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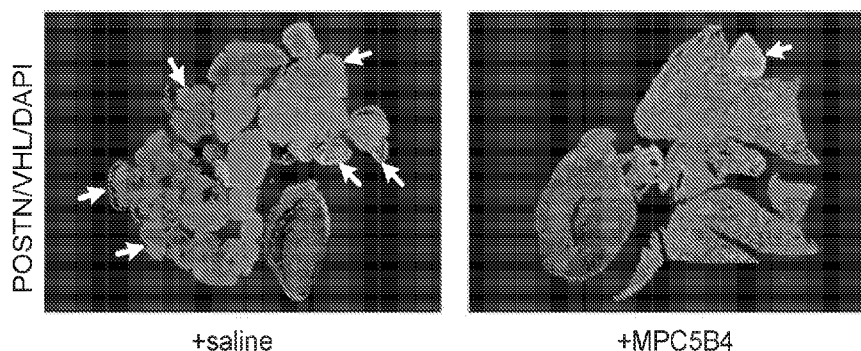


Figure 6C

(57) Abstract: Methods and pharmaceutical compositions for inhibiting metastasis of renal cell carcinoma (RCC) in a subject having RCC comprising reducing presence or biological activity of periostin. Methods for treating or preventing metastasis in a subject having RCC comprising administering to a primary RCC tumor site an anti-periostin (POSTN) monoclonal antibody and genetically modifying RCC tumor cells to delete the POSTN gene in RCC tumor cells.



METHODS AND COMPOSITIONS FOR INHIBITING PERIOSTIN-MEDIATED METASTATIC RENAL CELL CARCINOMA

GOVERNMENT SUPPORT

[001] This invention was made with government support under Grant Number CA216770, awarded by the National Institutes of Health. The government has certain rights in the invention.

CROSS-REFERENCE TO RELATED APPLICATIONS

[002] This application claims priority to and benefit of U.S. Provisional Application No. 62/810,192, filed February 25, 2019, which is hereby incorporated by reference in its entirety.

FIELD OF THE INVENTION

[003] Provided herein are pharmaceutical compositions and methods for treating, inhibiting and preventing periostin-mediated metastasis of renal cell carcinoma.

BACKGROUND OF THE INVENTION

[004] Renal cell carcinoma (RCC) is the most common cancer of the kidney, and it arises from the epithelial cells of renal cortex. RCC consistently ranks amongst the top ten most prevalent malignancies in the world, with over 62,000 and 250,000 newly diagnosed cases annually in US and worldwide, respectively. RCC is characterized by a wide range of histological subtypes with variable clinical behaviors. The clear cell subtype of RCC (ccRCC) makes up over 70% of RCC, and features tumor cells with abundant clear cytoplasm and acentric nuclei. Patients with localized disease are treated with nephrectomy and have a favorable 5-year survival of 73%. Unfortunately, approximately 30% of patients will develop metastatic disease, frequently spreading to distant vital organs, such as the lung. Despite the development of new targeted therapies, patients with metastatic ccRCC have a very poor outcome with a median survival of 13 months and a five-year survival rate of only 11%.

[005] Detailed studies of the von Hippel–Lindau (VHL) disease, a rare hereditary cancer syndrome manifested by renal, CNS, adrenal and pancreatic tumors, led to the identification and cloning of the VHL tumor suppressor gene. VHL plays an integral role in the pathogenesis of the

sporadic, non-familial form of ccRCC, as somatic mutations of this gene are reported to be seen in as high as 90% of cancer cases, the majority being missense or nonsense loss of function mutations. Seminal research in the last two decades have unraveled the VHL protein's intricate and important function as an E3 ubiquitin ligase that targets the degradation of the alpha subunit of hypoxia inducible transcription factors (HIF- α s) in an oxygen-dependent manner. The constitutive activation of the HIF pathway through loss of VHL function has implicated this pathway as an oncogenic driver and therapeutic target. Despite intensive investigative efforts, the precise oncogenic mechanism of VHL loss remains elusive. Numerous mouse models of renal tubule targeted deletion of VHL gene have failed to generate renal lesions beyond preneoplastic cysts, even when combined with deletion of other tumor suppressor genes such as PTEN or p53. It is clear that the loss of VHL function upregulates both HIF1 α and HIF2 α , however these two paralogs appear to have distinct and often contrary roles in their gene regulatory activities. Recent research suggests that HIF2 α plays a dominant oncogenic role, whereas HIF1 α is tumor suppressive in ccRCC. These opposing oncogenic roles of HIF1 α (HIF1A) and HIF2 α (HIF2A) are an active area of debate.

[006] The potential contribution of VHL loss or its downstream effectors to metastatic progression is also poorly defined. The analysis of numerous ccRCC clinical specimen was unable to find a significant correlation between VHL mutation status and clinical outcome. It has been reported that the silencing or deletion of the VHL gene consistently resulted in epithelial to mesenchymal transition (EMT). EMT is an embryonic program used by polarized epithelial cells to break away from cell-cell contacts and basement membrane attachment, enabling cellular migration to distant sites. It is highly reminiscent of the process that carcinomas adapt during metastatic spread, although the direct role of EMT in cancer metastasis is under debate.

[007] Accordingly, there exists a need for compositions and methods for treating periostin-mediated metastasis of renal cell carcinoma, in particular metastasis of clear cell renal cell carcinoma.

SUMMARY OF THE INVENTION

[008] In one aspect, the invention provides a method for inhibiting metastasis of renal cell carcinoma (RCC) in a subject having RCC, the method comprising reducing presence or biological activity of periostin, wherein reducing the presence or biological activity of periostin inhibits metastasis.

[009] In another aspect, the invention provides a method for treating or preventing metastasis in a subject having RCC, the method comprising administering to a primary RCC tumor site an anti-periostin (POSTN) monoclonal antibody and genetically modifying RCC tumor cells to delete the POSTN gene in RCC tumor cells.

[010] In a further aspect, the invention provides a pharmaceutical composition comprising an adenoviral vector comprising CRISPR/Cas9 and a guide RNA (gRNA), wherein the gRNA targets a nucleic acid encoding periostin and/or an anti-periostin (POSTN) monoclonal antibody.

[011] 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 belongs. Other features and advantages of the present invention will become apparent from the following detailed description examples and figures. It should be understood, however, that the detailed description and the specific examples while indicating certain embodiments of the invention are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description. It is also contemplated that whenever appropriate, any embodiment of the present invention can be combined with one or more other embodiments of the present invention, even though the embodiments are described under different aspects of the present invention.

BRIEF DESCRIPTION OF THE DRAWINGS

[012] **Figures 1A-1K** show that VHL-KO cells cooperate with VHL-WT cells to achieve metastasis. **Fig. 1A** shows mice were implanted with a total of 1×10^6 tumor cells, either RC-VHL-WT or RC-VHL-KO cells alone or a 1:1 mix of 2 cell types into their left kidney (n=6 per group). Images of 3 representative animals assessed by BLI on week 4 after implantation were shown. Lung metastasis indicated by elevated BLI signals over the chest were observed in the mixed tumor group (bar graph, right panel). **Fig. 1B** shows H&E stained sections (low magnification) and anti-VHL IHC staining of lung and heart in RC-VHL-WT tumor group, and **Fig. 1C** shows H&E stained sections and anti-VHL IHC staining of lung and heart in mixed tumor group. H&E stain showed a dramatic increase in lung metastases in the mixed tumor group. VHL stain showed numerous large VHL+ metastatic lesions in the lungs of a mouse with a mixed tumor, implanted with a 1:4 ratio of RC-VHL-WT to RC-VHL-KO cells. Arrowhead = heart. **Fig. 1D** shows Immunofluorescence (IF) staining of a small lung metastatic lesion from a 1:1 mixed tumor

bearing mouse. This lesion consisted of predominantly of HA+ (red) RC-VHL-WT cells with a few flag+ (green) VHL-KO cells dispersed centrally. **Fig. 1E** shows flow cytometry of disrupted lung tissue from the 1:1 mix tumor bearing mouse showed the great predominance of RC-VHL-WT cells (marked with HA-tagged mStrawberry) over RC-VHL-KO cells (marked with flag-tagged EGFP). **Fig. 1F** shows VHL-deleted ACHN human RCC line, AC-VHL-KO cells, underwent EMT with elevated expression of EMT markers, assessed by RT-PCR compared to the parental VHL+ ACHN (AC-VHL-WT) cells. **Fig. 1G** shows *in vitro* growth of AC-VHL-KO cells are significantly slower than AC-VHL-WT cells. **Fig. 1H** shows tumor established in Nu mice showed AC-VHL-WT and mixed tumor group established comparable primary tumor in the kidney. However, the lung volume of the mixed tumor group was greatly expanded. **Fig. 1I** shows growth of RC-VHL-KO tumors in mice is significantly slower than either RC-VHL-WT or the 1:1 mixed tumors, as assessed by BLI on week 4 after implantation. **Fig. 1J** shows flow cytometry was used to analyze the disrupted tumors at 4-weeks endpoint, enumerating the RC-VHL-WT cells marked with HA-tagged mStrawberry and RC-VHL-KO cells marked with flag-tagged EGFP. The number of cells in the VHL-KO tumor is significantly lower than the RC-VHL-WT and mixed tumor. **Fig. 1K** shows RC tumor growth was assessed in CAM tumor system. The growth of the 3 groups of CAM tumors was observed longitudinally. RC-VHL-KO tumors again grew poorly on CAM compared to RC-VHL-WT or 1:1 mixed tumors. Day 0 is the day of tumor cell implantation = day 7 post egg fertilization.

[013] **Figures 2A-2H** show human ccRCC tumor specimen consistently showed intratumoral heterogeneity of VHL expression. **Fig. 2A** shows H&E stain and **Fig. 2B** shows VHL IHC performed on parallel sections of tumor from a highly aggressive case of human ccRCC (case #22, **Table 1**). High magnification images show cytoplasmic VHL expression in tumor cells specifically in area (a), while VHL-expressing and non-expressing cells co-existed in area (b). VHL IHC of case #22 revealed extensive intratumoral heterogeneity with interspersed VHL+ and VHL- areas through the tumor. **Fig. 2C** shows H&E stain and **Fig. 2D** shows VHL IHC of the lung metastases of case #22 revealed a predominance of VHL+ tumor cells in the lung metastasis. **Fig. 2E** shows CAM tumors of case #22 were established by implanting small tumor chunks. Images of H&E stain and VHL IHC of the CAM tumor was shown. In **Fig. 2F** mutations calls from WES on common oncogenic driver genes known in ccRCC are shown for 4 loci of patient's tumor and derivative cell lines in the case #22. Point sizes represent variant allele frequencies. Values above

0.4 or 0.9 represent likely clonal mutations or clonal mutations combined with loss of heterozygosity respectively. Colors represent log2 copy number ratios (CNR) for each gene, with DNA gains in red and losses in blue. **Fig. 2G** shows density plot showing the CNR of the VHL locus after adjusting for both tumor purity and ploidy in the TCGA-KIRC cohort (n = 421). A CNR value of -1 represents a single copy loss of VHL (dotted line), suggesting that many TCGA samples have subclonal single copy loss. **Fig. 2H** shows density plot showing the VAF of somatic VHL mutations after adjusting for both tumor purity and ploidy in the TCGA-KIRC cohort (n = 66). The peak near VAF equal to 0.5 or 1 represents clonal somatic mutations, while other peaks represent subclonal mutations.

[014] **Figures 3A-3F** show VHL-KO cells induce proliferation, EMT and motility of VHL-WT cells. **Fig. 3A** shows a section of primary tumor with 1:1 mix of RC-VHL-KO and RC-VHL-WT cells implanted into mouse kidney was stained by IF to detect VHL (red), Ki67 (green) and nuclei (DAPI, blue). The dash lines demarcate VHL negative cell areas with intact nuclei. The % of Ki67 positivity is much higher in the VHL-positive area than VHL-negative area of mixed tumor lesion. **Fig. 3B** shows consecutive sections from a large lung metastatic lesion of a 1:1 mixed tumor stained with H&E, VHL and Ki67 revealed the prominence of RC-VHL-WT cells in the metastasis and they are more proliferative than the RC-VHL-KO cells. **Fig. 3C** shows the growth of RC-VHL-WT cells was enhanced under co-cultured with RC-VHL-KO cells (orange line) comparing to RC-VHL-WT cells cultured alone (red line). VHL-KO cells alone (green line) grew the slowest.. (*:p<0.05, **:p<0.01). **Fig. 3D** shows RC-VHL-WT cells grown in the presence of VHL-KO cells in transwell setting (VHL-WT +VHL-KO) express elevated EMT marker genes compared to VHL-WT cells grown alone. **Fig. 3E** shows RC-VHL-WT cells were marked with HA-tagged mStrawberry FP and RC-VHL-KO cells with flag-tagged EGFP. The motility of either RC-VHL-WT cells alone or VHL-KO cells alone or co-cultured of both cells (mixed) were monitored in a 2D scratch assay by time lapse live cell microscopy over 20 hrs. Migration speed of the 3 cells, VHL-WT, VHL-KO or VHL-WT in mixed culture were quantified (right graph). **Fig. 3F** shows the migration VHL-WT cells alone was also compared to VHL-WT cells with the addition of conditioned media from VHL-KO cells.

[015] **Figures 4A-4J** show the loss of VHL upregulates HIF1A and POSTN to induce EMT and motility. **Fig. 4A** shows the migration speed of VHL-WT cells (marked with mStrawberry) either co-culture with VHL-KO cells (marked with EGFP) or VHL/HIF1A-KO cells (marked with EGFP)

was assessed by time lapse microscopy in 2D scratch assay over 20 hrs. **Fig. 4B** shows human 786-O cells were transiently transfected with HIF1A or HIF2A encoded plasmid. The transfection resulted in the designed over expression of each HIF gene specifically (left panel). **Fig. 4C** shows the overexpression of HIF1A induced EMT markers assessed at the RNA level by RT-PCR (right panel) or at the protein expression level of MMP9. **Fig. 4D** shows VHL, HIF1A and POSTN protein expression in RC-VHL-WT, RC-VHL-KO and RC-VHL/HIF1A-KO double gene knockout cells was analyzed by Western immunoblot. **Fig. 4E** shows the promoter of POSTN from -2000 to -63 was cloned into pGL3-basic vector driving the firefly luciferase gene. Co-transfection of a HIF1A expressing vector significantly increase the POSTN promoter activity (left graph). No significant change was observed with co-transfecting HIF2A expressing vector (right graph). **Fig. 4F** shows the migration speed of VHL-WT cells (marked with mStrawberry) cocultured with VHL/POSTN-KO cells (marked with EGFP) was compared to VHL-WT cells cocultured with VHL-KO cells (marked with EGFP). **Fig. 4G** shows anti-POSTN mAb MPC5B4 was added at 1ug/ml to VHL-WT cells co-cultured with VHL-KO cells. The migration speed of VHL-WT cells without and with MPC5B4 was assessed. **Fig. 4H** shows the addition of recombinant POSTN protein increased the motility of VHL-WT cells and this enhancement was suppressed by the addition of Celentigide, an integrin inhibitor. **Fig. 4I** shows recombinant POSTN addition activates the FAK phosphorylation at Tyr 397 in VHL-WT cells, which is blocked by Cilengitide. **Fig. 4J** shows the addition of Cilengitide to mixed culture was able to abrogate the VHL-KO cells mediated motility enhancement on VHL-WT cells in a dose dependent manner. (*:p<0.05, **:p<0.01).

[016] **Figures 5A-5F** show VHL-KO cells cause vascular destruction to enhance intravasation. **Fig. 5A** shows sequential flow cytometric analysis of circulatory tumor cells at 2, 3, 4 and 6 weeks after implantation of VHL-WT or 1:1 mixed cells. **Fig. 5B** shows a 3D endothelial invasion assay was performed by placing a layer of tumor cells, either mStrawberry VHL-WT cells or EGFP marked VHL-KO cells or 1:1 mixed cells, over a layer of Matrigel® (~ 30 µm thick) right above an endothelial HUVEC cell layer (marked with tagBFP). HUVEC cell area were assessed 48 hrs. after co-culture (graph, right); **Fig. 5C** shows HUVECs were cocultured in transwell with VHL-WT or VHL-KO cells without or with anti-POSTN mAb MPC5B4. After 48 hrs of co-culture HUVEC cell extract harvested was analyzed by Western immunoblot for necroptosis and apoptosis associated proteins. **Fig. 5D** shows HUVEC cells co-cultured with VHL-WT or VHL-KO cells

for 48hrs and assessed for necroptosis by a reporter assay, scoring for uptake of EthD-III(+), normalized to Hoechst 33342(+) nuclei count. **Fig. 5E** shows apoptosis caspase 3/7 glow luminescence reporter assay was used to evaluate HUVECs co-cultured with VHL-WT or VHL-KO cells or VHL-KO cells plus 1ug/ml of MPC5B4 anti-POSTN mAb. **Fig. 5F** shows tumor vascular leakage was assessed by the Miles assay on VHL-WT or 1:1 mixed CAM tumors. Evans Blue dye was injected IV into the chick embryo. The extent of tumor vascular leakage was scored by the amount of dye leaked and retained in the tumor. (*:p<0.05, **:p<0.01)

[017] **Figures 6A-6G** show inhibition of POSTN blocks metastasis. **Fig. 6A** shows intrarenal co-implantation of 1×10^6 total cells of RC-VHL-WT and RC-VHL-KO cells or RC-VHL-WT and RC-VHL/POSTN-KO cells at 1:1 ratio. BLI at 4 weeks post implantation showed that the VHL-KO cells can induce lung metastasis but not the POSTN and VHL double gene knockout (VHL/POSTN-KO) cells. **Fig. 6B** shows mice received renal implantation of RC-VHL-WT and RC-VHL-KO cells were treated with either control IgG or MPC5B4 anti-POSTN mAb. Primary tumors and lungs harvested at 4 weeks after tumor implantation are shown. **Fig. 6C** shows immunofluorescent stain of lung lobes and heart from control- or MPC5B4-treated tumor bearing animals were shown. POSTN stained in red, VHL stained green and DAPI in blue. White arrows indicate some of the lung metastases. **Fig. 6D** shows H&E stain of the same tumor sections shown in **Fig. 6C**. **Fig. 6E** shows suppression of lung metastasis by MPC5B4 treatment examination of lung weights from the animal subjects. **Fig. 6F** shows a summary of paracrine pro-metastatic functions mediated by POSTN expressed by VHL-KO cells. POSTN can induce the motility and EMT on the epithelial VHL-WT cells. POSTN can also induce apoptosis on endothelial cells and cause vascular leakage. **Fig. 6F** shows the cooperative metastatic mechanism uncovered by the inventors' model's paracrine interactions between VHL-KO and VHL-WT cells at the primary tumor, promoted the aggression of VHL-WT cells. **Fig. 6G** shows POSTN was identified as a soluble metastatic mediator that augments the intravasation step (1). Increased tumor cell survival in circulation (step 2) and enhancing metastatic colonization (step 3) could be additional steps to augment metastasis.

