibody.

In another embodiment of the invention, the binding fragment comprises an Fab fragment of the antibody.

In another embodiment of the invention, the antibody is a fully human monoclonal antibody.

In another embodiment of the invention, the antibody is a humanized monoclonal antibody.

In another embodiment of the invention, the aforementioned antibody, or binding fragment thereof, binds to a cancer cell expressing clathrin heavy chain (CHC).

In another embodiment of the invention, the isolated antibody or binding fragment is labeled with a detectable compound or an enzyme.

In another embodiment of the invention, the isolated antibody or binding fragment is encapsulated within a liposome.

In another aspect, the invention relates to a method for inhibiting tumor cell growth and/or tumor angiogenesis, which comprises administering to a subject in need thereof a composition comprising the aforementioned purified monoclonal antibody, or antigen-binding portion thereof, and a pharmaceutically acceptable carrier.

Further in another aspect, the invention relate to a method for inhibiting tumor growth and/or tumor angiogenesis, which comprises: administering to a subject in need thereof a composition comprising the aforementioned isolated monoclonal antibody, or binding fragment thereof, and a pharmaceutically acceptable carrier.

In one embodiment of the invention, the tumor cell expresses clathrin heavy chain (CHC). In another embodiment of the invention, the tumor cell is selected from the group consisting of pancreatic cancer cells, breast cancer cells, lung cancer cells, ovary cancer cells, oral cancer cells, and tumor-associated endothelial cells.

Further in another aspect, the invention relate to an isolated single-chain variable fragment comprising: (a) the heavy chain variable region (SEQ ID NO: 2) and the light chain variable region (SEQ ID NO: 3) of the isolated antibody or binding fragment as aforementioned; and (b) a linker peptide connecting the heavy chain variable region (SEQ ID NO: 2) and the light chain variable region (SEQ ID NO: 3).

Further in another aspect, the invention relate to a method for detecting cancer in a subject, which comprises: (a) applying the isolated monoclonal antibody, or binding fragment thereof, of claim 4 to a cell or tissue sample obtained from the subject; and (b) assaying the binding of the isolated monoclonal antibody, or binding fragment thereof to the cell or the tissue sample; and (c) comparing the binding with a normal control to determine the presence of the cancer in the subject, wherein the cancer expresses human clathrin heavy chain.

Yet in another aspect, the invention relates to a composition comprising: (a) the aforementioned isolated monoclonal antibody or binding fragment thereof and (b) a pharmaceutically acceptable carrier.

Alternatively, the invention relates to a composition comprising: (a) the aforementioned purified monoclonal antibody or antigen-binding portion thereof; and (b) a pharmaceutically acceptable carrier.

In one embodiment of the invention, the composition further comprises an anticancer agent. The anticancer agent may be a small molecule drug including, but not limited to, gemcitabine.

In another embodiment of the invention, the aforementioned isolated monoclonal antibody, or an antigen-binding portion thereof, exhibits at least one, two, three, four, five, six, seven, or all eight of the following properties: (a) specifically binds to pancreatic adenocarcinoma cells; (b) binds to the cell surface and cytosol of cancer cells and tumor blood vessels; (c) is internalized by CHC-expressing cells; (d) inhibiting tumor growth, invasion ability, migration, and angiogenesis; (e) inducing apoptosis in cancer cells and human umbilical vein endothelial cells (HUVECs); (f) inhibiting tumor growth and tumor blood vessels in pancreatic cancer in vivo; (g) suppressing epidermal growth factor (EGF), transferrin (Tf), and VEGF internalizations by cancer cells; or (h) suppressing hypoxia-inducible factor-1 α (HIF-1 α) expression and vascular endothelial growth factor (VEGF) secretion.

EXAMPLES

Without intent, to limit the scope of the invention, exemplary instruments, apparatus, methods and their related results according to the embodiments of the present invention are given below. Note that titles or subtitles may be used in the examples for convenience of a reader, which in no way should limit the scope of the invention. Moreover, certain theories are proposed and disclosed herein; however, in no way they, whether they are right or wrong, should limit the scope of the invention so long as the invention is practiced according to the invention without regard for any particular theory or scheme of action.

Materials and Methods

Cell Lines and Culture

Human pancreatic adenocarcinoma cell lines (MIA PaCa-2 and AsPC-1), human breast carcinoma cell lines (MDA-MB-231), human ovarian cancer cell lines (SKOV3), human colon cancer cell lines (COLO 205), and a human skin fibroblast cell line (CCD-112Sk), 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 nasal mucosal (NNM) epithelia were a primary culture derived from a nasal polyp. Human umbilical vein endothelial cells (HUVECs) were purchased (LONZA™) and grown in EBM-2 medium (LONZA™). The cell lines, including were purchased from American Type Culture Collection (ATCC®), Human oral cancer cell lines (SAS) were obtained from the Japanese Cancer Research Resources Bank. These cells were cultured by cell bank protocols and had been passaged for fewer than 6 months after resuscitation.

Generation of Monoclonal Antibodies

Monoclonal antibodies against MIA PaCa-2 were generated following a standard procedure with slight modifications. Briefly, female BALB/cJ 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 NS1/1-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/cJ mice and mAbs purified with protein G Sepharose 4G gel (GE).

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. 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. The digested peptides were analyzed by LC-MS/MS sequencing in the Core Facility for Proteomics and Structural Biology Research at Academia Sinica (Taipei).

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-1 α antibody (BD), then analyzed by immunoblotting. The signals were developed using enhanced chemiluminescence reagents (ECL) (Thermo).

Enzyme-Linked Immunosorbent Assay and Flow Cytometric Analysis

Cells were cultured in 96-well polystyrene plates for 3 days and then fixed with 2% paraformaldehyde. After blocking and washing twice with PBS/0.1 % Tween 20 (PBST0.1), the hybridoma supernatants were added and the plates were incubated for 2 h at room temperature. This was followed by washing and incubating with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG for 1 h. The colorimetric reaction was developed with the substrate ortho-phenylenediamine (OPD) according to the manufacturer's instructions (Sigma), and was stopped by adding 3 N HCl. Absorbance at 490 nm was measured using a microplate reader. Each control and test group was tested in triplicate. Each experiment was repeated at least three times. To analyse binding, cells (1×10^5 cells per tube) were prepared, washed, centrifuged, and resuspended in 100 μ l of PBS/1% FCS containing Pa65-2 or NM-IgG. Cells were incubated at 4° C. for 1 h, washed twice with PBS/1% FCS and stained with fluorescent isothiocyanate (FITC)-labeled goat anti-mouse IgG at 4° C. for 20 min. The stained cells were washed twice and fluorescence signals were measured using a FACScan (BD). At least 5×10^3 cells were acquired by list mode, measurements were performed on a single cell basis, and were displayed as frequency distribution histograms or dot histograms.

MTT assay

Cells were cultured in 96-wells plates. To reduce cancer growth, the cells were culture in Dulbecco's modified Eagle's medium (DMEM) containing 50 μ g/ml Pa65-2 or NM-IgG. After incubating for 0, 1, 2, 3 days, the cells were subjected to an MTT assay. The absorbance was determined with a microplate reader at 540 nm. Each assay was repeated for three times.