[018] **Figures 7A(a)-7F** show segregated expression of HIF1A and HIF2A that informed on VHL-expression status and cellular proliferation in metastatic ccRCC. **Fig. 7A(a)-7A(b)** VHL-IHC and Ki-67-immunofluorescence staining in parallel sections of human ccRCC (case #22, Table I). A high magnification image shows cytoplasmic VHL expression specifically in area (**Fig.**

7A(b)) and not in area (**Fig. 7A(a)**). The bar graph shows the average percentage of Ki-67 in the VHL-positive and VHL-negative regions. **Fig. 7B** shows spatial relationship of VHL and Ki-67 expression in case #22 was assessed by double IF staining. Fluorescent images were analyzed by the HALO (Indica, USA) software. In the first panel and fifth panel, VHL positive cells and Ki-67 positive cells are represented by blue dots. The second panel shows a heatmap of VHL+ cells intensity. The third panel reveals a boundary map of VHL+ tumor regions with topographic contour lines indicating the distance from the tumor boundary. For distance measurements of Ki67+ cells, contour lines were placed up to 2000 μ m from the tumor edge towards the inside of the tumor and up to 4000 μ away from the tumor edge of VHL+ tumor regions. Regions in between the contour lines are shown as different colors from the innermost red to farthest blue. Ki-67+ cells in each region were counted, normalized to the area and plotted in the histogram in the fourth panel. The greatest density of Ki-67+ cells is indicated by the peak at the boundary VHL+ tumor regions. IF stained tissue sections from **Fig. 7C** shows a primary renal tumor and **Fig. 7D** shows lung metastatic lesion from a mouse implanted with 1:4 RC-VHL-WT: RC-VHL-KO tumor cells. VHL, HIF1A, HIF2A and nuclei (DAPI) is marked by red, white, green and blue fluorescence, respectively. **Fig. 7E** shows a boundary map of HIF2A+ tumor regions in case #22 with topographic contour lines indicating the distance from the tumor boundary. Quantitative analysis by HALO software showed that HIF2A and HIF1A expression is located in different tumor cells in distinct area of the tumor, such that area with high HIF2A expression is low in HIF1A expression and vice versa. **Fig. 7F** shows representative images of IF stain detecting VHL, HIF1A, HIF2A expression and nuclei in the primary tumor of case #22 are shown.

[019] **Figures 8A-8G** show the upregulation of POSTN in VHL negative areas of human ccRCC tumors. **Fig. 8A** shows consecutive sections from a large lung metastatic lesion developed from 1:1 VHL-WT and VHL-KO mixed tumor were stained with H & E, against HA tag (VHL-WT cells), flag tag (VHL-KO cells) and anti-POSTN. POSTN expression correlated to VHL-KO cells but is excluded from the VHL-WT cells. **Fig. 8B** shows tissue microarray (TMA) of over 300 cases of RCC patients were assessed for VHL and POSTN expression. Representative images from 16 cases showing inverse correlated expression pattern between VHL and POSTN. **Fig. 8C** shows IHC stain for VHL and POSTN were performed on consecutive sections for the tumor tissue of case #22. Higher magnification of the boxed area a and b were shown on the right. **Fig. 8D** shows the multiplex IF stain, analyzed by HALO software, revealed the cellular distribution of

VHL+POSTN- and VHL-POSTN+ cells in the case #22. **Fig. 8E** shows that by utilizing the cell coordinates produced in **Fig. 8D** and by similar approach described in **Fig. 7B**, the VHL+POSTN- cells and VHL-POSTN+ cells in each evenly divided areas with respect to the interface border of VHL+POSTN- area were counted, normalized to each area and plotted in the rightmost curve. The leftmost graph indicates the VHL+POSTN- cell distribution heatmap and its area definition (red line). The middle graph illustrates the VHL-POSTN+ cell distribution. The warmer color (orange) in the heatmap encircles areas of denser compacted cells and cooler color (dark blue) signifies areas with sparser cells. The rightmost plot shows VHL+POSTN- cells (red curve, left y-axis) and VHL-POSTN+ cells (green curve, right y-axis) cluster with their own kinds of cells, and spatially distributed reciprocally, indicating these two populations are spatially separated. **Fig. 8F** shows the lung metastatic lesions of case #22 and **Fig. 8G** shows the retroperitoneal metastatic lesion of case #17 (see **Table 1**) were stained by H&E or by IF to detect VHL(red), POSTN(green) and nucleus (DAPI, blue). Low (upper row) and high magnification (lower row) views of the boxed area were shown.

[020] **Figures 9A-9J** further show metastasis requires cooperation between VHL-KO and VHL-WT RCC cells. **Fig. 9A** shows lung metastases from RVN tumors consisted largely of VHL expressing cells with minor pockets of VHL-deleted, MMP-9+ cells. **Fig. 9B** shows mice bearing mixed renal tumors suffered tumor cachexia with significant weight loss. **Figs. 9C, 9D** show that due to retarded primary tumor growth no lung metastases were observed in the VHL-KO tumor group. **Fig. 9E** shows consistent with the findings in the RC model, a clonal AC-VHL-KO line exhibited EMT cell morphology slower growth as compared to AC-VHL-WT cells shown in **Fig. 9F**. The *in vivo* metastatic behavior of this AC model also reproduced that observed in the RC model. **Fig. 9G** shows a clonal AC-VHL-KO line exhibited EMT cell morphology. **Fig. 9H** shows lung metastases were observed only in the mixed tumor group as assessed by detailed histology. **Fig. 9I** shows the CAM tumor model not only substantiated the poor growth phenotype of VHL-KO tumors, compared to VHL-WT and 1:1 mixed tumors. **Fig. 9J** shows the significant increase of metastatic tumor cells in circulation of the mixed tumor group was demonstrated.

[021] **Figures 10A-10C** further describe intratumoral heterogeneity of VHL expression occurs in human ccRCC. **Fig. 10A** shows the analysis of a total of 26 cases of ccRCC, a total of 26 cases of ccRCC, the first 16 cases were collected from paraffin embedded samples analyzed a total of 26 cases of ccRCC, a total of 26 cases of ccRCC, the first 16 cases were collected from paraffin

embedded samples. **Fig. 10B** shows the most recent 10 cases are freshly harvested tumor specimens for H&E stain. **Fig. 10C** shows representative images of VHL stain of case #18, 21, 24 and 25 are shown.

[022] **Figures 11A-11I** describe further investigation of the relationship between VHL mutational status and the metastatic process. **Fig. 11A** shows similar to the 3D migration assay, a layer of either VHL-WT or VHL-KO or mixed 1:1 cells were placed above a layer of HUVEC endothelial cells, separated by a thin layer of Matrigel®. **Fig. 11B** shows POSTN is upregulated in kidney cancer and is a poor prognostic indicator for RCC. **Figs. 11C, 11D** show POSTN was upregulated in VHL-deleted RC cells and the knockdown of HIF1A reduced POSTN expression at the RNA level. **Fig. 11E** shows this finding suggests these VHL+, HIF2A+ tumor cells are the more proliferative population in the tumor. **Fig. 11F** shows the addition of recombinant POSTN to VHL-WT cells significantly promoted their motility and the addition of the cyclic peptide integrin inhibitor, Cilengitide, blocked this POSTN-mediated motility enhancement. **Fig. 11G** shows as a secreted factor, POSTN's impact is unlikely to be limited just to VHL-WT cells, as Cilengitide also inhibited the motility of VHL-KO cells. **Fig. 11H** shows further analysis of POSTN's mechanism of action showed that either co-culture with VHL-KO cells or the addition of recombinant POSTN was able to induce the phosphorylation of FAK at Tyr 397 in HUVEC cells and Cilengitide inhibited this FAK activation. **Fig. 11I** shows further analysis of POSTN's mechanism of action showed that either co-culture with VHL-KO cells or the addition of recombinant POSTN was able to induce the phosphorylation of FAK at Tyr 397 in HUVEC cells and Cilengitide inhibited this FAK activation.

[023] **Figures 12A-12E** show results of tests on a highly aggressive case of human ccRCC (case cell line #22). **Fig. 12A** shows whole exome sequencing (WES) of this #22 cell line and the parental tumor showed they shared higher than 80% of the mutations, and in **Fig. 12B**, leading to amino acid substitution of L169P. **Fig. 12C** shows this L169P substituted VHL functioned expectedly as HIF1A protein in this #22 line was degraded in normoxia and stabilized upon its deletion. **Fig. 12D** shows that a primary cell line generated from case #24 showed prominent lipid droplet in cytoplasm (Oil Red O stain). **Fig. 12E** show amongst the primary tumor cell lines (#21, 22, 23, 24) that were generated, VHL- and HIF2A-expressing cells predominate

DETAILED DESCRIPTION OF THE INVENTION

[024] In the following detailed description, numerous specific details are set forth in order to provide a thorough understanding of the invention. However, it will be understood by those skilled in the art that the present invention may be practiced without these specific details. In other instances, well-known methods, procedures, and components have not been described in detail so as not to obscure the present invention.

[025] In one aspect, the invention provides a method for inhibiting metastasis of renal cell carcinoma (RCC) in a subject having RCC, the method comprising reducing presence or biological activity of periostin, wherein reducing the presence or biological activity of periostin inhibits metastasis.

[026] In some embodiments, the reducing the presence or biological activity of periostin is provided at a primary tumor site of the RCC and/or in a stromal fibroblast in proximity to the primary tumor site. In various some embodiments, the reducing the presence of biological activity of periostin comprises reducing, silencing or eliminating the expression of periostin. In certain embodiments, the reducing the presence or biological activity of periostin comprises genetically modifying an RCC tumor cell to delete a nucleic acid encoding periostin. In particular embodiments, the genetic modification comprises administering to the subject an adenoviral vector comprising CRISPR/Cas9 and a guide RNA (gRNA), wherein the gRNA targets the nucleic acid encoding periostin.

[027] In some embodiments, the reducing the presence or biological activity of periostin comprises administering to the subject an anti-periostin (POSTN) monoclonal antibody. In certain embodiments, the reducing the presence or biological activity of periostin comprises administering to the subject an antisense oligonucleotide, shRNA, siRNA. In various embodiments, the reducing the presence or biological activity of periostin comprises administering to the subject an POSTN receptor antagonist. In some embodiments, the POSTN receptor antagonist is an $\alpha_v\beta_5$ integrin ($\alpha_v\beta_5$) and/or an $\alpha_v\beta_3$ integrin ($\alpha_v\beta_3$).

[028] In particular embodiments of the herein provided methods, the methods further comprise detecting a deletion of Von Hippel Lindau (VHL) tumor suppressor gene (VHL-KO) in the RCC tumor cell prior to the reducing the presence or biological activity of periostin.

[029] In some embodiments, of the herein provided methods, the methods further comprise detecting overexpression of POSTN in the RCC tumor cell prior to the reducing the presence or biological activity of periostin.

[030] In certain embodiments of the herein provided methods, the methods further comprise administering to the subject an antiangiogenic drug, a tyrosine-kinase inhibitor (TKI), interleukin-2, or a combination thereof.

[031] In some embodiments, the CRISPR/Cas9 targets a codon of the nucleic acid encoding periostin for base editing into a nonsense codon. In certain embodiments, the base editing is performed after detection of overexpression of POSTN in the RCC tumor cell. In various embodiments, the base editing inhibits expression of periostin.

[032] In particular embodiments, the inhibition of the expression of the periostin decreases or prevents development of lung metastasis in the subject. In some embodiments, the expression of periostin is inhibited by 50% to 100%.

[033] In some embodiments of the herein provided methods, the methods further comprise administering to the subject a second adenoviral vector, wherein the second adenoviral vector comprises a nucleic acid encoding a VHL tumor suppressor.

[034] In various embodiments, the biological activity of periostin comprises cell adhesion, cell motility, growth, migration and invasion of cancer cells, binding to one or more integrin, regulation of epithelial-mesenchymal transition (EMT) and/or induction of apoptosis of endothelial cells.

[035] In another aspect, the invention provides a method for treating or preventing metastasis in a subject having RCC, the method comprising administering to a primary RCC tumor site an anti-periostin (POSTN) monoclonal antibody and genetically modifying RCC tumor cells to delete the POSTN gene in RCC tumor cells.

[036] In some embodiments, genetically modifying the RCC tumor cells comprises administering to the subject an adenoviral vector comprising CRISPR/Cas9 and a gRNA, wherein the gRNA targets the nucleic acid encoding periostin.

[037] In further embodiments, genetically modifying the RCC tumor cells reduces, silences or eliminates expression of periostin therein. In some embodiments, the reduction, silencing or elimination of periostin expression decreases or prevents development of lung metastasis in the subject. In particular embodiments, the periostin expression is reduced by 50% to 100%.

[038] In some embodiments of the herein provided methods, the methods further comprise detecting a deletion of Von Hippel Lindau (VHL) tumor suppressor gene (VHL-KO) in the RCC tumor cells prior to genetically modifying the RCC tumor cells.

[039] In certain embodiments of the herein provided methods, the methods further comprise detecting overexpression of POSTN in the RCC tumor cells prior to genetically modifying the RCC tumor cells.

[040] In various embodiments, the herein proved methods further comprise administering to the subject an antiangiogenic drug, a tyrosine-kinase inhibitor (TKI), interleukin-2, or a combination thereof.

[041] In certain embodiments, the herein proved methods further comprise administering to the subject an antisense oligonucleotide, shRNA, siRNA.

[042] In a further aspect, the invention provides a pharmaceutical composition comprising an adenoviral vector comprising CRISPR/Cas9 and a guide RNA (gRNA), wherein the gRNA targets a nucleic acid encoding periostin and/or an anti-periostin (POSTN) monoclonal antibody.

[043] In some embodiments, the pharmaceutical composition further comprises a pharmaceutically acceptable carrier. In various embodiments, the pharmaceutical composition is formulated for intra-tumoral injection or intravenous injection. In particular embodiments, the pharmaceutical composition further comprises a second adenoviral vector, wherein the second adenoviral vector comprises a nucleic acid encoding a VHL tumor suppressor.

[044] The following examples are presented in order to more fully illustrate the preferred embodiments of the invention. They should in no way be construed, however, as limiting the broad scope of the invention.

EXAMPLES

METHODS AND MATERIALS

Cells, Plasmids and Reagents

[045] RENCA (RC) cell line was purchased from ATCC and it is maintained in RPM-1640 supplemented with 10% FBS and 1x Penicillin/Streptomycin. All CRISPR/Cas9 mediated knockout RC cell lines were selected with puromycin and clonally purified via single cell cloning in a 96-well plate. The lentiviral vectors encoding HA-tagged mStrawberry FP, modified from pSicoR (Addgene, #11579), was used to label RC-VHL-WT cells, while the vector encoding flag-tagged EGFP was used to label RC-VHL-KO or RC-VHL/HIF1A-KO or RC-VHL /POSTN-KO

cells. In addition, for *in vivo* studies all cell lines were also marked with lentivirus expressing firefly luciferase, enabling bioluminescence imaging (BLI). pGL3-basic was from Promega Corp. (Cat#E1751) and was enzymatically digested with MluI and XhoI. Periostin promoter was cloned via primers (Forward: CGACGCGTTAAGGTGGACAGTGAGGAAGACACA [SEQ ID NO:1]; Reverse: CCGCTCGAGTTGAGAAGAACGAGAGTAGAGATTTTAGG [SEQ ID NO:2]) from genomic DNA RENCA cells. The control renilla luciferase vector was pRL-TK from Promega Corp. (Cat# E2231). HIF1A overexpressing plasmid was from Addgene (Cat# 44028) and is constitutively active.

Time lapse microscopy for 2D scratch assay and 3D migration assay

[046] 1×10^5 tumor cells in total (e.g. 5×10^4 cells each of VHL-WT and VHL-KO cells) were grown on the 24-well plate until 90% confluence. One margin of 200ul tips were used to scratch the bottom of each well to form a gap. The cell migration was monitored continuously in the Nikon Eclipse Ti-E time lapse microscope under a 10x objective lens, with the stage kept at 37°C, supplied with 5% CO₂ and humidified. Selected fields of interest were set and recorded, each frame at 15min intervals for 20 hours with FITC and TRITC channel. The Nikon elements software was used to measure the migration speed of cell in each group.

[047] Transwell chambers (0.4μm pore size, CLS3470-48EA, Thermofisher) were assembled in a 24-well plate. 1ml of RPMI-1640 media supplemented with 10% fetal bovine serum and 50ng/ml EGF were added into the bottom chamber. 1×10^5 HUVEC (human umbilical endothelial cells) were seeded on the bottom of the transwell chamber. On day 2, a layer of 100ul (for migration assay, as shown in **Figure 11A**) or 30ul (for 3D in-vitro intravasation assay as shown in **Figure 11A**, lower panel) Matrigel® (356234, Corning Corp.) was coated on top of the layer of HUVEC cells and placed back in a 37°C incubator to solidify. 1×10^5 tumor cells in total were then seeded on top of Matrigel®. Nikon Eclipse Ti-E time lapse confocal microscope was used to image the cell migration as above. The z-step parameters were set with the HUVEC cell layer as bottom; and tumor cell layer as top with ~200 stepwise stacks for each scanning every 15min for 48 hours.

Western immunoblot, necroptosis and apoptosis reporter assay

[048] For western blot, 1×10^6 HUVEC cells were seeded on the bottom of transwell chambers (1μm pore size, 353102, Falcon) of a 6-well plate chamber with 1×10^6 tumor cells total seeded on the top chamber, with or without 1μg/ml anti-POSTN MPC5B4 mAb, with or without Cilengitide in concentrations as indicated in the figure legends. HUVEC cells were harvested after 48 hours

for whole cell lysate protein extraction with RIPA buffer (89901, Thermofisher) supplemented with proteinase inhibitors (78430, Thermofisher), boiled for 10min and loaded with 6x SDS loading buffer. Blots were probed with anti-phospho-RIP (Ser166), anti-RIP, anti-phospho-MLKL(Ser358), anti-MLKL from Apoptosis/Necroptosis Antibody Sampler Kit (92570, Cell Signaling Technology and anti-Caspase-3, anti-Cleaved Caspase-8 and anti-Caspase-8 from Apoptosis/Necroptosis Antibody Sampler Kit (92570, Cell Signaling Technology). Blots were imaged and analyzed on a chemiDoc XRS+ with associated ImageLab software (BioRad).

[049] For necroptosis reporter assay, transwell chambers assembled in a 24-well plate (0.4 μ m pore size, CLS3470-48EA, Thermofisher) were seeded with 1×10^5 HUVEC cells on the bottom and 1×10^5 tumor cells total on the top chamber with or without 1 μ g/ml anti-POSTN MPC5B4 mAb. After 48 hours, HUVEC cells were washed with PBS once and solution of 1.6 μ M Ethidium Homodimer III (EthD-III, Cat#400050, Biotium) and 2 μ M Hoechst33342 (#40045, Biotium) was added to cells and incubated at humidified, 5% CO₂ incubator at 37°C for 15 min. Using the Nikon Ti-E live cell microscope, images of each well were taken with 5 random fields under 10x object lens at DAPI and Tritc channel. Images were quantified by ImageJ and analyzed with graphpad.

[050] For apoptosis evaluation, HUVEC cells were cultured in transwell chambers as noted above. After 48 hours, the plates were equilibrated at room temperature for 10min and 200 μ l of Caspase-Glo 3/7 reagent (G8090, Promega) added to each well, placed on a shaker at 300-500rpm for 30 seconds, incubated at room temperature for 1 hour, and then analyzed for luminescence by Synergy HT microplate reader (BioTek).

Cell proliferation assay

[051] Cell proliferation was measured using MTS assay and direct cell counting. For both assays, cells in log phase were counted and seeded at the density of 1000 cells/well in 96-well plate on day 0, or 500 cells/well in 384-well plate. For the MTS assay, cell numbers were evaluated every 24 hours on days 1, 2, 3, 4, 5 and 6 using the MTS kit (Promega, USA) and measured with Multiskan MK3 microplate reader (Thermo, USA). For direct cell counting, ImageXpress workstation was used to photograph each well in 384-well plate and count the DAPI stained cells.

Avian chorioallantoic membrane (CAM) tumor xenograft model

[052] To prepare the CAM for cancer cell implantation, fertilized chicken eggs were purchased from AA Lab Eggs Inc. (Los Angeles, CA) and maintained in egg incubator with turner (Incubator Warehouse, USA) at 38°C, 60~70% humidity. On day 7 after fertilization, a marking pen was used

to label an area in the middle part of live eggs with thick blood vessels. Then, the air pocket on one end of egg was removed to the designated area via #15 syringe needle and pipets. Also, the egg shell above the new air pocket was secured by a piece of packing tape in appropriate size and a central area of 1.5x1.5cm was carefully removed by tweezers with fine tips. To help maintain the moisture of the new air pocket, a piece of Tegaderm membrane (Cat#21272, Moore Medical Inc., USA) was applied covering the window of egg shell.

[053] The 2nd day after the window of egg shell was open, dead eggs were excluded and the remaining eggs were randomly grouped for tumor cell implantation. On the 3rd day after the egg shell window was open, tumor cells were prepared by trypsinization and washed with PBS. The cells were counted for resuspension in RPMI-1640 with L-glutamine diluted Matrigel® (356234, Corning Corp.) (2:1 ratio) to reach a density 1.5×10^7 cells/ml of either VHL-WT cells, VHL-KO cells or mixture of VHL-WT and VHL-KO cells (1:1). To pre-solidify the cell suspension in Matrigel®, 200ul of each cell type were prepared in 200ul tips and allowed to sit in a cell incubator for 15min. Then each egg was implanted with 200µl cell suspension on the CAM surface in the window. Cells were allowed to grow for over 10 days and photographed every 2 days. On day 10 after implantation, chicken blood was collected via heparinized 10ml syringe and the embryos were sacrificed by incubation in ice for 10 minutes. Tumors were dissected, weighed and fixed in 4% paraformaldehyde overnight for paraffin-wax embedding. Slides were cut for staining.

Orthotopic tumor studies and anti-Periostin antibody treatment in mice.

[054] Intrarenal implantation of RC or AC tumor cells of 1×10^6 tumor cells total was performed as described previously. One week after implanting 1:1 mixture of VHL-WT and VHL-KO cells, MPC5B4 mAb was injected via tail vein at 10mg/kg each, 3 times a week for 4 weeks. The animals were imaged and sacrificed. Tissues were harvested and fixed and paraffin embedded and cut for histological analyses.

Immunohistochemistry and immunofluorescence staining

[055] Slides were baked at 65°C for 20min and deparaffinized through 3 times of xylene and rehydrated from 100% ethanol to water. Citrate buffer was used for antigen retrieval in vegetable steamer for 25 min. 1% BSA was used for blocking and primary antibodies were used for overnight incubation at 4°C including anti-VHL (1:200, ab135576, Abcam, USA), anti-flag (1:200, Cat# 14-6681-82, eBioscience, USA), anti-HA (1:200, Cat#sc805, Santa Cruz Biotechnology, USA) and anti-Ki67 (1:200, Cat# VP-RM04, Vector Laboratories, USA). After 3 times of wash at TBST (7

min each), slides were incubated with secondary antibody (Goat-anti-Rabbit, Cat#111-035-045; Goat-anti-Mouse, Cat#115-035-062, both from Jackson ImmunoResearch Laboratories, USA) at 1:200 dilution ratio. For immunohistochemistry staining, slides were washed 3 times with TBST (7 min each) and applied with DAB reagents (Cat# DB801R, Biocare Medical, USA) and counterstained with hematoxylin. While for immunofluorescence staining, slides were washed 3 times with TBST (7 min each) and applied with FITC-conjugated TSA (SAT701001EA, Perkin Elmer, USA), or CY3-conjugated TSA (NEL744001KT, Perkin Elmer, USA) for the first color labeling and can then be stained with an additional color with respect to another antibody, as indicated by the TSA kit protocol. After the TSA staining, Hoechst 33342 were applied on slides for nucleus staining and slides are ready for scanning at TPCL (UCLA) after slides were sealed with glycerol.

Flow Cytometry

[056] Primary tumors or lungs of mice were dissected, minced into small pieces of chunks and digested with 0.2% Collagenous II at 37°C, 100rpm shaker. The cell suspensions were passed through 70µm cell strainers. The digested cells were stained with Hoechst 33342 for 15min and sent for flow cytometry analysis. Similarly, chicken blood and mouse blood were collected and processed with red blood cell lysis buffer (Cat# 555899, BD Bioscience, USA). Then cells were analyzed by flow cytometry for mStrawberry and EGFP expression.

Isolation and cultivation primary ccRCC tumor cells.

[057] With the consent of patients, the primary ccRCC tumor sample was collected and chopped into pieces by sterile scissors and surgical knives in RPMI-1640 media. All the tissue chunks were collected into a 15ml conical tube for centrifuge at 300g and room temperature for 5min. The supernatant was discarded carefully and the tissue pellet was resuspended with 2.6ml prediluted 3u/L Liberase TM (5401119001, Sigma-Aldrich) in RPMI-1640 media. Then the 15ml conical tube was put on a shaker at 100rpm, 37°C for 1 hour. When the tissue was fully digested and no chunks visible, cells were spun down at 300g and room temperature for 5min. The pellet was further treated with prediluted 1x red blood cell lysis buffer (555899, BD biosciences) in sterile water for 15min and washed once with PBS. Cells were resuspended in RPMI-1640 supplemented with 10% fetal bovine serum and 1x penicillin/Streptomycin (15140122, Thermofisher) and cultured in a humidified, 5% CO₂ incubator at 37°C.

Human ccRCC patient specimen.

[058] Tissue microarray was constructed from a cohort of 357 patients who underwent nephrectomy for sporadic RCC at UCLA between 1989 and 2000, as previously described. Clinical data, including age, gender, and Eastern Cooperative Oncology Group performance status (ECOG PS) and pathologic data, including tumor-node-metastasis stage, histologic subtype, and Fuhrman grade have all been collected on these cases and the study has been approved by UCLA Institutional Review Board.

[059] Large tumor tissues from primary tumors and/or corresponding local invasion/metastases were obtained from 26 patients who received radical nephrectomy in the Department of Urology at Ronald Regan Medical Center, UCLA, from 2015 to 2018. All patients involved consented to participate in the study before surgery and all experiments were performed according to the approved guidelines, complying with the principles for the use of human tissues in the Declaration of Helsinki. This study was approved by the Institutional Review Board of UCLA.

Statistics

[060] Each experiment was performed at least in triplicate unless otherwise stated. Data are presented as mean $\pm$ standard deviation (SD). Significance was determined by a paired, Student's T-test when there were two groups or by a one-way ANOVA when there were three or more groups (Graphpad Prism ver6.0). A p-value cutoff of 0.05 was used to establish significance.

RESULTS**Metastasis requires cooperation between VHL-KO and VHL-WT RCC cells**

[061] The CRISPR/cas9 system was used to delete the *VHL* gene in the VHL+ murine RENCA (RC) RCC cell line in an attempt to recreate a more clinically relevant model, as described by Schokrpur, S., et al. CRISPR-Mediated VHL Knockout Generates an Improved Model for Metastatic Renal Cell Carcinoma. *Scientific reports* **6**, 29032 (2016), which is incorporated by reference herein in its entirety. The first VHL-deleted cell line (denoted as RVN) was created by transducing RC cells with VHL targeted lentiCRISPR. The RVN cells underwent EMT and developed rampant lung metastases upon intrarenal implantation, much more aggressively than the parental RENCA cells. Unexpectedly, lung metastases from RVN tumors consisted largely of VHL expressing cells with minor pockets of VHL-deleted, MMP-9+ cells (**Figure 9A**). Since the RVN line consists of a mixed population of VHL negative and positive cells, this result indicated the possibility that deletion of the *VHL* gene might not be a direct cause of tumor metastasis. Thus,

several clones were selected with bi-allelic *VHL* gene deletion, generated through transient expression of CRISPR/Cas9, as described in Hu, et al., Mol Ther Methods Clin Dev. 2018 9:203-210, which is incorporated by reference herein in its entirety. The clonal VHL knockout line was denoted as RC-VHL-KO while the parental VHL+ control line was denoted as RC-VHL-WT.

Next, renal tumors were established with either RC-VHL-WT cells or RC-VHL-KO cells alone or with a 1:1 mixture of the two cell lines. The growth and dissemination of these tumors in mice were monitored by bioluminescence imaging (BLI) of the firefly luciferase marker gene. As previously reported by Hu et al. Mol Ther Methods Clin Dev. 2018 9:203-210, which is incorporated by reference in its entirety, RC-VHL-WT primary tumors grew well, while VHL-KO tumors hardly grew at all (**Figure 1A**). Interestingly, the primary tumors from 1:1 cell mixture not only grew but also exhibited metastasis with prominent thoracic BLI signals (**Figure 1A**). In addition, mice bearing mixed renal tumors suffered tumor cachexia with significant weight loss (**Figure 9B**). Due to retarded primary tumor growth no lung metastases were observed in the VHL-KO tumor group (**Figures 9C, 9D**).

[062] The metastatic lung lesions were further examined by detailed histological analyses. The 1:1 mixed tumor group exhibited greatly increased numbers and sizes of lung metastases compared to the VHL-WT tumor group, which could be easily appreciated even at low magnification (**Figures 1B, 1C**) as reported previously by Hu et al. Mol Ther Methods Clin Dev. 2018 9:203-210, which is incorporated herein by reference in its entirety. To recapitulate human ccRCC tumors consisting predominantly of tumor cells with loss of VHL, the inventors established renal tumor with VHL-WT and VHL-KO cells at 1:4 ratio in syngeneic BALB/C mice, as previously described by Hu et al. Mol Ther Methods Clin Dev. 2018 9:203-210, which is incorporated herein by reference in its entirety. Similar to the RVN or 1:1 mixed tumors, this 1:4 mixed tumor group also metastasized rampantly to the lungs (**Figure 1C**). Furthermore, VHL immunohistochemistry (IHC) stain revealed mostly VHL+ cells with small rims of VHL-negative cells (**Figure 1C**). The VHL-WT and VHL-KO cells were labeled with HA-tagged mStrawberry FP and HA-tag EGFP respectively, to facilitate the *in vivo* tracing of these cells. Upon close inspection, a small metastatic lesion was predominantly composed of VHL-WT, with only a small aggregate of VHL-KO cells in the center and spindle-shaped VHL-KO cells scattered elsewhere (**Figure 1D**). By flow cytometry of a large number of lung metastases, The proportion of VHL-KO cells in lung metastases was estimated to be less than 1% by flow cytometry (**Figure 1E**).

[063] Next, the inventors ascertained whether this novel cooperative mechanism of metastasis could be operating in human ccRCC. The same CRISPR/Cas9 lentiviral system was applied in to knockout the VHL gene in the human ACHN (AC) cell line, a widely used human RCC cell line known to express wildtype VHL protein, as described by Giard, D.J., *et al.* In Vitro Cultivation of Human Tumors: Establishment of Cell Lines Derived From a Series of Solid Tumors2. *JNCI: Journal of the National Cancer Institute* 51, 1417-1423 (1973), which is incorporated by reference in its entirety. Consistent with the findings in the RC model, as described in Schokrpur, S., *et al.* CRISPR-Mediated VHL Knockout Generates an Improved Model for Metastatic Renal Cell Carcinoma. *Scientific reports* 6, 29032 (2016), which is incorporated by reference in its entirety, a clonal AC-VHL-KO line exhibited EMT cell morphology (**Figure 9G**), elevated expression of EMT markers (**Figures 1F**) and slower growth as compared to AC-VHL-WT cells (**Figure 1G**). Both AC-VHL-WT and 1:1 VHL-WT and VHL-KO tumor grew well after intrarenal implantation (**Figure 1H**). However, lung metastases were observed only in the mixed tumor group as assessed by gross tissue inspection (**Figure 1H**) and detailed histology (**Figure 9H**), reproducing the results observed in the RC model. Of note, the AC cells grew more slowly than RC cells and hence, the primary tumor and lung metastatic lesions were much smaller, as well.

[064] The EMT+ VHL-KO cells derived from the murine RC or the human AC model consistently grow slower than their parental VHL-WT cells in cell culture (**Figure 1G**) and in mice. As assessed by BLI and flow cytometry, RC-VHL-KO tumors were significantly growth retarded compared to the VHL-WT or 1:1 mixed tumors (**Figures 1A, 1I, 1J**). The *in vivo* tumor growth behavior of the RC cell lines was further verified in the avian chorioallantoic membrane (CAM) tumor system, as described by Ribatti, D. The chick embryo chorioallantoic membrane as a model for tumor biology. *Experimental cell research* 328, 314-324 (2014); and Hagedorn, M., *et al.* Accessing key steps of human tumor progression—in vivo—by using an avian embryo model. *Proceedings of the National Academy of Sciences of the United States of America* 102, 1643-1648 (2005), each of which is incorporated by reference in its entirety. The CAM tumor model not only substantiated the poor growth phenotype of VHL-KO tumors, compared to VHL-WT and 1:1 mixed tumors, (**Figure 1K** and **Figure 9I**), but also demonstrated the significant increase of metastatic tumor cells in circulation of the mixed tumor group (**Figures 9J, 1K**).

Taken together, the inventors' VHL-deleted ccRCC models reveal a novel metastatic mechanism that relies on cooperative interactions between two distinct populations of tumor cells, namely

the VHL-KO and VHL-WT cells. An immediate relevant issue to address is whether this novel cooperative mechanism could be at play in the clinical disease.

Intratumoral heterogeneity of VHL expression occurs in human ccRCC

[065] The heterogeneous genomic landscape of ccRCC, has been well documented and linked to disease progression and metastasis. However, the heterogeneity of loss or mutation at the VHL locus remains unclear despite the loss of VHL function has been recognized as a trunk lesion in the great majority of ccRCC cases, as described in Gossage L et al. Nat Rev Cancer 15, 55-64 2015, which is incorporated by reference in its entirety. If the cooperative crosstalk observed in the preclinical metastasis model described herein operates in human cancers, it is expected to observe heterogeneity of VHL protein expression in patient's tumor cells. To address this question, the inventors examined VHL protein expression in large tissue sections of primary tumors and local invasion, collected from surgery at the inventors' institution over the past 3 years. As tabulated in **Table 1**, the inventors have analyzed a total of 26 cases of ccRCC, a total of 26 cases of ccRCC, the first 16 cases were collected from paraffin embedded samples (**Figure 10A**). Case #1 to 10 are specimens from local invasion or lymph node metastasis and case #11 to 16 are paired primary tumor and metastasis. The most recent 10 cases (#17 to 26) are freshly harvested tumor specimens collected from consecutive surgeries performed by a single surgeon (see **Table 1** for annotation and **Figure 10B** for H&E stain). IHC analyses were performed and showed that all 26 cases exhibit heterogeneity in VHL protein expression, with VHL positive area ranging from 10% to 90% in each tumor (**Table 1**). None of the cases examined sustained a uniform loss of VHL protein throughout the entire tumor. Representative images of VHL stain of case #18, 21, 24 and 25 are shown in **Figure 10C**.

[066] To further investigate the relationship between VHL mutational status and the metastatic process, the inventors focused on the most aggressive case of metastatic ccRCC amongst the recent cases, case #22 (see **Table 1** for details). This patient presented with a large 10 cm Fuhrman grade 4 primary tumor and bilateral lung metastases and succumbed to the disease within 1 year of nephrectomy despite multiple surgical and pharmacological interventions. As shown in **Figures 2A** and **2B**, VHL expression in the primary tumor of #22 was highly heterogeneous, with VHL-positive cells immediately juxtaposed to VHL-negative cells. Interestingly, the lung metastatic lesions of this patient, showed very high prevalence of VHL-positive cells (**Figures 2C, 2D**). This

is highly reminiscent of the lung metastatic lesions of our RENCA mixed tumors (**Figures 1C, 1D**).

VHL+ cells in ccRCC tumors are frequently assigned as normal host cells in the microenvironment. To investigate VHL and its regulation in detail, the inventors generated primary tumor cell lines and patient derived xenografts (PDXs) in CAM from the freshly harvested specimen, case #18-26, with a 80% success rate (as described by Hu et al., Establishment of Xenografts of Urological Cancers on Chicken Chorioallantoic Membrane (CAM) to Study Metastasis. Precision Clinical Medicine, 2019 Oct 1; 2(3): 140–151, which is incorporated herein by reference in its entirety.

[067] The CAM PDXs established from small tumor pieces of case #22 (**Figure 2E**) revealed the presence of VHL-positive and -negative tumor cells. A VHL+ primary cell line was also generated from the primary tumor of #22. Whole exome sequencing (WES) of this #22 cell line and the parental tumor showed they shared higher than 80% of the mutations (**Figure 12A**) and its VHL gene sequence revealed an inframe T506C transition, leading to amino acid substitution of L169P (**Figure 12B**). This L169P substituted VHL functioned expectedly as HIF1A protein in this #22 line was degraded in normoxia and stabilized upon its deletion (**Figure 12C**). Common ccRCC driver mutations such as VHL, TP53, BAP1, PBRM1, SETD2, were analyzed by WES in 4 different loci of primary tumor of #22 and its derivative cell line. Variant allele frequencies (VAF) and copy number ratios (CNR) showed clonal missense and frameshift mutation in the VHL and BAP1 gene, respectively, in the #22 cell line. But these 2 mutations are subclonal in the 4 tumor loci, suggesting cellular heterogeneity exists in the primary tumor. These results suggest the VHL+ cells in case #22 are unlikely to be normal cells but they are proliferative tumor cells (see **Figure 7** below for further information).

[068] Next, the VHL expression heterogeneity issue was further investigated in the TCGA database. After adjusting for both tumor purity and ploidy, the CNR at the VHL locus was analyzed in the TCGA KIRC cohort (n = 421). The peak CNR value is between -1 and 0.5, suggesting that many TCGA samples have subclonal single copy loss of VHL as a value of -1 represents a single copy loss (dotted line, **Figure 2G**). The VAF of somatic VHL mutations after adjusting for both tumor purity and ploidy in the TCGA-KIRC cohort (n = 64) also displayed multiple peaks between 0.5 and 1, indicating they are subclonal mutations (**Figure 2H**).

[069] Collectively, the IHC analyses and genomic profiling revealed that VHL protein expression and gene mutation displays cell-to-cell heterogeneity within individual human ccRCC tumors.

This finding supports that the observed metastatic crosstalk between VHL-KO and VHL-WT cells in our preclinical models could be operating in patients' tumors.