Inhibition of HUVEC Internalization by Pa65-2

HUVEC were washed with serum-free medium, incubated in 1% BSA in serum-free medium at 37° C., and pretreated with Pa65-2 or NM-IgG (50 μ g/ml) at 37° C. They were then incubated with or without VEGF-A (40 ng/ml) at 37° C. or 4° C. After cells were washed, fixed and made permeable. The permeable cells were incubated with anti VEGF-A antibody (A-20, Santa Cruz). Finally, cells were stained using Rhodamine-conjugated secondary antibodies.

Cell Proliferation Analysis and Invasion Assays

RT-CES (ACEA), a microelectronic cell sensor system, was used to count tire number of living cells. Cells (5×10^3) were seeded into each sensor-containing well in microliter 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 μ m; Millipore) according to the manufacturer's directions. Cells were counted under a microscope in five predetermined fields. Assays were performed in triplicate.

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. The stable transfectants were established by puromycin selection. The VEGF reporter plasmids contains nucleotides -2274 to +379 of the VEGF gene inserted into luciferase reporter pGL2-Basic (PROMEGA™) as previously described (Forsythe et al (1996) Activation of vascular endothelial growth factor gene transcription by hypoxia-inducible factor 1. *Mol Cell Biol*, 16, 4604-13), VEGF promoter primer sequences are presented in Table 3. 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). 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 μ g of total RNA by SuperScript III reverse transcriptase (INVITROGEN™). CLTC, VEGF, HIF-1 α , erythropoietin (EPO), platelet-derived growth factor- β (PDGF- β) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers sequences are presented in Table 4. 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.

Hypoxia Assay

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

Chromatin Immunoprecipitation (ChIP)

The protocol for chromatin immunoprecipitation (ChIP) has been described previously. 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-1 α (Abcam) or NM-IgG (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 $\Delta\Delta C_1$ method with C₁ 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 Table 5.

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 AFS (Leica), in which they were dehydrated by methanol at -91° C. for 4-5 days. Cells were later warmed to -50° C., embedded in Lowicryl HM20, and polymerized at -50° C. by UV. Ultrathin sections of 90 nm thickness were obtained using an Ultracut UC7 (Leica). The sections were incubated with the Pa65-2 mouse IgG or anti-HIF-1 α rabbit IgG. The secondary antibodies (Jackson), goat anti-mouse IgG (F(ab')₂ fragment) conjugated with 18 nm gold particles, or goat anti-rabbit IgG (F(ab')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).

Inhibition of Cell Internalization by Pa65-2

EGF and Tf uptake assays were carried out using a fluorescence-based approach, as previously described (26). 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-IgG (50 μ g/ml) at 37° C. They were then incubated with or without Alex 555-EGF (1 μ g/ml, INVITROGEN™) or Alex 555-transferrin (50 μ g/ml) at 37° C. or 4° C. The cells were imaged using a Leica TCS SP confocal microscope (Leica).

Immunofluorescent Staining

Cells were incubated with anti-Pa65-2 and anti-HIF-1 α antibodies, and then with FITC- or Rhodamine-conjugated secondary antibodies (Jackson). Images were captured by confocal microscopy (Leica).

Apoptosis Assay

Apoptosis of cultured cells was verified through the detection of caspase activity using sulforhodamine FLICA apoptosis detection kit (Immunochemistry Technologies). Cells in 96-well culture plates were treated with Pa65-2 or NM-IgG for 24 h. After incubating with FLICA, cells were read at ex/era of 550/595 nm with fluorescence plate reader (Molecular Devices).

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 Ethics of Animal Experiments of Academia Sinica (Taipei). 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 mm³) were intravenously injected in the tail vein with Pa65-2, or gemcitabine, 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 $\times$ (width)² $\times$ 0.52. Animals were treated following the guidelines established by Academia Sinica (Taipei).

CD31 Staining and Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick End Labeling (TUNEL) Assay

CD31 staining and TUNEL assays were carried out as described previously. The slides were then visualized under a fluorescent microscope and analyzed with MetaMorph software.

Analysis of Tissue Samples

The human pancreatic cancer gene expression arrays (Origene) were analyzed by an ABI9600 thermocycler (Applied Biosystems). Tissue arrays were purchased from Pantomics Inc. Tissue sections were stained with antibodies specific for CHC (Pa65-2), VEGF (GeneTex), HIF-1 α (Millipore), NM-

IgG, and UEA-1-FITC (Vector). Quantification of DAB intensity was by HistoQuest software (TissueGnostics). The protocol was approved by the institutional Review Board of Human Subjects Research Ethics Committee of Academia Sinica (Taipei).

cDNA Synthesis and Amplification of Variable Region

Total mRNA was extracted from Pa65-2-producing hybridoma cells using the FastTrack mRNA isolation kit (INVITROGEN™). The cDNA synthesis was based on Orlandi et al. (1989) "Cloning immunoglobulin variable domains for expression by the polymerase chain reaction" *Proc Natl Acad Sci USA*, 86, 3833-7. A reaction mixture containing 10 µg of mRNA, 20 pmol of VH1FOR primer or VK1FOR primers sequences are presented in Supplementary Table 2, 250 µM of each dNTP, 50 mM Tris-HCl pH 8.3, 140 mM KCl, 10 mM MgCl₂, 10 mM dithiothreitol, and 20 units of RNasin (PHARMACIA™/LKB Biotechnology) was heated at 70° C. for 10 min and cooled. Reverse transcriptase (Anglian Biotech) was added and the reaction incubated at 42° C. for 1 hr. For amplification by Taq DNA polymerase (PROMEGA™), a reaction mixture was made containing 5 µl of the c-DNA-RNA hybrid, 25 pmol of primers VH1FOR or VK1FOR and VH1BACK or VK1BACK primers sequences are presented in Supplementary Table 2. 250 µM of each dNTP, 67 mM Tris chloride (pH 8.8), 17 mM (NH₄)₂SO₄, 10 mM MgCl₂, 200 µg of gelatine per ml, and 2 units of Taq DNA polymerase PCR was performed for 45 cycles (1', 95° C.; 1', 55° C.; 2', 72° C.) followed by gel purification. The PCR products were then cloned into TA cloning vector pCR2.1 (INVITROGEN™) according to the manufacturer's protocol and the vector was transfected into the DH5α strain of *Escherichia coli* (GIBCO BRL™) and sequenced.

Statistical Analyses

Statistical analyses were done using unpaired Student's t-tests where appropriate. *, p<0.05, **, p<0.01 were considered significant.

Enzyme-linked Immunosorbent Assay and Flow Cytometric Analysis

3×10⁵ cells were cultured into each well of 96-well polystyrene plates for 3 days and fixed with 2% paraformaldehyde. After blocking and washing twice with PBS/0.1% Tween 20 (PBST0.1), the hybridoma supernatants were added and the plates were incubated for 2 hr at room temperature. This was followed by washing and incubation with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG for 1 hr. The colorimetric reaction was developed with the substrate ortho-phenylene-diamine (OPD) according to manufacturer's instructions (Sigma, St. Louis, Mo.), and was stopped by adding 3 N HCl. Absorbance at 490 nm was measured using a microplate reader. Each control and test group was tested in triplicate. Each experiment was repeated at least three times. To analyse binding, cells (1×10⁵ cells per tube) were prepared, washed, centrifuged, and resuspended in 100 µl of PBS/1% PCS containing Pa65-2 or NM-IgG. Cells were incubated at 4° C. for 1 hr, washed twice with PBS/1% FCS and stained with fluorescent isothiocyanate (FITC)-labeled goat anti-mouse IgG at 4° C. for 20 min. The stained cells were washed twice and fluorescence signals were measured using a FACScan (BD Biosciences, San Jose, Calif.). At least 5×10³ cells were acquired by list mode, measurements were performed on a single cell basis, and were displayed as frequency distribution histograms or dot histograms.