Table 1: VHL status summary of human ccRCC samples

		VHL status		Pathology	Stage	Size	Sample Location	Stage at specimen	Course	VHL mutation
		Primary tumor	Metastasis [1]							
#1	Case 1	N/A	+	ccRCC	N/A	N/A	N/A	N/A	N/A	N/A
#2	Case 2	N/A	+	ccRCC	N/A	N/A	N/A	N/A	N/A	N/A
#3	Case 3	N/A	+	ccRCC	N/A	N/A	N/A	N/A	N/A	N/A
#4	Case 4	N/A	++	ccRCC	N/A	N/A	N/A	N/A	N/A	N/A
#5	Case 5	N/A	+++	ccRCC	N/A	N/A	N/A	N/A	N/A	N/A
#6	Case 6	N/A	++++	ccRCC	N/A	N/A	N/A	N/A	N/A	N/A
#7	Case 7	N/A	++++	ccRCC	N/A	N/A	N/A	N/A	N/A	N/A
#8	Case 8	N/A	+	ccRCC	N/A	N/A	N/A	N/A	N/A	N/A
#9	Case 9	N/A	++++	ccRCC	N/A	N/A	N/A	N/A	N/A	N/A
#10	Case 10	N/A	++	ccRCC	N/A	N/A	N/A	N/A	N/A	N/A
#11	S09-2265	+++	++++	ccRCC	N/A	N/A	N/A	N/A	N/A	N/A
#12	S12-7574	+	++++	ccRCC	N/A	N/A	N/A	N/A	N/A	N/A
#13	S13-5071	+++	++	ccRCC	N/A	N/A	N/A	N/A	N/A	N/A
#14	S14-25301	++	++	ccRCC	N/A	N/A	N/A	N/A	N/A	N/A
#15	S15-2908	+++	++	ccRCC	N/A	N/A	N/A	N/A	N/A	N/A
#16	S15-7490	+	++	ccRCC	N/A	N/A	N/A	N/A	N/A	N/A
#17	AC08112017	N/A	+++	ccRCC	T1a>N1M1	3.3cm	Metastatic	Metastatic	[2]	-
#18	AC10062017	++	N/A	ccRCC, Fuhrman 3/4	T2	8.5cm	Primary	Localized	NED [3]	c.51_623del
#19	AC12132017	+	N/A	ccRCC, Fuhrman 2/4	T1a	2.9cm	Primary	Localized	NED [3]	-
#20	AC12152017	+	N/A	ccRCC, Fuhrman 3/4	T1b	6.7cm	Primary	Localized	NED [3]	-
#21	AC01192018	++	N/A	ccRCC, Fuhrman 2/4	T1a	2.7cm	Primary	Localized	NED [3]	No mutation
#22	AC01272018	++	++++	ccRCC, Fuhrman 4/4	T3aN0M2	10.1cm	Primary	Metastatic	[4]	c.506T>C
#23	AC02152018	++++	N/A	Oncocytoma	T2	8cm	Primary	Localized	N/A	c.51_623del

		VHL status		Pathology	Stage	Size	Sample Location	Stage at specimen	Course	VHL mutation
		Primary tumor	Metastasis [1]							
#24	AC02212018	+	N/A	ccRCC, Fuhrman 3/4	T2	7.2cm	Primary	Metastatic	[5]	c.564_565insT
#25	AC04052018	++	N/A	ccRCC, Fuhrman 2/4	T1a	3.3cm	Primary	Localized	NED [3]	c.51_623del
#26	AC09252018	++	N/A	ccRCC, Fuhrman 2/4	T1a	3.4cm	Primary	Localized	NED [3]	c.240T>A

+	++	+++	++++	+++++
(0, 20%)	(20%, 40%)	(40%, 60%)	(60%, 80%)	(80%, 100%)

[1]: Metastasis as mentioned in this form includes both local invasion and lymph node metastasis

[2]: 65 y/o, female, the sample is a recurrent retroperitoneal lymph node metastasis resected 3 months after the patient received partial nephrectomy for her left renal mass

[3]: No evidence disease

[4]: 59 y/o, male, the sample is a primary renal mass from the right kidney in the first surgery. The patient presented with 10cm right renal mass and bilateral pulmonary nodules that were biopsy proven lung metastases. He underwent cytoreductive nephrectomy in the first surgery and received 5 months of cabozantinib until progression, then axitinib and ipilimumab/nivolumab for another 4 cycles and nivolumab maintenance therapy until death 1 year after the first surgery.

[5]: the patient is stable on cabozantinib

VHL-KO cells induce the growth and EMT/motility of VHL-WT tumor cells

[070] Next, the inventors further investigated the crosstalk signal involved in metastasis and pondered if the non-proliferative VHL-KO cells could induce the proliferation of VHL-WT cells in the same tumor. VHL-KO cells consistently exhibit retarded growth rate in cell culture and in animal models (**Figures 1A, 1G, 1I, 1K**). But, mixed tumors that consisted of only 20% of VHL-WT cells and 80% of VHL-KO cells (1:4 mixed tumor) grew robustly. Thus, cellular proliferation was assessed in primary tumors or lung metastases by Ki67 staining. In a 1:1 mixed RC renal tumor, the VHL-WT cells are more proliferative than VHL-KO cells (**Figure 3A**), which is also the case in lung metastases (**Figure 3B**). When the proliferative crosstalk was analyzed in transwell cocultures, factors released by VHL-KO cells significantly increased the growth rate of VHL-WT cells compared to VHL-WT cells cultured alone (**Figure 3C**).

[071] It was previously reported that VHL gene deletion caused RCC tumor cells to undergo EMT with increased motility. A possible paracrine impact of VHL-KO cells could be to induce EMT and the motility of VHL-WT cells. Co-culture of VHL-KO cells with VHL-WT cells appeared to induce the epithelial VHL-WT cells to an EMT-like state with upregulation of EMT markers such as N-Cad, MMP-9 and SMA and a concomitant lowering of E-Cad expression (**Figure 3D**). The motility of clonally purified lines, marked with mStrawberry FP (VHL-WT) or EGFP (VHL-KO), was measured using real time using time-lapse live cell microscopy. VHL-KO cells migrated faster than VHL-WT cells (**Figure 3E**). The migration of VHL-WT cells was greatly enhanced in co-culture with VHL-KO cells (**Figure 3E**). The migration was further performed in a 3D system allowing cancer cells to migrate through an extracellular matrix. Paralleling the results of the 2D system, VHL-KO cells also migrated faster than VHL-WT cells in 3D (**Figure 11A**). Conditioned media of VHL-KO cells was able to enhance the motility of VHL-WT cells, albeit to a lesser extent than co-culture (**Figure 3F**).

[072] Together, these results show that the EMT+ VHL-KO cells are able to increase the proliferation and the mobility of neighboring VHL-WT cells via soluble factors and cell-cell contact, promoting the aggressive and metastatic behavior of the tumor.

Periostin, a HIF1A-dependent factor secreted by VHL-KO, promotes metastasis

[073] Next the inventors sought to clarify signals downstream of VHL loss that could be governing metastasis. HIF1A and HIF2A are known to be upregulated upon the loss of VHL

function. But, these two paralogues often have contrasting functions. The data consistently support HIF1A exerts the EMT paracrine effects. For instance, the deletion of HIF1A in VHL-KO cells, denoted as VHL/HIF1A-KO, abrogated the effect of VHL deletion to promote the motility of VHL-WT cells (**Figure 4A**). Besides the RC and AC model, the HIF1A mediated induction of EMT was also observed in the human 786-O cell line. As shown in **Figure 4B**, transfection of HIF1A but not HIF2A promoted EMT markers as analyzed at the RNA level and at the protein expression level (**Figure 4C**). To seek out novel gene downstream of HIF1A that could regulate metastasis, we performed RNA-sequencing from our VHL-deleted RVN cells and identified 4 HIF1A-regulated genes, which when coordinately upregulated predicts a very poor patient survival in the TCGA RCC (KIRC) database. Amongst the 4 identified HIF1A-regulated genes, we decided to focus on POSTN as it encodes a secreted cell adhesion protein upregulated in EMT, with known paracrine activity that confers aggressive and metastatic behavior). POSTN is upregulated in kidney cancer and is a poor prognostic indicator for RCC (**Figure 11B**). However, the function role of POSTN in RCC tumorigenesis has not been defined. POSTN was upregulated in VHL-deleted RC cells and the knockdown of HIF1A reduced POSTN expression at the RNA (**Figures 11C, 11D**) and protein level (**Figure 3D**). The inventors further verified that HIF1A but not HIF2A is a direct transcriptional activator of POSTN. As shown in **Figure 3E**, transfection of a HIF1A-containing plasmid, but not HIF2A, was able to enhance the expression of luciferase reporter gene controlled by the POSTN promoter. To assess the contribution of POSTN to cell motility, a VHL and POSTN double gene knockout (RC-VHL/POSTN-KO) line was constructed and showed that this line exhibited significantly decreased ability to augment the motility of VHL-WT cells (**Figure 3F**). To further pinpoint the role of POSTN as the soluble mediator of enhanced cell motility, a monoclonal anti-POSTN neutralizing antibody (MPC5B4) that disrupts the interactions of POSTN with integrin $\alpha V\beta 3(43)$ was employed. Blocking POSTN with mAb MPC5B4 reversed the augmentation of VHL-WT motility that is induced by VHL-KO cells in coculture (**Figure 4G**).

[074] The mechanism of action of POSTN was further investigated by add-back experiments. The addition of recombinant POSTN to VHL-WT cells significantly promoted their motility and the addition of the cyclic peptide integrin inhibitor, Cilengitide, blocked this POSTN-mediated motility enhancement (**Figure 4H** and **Figure 11F**). The addition of recombinant POSTN to VHL-WT cells activated the focal adhesion kinase (FAK) via phosphorylation at Tyr 397, which

is blocked by Cilengitide (**Figure 4I**). In the co-culture of VHL-WT and VHL-KO cells, Cilengitide was able to disrupt the VHL-KO cells mediated dose dependent motility enhancement of VHL-WT cells (**Figure 4J**). As a secreted factor, POSTN's impact is unlikely to be limited just to VHL-WT cells, as Cilengitide also inhibited the motility of VHL-KO cells (**Figure 11G**). Taken together, the results herein strongly support that HIF1A-dependent overexpression and secretion of POSTN by the VHL-KO cells enhances the motility of VHL-WT cells through an integrin-mediated signaling pathway.

VHL-KO cells cause vascular destruction to promote tumor cell intravasation

[075] The inventors surmised that VHL-KO cells could exert their paracrine influence in other steps in the metastatic cascade. Thus, the kinetics of tumor cell escape into the circulation (i.e. circulatory tumor cells, CTCs) were carefully examined to inform on the vascular intravasation process. To track the two cell populations, the VHL-WT and VHL-KO cells were again marked with mStrawberry FP and EGFP, respectively. CTCs from the VHL-WT or mixed tumors were serially monitored starting from week 2 to 6 weeks after tumor cell implantation (**Figure 5A**). In the mixed tumor bearing animals, the motile VHL-KO cells appeared to be the first cells to escape into the circulation, starting as early as week 2, and increasing through week 3. No VHL-WT CTCs were detected in circulation until week 3. Strikingly, in comparison to VHL-WT tumors, the presence of VHL-KO cells in the mixed tumors enhanced the number of VHL-WT cells escaping into the circulation (**Figure 5A**). Further, the augmentation of tumor cell escape increased progressively over time as the VHL-WT CTCs were 1.3-, 2.7- and 5.1-fold higher in the mixed group at week 3, 4 and 6, respectively (**Figure 4A**). Intriguingly, the number of VHL-KO CTCs peaked at week 3 and were barely detectable at 6 weeks (**Figure 4A**). The poor proliferative phenotype of VHL-KO cells could be a reason for their diminishing presence over time not only in the circulation but also in metastases (**Figures 1C, 1D, 1E** and **Figures 9A, 9D**).

[076] The promotion of vascular intravasation raises the possibility that VHL-KO cells could disrupt the endothelial cell barrier as described in a recent study by Strilic et al., Tumour-cell-induced endothelial cell necroptosis via death receptor 6 promotes metastasis. *Nature*. 2016;536(7615):215-8, which is incorporated herein by reference in its entirety. Strilic et al. reported that the destruction of endothelial cells by tumor cells promoted tumor metastasis. To examine the possible role of this mechanism in our tumor model, a 3D system to mimic the intravasation step was established. Similar to the 3D migration assay (**Figure 11A**), a layer of

either VHL-WT or VHL-KO or mixed 1:1 cells were placed above a layer of HUVEC endothelial cells, separated by a thin layer of Matrigel®. After 48 hours the integrity of the endothelial cell layer was tabulated, which showed a significant destruction of endothelial cells in the co-culture with either VHL-KO or 1:1 mixed cells but not with VHL-WT cells alone (**Figure 5B**). The molecular signals involved in the destruction of endothelial cells by the VHL-KO cells was further investigated. As reported a wide range of tumor cell models appear to induce necroptosis in endothelial cells, but no RCC model was investigated by Strilic et al. Thus, the inventors examined if the RC cells were able to induce either necroptosis or apoptosis in HUVEC endothelial cells. The results in **Figure 5C** revealed that co-culture with VHL-WT or VHL-KO cells did not activate the necroptosis markers MLKL or RIP in HUVEC cells. However, VHL-KO cells and to a lesser extent VHL-WT cells were able to induce a robust apoptotic response in endothelial cells as indicated by caspase 3 cleavage. This result was further supported by using a necroptosis (**Figure 5D**) and an apoptosis (**Figure 5E**) reporter assay. Interestingly, the addition of the anti-POSTN MPC5B4 mAb was able to abrogate the VHL-KO cells induced apoptosis in HUVEC cells (**Figures 5C and 5E**). Further analysis of POSTN's mechanism of action showed that either co-culture with VHL-KO cells or the addition of recombinant POSTN was able to induce the phosphorylation of FAK at Tyr 397 in HUVEC cells and Cilengitide inhibited this FAK activation (**Figure 11H**). But recombinant POSTN alone was unable to induce apoptosis in HUVEC cells (**Figure 11I**). To support this vascular destruction effect could be operating *in vivo*, the CAM tumor system was used to assess vascular leakage by the Miles assay. As shown in **Figure 5F**, the vasculature of the mixed tumors was leakier than the VHL-WT tumors.

[077] In total, these results highlight that VHL-KO cells could be augmenting the escape of tumor cells into the blood circulation by destroying the endothelial cell barrier. The POSTN protein secreted by VHL-KO cells is necessary but not sufficient to induce apoptosis in endothelial cells. Other factor(s) produced by VHL-KO cells are required to work in concert with POSTN to enable the vascular destruction process.

Inhibition of POSTN blocks metastasis in a preclinical ccRCC model

[078] Given the multiple paracrine influences exerted by POSTN on the metastatic cascade, the inventors assessed the therapeutic impact of blocking POSTN by a genetic and a pharmacological approach. The RC-VHL/POSTN-KO double gene deleted cell line exhibited a greatly diminished ability to promote the motility of VHL-WT cells in co-culture (**Figure 4F**). Here the inventors

established renal tumors in mice with either a 1:1 mixture of VHL-WT and VHL-KO cells or VHL-WT and VHL/POSTN-KO cells. Comparing to these two mixed tumor groups, the group bearing tumor with VHL-WT and VHL/POSTN-KO cells showed no evidence of lung metastasis as monitored by BLI while the tumor group with VHL-WT and VHL-KO cells metastasized readily to the lung (**Figure 6A**). The inventors further assessed if the MPC5B4 mAb could serve as a therapeutic agent to inhibit lung metastasis. As shown in **Figure 6B**, MPC5B4 treatment was able to greatly suppress the development of lung metastasis, without a significant impact on the growth of the primary tumor. The suppression of lung metastasis by MPC5B4 treatment can be appreciated by gross examination of lungs from the animal subjects (**Figure 6B**), and IF analyses and H&E stain of the lungs (**Figures 6C, 6D**) and lung weight (**Fig. 6E**). Taken together, the data herein reveal that POSTN is a critical paracrine factor secreted by VHL-KO cells that promotes a multitude of pro-metastatic functions such as the EMT and motility of VHL-WT cells and the destruction of adjacent blood vessels (**Figure 6F**). Thus, targeted blockade of POSTN appears to be a promising treatment to inhibit the deadly metastatic process in ccRCC.

The relationship of VHL, HIF1A and POSTN expression in human ccRCC tumors

[079] Clearly, the results accumulated from the inventors' metastatic ccRCC model thus far support that further exploration of blocking POSTN as an anti-metastasis treatment in the clinic is warranted. In pursuing this line of investigation, it was first queried if the tumor characteristics and the novel pathways downstream of VHL loss that were discovered could also be at play in clinical settings.

[080] It was observed that in mixed tumors the VHL-WT tumor areas were consistently more proliferative than the VHL-KO areas (**Figures 3A, 3B**). In assessing the most aggressive and metastatic human ccRCC, case #22, the inventors again observed that the level of cellular proliferation, denoted by Ki67-positivity, was much higher in VHL-positive tumor areas than the VHL-negative regions (**Figure 7A, Table 1**). Using the HALO infiltration analysis module to decipher the spatial relationship between the respective cell populations, the highest concentration of Ki-67-positive cells resided in the border of VHL-positive areas that were in closest proximity to and under the greatest paracrine influence by the VHL-negative cells (**Figure 7B**). The finding is supportive of VHL-negative cells exerting a positive influence on the proliferation of VHL-positive cells in case #22. Next, examined was the differential functional role of HIF1A and HIF2A in further detail by IF analyses to decipher their special localization. The primary kidney tumor

and lung metastases of our RC mixed tumor consistently showed that HIF1A expression localized to VHL-negative areas while HIF2A expression predominantly localized to VHL-positive area, especially at the interface with VHL-negative areas (**Figures 7C, 7D**). Paralleling the inventors' preclinical model, IF analyses of primary tumor of case #22 revealed that HIF1A and HIF2A expression were localized to spatially distinct tumor areas and HIF2A expression coincided prominently in the VHL+ area (**Figures 7E, 7F**). Of interest, amongst the primary tumor cell lines (#21, 22, 23, 24) that were generated, VHL- and HIF2A-expressing cells predominate. This finding suggests these VHL+, HIF2A+ tumor cells are the more proliferative population in the tumor (**Figure 12E**).

[081] Next examined was whether POSTN expression in ccRCC human tumors also follows the pattern observed in our preclinical model. Staining parallel sections of a large metastatic lesion from a mixed tumor to identify VHL-WT and VHL-KO cells and POSTN revealed that POSTN expression coincided closely with the location of VHL-KO cells and not VHL-WT cells (**Figure 8A**). Next it was inquired if this relationship is maintained in patients' tumor by IHC in a tissue microarray (TMA) constructed from over 300 ccRCC patients who underwent nephrectomy at the inventors' institution, as described by Klatte T, et al. Hypoxia-inducible factor 1 alpha in clear cell renal cell carcinoma. *Clinical cancer research : an official journal of the American Association for Cancer Research*. 2007;13(24):7388-93, which is incorporated herein by reference in its entirety. Due to small sampling area of TMA, many samples were non-informative. **Figure 8B** showed images from 16 informative cases which revealed the reciprocal relationship that the inventors have observed. IHC analyses in the primary tumor of the most aggressive case #22 again verified this inverse relationship of high VHL expression showed low POSTN expression and low VHL areas expressed high levels of POSTN (**Figure 8C**). Furthermore, quantitative analysis of VHL+POSTN- and VHL-POSTN+ cells in case #22 using the HALO image analysis software (Indica Labs, USA) confirmed the distinct spatial distribution of these two populations (**Figure 8D, 8E**). IF stain of the lung metastatic lesions of case #22 clearly showed the predominance of VHL-positive cells (red fluorescence), which were excluded from the POSTN expressing cells (green fluorescence, **Figure 8F**). This reciprocal relationship was further confirmed in the retroperitoneal lymph node metastasis of case #17 (**Figure 8G**). At 100x magnification, it is clear that VHL (red fluorescence) and POSTN (green fluorescence) expression were mutually excluded from each other with no co-localization. This spatial heterogeneity of the VHL+ and VHL-

populations observed in clinical specimens reiterated the findings in our RENCA metastatic ccRCC model (**Figures 1C, 5A, Figures 9A, 9E**).