Quantitative Reverse Transcription Polymerase Chain Reaction (RT-PCR)

RNA extractions were performed using the RNeasy Mini kit (QIAGEN, Valencia, Calif.) according to the manufacturer's instructions. First strand cDNA was synthesized from 1.0

µg of total RNA by Superscript III reverse transcriptase (INVITROGEN™, Carlsbad, Calif.). The primers used for PCR amplification were: GAPDH-F, 5'-CTTACCACCATG-GAGGAGGC-3' (SEQ ID NO: 30); GAPDH-R, 5'-GGCATGGACTGTGGTCATGAG-3' (SEQ ID NO: 31); and CLTC-F, 5'-GACAAAGGTGGATAAATTAGATGC-3' (SEQ ID NO: 20); CLTC-R, 5'-TAAACAATGGGTTGT-GTCTCTGTA-3' (SEQ ID NO: 21). Real-time PCR was performed using a LightCycler 480 System (Roche, Indianapolis, Ind.). 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.

cDNA Synthesis and Amplification of Variable Region

Total mRNA was extracted from Pa65-2-producing hybridoma cells using the FastTrack mRNA isolation kit (INVITROGEN™, Carlsbad, Calif.). The cDNA synthesis was based on Orlandi et al. (1989) "Cloning immunoglobulin variable domains for expression by the polymerase chain reaction" *Proc Natl Acad Sci USA*, 86, 3833-7. A reaction mixture containing 10 µg of mRNA, 20 pmol of VH1FOR primer 5'-d(TGAGGAGACGGTGACCGTGGTCCCTTG-GCCCCAG-3' (SEQ ID NO: 36) or VH1FOR primer 5'-d(GTTAGATCTCCAGCTTGGTCCC-3' (SEQ ID NO: 37), 250 µM of each dNTP, 50 mM Tris-HCl pH 8.3, 140 mM KCl, 10 mM MgCl₂, 10 mM dithiothreitol, and 20 units of RNasin (PHARMACIA™/LKB Biotechnology, Inc., Piscataway, N.J.) was heated at 70° C. for 10 min and cooled. Reverse transcriptase (Anglian Biotech, Colchester, U.K.) was added and the reaction incubated at 42° C. for 1 hr. For amplification by Taq DNA polymerase (PROMEGA™, Madison, Wis., USA), a reaction mixture was made containing 5 µl of the cDNA-RNA hybrid, 25 pmol of primers VH1FOR or VH1FOR and VHIBACK 5'-d(AGGTSMARCTGCAG-SAGTCWGG-3' (in which S=C or G, M=A or C, R=A or G, and W=A or T) (SEQ ID NO: 38) or VK1BACK 5'-d(GA-CATTCAGCTGACCCAGTCTCCA-3' (SEQ ID NO: 39) as appropriate, 250 µM of each dNTP, 67 mM Tris chloride (pH 8.8), 17 mM (NH₄)₂SO₄, 10 mM MgCl₂, 200 µg of gelatine per ml, and 2 units of Taq DNA polymerase. PCR was performed for 45 cycles (1', 95° C.; 1', 55° C.; 2', 72° C.) followed by gel purification. The PCR products were then cloned into TA cloning vector pCR2.1 (INVITROGEN™, Carlsbad, Calif.) according to the manufacturer's protocol and the vector was transfected into the DH5α strain of *Escherichia coli* (GIBCO BRL™, Gaithersburg, Md., USA) and sequenced.

Wound Scratch Assay

1×10⁵ CL1-5 cells were plated onto tissue culture dishes. After 24 hr incubation, the cells were scratched in a standardized manner with a plastic apparatus to create a cell-free zone in each well, 2 mm in width. The cells were then incubated for 14 hr at 37° C. Migration of the cells into the scratch was observed at time points of 7 hr and 14 hr.

Results

Generation and Characterization of mAbs Against Pancreatic Cancer

To obtain a potential target for pancreatic adenocarcinoma therapy, BALB/cMice were immunized with MIA PaCa-2 cells. 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 clones that exhibited higher reactivities against MIA PaCa-2 cells were selected (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 (FIGS.

7A-C). Immuno-electron microscopy showed cellular localization of Pa65-2 on the plasma membrane of MIA PaCa-2 cells (FIG. 7D). Pancreatic adenocarcinoma cell lines, MIA PaCa-2 and AsPC-1, and normal cell lines, HUVEC, NNM, and CCD-112Sk, 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 cancer cell lines (FIGS. 1A-B).

All 46 specimens of pancreatic cancer tissues were positively stained by Pa65-2, while two normal pancreatic tissues were not, as shown by the pancreatic cancer tissue arrays (FIG. 1C and Table 2). Notably; the target antigen of Pa65-2 is present not only in tumor tissues but also in blood vessel-like structures (FIG. 1C, arrow). Immunofluorescent analysis further confirmed that the antigen of Pa65-2 was colocalized with the endothelial cell marker Ulex europeus agglutinin-1 (UEA-1) in the blood vessels of human pancreatic cancer tissues (FIG. 8). Pa65-2 can also bind to other types of cancer cell lines, including MDAMB-231 (breast cancer), CL1-5 (lung cancer), SKOV3 (ovary cancer) and SAS (oral cancer), as shown by ELISA and Western blot analysis (FIG. 9).

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) (FIG. 10). 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, whose CDR is unknown (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 recognized CHC. Three complementarity-determining regions (CDRs) in the heavy and light chains of Pa65-2 are shown in Table 1.

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 knockdown 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 the proliferation rate of the cells (FIG. 11). 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 (FIGS. 12A-E). Furthermore, TUNEL staining showed that CHC knockdown increased apoptosis of the tumor cells (FIG. 2H). These results indicate that CHC may play a role in the regulation of tumorigenesis.

CHC Regulates VEGF Expression in Pancreatic Cancer

Knockdown of CHC markedly reduced tumor growth (FIGS. 2E-G). 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-1 α -mediated VEGF axis plays an important role in tumor angiogenesis, we further investigated whether knockdown of CHC had an impact on HIF-1 α and VEGF expressions. Results showed that the protein expression, levels of CHC, VEGF, and HIF-1 α in the tumor xenografts were significantly reduced in the CHC knockdown group (FIG. 3B and FIG. 13), 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-1 α in CHC knockdown xenograft tumor was observed only in protein level (FIG. 3B and FIG. 13) but not in the mRNA level (FIG. 3C). Upon further evaluation of CHC and VEGF mRNA levels in nineteen human pancreatic cancer panels, we found a correlation between the expressions of CHC and VEGF (coefficient = 0.299; minor correlation, $P=0.02$) (FIG. 3D).