[082] Taken together, the VHL-deleted ccRCC models herein reveal a novel metastatic mechanism that relies on cooperative interactions between two distinct populations of tumor cells, namely the VHL-KO and VHL-WT cells. The VHL-KO cells display an EMT and highly motile phenotype, but alone they grow poorly *in vivo*. However, in conjunction with VHL-WT cells, VHL-KO cells appeared to induce an aggressive behavior that promotes rampant lung metastases composed predominantly of VHL-WT cells. Many of the phenotype observed in the inventors' preclinical model was observed in clinical tumor specimens, in particular the loss of VHL function leading to upregulation of HIF1A and POSTN. It is believed that these results lend credibility that the cooperative metastasis mechanism discovered in the preclinical metastatic models could be operational in human ccRCC. Further unraveling and confirmation of the critical metastatic crosstalk at play could lead to novel therapeutic avenues to control the lethal metastatic stage of ccRCC.

DISCUSSION

[083] In RCC and other epithelial cancers, metastasis is the major cause of mortality. Its complex nature coupled with an incomplete understanding of the mechanism of metastasis pose a significant challenge to devise effective treatment. For the last three decades, the progression model is the most common, prevailing concept of how cancer metastasis occurs. This model postulates that multiple and progressive mutational events occur in order for a small fraction of cells to acquire full metastatic potential. Subsequent studies showed that clonal evolution and selection can enhance not only metastatic potential but also achieve metastatic site specificity. The cooperative model of metastasis uncovered here proposes a distinctly different mechanism, in that a relay of signals between 2 teams of tumor cells, rather than a clonal progression of a single team, is needed to achieve metastasis.

[084] In the metastatic ccRCC model reported here (summarized in **Figure 6F**), the prerequisite pro-metastatic interactions occur between the team of VHL-KO and VHL-WT cells. Intriguingly, the EMT+ VHL-KO cells are poorly proliferative themselves, but they serve as the metastatic driver to induce aggressive behaviors in the normally non-metastatic VHL-WT cells. The inventors further elucidated the loss of VHL function in the VHL-KO cells upregulates HIF1A, which in turn stimulates the production of POSTN. POSTN serves as the critical paracrine

metastasis-promoting factor by not only inducing the EMT state and motility of neighboring VHL-WT tumor cells but it also causes vascular destruction to facilitate the escape of tumor cells into the circulation. Given that POSTN can impact the metastatic process in multiple ways (**Figure 6F**), the inventor showed that blocking POSTN's function can indeed halt lung metastasis in the preclinical models herein. Clinical relevance of the cooperative mechanism of metastasis is supported by the fact that human tumor samples consistently show intratumoral heterogeneity with the co-existence of VHL-negative and VHL-positive tumor cell clusters in individual cases of ccRCC. Furthermore, the investigations herein demonstrate that the path of POSTN overexpression due to VHL loss and HIF1A upregulation is also followed in the clinical setting akin to our preclinical model.

[085] The identification of POSTN as a critical factor playing a direct and instrumental role in metastasis is a novel and significant finding. POSTN, also known as osteoblast-specific factor 2, is a ubiquitous secreted stromal protein that promotes integrin-dependent cell adhesion and motility during bone and cardiac development. The overexpression of POSTN is observed under EMT and hypoxic conditions, which corresponds to the conditions of our VHL-KO cells. POSTN was reported to bridge the colonization of breast cancer cells to their terminal lung metastatic site, placing POSTN's involvement at the distal end of the metastatic cascade (step 3, **Figure 6G**). This result differs from the inventors' results that show POSTN acts at the tumor proximal and intravasation step (step 1). POSTN's role in the latter steps of the metastatic cascade in the ccRCC model described herein has not been fully investigated. The use of anti-POSTN neutralizing antibody has also been shown to be a fruitful therapeutic approach to block metastatic progression in an ovarian cancer model. The clinical applicability of POSTN-targeted therapeutic approach to block metastasis clearly warrants further investigation.

[086] Although HIF1A and HIF2A are both known to be upregulated upon the loss of VHL gene function, the inventors believe these two isoforms likely play different roles in the growth and metastasis of ccRCC tumors. First, HIF1A and HIF2A is expressed in different cell populations in distinct areas within a tumor. Consistently, elevated HIF1A expression tracks with the poorly proliferative RC-VHL-KO cells of the model herein or the VHL-negative cells in clinical tumor samples, while the expression of HIF2A is more notable in the more proliferative VHL-WT or VHL-positive cells. Currently, the precise functional role of these 2 HIF isoforms in metastatic ccRCC remains unclear. However, the data herein does not support HIF1A functioning as a tumor

suppressor. It is believed that HIF1A upregulation contributes to the EMT and growth retardation phenotype of the VHL-KO cells. The HIF1A-mediated cell cycle arrest by upregulation of p21 and p27 through a Myc-directed derepression of these CDK inhibitors has been extensively studied. Despite its growth suppressive actions, HIF1A induces the expression of POSTN and its associated pro-metastatic functions (**Figure 4E**). Deletion of the HIF1A gene in addition to the VHL gene abrogated the prometastatic effects of VHL-KO cells. These results support that HIF1A acts to promote metastasis rather than to squelch tumor development. As such, these prometastatic activities of HIF1A fit the recent extensive tumor genetic analyses that support tumor hypoxia as an important driving force of tumor aggression in human ccRCC and many other cancers. Clearly, further investigation of the differential role of HIF1A and HIF2A in ccRCC metastasis is warranted.

[087] The cooperative “team work” concept working between distinct populations of tumor cells to advance the disease has also been reported in a recent study in breast cancer. In this study, EMT in breast cancer cells, induced by overexpression of EMT transcription factors, activate Hedgehog/GLI signaling to promote the aggressive behavior of non-EMT cells in a paracrine manner. The cooperation between EMT and non-EMT cells reported is highly reminiscent of the crosstalk in our VHL-KO and VHL-WT model, but POSTN is a functional mediator in the inventors’ model. The cooperative metastatic model proposed here and by others emphasizes the need for different strategies to search for novel therapeutic targets. The clinicians should determine if tumor heterogeneity exists for the crosstalk between different populations to occur. Intratumoral gene expression heterogeneity is widely recognized in ccRCC and high power gene sequencing technologies and bioinformatics have been applied to study this disease. Despite these advances in the study of ccRCC, the untangling of the signaling pathways to distill the key crosstalk signals remains very challenging. A potential prudent approach could be to first separate the distinct populations based on cellular morphology or protein biomarker(s) and then pursue the expression interrogation of the distinct populations. Furthermore, in pathways such as VHL and HIF1A that involve extensive post-transcriptional regulations of protein stability, it would be prudent to integrate protein expression in conjunction with the gene expression analyses to gain a comprehensive view of the tumor biology. In total, the study reported here provides an alternate idea of how the complex task of metastatic dissemination could be achieved by a heterogeneous tumor like ccRCC. This cooperative model can guide the search for better and more effective metastatic blocking treatment and address a clear unmet need in the field of cancer research.

[088] All publications, accession numbers, patents, and patent applications cited in this specification are herein incorporated by reference as if each was specifically and individually indicated to be incorporated by reference.

[089] While certain features of the invention have been illustrated and described herein, many modifications, substitutions, changes, and equivalents will now occur to those of ordinary skill in the art. It is, therefore, to be understood that the appended claims are intended to cover all such modifications and changes as fall within the true spirit of the invention.

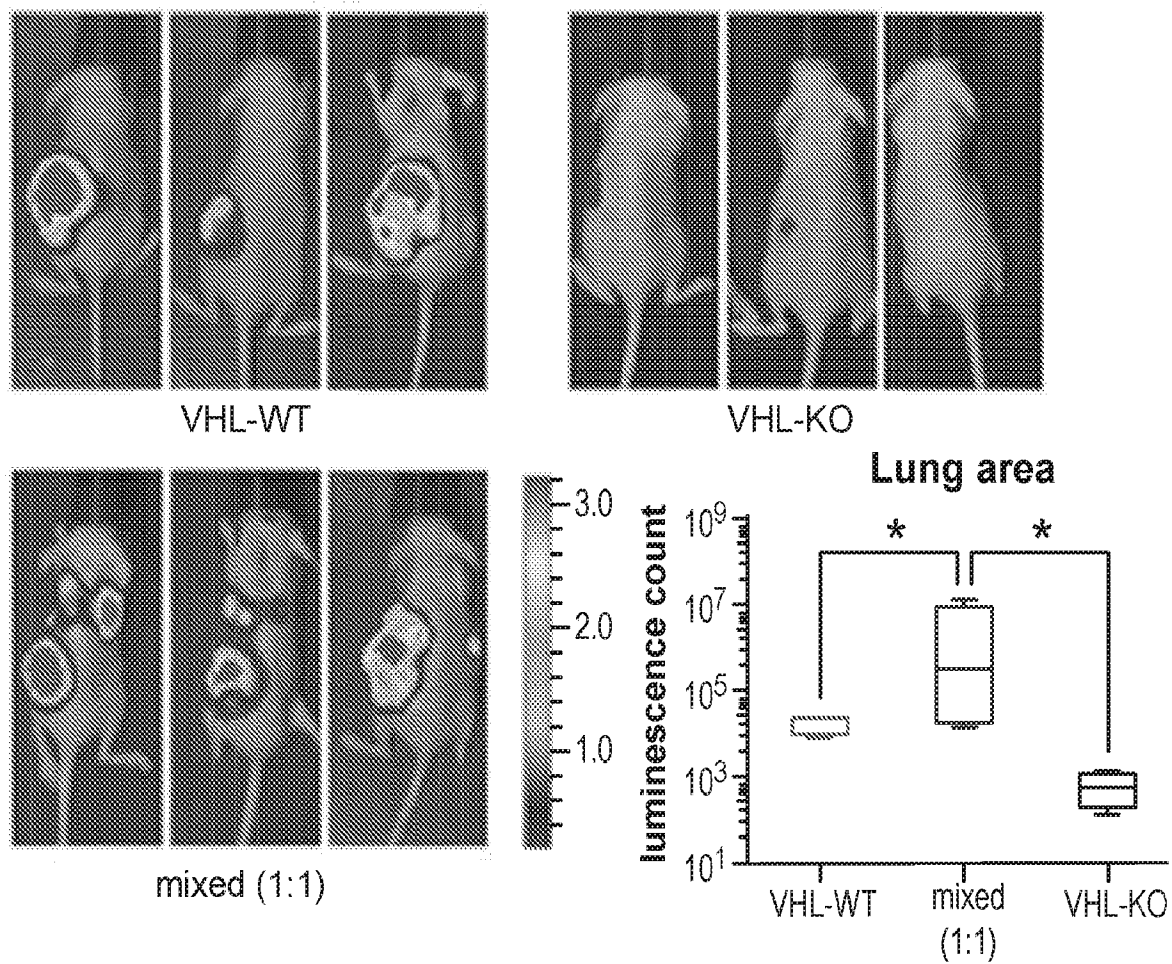
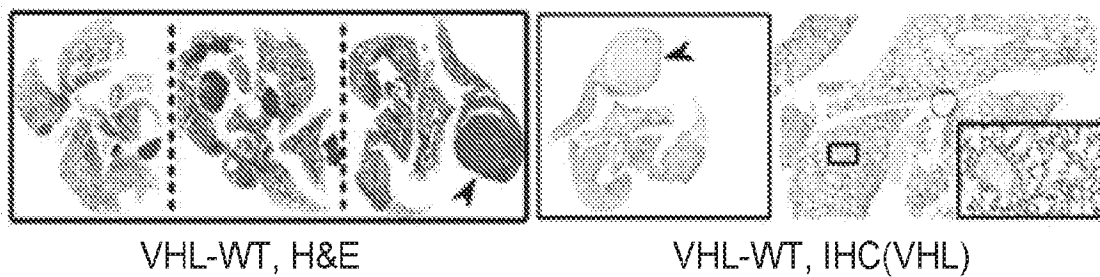
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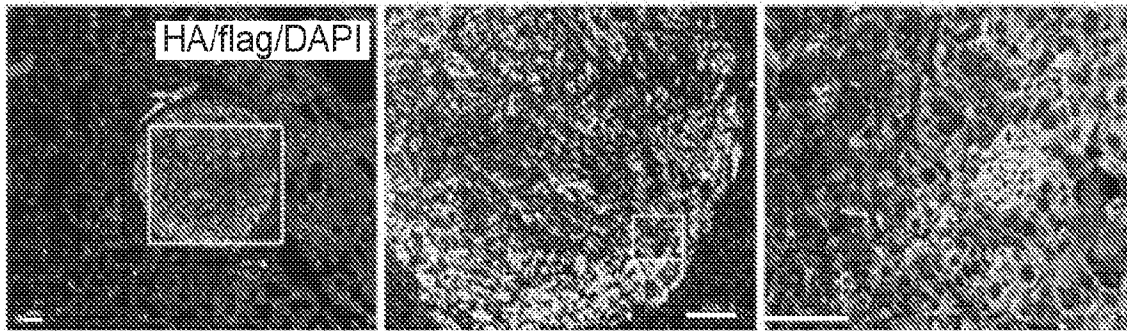
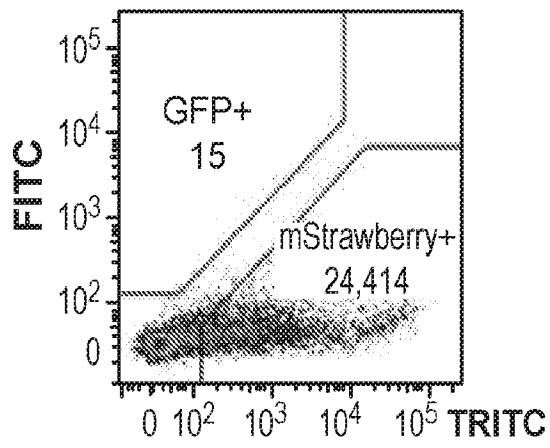
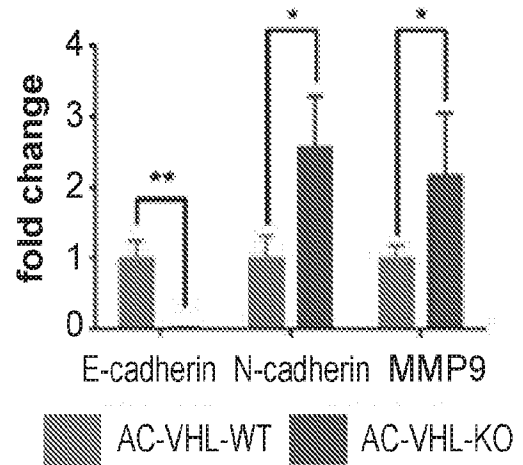
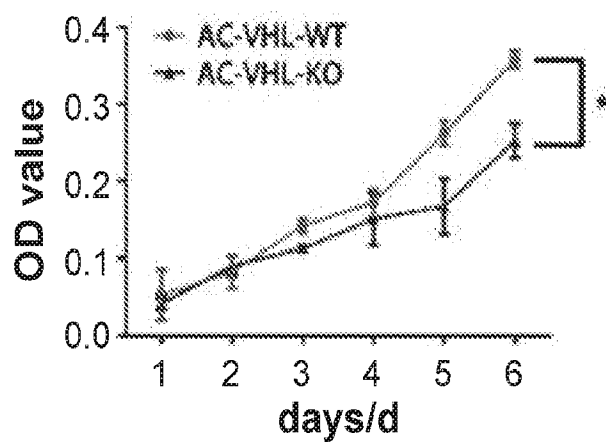
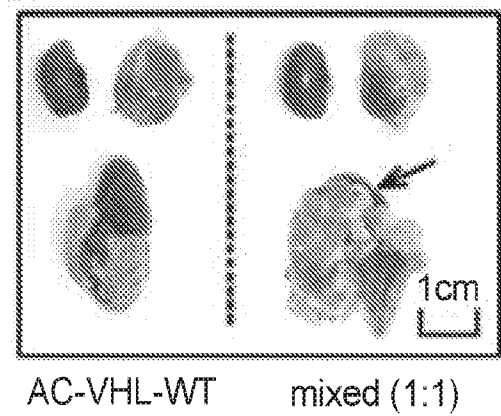
WHAT IS CLAIMED IS:

1. A method for inhibiting metastasis of renal cell carcinoma (RCC) in a subject having RCC, the method comprising reducing presence or biological activity of periostin, wherein reducing the presence or biological activity of periostin inhibits metastasis.
2. The method of claim 1, wherein the reducing the presence or biological activity of periostin is provided at a primary tumor site of the RCC and/or in a stromal fibroblast in proximity to the primary tumor site.
3. The method of claim 1, wherein reducing the presence of biological activity of periostin comprises reducing, silencing or eliminating the expression of periostin.
4. The method of claim 1, wherein reducing the presence or biological activity of periostin comprises genetically modifying an RCC tumor cell to delete a nucleic acid encoding periostin.
5. The method of claim 4, wherein genetic modification comprises administering to the subject an adenoviral vector comprising CRISPR/Cas9 and a guide RNA (gRNA), wherein the gRNA targets the nucleic acid encoding periostin.
6. The method of claim 1, wherein reducing the presence or biological activity of periostin comprises administering to the subject an anti-periostin (POSTN) monoclonal antibody.
7. The method of claim 1, wherein reducing the presence or biological activity of periostin comprises administering to the subject an antisense oligonucleotide, shRNA, siRNA.
8. The method of claim 1, wherein reducing the presence or biological activity of periostin comprises administering to the subject an POSTN receptor antagonist.
9. The method of claim 8, wherein, the POSTN receptor antagonist is an alpha-v/beta-5 integrin ($\alpha_v\beta_5$) and/or an alpha-v/beta-3 integrin ($\alpha_v\beta_3$).
10. The method of claim 1, further comprising detecting a deletion of Von Hippel Lindau (VHL) tumor suppressor gene (VHL-KO) in the RCC tumor cell prior to the reducing the presence or biological activity of periostin.
11. The method of claim 1, further comprising detecting overexpression of POSTN in the RCC tumor cell prior to the reducing the presence or biological activity of periostin.

12. The method of claim 1, further comprising administering to the subject an antiangiogenic drug, a tyrosine-kinase inhibitor (TKI), interleukin-2, or a combination thereof.
13. The method of claim 5, wherein the CRISPR/Cas9 targets a codon of the nucleic acid encoding periostin for base editing into a nonsense codon.
14. The method of claim 13, wherein the base editing is performed after detection of overexpression of POSTN in the RCC tumor cell.
15. The method of claim 13, wherein the base editing inhibits expression of periostin.
16. The method of claim 15, wherein inhibition of the expression of the periostin decreases or prevents development of lung metastasis in the subject.
17. The method of claim 16, wherein expression of periostin is inhibited by 50% to 100%.
18. The method of claim 5, further comprising administering to the subject a second adenoviral vector, wherein the second adenoviral vector comprises a nucleic acid encoding a VHL tumor suppressor.
19. The method of claim 1, wherein the biological activity of periostin comprises cell adhesion, cell motility, growth, migration and invasion of cancer cells, binding to one or more integrin, regulation of epithelial-mesenchymal transition (EMT) and/or induction of apoptosis of endothelial cells.
20. A method for treating or preventing metastasis in a subject having RCC, the method comprising administering to a primary RCC tumor site an anti-periostin (POSTN) monoclonal antibody and genetically modifying RCC tumor cells to delete the POSTN gene in RCC tumor cells.
21. The method of claim 20, wherein genetically modifying the RCC tumor cells comprises administering to the subject an adenoviral vector comprising CRISPR/Cas9 and a gRNA, wherein the gRNA targets the nucleic acid encoding periostin.
22. The method of claim 20, wherein genetically modifying the RCC tumor cells reduces, silences or eliminates expression of periostin therein.
23. The method of claim 22, wherein reduction, silencing or elimination of periostin expression decreases or prevents development of lung metastasis in the subject.