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. 3E). Luciferase assay also showed that CHC can participate in the regulation of VEGF-A promoter activity (FIG. 3F). 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. It was found that CHC bound to the VEGF-A promoter region (FIG. 3G) and that there was a significant decrease in the binding of CHC and HIF-1 α to the VEGF-A promoter region in CHC knockdown cells (FIG. 3G-H). These observations suggest that CHC may interact with HIF-1 α and co-localize onto the HRE of the VEGF-A promoter, which can then induce VEGF-A expression. CHC Interacts with and Stabilizes HIF-1 α in MIA PaCa-2 Cells

To investigate whether CHC could interact with HIF-1 α , we assessed the co-localization of CHC and HIF-1 α by confocal microscopy. Interestingly, CHC was found in 80% of the nuclei of the MIA PaCa-2 cells, while HIF-1 α was found in 83% of nuclei of MIA PaCa-2 cells during hypoxia. Together, 70% the nuclei of the MIA PaCa-2 cells contained both CHC and HIF-1 α during hypoxia (FIG. 4A and FIGS. 14A-C). However, when CHC was knocked down, a low level of HIF-1 α was detected, in either cytoplasm or nuclei (FIG. 4A and FIGS. 14A-C). The same results were observed in CL1-5 lung cancer cells (FIG. 12F). Subsequently, immunoprecipitation and immuno-electron microscopy were applied to verify protein interaction between CHC and HIF-1 α . As shown in FIG. 4B, HIF-1 α and CHC were co-immunoprecipitated with each other. The double labeling immuno-electron microscopy data further showed that CHC and HIF-1 α were present in the nuclei as well as in the cytoplasm of MIA PaCa-2 cells. The co-localization of CHC and HIF-1 α was particularly noticeable inside the nuclei in both mock and control cells (FIG. 4C), suggesting an interaction between, the two proteins.

It was noticed that the protein level of HIF-1 α , which was induced by hypoxia, was decreased in CHC knockdown cells (FIG. 4C and FIG. 14D), whereas the mRNA level of HIF-1 α was not affected by CHC knockdown (FIG. 14E), suggesting

that CHC may influence HIF-1 α protein stability. To test whether knockdown of CHC decreased the stability of HIF-1 α protein, cells were treated with cycloheximide (CHX) to block de novo protein synthesis. The results suggested that suppression of CHC decreased HIF-1 α protein stability (FIG. 14F). However, the decreased protein level of HIF-1 α in CHC knockdown, cells was rescued with the presence of MG132 (a proteosome inhibitor) (FIG. 4D). We further analyzed the expressions of HIF-1 α -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-1 α may protect HIF-1 α protein stability. Inhibition of Ligand Internalization and Cell Growth by Pa65-2

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 administrated 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 FIG. 16A). When analyzing the tumor sections, it was 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 (FIGS. 6A and 16B). These findings show that Pa65-2 possesses the ability to inhibit pancreatic tumor growth and angiogenesis.

TABLE 1

V _H domains (SEQ ID NO: 2)			
FW1	CDR1	FW2	CDR2
EVKLVESGGGLVKPGG SLNISCASGETES (SEQ ID NO: 10)	NYVMS (SEQ ID NO: 4)	WVRQTPEKRLEW VA (SEQ ID NO: 11)	TISSGDNMYYPDS VKG (SEQ ID NO: 5)
FW3	CDR3	FW4	
RFTISSDNAKNTLFLQM SSLRSEDALNYCAR (SEQ ID NO: 12)	HFDNYEGNSMDY (SEQ ID NO: 6)	WGQGTSTVSSA KTTPPSDYPLA (SEQ ID NO: 13)	
V _L domains (SEQ ID NO: 3)			
FW1	CDR1	FW2	CDR2
DIVLTQSPATLSVTPGD SVSLSC (SEQ ID NO: 14)	RASQSIRSNLH (SEQ ID NO: 7)	WYQQKSHESPRL LIK (SEQ ID NO: 15)	YASQSIIS (SEQ ID NO: 8)
FW3	CDR3	FW4	
GIPSRFSGSGSGTDFILSI NSVATEDECNYFC (SEQ ID NO: 16)	QQSNWPL (SEQ ID NO: 9)	TFGAGTKLELKR ADAAPTVS (SEQ ID NO: 17)	

Complementarity-determining regions 1-3 (CDR1-3), and framework regions 1-4 (FW1-4) for both the V_H and V_L domains are shown. The V domain families were aligned by igblast database.

Clathrin-mediated endocytosis (CME) is a major mechanism for the internalizations of plasma-membrane receptors. We evaluated the effect of Pa65-2 on the internalization of epidermal growth factor (EGF) and transferrin (Tf), known ligands for CME (30,31). As shown in FIG. 5A, treatment with Pa65-2 blocked EGF and transferrin 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-1 α expression and VEGF secretion (FIG. 5E). However, treatment with normal mouse IgG (NM-IgG) showed no inhibitory activities (FIGS. 5A-E). Moreover, it was also found that Pa65-2 suppressed VEGF internalization and induced apoptosis in HUVECs (FIG. 15). Pa65-2 Suppression of Pancreatic Xenograft Tumor Growth Since CHC shRNA knockdown severely affected xenograft tumor growth in vivo, we investigated whether Pa65-2 could be used to directly inhibit tumor angiogenesis

Table 1 shows the amino acid sequences of V_H and V_L domains of anti-CMC monoclonal antibody, which is related to the SEQ ID NO: 1 shown in FIG. 10. Table 2 shows CHC expression in human pancreas cancer tissue arrays. We modified the “Quick Score” methods by reducing the original scales (Siegel et al. (2012) “Cancer statistics 2012” *CA Cancer J Clin*, 62, 10-29). Two indices were used in evaluating the staining results of tumors. Labeling Index (LI) indicates the number of tumor cells that were immunostained (or “positive cells”) as a proportion of the overall number of cells. A score on a scale of 0-2 was assigned; 0-10%=0, 11-49%=1, 50-100% =2. The second index is the Intensity Index (II), which measures the average intensity of the immunostaining. A score on a scale of 0-2 was assigned; no staining=0, weak staining=1, and strong staining=2. The scores from these two indices were added to give the tumor Labeling Score (LS), which ranges from 0-4.

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TABLE 2

Pancreas Cancer	Stage	Labeling Score
Normal Pancreas		0
Adenocarcinoma I	T2N0M0	3
	T2N1M0	2
	T3N0M0	3
	T3N1M0	3
Adenocarcinoma II	T2N0M0	2
	T3N0M0	3
	T3N1MX	3
	T4N1M1	4
Adenocarcinoma III	T3N0M0	3
	T3N1MX	3

Table 3 shows the list of primers for cloning. Table 4 shows the list of primers for Quantitative RT-PCR. Table 5 shows list of primers for ChIP, Table 6 shows the list of primers for cDNA synthesis and amplification of variable region.

TABLE 3

Name	Sequence (5'-3')
VEGF f	GGTACCGGGCCACGGAGTGACTGGTGAT (SEQ ID NO: 18)
VEGF r	CTCGAGGGAAGAGAGACGGGGTCAGAGAGA (SEQ ID NO: 19)

TABLE 4

Name	Sequence (5'-3')
CLTC f	GACAAAGGTGGATAAATTAGATGC (SEQ ID NO: 20)
CLTC r	TAAACAATGGGTTGTGTCTCTGTA (SEQ ID NO: 21)
VEGF f	ACATCTTCCAGGAGTACCC (SEQ ID NO: 22)
VEGF r	CTTGGTGAGTTTGATCCG (SEQ ID NO: 23)
HIF-1 α f	GGTTCACTTTTCAAGCAGTAGG (SEQ ID NO: 24)
HIF-1 α r	GTGGTAATCCACTTTCATCCATT (SEQ ID NO: 25)
EPO f	AGCAGGAAGCATTCAGAGA (SEQ ID NO: 26)
EPO r	AGGTAAATCGCCTCCAAAG (SEQ ID NO: 27)
PDGF- β f	CTGGCATGCAAGTGTGAGAC (SEQ ID NO: 28)
PDGF- β r	CGAATGGTCACCCGAGTTT (SEQ ID NO: 29)
GAPDH f	CTTACCACCATGGAGGAGGC (SEQ ID NO: 30)
GAPDH r	GGCATGGACTGTGGTCATGAG (SEQ ID NO: 31)