24. The method of claim 23, wherein the periostin expression is reduced by 50% to 100%.
25. The method of claim 20, further comprising detecting a deletion of Von Hippel Lindau (VHL) tumor suppressor gene (VHL-KO) in the RCC tumor cells prior to genetically modifying the RCC tumor cells.
26. The method of claim 20, further comprising detecting overexpression of POSTN in the RCC tumor cells prior to genetically modifying the RCC tumor cells.
27. The method of claim 20, further comprising administering to the subject an antiangiogenic drug, a tyrosine-kinase inhibitor (TKI), interleukin-2, or a combination thereof.
28. The method of claim 20, further comprising administering to the subject an antisense oligonucleotide, shRNA, siRNA.
29. A pharmaceutical composition comprising an adenoviral vector comprising CRISPR/Cas9 and a guide RNA (gRNA), wherein the gRNA targets a nucleic acid encoding periostin and/or an anti-periostin (POSTN) monoclonal antibody.
30. The pharmaceutical composition of claim 29, further comprising a pharmaceutically acceptable carrier.
31. The pharmaceutical composition of claim 29, formulated for intra-tumoral injection or intravenous injection.
32. The pharmaceutical composition of claim 29, further comprising a second adenoviral vector, wherein the second adenoviral vector comprises a nucleic acid encoding a VHL tumor suppressor.

*Figure 1A**Figure 1B**Figure 1C*

*Figure 1D**Figure 1E**Figure 1F**Figure 1G**Figure 1H*

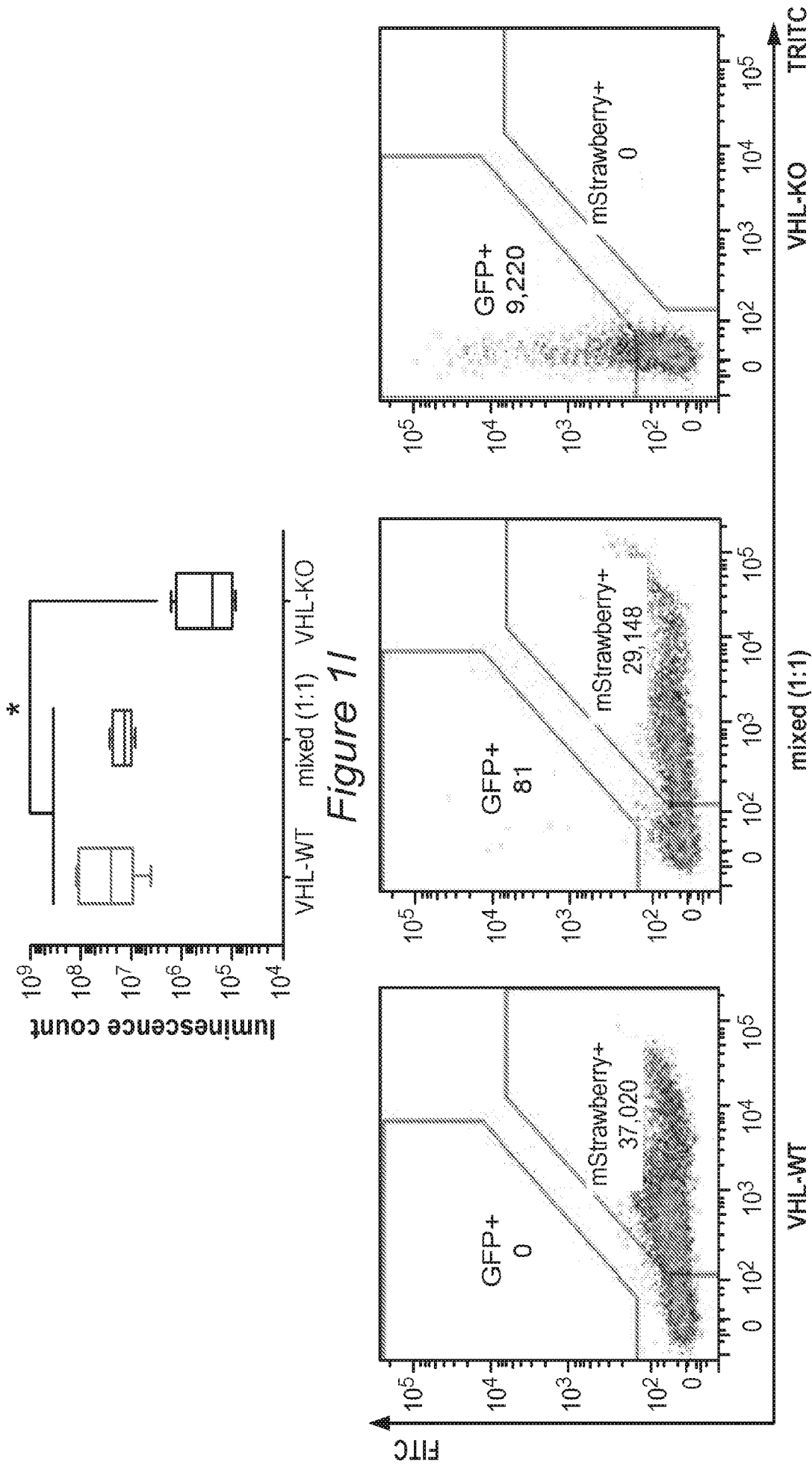


Figure 1I

Figure 1J

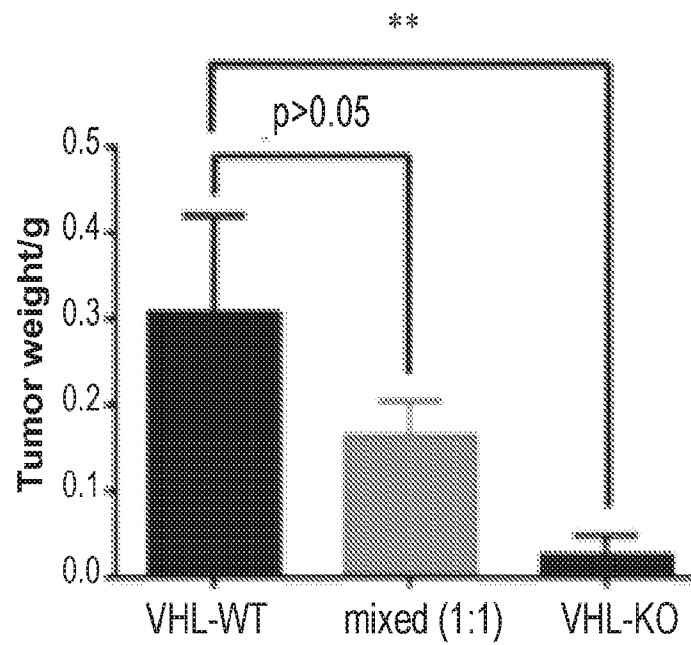
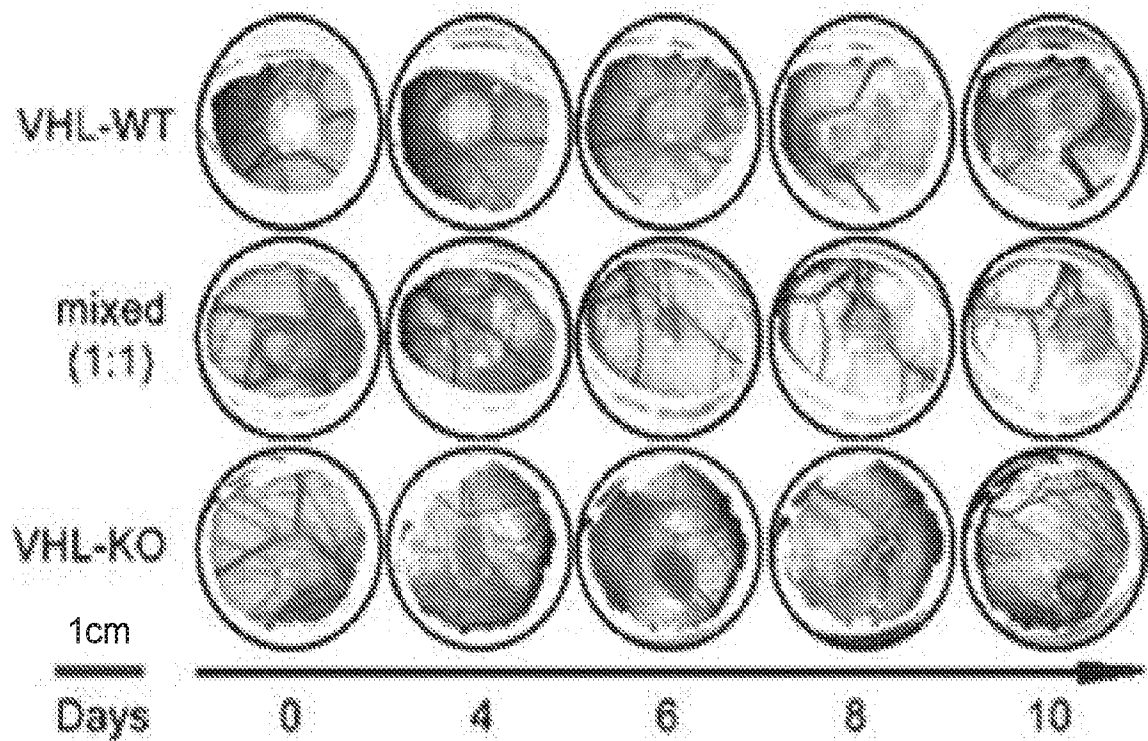
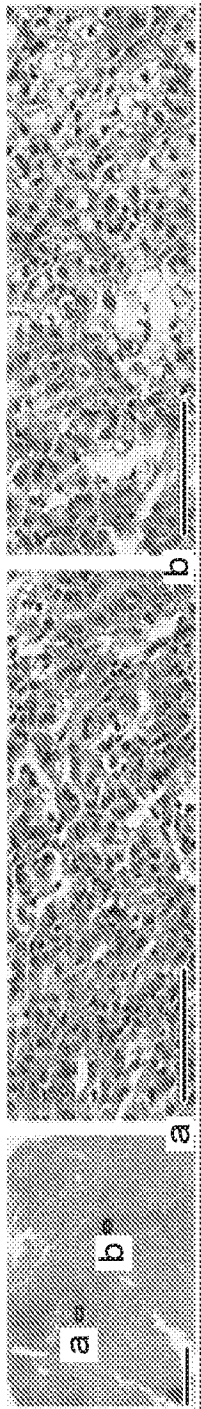
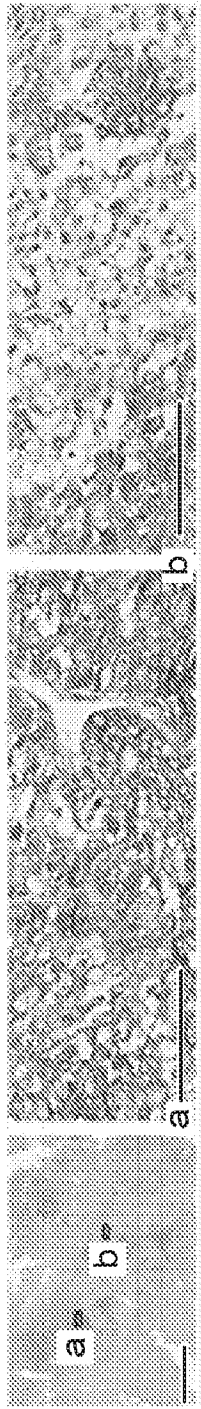


Figure 1K



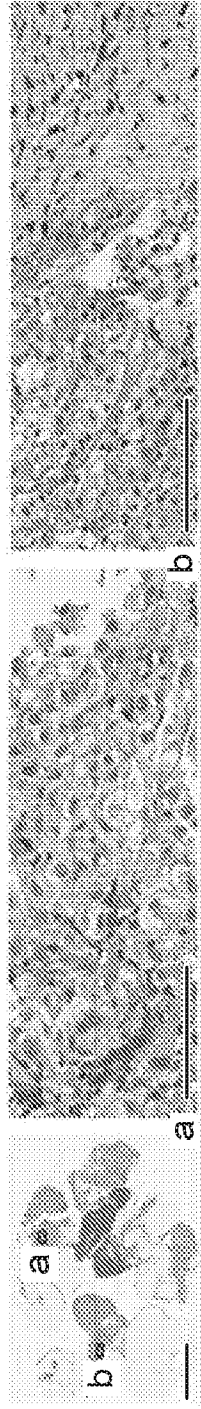
Tumor, #22(H&E)

Figure 2A



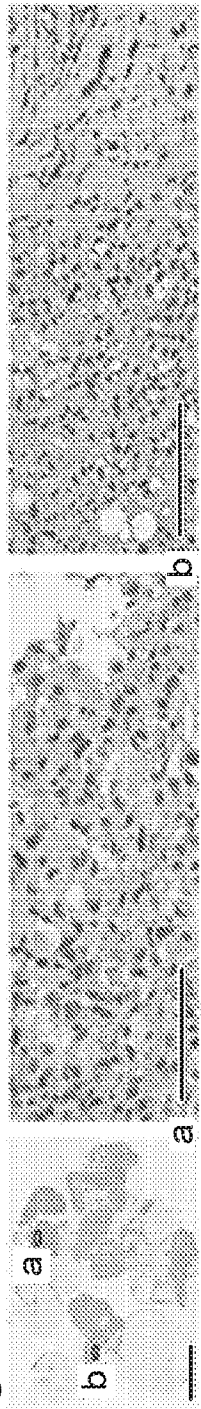
Tumor, #22(IHC, anti-VHL)

Figure 2B



Lung metastasis biopsy, #22(H&E)

Figure 2C



Lung metastasis biopsy, #22(IHC, anti-VHL)