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TABLE 5

Name	Sequence (5'-3')
VEGF f	GAGCCCGCGCCCGGAGG (SEQ ID NO: 32)
VEGF r	CAGCCAGAAAGTTGGAC (SEQ ID NO: 33)
GAPDH f	AGGTGAAGGTCGGAGTCAAC (SEQ ID NO: 34)
GAPDH r	TCTTCTGGGTGGCAGTGATG (SEQ ID NO: 35)

TABLE 6

Name	Sequence (5'-3')
VH1FOR	d(TGAGGAGACGGTGACCGTGGTCCCTGGCCCCAG) (SEQ ID NO: 36)
VK1FOR	d(GTTAGATCTCCAGCTTGGTCCC) (SEQ ID NO: 37)
VH1BACK	d(AGGTSMARCTGCAGSAGTCWGG-3* (in which S = C or G, M = A or C, R = A or G, and W = A or T) (SEQ ID NO: 38)
VK1RACK	d(GACATTAGCTGACCCAGTCTCCA) (SEQ ID NO: 39)

Table 6 shows the list of primers for cDNA synthesis and amplification of the variable region of IgG. As shown in Table 6, the primer VH1BACK includes 2⁵ different sequences. The sequence AGGTCAAACCTCCAGSAGTCAGG (SEQ ID NO: 38) is a representative sequence of them.

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-1 α and participated in the regulation of VEGF transcription. Knockdown of CHC expression also decreased the protein stability of HIF-1 α . To our knowledge, this is the first study to demonstrate that CHC promotes angiogenesis and tumor growth by stabilizing HIF-1 α , followed by upregulating the expression of VEGF.

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. Diminishing CHC levels by shRNA resulted in decreased protein stability of HIF-1 α and decreased expressions of HIF-1 α down-stream genes.

Based on the studies, it was proposed 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-1 α 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, Tf 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 cancer cell invasion, migration and VEGF expression, and to inhibit tumor growth and angiogenesis.

In summary, Pancreatic adenocarcinoma is an aggressive disease with a high mortality rate. Currently, treatment options are limited. In an effort to improve the efficacy of treatments for pancreatic adenocarcinoma, we have generated a monoclonal antibody (mAb), Pa65-2, which specifically binds to pancreatic cancer cells and tumor blood vessels but not to normal cells. The target protein of Pa65-2 is identified as human clathrin heavy chain (CHC; SEQ ID NO: 1). We found that knockdown of CHC or Pa65-2 treatment not only reduced cancer cell proliferation, colony formation and invasion, but it also induced cancer cell apoptosis. 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)-1 α protein, increasing the stability of this protein and facilitating its nuclear translocation and hypoxia responsive element (HRE) promoter binding, thereby regulating the expression of vascular endothelial growth factor (VEGF). Knockdown of CHC results in downregulations of both HIF-1 α and its downstream target gene expressions, such as VEGF, erythropoietin (EPO) and platelet-derived growth factor- β (PDGF- β). Pa65-2 treatment blocked cancer cells' uptake of epidermal growth factor (EGF) and transferin (Tf), inhibited cancer cell proliferation, invasion, and

induced cancer cell apoptosis. 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.

The foregoing description of the exemplary embodiments of the invention has been presented only for the purposes of illustration and description and is not intended to be exhaustive or to limit the invention to the precise forms disclosed. Many modifications and variations are possible in light of the above teaching.

The embodiments and examples were chosen and described in order to explain the principles of the invention and their practical application so as to enable others skilled in the art to utilize the invention and various embodiments and with various modifications as are suited to the particular use contemplated. Alternative embodiments will become apparent to those skilled in the art to which the present invention pertains without departing from its spirit and scope. Accordingly, the scope of the present invention is defined by the appended claims rather than the foregoing description and the exemplary embodiments described therein.

Some references, which may include patents, patent applications and various publications, are cited and discussed in the description of this invention. The citation and/or discussion of such references is provided merely to clarify the description of the present invention and is not an admission that any such reference is "prior art" to the invention described herein. All references cited and discussed in this specification are incorporated herein by reference in their entireties and to the same extent as if each reference was individually incorporated by reference.

SEQUENCE LISTING

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<213> ORGANISM: Homo sapiens

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20 25 30

Glu Ser Asp Lys Phe Ile Cys Ile Arg Glu Lys Val Gly Glu Gln Ala
35 40 45

Gln Val Val Ile Ile Asp Met Asn Asp Pro Ser Asn Pro Ile Arg Arg
50 55 60

Pro Ile Ser Ala Asp Ser Ala Ile Met Asn Pro Ala Ser Lys Val Ile
65 70 75 80

Ala Leu Lys Ala Gly Lys Thr Leu Gln Ile Phe Asn Ile Glu Met Lys
85 90 95

Ser Lys Met Lys Ala His Thr Met Thr Asp Asp Val Thr Phe Trp Lys
100 105 110

Trp Ile Ser Leu Asn Thr Val Ala Leu Val Thr Asp Asn Ala Val Tyr
115 120 125

His Trp Ser Met Glu Gly Glu Ser Gln Pro Val Lys Met Phe Asp Arg
130 135 140

His Ser Ser Leu Ala Gly Cys Gln Ile Ile Asn Tyr Arg Thr Asp Ala
145 150 155 160

-continued

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595						600						605					
Asp	Arg	Ala	His	Ile	Ala	Gln	Leu	Cys	Glu	Lys	Ala	Gly	Leu	Leu	Gln		
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Val	His	Thr	His	Leu	Leu	Asn	Pro	Glu	Trp	Leu	Val	Asn	Tyr	Phe	Gly		
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Ser	Leu	Ser	Val	Glu	Asp	Ser	Leu	Glu	Cys	Leu	Arg	Ala	Met	Leu	Ser		
660						665						670					
Ala	Asn	Ile	Arg	Gln	Asn	Leu	Gln	Ile	Cys	Val	Gln	Val	Ala	Ser	Lys		
675						680						685					
Tyr	His	Glu	Gln	Leu	Ser	Thr	Gln	Ser	Leu	Ile	Glu	Leu	Phe	Glu	Ser		
690						695						700					
Phe	Lys	Ser	Phe	Glu	Gly	Leu	Phe	Tyr	Phe	Leu	Gly	Ser	Ile	Val	Asn		
705						710						715					
Phe	Ser	Gln	Asp	Pro	Asp	Val	His	Phe	Lys	Tyr	Ile	Gln	Ala	Ala	Cys		
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Cys	Tyr	Asp	Pro	Glu	Arg	Val	Lys	Asn	Phe	Leu	Lys	Glu	Ala	Lys	Leu		
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Thr	Asp	Gln	Leu	Pro	Leu	Ile	Ile	Val	Cys	Asp	Arg	Phe	Asp	Phe	Val		
770						775						780					
His	Asp	Leu	Val	Leu	Tyr	Leu	Tyr	Arg	Asn	Asn	Leu	Gln	Lys	Tyr	Ile		
785						790						795					
Glu	Ile	Tyr	Val	Gln	Lys	Val	Asn	Pro	Ser	Arg	Leu	Pro	Val	Val	Ile		
805						810						815					
Gly	Gly	Leu	Leu	Asp	Val	Asp	Cys	Ser	Glu	Asp	Val	Ile	Lys	Asn	Leu		
820						825						830					
Ile	Leu	Val	Val	Arg	Gly	Gln	Phe	Ser	Thr	Asp	Glu	Leu	Val	Ala	Glu		
835						840						845					
Val	Glu	Lys	Arg	Asn	Arg	Leu	Lys	Leu	Leu	Leu	Pro	Trp	Leu	Glu	Ala		
850						855						860					
Arg	Ile	His	Glu	Gly	Cys	Glu	Glu	Pro	Ala	Thr	His	Asn	Ala	Leu	Ala		
865						870						875					
Lys	Ile	Tyr	Ile	Asp	Ser	Asn	Asn	Asn	Pro	Glu	Arg	Phe	Leu	Arg	Glu		
885						890						895					
Asn	Pro	Tyr	Tyr	Asp	Ser	Arg	Val	Val	Gly	Lys	Tyr	Cys	Glu	Lys	Arg		
900						905						910					
Asp	Pro	His	Leu	Ala	Cys	Val	Ala	Tyr	Glu	Arg	Gly	Gln	Cys	Asp	Leu		
915						920						925					
Glu	Leu	Ile	Asn	Val	Cys	Asn	Glu	Asn	Ser	Leu	Phe	Lys	Ser	Leu	Ser		
930						935						940					
Arg	Tyr	Leu	Val	Arg	Arg	Lys	Asp	Pro	Glu	Leu	Trp	Gly	Ser	Val	Leu		
945						950						955					
Leu	Glu	Ser	Asn	Pro	Tyr	Arg	Arg	Pro	Leu	Ile	Asp	Gln	Val	Val	Gln		
965						970						975					
Thr	Ala	Leu	Ser	Glu	Thr	Gln	Asp	Pro	Glu	Glu	Val	Ser	Val	Thr	Val		
980						985						990					
Lys	Ala	Phe	Met	Thr	Ala	Asp	Leu	Pro	Asn	Glu	Leu	Ile	Glu	Leu	Leu		
995						1000						1005					

-continued

Glu Lys	Ile Val	Leu Asp	Asn	Ser Val	Phe Ser	Glu	His Arg	Asn	
1010			1015			1020			
Leu Gln	Asn Leu	Leu Ile	Leu	Thr Ala	Ile Lys	Ala	Asp Arg	Thr	
1025			1030			1035			
Arg Val	Met Glu	Tyr Ile	Asn	Arg Leu	Asp Asn	Tyr	Asp Ala	Pro	
1040			1045			1050			
Asp Ile	Ala Asn	Ile Ala	Ile	Ser Asn	Glu Leu	Phe	Glu Glu	Ala	
1055			1060			1065			
Phe Ala	Ile Phe	Arg Lys	Phe	Asp Val	Asn Thr	Ser	Ala Val	Gln	
1070			1075			1080			
Val Leu	Ile Glu	His Ile	Gly	Asn Leu	Asp Arg	Ala	Tyr Glu	Phe	
1085			1090			1095			
Ala Glu	Arg Cys	Asn Glu	Pro	Ala Val	Trp Ser	Gln	Leu Ala	Lys	
1100			1105			1110			
Ala Gln	Leu Gln	Lys Gly	Met	Val Lys	Glu Ala	Ile	Asp Ser	Tyr	
1115			1120			1125			
Ile Lys	Ala Asp	Asp Pro	Ser	Ser Tyr	Met Glu	Val	Val Gln	Ala	
1130			1135			1140			
Ala Asn	Thr Ser	Gly Asn	Trp	Glu Glu	Leu Val	Lys	Tyr Leu	Gln	
1145			1150			1155			
Met Ala	Arg Lys	Lys Ala	Arg	Glu Ser	Tyr Val	Glu	Thr Glu	Leu	
1160			1165			1170			
Ile Phe	Ala Leu	Ala Lys	Thr	Asn Arg	Leu Ala	Glu	Leu Glu	Glu	
1175			1180			1185			
Phe Ile	Asn Gly	Pro Asn	Asn	Ala His	Ile Gln	Gln	Val Gly	Asp	
1190			1195			1200			
Arg Cys	Tyr Asp	Glu Lys	Met	Tyr Asp	Ala Ala	Lys	Leu Leu	Tyr	
1205			1210			1215			
Asn Asn	Val Ser	Asn Phe	Gly	Arg Leu	Ala Ser	Thr	Leu Val	His	
1220			1225			1230			
Leu Gly	Glu Tyr	Gln Ala	Ala	Val Asp	Gly Ala	Arg	Lys Ala	Asn	
1235			1240			1245			
Ser Thr	Arg Thr	Trp Lys	Glu	Val Cys	Phe Ala	Cys	Val Asp	Gly	
1250			1255			1260			
Lys Glu	Phe Arg	Leu Ala	Gln	Met Cys	Gly Leu	His	Ile Val	Val	
1265			1270			1275			
His Ala	Asp Glu	Leu Glu	Glu	Leu Ile	Asn Tyr	Tyr	Gln Asp	Arg	
1280			1285			1290			
Gly Tyr	Phe Glu	Glu Leu	Ile	Thr Met	Leu Glu	Ala	Ala Leu	Gly	
1295			1300			1305			
Leu Glu	Arg Ala	His Met	Gly	Met Phe	Thr Glu	Leu	Ala Ile	Leu	
1310			1315			1320			
Tyr Ser	Lys Phe	Lys Pro	Gln	Lys Met	Arg Glu	His	Leu Glu	Leu	
1325			1330			1335			
Phe Trp	Ser Arg	Val Asn	Ile	Pro Lys	Val Leu	Arg	Ala Ala	Glu	
1340			1345			1350			
Gln Ala	His Leu	Trp Ala	Glu	Leu Val	Phe Leu	Tyr	Asp Lys	Tyr	
1355			1360			1365			
Glu Glu	Tyr Asp	Asn Ala	Ile	Ile Thr	Met Met	Asn	His Pro	Thr	
1370			1375			1380			
Asp Ala	Trp Lys	Glu Gly	Gln	Phe Lys	Asp Ile	Ile	Thr Lys	Val	
1385			1390			1395			

-continued

Leu Gln Met Ser Ser Leu Arg Ser Glu Asp Thr Ala Leu Tyr Tyr Cys
 85 90 95

Ala Arg His Phe Asp Asn Tyr Glu Gly Asn Ser Met Asp Tyr Trp Gly
 100 105 110

Gln Gly Thr Ser Val Thr Val Ser Ser Ala Lys Thr Thr Pro Pro Ser
 115 120 125

Asp Tyr Pro Leu Ala
 130

<210> SEQ ID NO 3
 <211> LENGTH: 116
 <212> TYPE: PRT
 <213> ORGANISM: Homo sapiens