Figure 2D

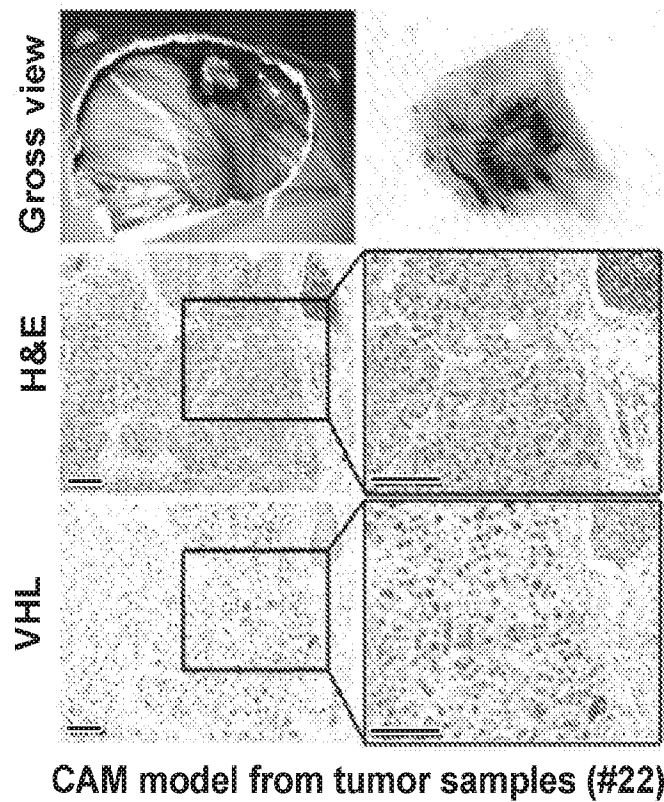


Figure 2E

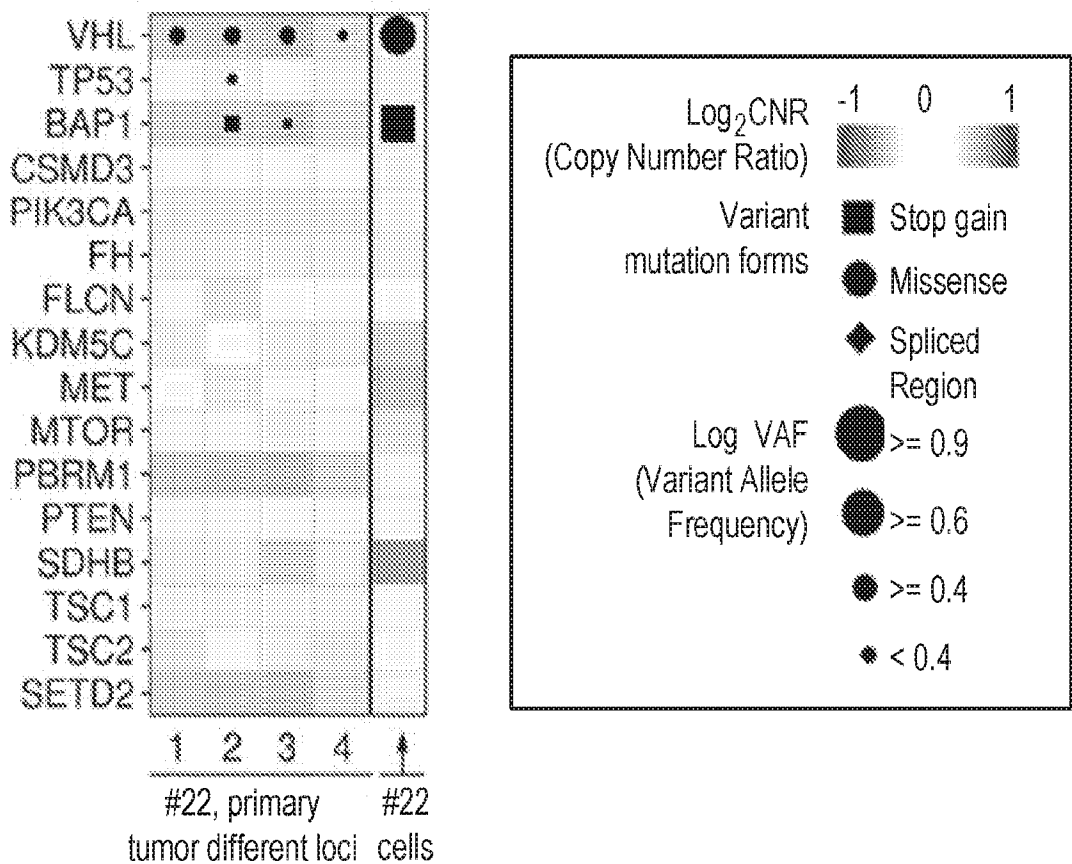
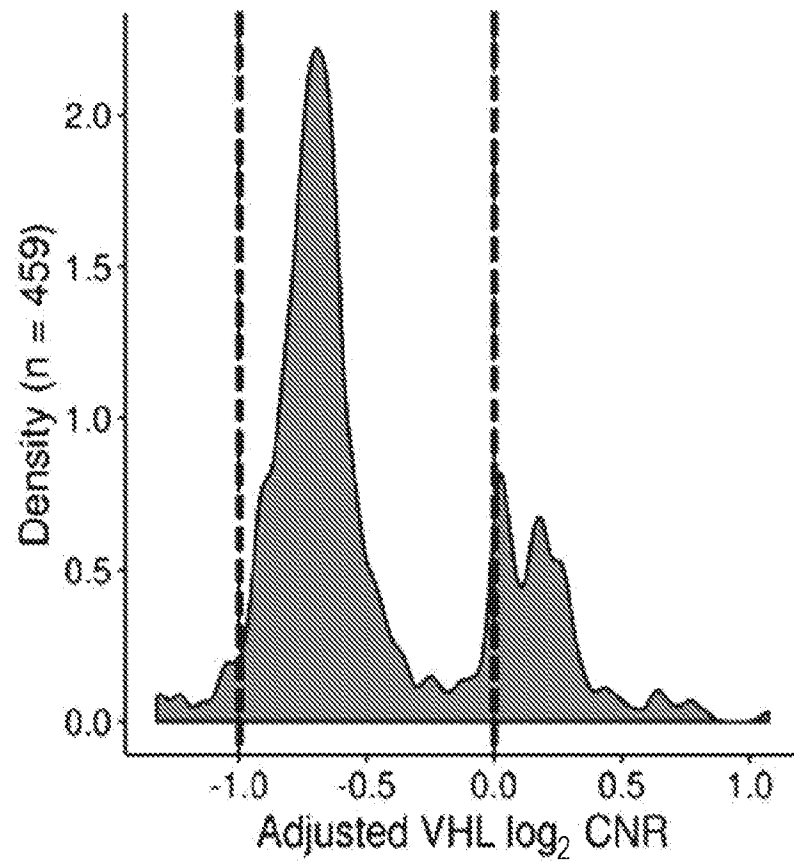
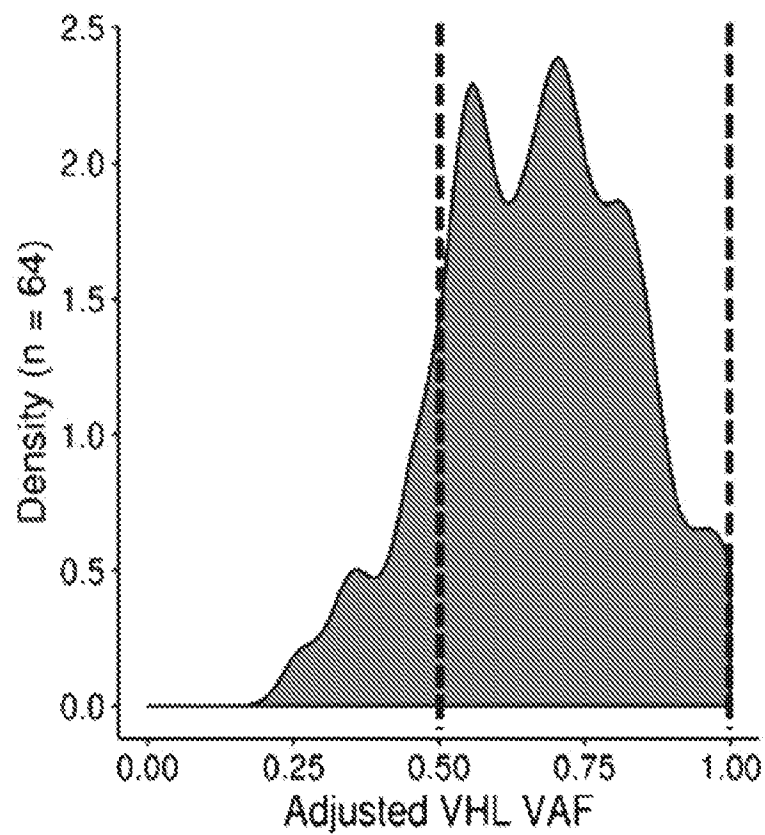
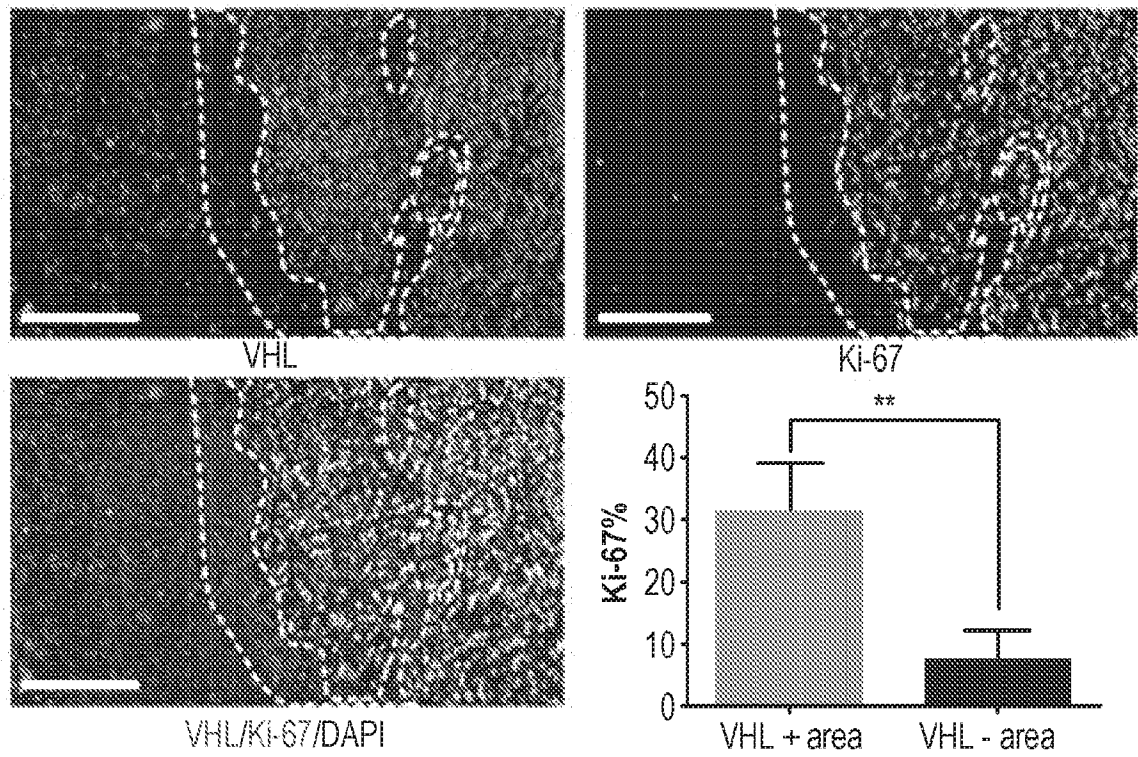


Figure 2F

*Figure 2G**Figure 2H*



Primary tumor (mixed, VHL negative cell area encircled in dashline)

Figure 3A

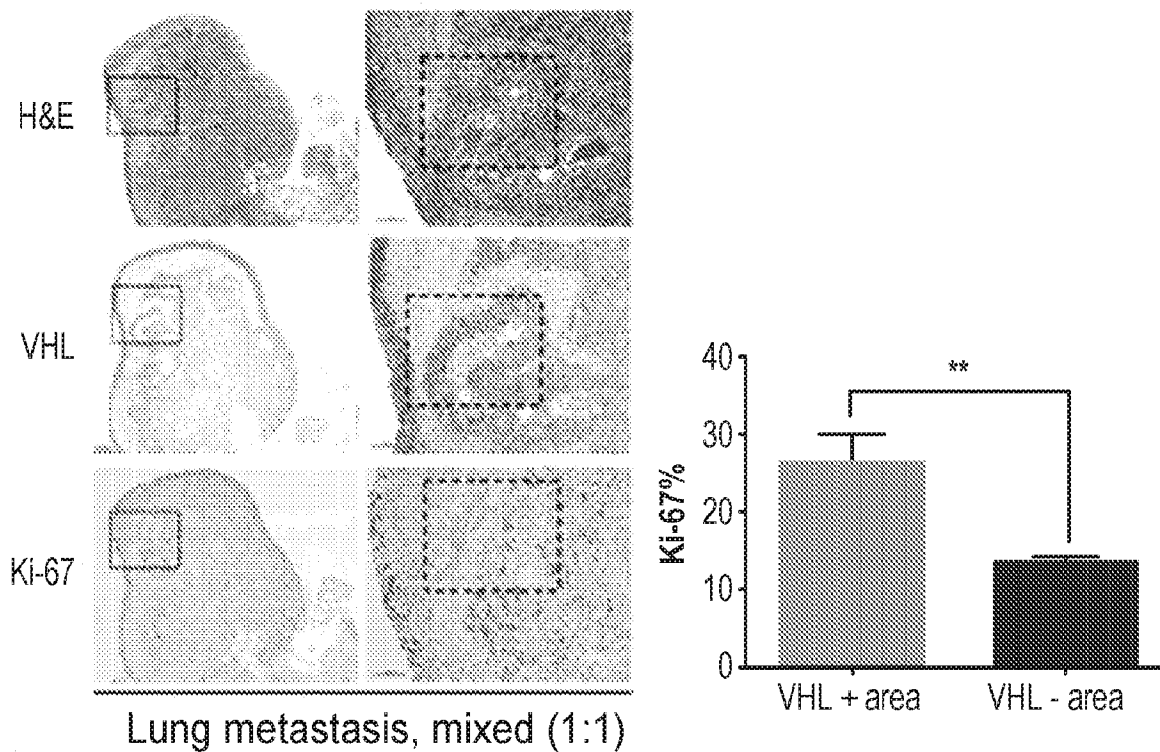
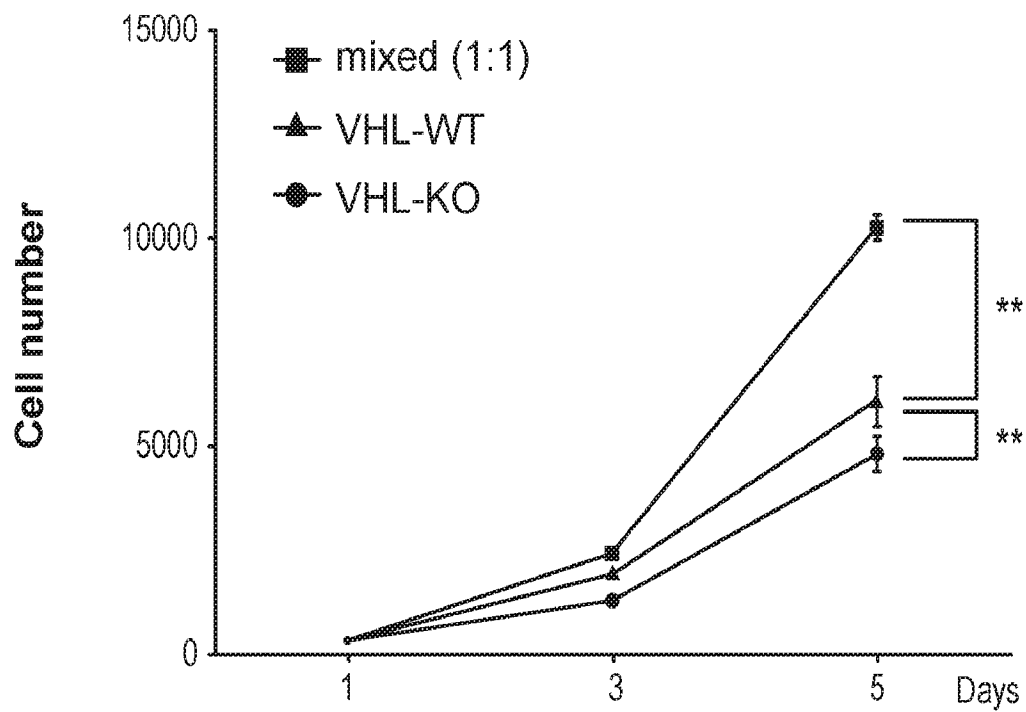
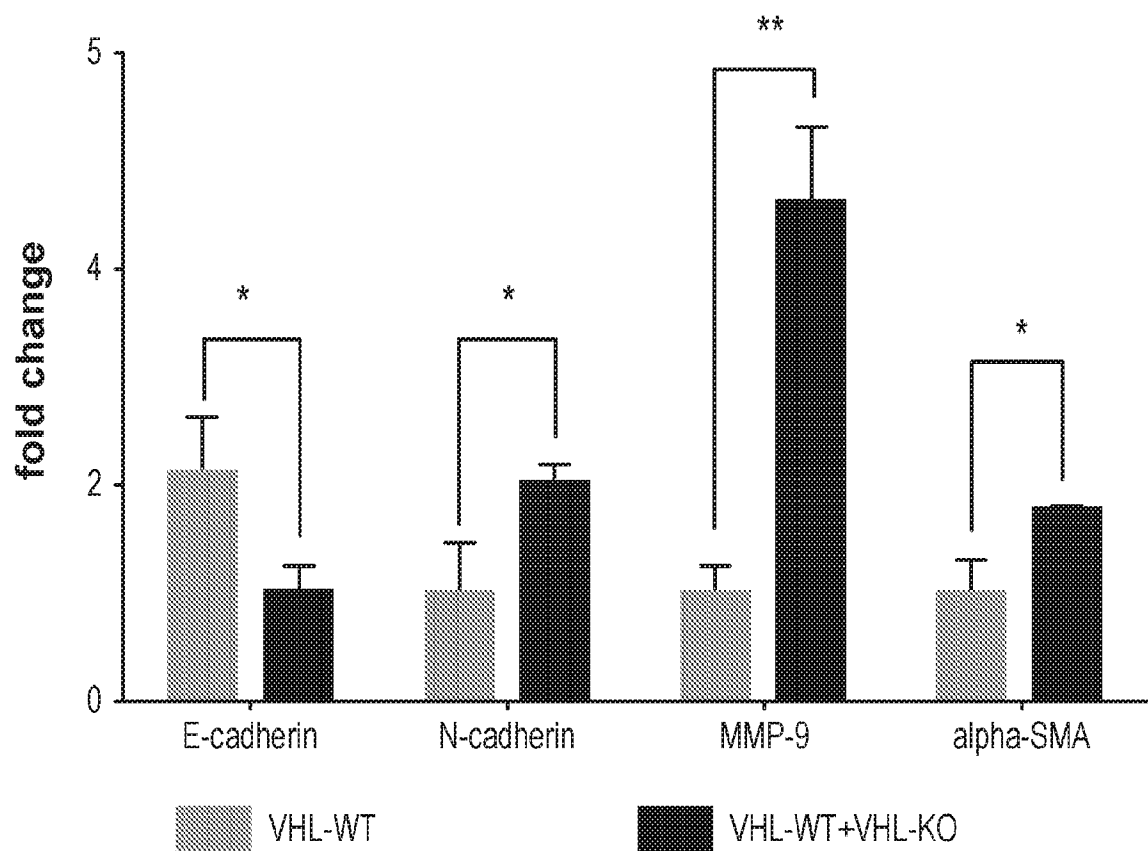


Figure 3B

*Figure 3C**Figure 3D*

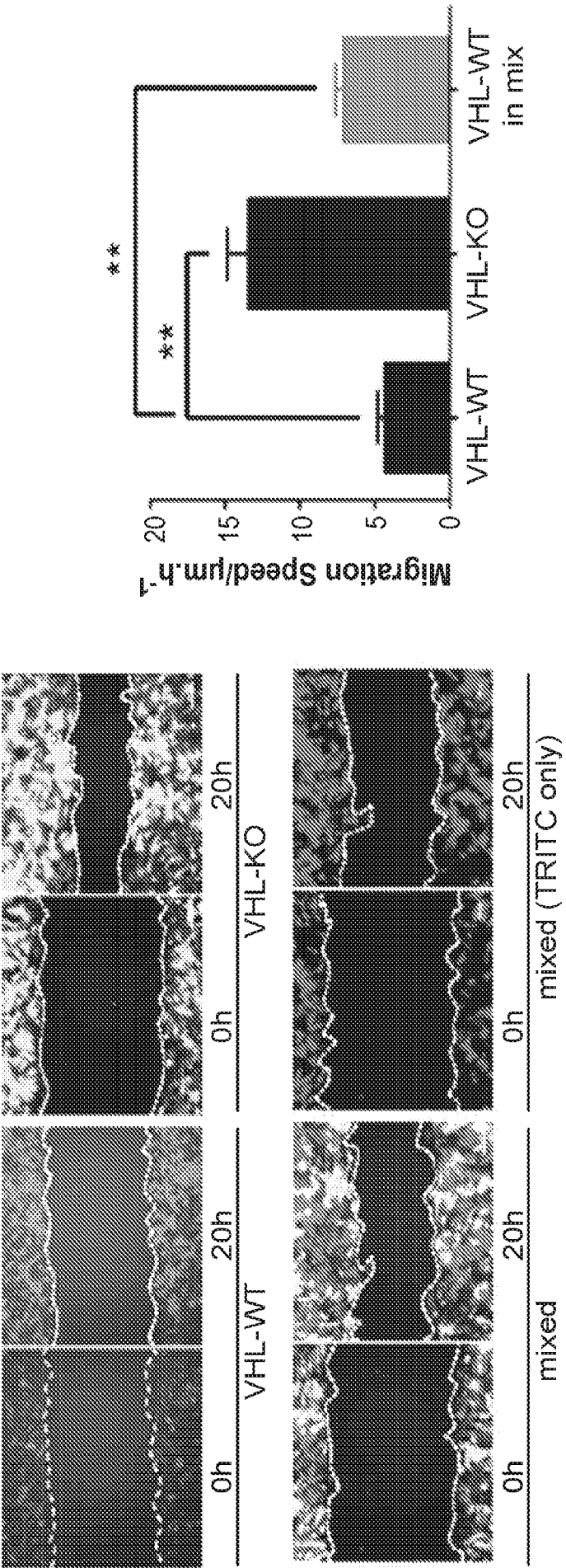


Figure 3E

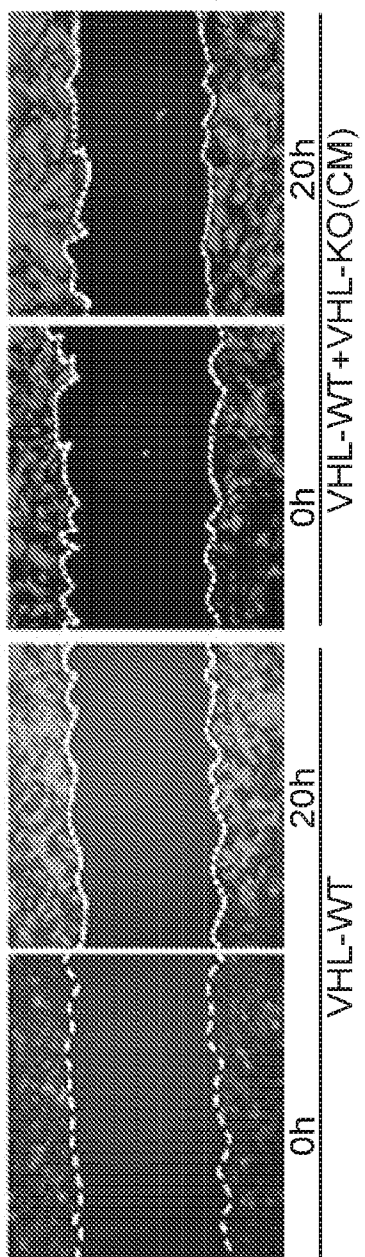
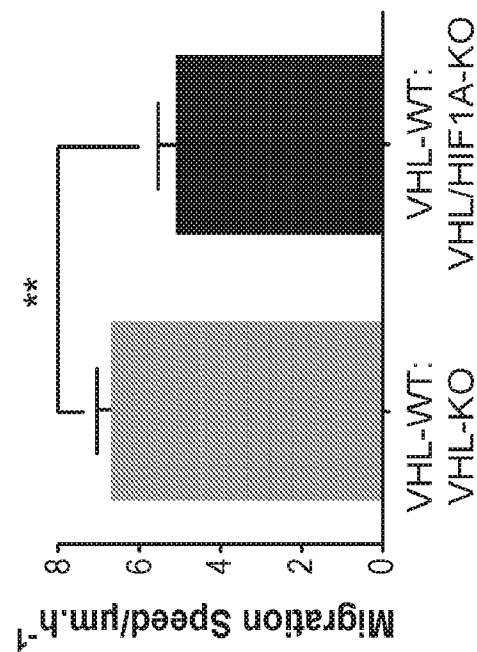
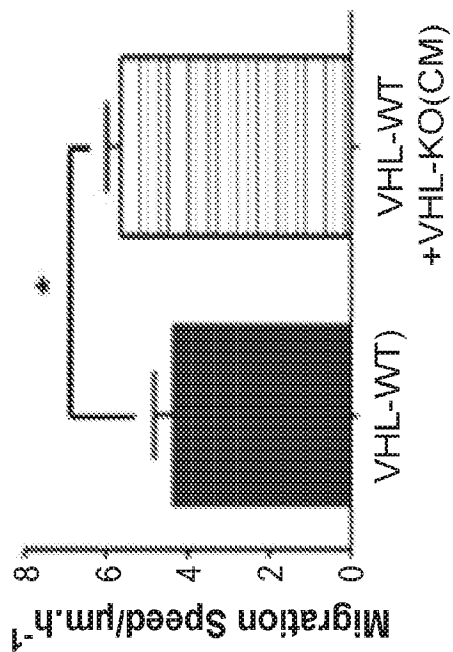