<400> SEQUENCE: 3

Asp Ile Val Leu Thr Gln Ser Pro Ala Thr Leu Ser Val Thr Pro Gly
 1 5 10 15

Asp Ser Val Ser Leu Ser Cys Arg Ala Ser Gln Ser Ile Arg Ser Asn
 20 25 30

Leu His Trp Tyr Gln Gln Lys Ser His Glu Ser Pro Arg Leu Leu Ile
 35 40 45

Lys Tyr Ala Ser Gln Ser Ile Ser Gly Ile Pro Ser Arg Phe Ser Gly
 50 55 60

Ser Gly Ser Gly Thr Asp Phe Ile Leu Ser Ile Asn Ser Val Ala Thr
 65 70 75 80

Glu Asp Phe Gly Val Tyr Phe Cys Gln Gln Ser Asn Ser Trp Pro Leu
 85 90 95

Thr Phe Gly Ala Gly Thr Lys Leu Glu Leu Lys Arg Ala Asp Ala Ala
 100 105 110

Pro Thr Val Ser
 115

<210> SEQ ID NO 4
 <211> LENGTH: 5
 <212> TYPE: PRT
 <213> ORGANISM: Homo sapiens

<400> SEQUENCE: 4

Asn Tyr Val Met Ser
 1 5

<210> SEQ ID NO 5
 <211> LENGTH: 17
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 <213> ORGANISM: Homo sapiens

<400> SEQUENCE: 5

Thr Ile Ser Ser Gly Asp Asn Tyr Met Tyr Tyr Pro Asp Ser Val Lys
 1 5 10 15

Gly

<210> SEQ ID NO 6
 <211> LENGTH: 12
 <212> TYPE: PRT
 <213> ORGANISM: Homo sapiens

<400> SEQUENCE: 6

His Phe Asp Asn Tyr Glu Gly Asn Ser Met Asp Tyr
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<210> SEQ ID NO 7
<211> LENGTH: 11
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 7

Arg Ala Ser Gln Ser Ile Arg Ser Asn Leu His
1 5 10

<210> SEQ ID NO 8
<211> LENGTH: 7
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 8

Tyr Ala Ser Gln Ser Ile Ser
1 5

<210> SEQ ID NO 9
<211> LENGTH: 8
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 9

Gln Gln Ser Asn Ser Trp Pro Leu
1 5

<210> SEQ ID NO 10
<211> LENGTH: 30
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 10

Glu Val Lys Leu Val Glu Ser Gly Gly Gly Leu Val Lys Pro Gly Gly
1 5 10 15

Ser Leu Asn Ile Ser Cys Ala Ala Ser Gly Phe Thr Phe Ser
20 25 30

<210> SEQ ID NO 11
<211> LENGTH: 14
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 11

Trp Val Arg Gln Thr Pro Glu Lys Arg Leu Glu Trp Val Ala
1 5 10

<210> SEQ ID NO 12
<211> LENGTH: 32
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 12

Arg Phe Thr Ile Ser Ser Asp Asn Ala Lys Asn Thr Leu Phe Leu Gln
1 5 10 15

Met Ser Ser Leu Arg Ser Glu Asp Thr Ala Leu Tyr Tyr Cys Ala Arg
20 25 30

<210> SEQ ID NO 13
<211> LENGTH: 23
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

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<400> SEQUENCE: 13

Trp	Gly	Gln	Gly	Thr	Ser	Val	Thr	Val	Ser	Ser	Ala	Lys	Thr	Thr	Pro
1				5					10					15	

Pro	Ser	Asp	Tyr	Pro	Leu	Ala
			20			

<210> SEQ ID NO 14

<211> LENGTH: 23

<212> TYPE: PRT

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 14

Asp	Ile	Val	Leu	Thr	Gln	Ser	Pro	Ala	Thr	Leu	Ser	Val	Thr	Pro	Gly
1				5					10					15	

Asp	Ser	Val	Ser	Leu	Ser	Cys
				20		

<210> SEQ ID NO 15

<211> LENGTH: 15

<212> TYPE: PRT

<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 15

Trp	Tyr	Gln	Gln	Lys	Ser	His	Glu	Ser	Pro	Arg	Leu	Leu	Ile	Lys
1				5					10					15

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<211> LENGTH: 32

<212> TYPE: PRT

<213> ORGANISM: Homo sapiens

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Gly	Ile	Pro	Ser	Arg	Phe	Ser	Gly	Ser	Gly	Ser	Gly	Thr	Asp	Phe	Ile
1				5					10					15	

Leu	Ser	Ile	Asn	Ser	Val	Ala	Thr	Glu	Asp	Phe	Gly	Val	Tyr	Phe	Cys
			20					25					30		

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<211> LENGTH: 20

<212> TYPE: PRT

<213> ORGANISM: Homo sapiens

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Thr	Phe	Gly	Ala	Gly	Thr	Lys	Leu	Glu	Leu	Lys	Arg	Ala	Asp	Ala	Ala
1				5					10					15	

Pro	Thr	Val	Ser
			20

<210> SEQ ID NO 18

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<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Cloning VEGF f

<400> SEQUENCE: 18

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28

<210> SEQ ID NO 19

<211> LENGTH: 32

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

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<223> OTHER INFORMATION: Cloning VEGF r

<400> SEQUENCE: 19

ctcgagggaa gagagagacg gggtcagaga ga

32

<210> SEQ ID NO 20

<211> LENGTH: 24

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: RT-PCR CLTC f

<400> SEQUENCE: 20

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24

<210> SEQ ID NO 21

<211> LENGTH: 24

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: RT-PCR CLTC r

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24

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<211> LENGTH: 19

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: RT-PCR VEGF f

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19

<210> SEQ ID NO 23

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<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: RT-PCR VEGf r

<400> SEQUENCE: 23

cttggtgagg ttgatccg

19

<210> SEQ ID NO 24

<211> LENGTH: 23

<212> TYPE: DNA

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<223> OTHER INFORMATION: RT-PCR HIF-1alpha f

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ggttcacttt ttcaagcagt agg

23

<210> SEQ ID NO 25

<211> LENGTH: 23

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: RT-PCR HIF-1alpha r

<400> SEQUENCE: 25

gtggtaatcc accttcatcc att

23

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<210> SEQ ID NO 26
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: RT-PCR EPO f

<400> SEQUENCE: 26

agcaggaagc attcagaga 19

<210> SEQ ID NO 27
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<223> OTHER INFORMATION: RT-PCR EPO r

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aggtaaatcg cctccaaag 19

<210> SEQ ID NO 28
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<223> OTHER INFORMATION: RT-PCR PDGF-beta f

<400> SEQUENCE: 28

ctggcatgca agtgtgagac 20

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cgaatgggtca cccgagttt 19

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cttcaccacc atggaggagg c 21

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<223> OTHER INFORMATION: RT-PCR GAPDH r

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<210> SEQ ID NO 32
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<220> FEATURE:
<223> OTHER INFORMATION: ChIP VEGF f

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<400> SEQUENCE: 32

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17

<210> SEQ ID NO 33

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<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: CHIP VEGF r

<400> SEQUENCE: 33

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<223> OTHER INFORMATION: CHIP GAPDH f

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<210> SEQ ID NO 35

<211> LENGTH: 20

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: CHIP GAPDH r

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20

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<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: VH1FOR

<400> SEQUENCE: 36

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34

<210> SEQ ID NO 37

<211> LENGTH: 22

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: VK1FOR

<400> SEQUENCE: 37

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<210> SEQ ID NO 38

<211> LENGTH: 22

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: VH1BACK

<400> SEQUENCE: 38

aggtcaaaact gcagsagtca gg

22

<210> SEQ ID NO 39

<211> LENGTH: 24

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<212> TYPE: DNA
 <213> ORGANISM: Artificial Sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: VK1BACK

<400> SEQUENCE: 39

gacattcagc tgaccagtc tcca

24

What is claimed is:

1. A purified monoclonal antibody or an antigen-binding fragment thereof which specifically binds to human clathrin heavy chain (CHC) comprising the amino acid sequence of SEQ ID NO: 1 and comprises a heavy chain variable region comprising the amino acid sequence of SEQ ID NO: 2.
2. The purified monoclonal antibody or antigen-binding fragment thereof of claim 1, wherein the purified monoclonal antibody comprises a light chain variable region comprising the amino acid sequence of SEQ ID NO: 3.
3. The purified monoclonal antibody or antigen-binding fragment thereof of claim 1, which binds to cells selected from the group consisting of pancreatic cancer cells, breast cancer cells, lung cancer cells, ovary cancer cells, oral cancer cells, and tumor-associated endothelial cells.
4. An isolated monoclonal antibody or an antigen binding fragment thereof binding to human clathrin heavy chain (CHC) and comprising:
 - (a) a heavy chain variable region, comprising:
 - (i) complementarity determining region 1 (CDR1) comprising SEQ ID NO: 4;
 - (ii) complementarity determining region 2 (CDR2) comprising SEQ ID NO: 5; and
 - (iii) complementarity determining region 3 (CDR3) comprising SEQ ID NO: 6; and
 - (b) a light chain variable region, comprising.
 - (i) CDR1 comprising SEQ ID NO: 7;
 - (ii) CDR2 comprising SEQ ID NO: 8; and
 - (iii) CDR3 comprising SEQ ID NO: 9.
5. The isolated antibody or binding fragment of claim 4, wherein the binding fragment comprises an Fv fragment of the antibody.
6. The isolated antibody or binding fragment of claim 4, wherein the binding fragment comprises an Fab fragment of the antibody.
7. The isolated antibody or binding fragment of claim 4, wherein the antibody is a humanized monoclonal antibody.
8. The isolated antibody or binding fragment of claim 4, which binds to a cancer cell expressing clathrin heavy chain (CHC).
9. The isolated antibody or binding fragment claim 4, which is labeled by a detectable compound or an enzyme.
10. The isolated antibody or binding fragment of claim 4, which is encapsulated within a liposome.
11. A method for inhibiting tumor cell growth and/or tumor angiogenesis, comprising: administering to a subject in need thereof a composition comprising, the purified monoclonal antibody or antigen-binding portion thereof of claim 1 and a pharmaceutically acceptable carrier, wherein the tumor expresses human clathrin heavy chain.
12. A method for inhibiting tumor growth and/or tumor angiogenesis, comprising: administering to a subject in need thereof a composition comprising the isolated monoclonal antibody, or binding fragment thereof of claim 4 and a pharmaceutically acceptable carrier, where in tumor express human clathrin heavy chain.
13. The method of claim 11, wherein the tumor cell is selected from the group consisting of pancreatic cancer cells, breast cancer cells, lung cancer cells, ovary cancer cells, oral cancer cells, and tumor-associated endothelial cells.
14. An isolated single-chain antibody comprising:
 - (a) the heavy chain variable region (SEQ ID NO: 2) and the light chain variable region (SEQ ID NO: 3) of the isolated antibody or binding fragment of claim 2; and
 - (b) a linker peptide connecting the heavy chain variable region (SEQ ID NO: 2) and the light chain variable region (SEQ ID NO: 3).
15. A composition comprising:
 - (a) the isolated monoclonal antibody or binding fragment of claim 4; and
 - (b) a pharmaceutically acceptable carrier,
16. The composition of claim 15, further comprising an anti-cancer agent
17. A method for detecting cancer in a subject, comprising:
 - (a) applying the isolated monoclonal antibody or binding fragment thereof of claim 4 to a cell or tissue sample obtained from the subject; and
 - (b) assaying the binding of the isolated monoclonal antibody or binding fragment thereof to the cell or the tissue sample; and
 - (c) comparing the binding with a normal control to determine the presence of the cancer in the subject, wherein the cancer expresses human clathrin heavy chain.
18. The isolated monoclonal antibody or an antigen-binding fragment thereof of claim 1, which exhibits at least one, two, three, four, five, six, seven, or all eight of the following properties:
 - (a) specifically binds to pancreatic adenocarcinoma cells;
 - (b) binds to the cell surface of cancer cells and tumor blood vessels;
 - (c) is internalized by CHC-expressing cells upon binding;
 - (d) inhibiting tumor growth, invasion, migration, and angiogenesis;
 - (e) inducing apoptosis in cancer cells and human umbilical vein endothelial cells (HUVECs);
 - (f) inhibiting tumor growth and tumor blood vessel formation in pancreatic cancer in vivo;
 - (g) suppressing epidermal growth factor (EGF), transferrin (Tf) and VEGF internalizations by cancer cells; and
 - (h) suppressing hypoxia-inucible factor-1 α (HIF-1 α) expression and vascular endothelial growth factor (VEGF) secretion.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE
CERTIFICATE OF CORRECTION

PATENT NO. : 8,969,530 B2
APPLICATION NO. : 13/644311
DATED : March 3, 2015
INVENTOR(S) : Han-Chung Wu and Kuo-Hua Tung

Page 1 of 1

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

In the Claims

Column 45, Line 29, Claim 1 delete “binging” and substitute --binding--

Column 46, Line 15, Claim 12 delete “antibody, or” and substitute --antibody or--

Column 46, Line 17, Claim 12 delete “carrier, where in tumor express” and substitute --carrier,
wherein the tumor expresses--

Signed and Sealed this
Twenty-first Day of July, 2015



Michelle K. Lee
Director of the United States Patent and Trademark Office

专利名称(译)	用于抑制肿瘤血管生成和生长的抗网格蛋白重链单克隆抗体及其应用		
公开(公告)号	US8969530	公开(公告)日	2015-03-03
申请号	US13/644311	申请日	2012-10-04
[标]申请(专利权)人(译)	中央研究院		
申请(专利权)人(译)	中央研究院		
当前申请(专利权)人(译)	中央研究院		
[标]发明人	WU HAN CHUNG TUNG KUO HUA		
发明人	WU, HAN-CHUNG TUNG, KUO-HUA		
IPC分类号	C07K16/00 A61K39/00 A61K39/395 G01N33/53 C12P21/08 C07K16/18 C07K16/30		
CPC分类号	C07K16/18 C07K16/303 C07K2317/73		
优先权	61/543118 2011-10-04 US		
其他公开文献	US20130084298A1		
外部链接	Espacenet USPTO		

摘要(译)

纯化的单克隆抗体，或。公开了其特异性结合人网状蛋白重链（CHC）的抗原结合部分。抗体或其抗原结合部分表现出以下性质中的至少一种，两种，三种，四种，五种，六种，七种或全部八种：（a）特异性结合胰腺癌细胞；（b）与癌细胞和肿瘤血管的细胞表面和胞质溶胶结合；（c）由表达CHC的细胞内化；（d）抑制肿瘤生长，侵袭能力，迁移和血管生成；（e）诱导癌细胞和人脐静脉内皮细胞凋亡；（f）体内抑制胰腺癌中的肿瘤生长和肿瘤血管；（g）抑制癌细胞表皮生长因子，转铁蛋白和VEGF内化；（h）抑制缺氧诱导因子-1 α 表达和血管内皮生长因子分泌。还公开了抑制受试者中肿瘤细胞生长和/或血管生成以及检测癌症的方法。

FIG. 1