Figure 3F

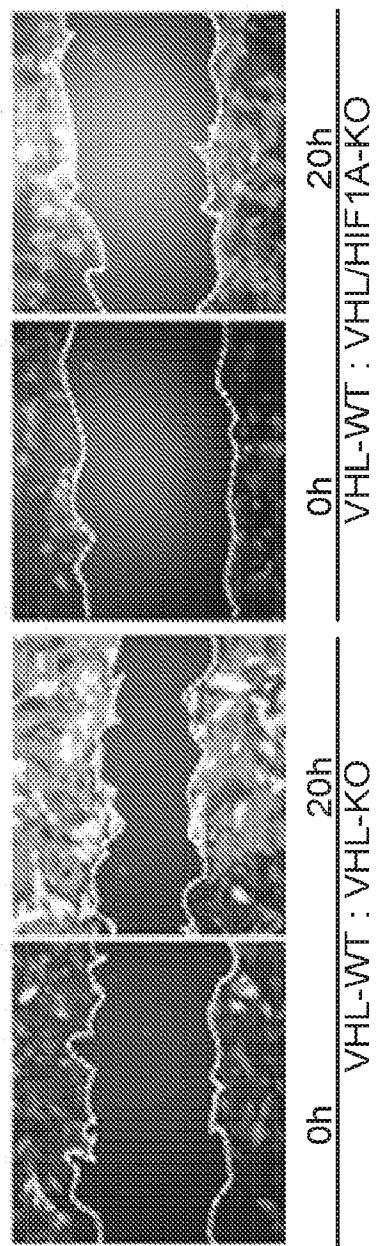
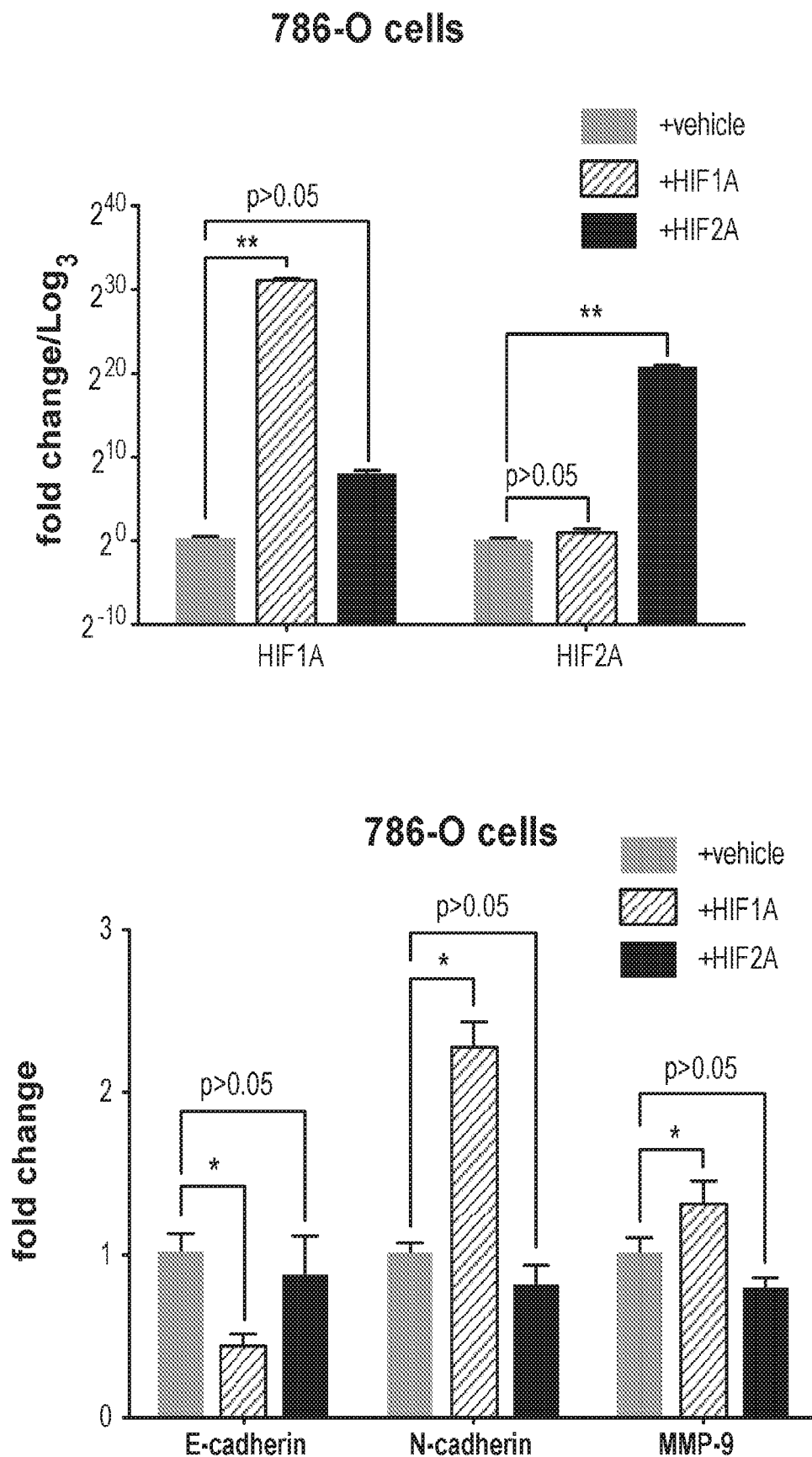
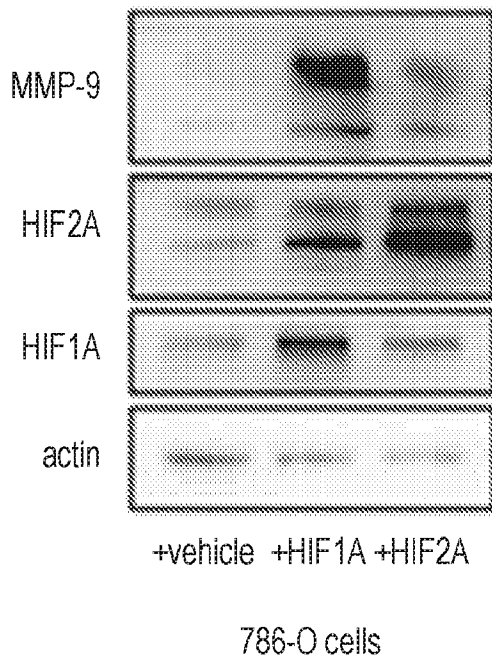
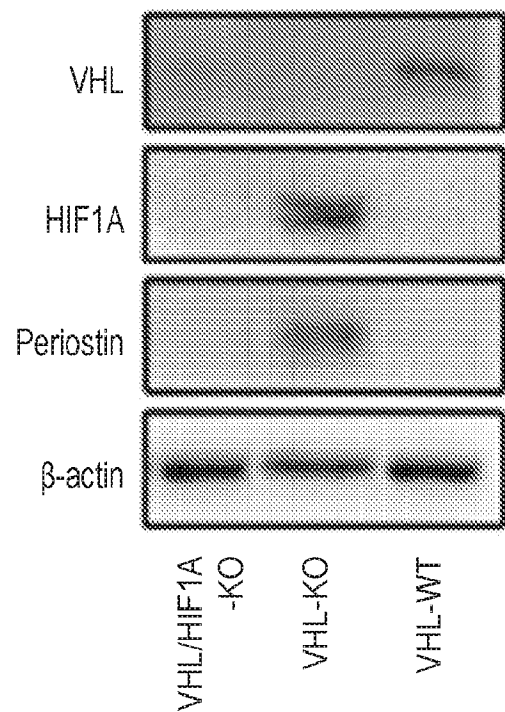
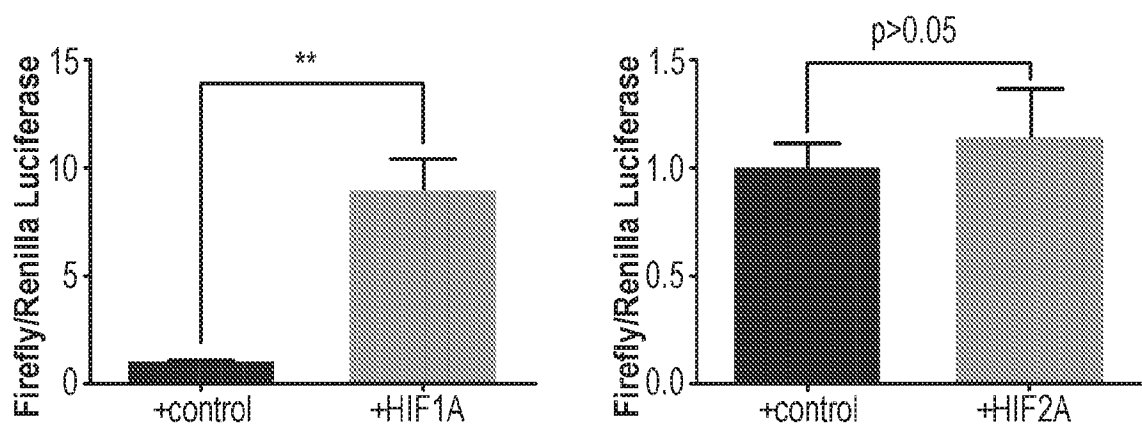


Figure 4A

*Figure 4B*

*Figure 4C**Figure 4D**Figure 4E*

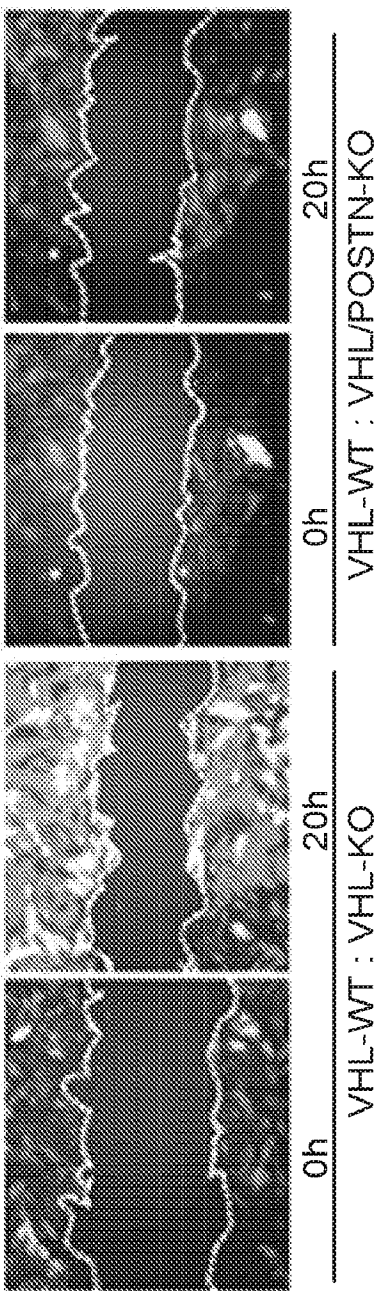
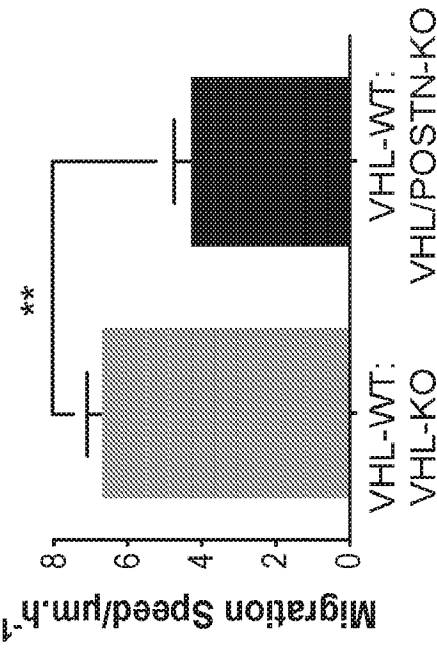


Figure 4F

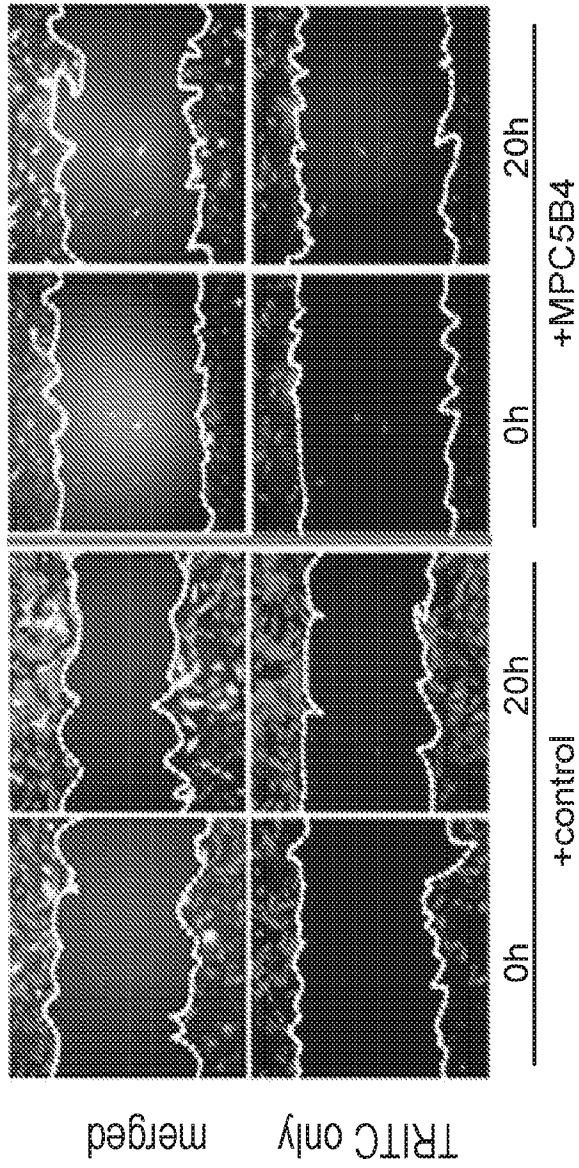
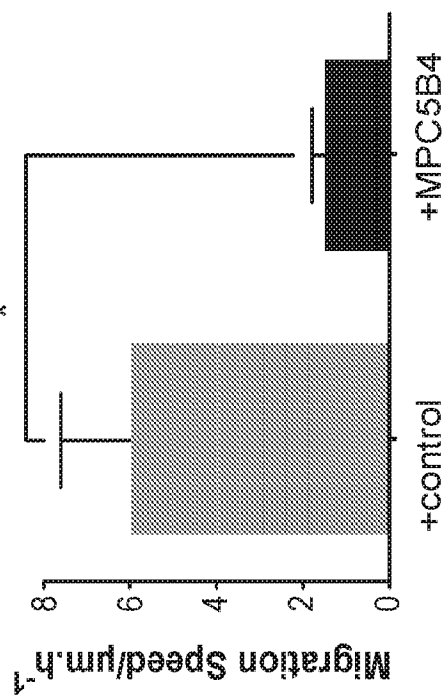
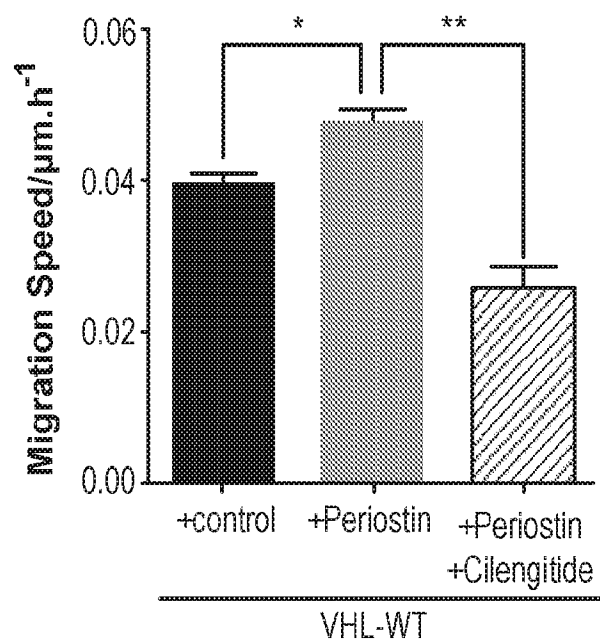
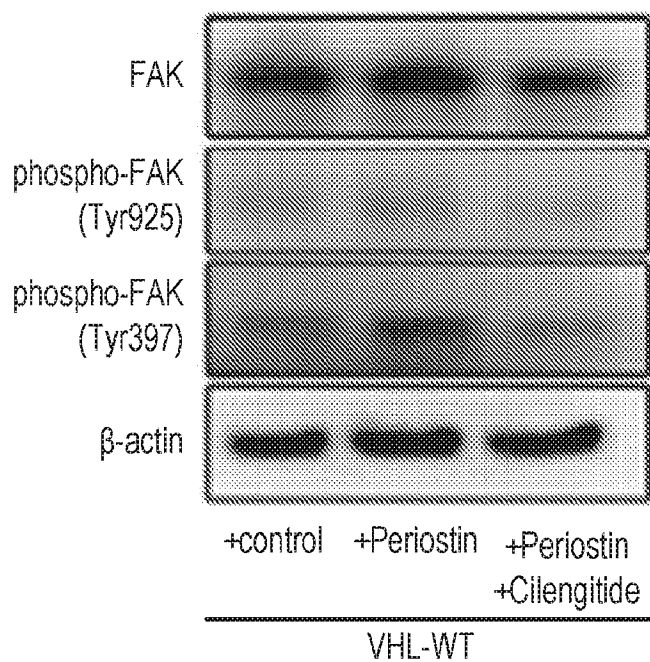


Figure 4G

*Figure 4H**Figure 4I*

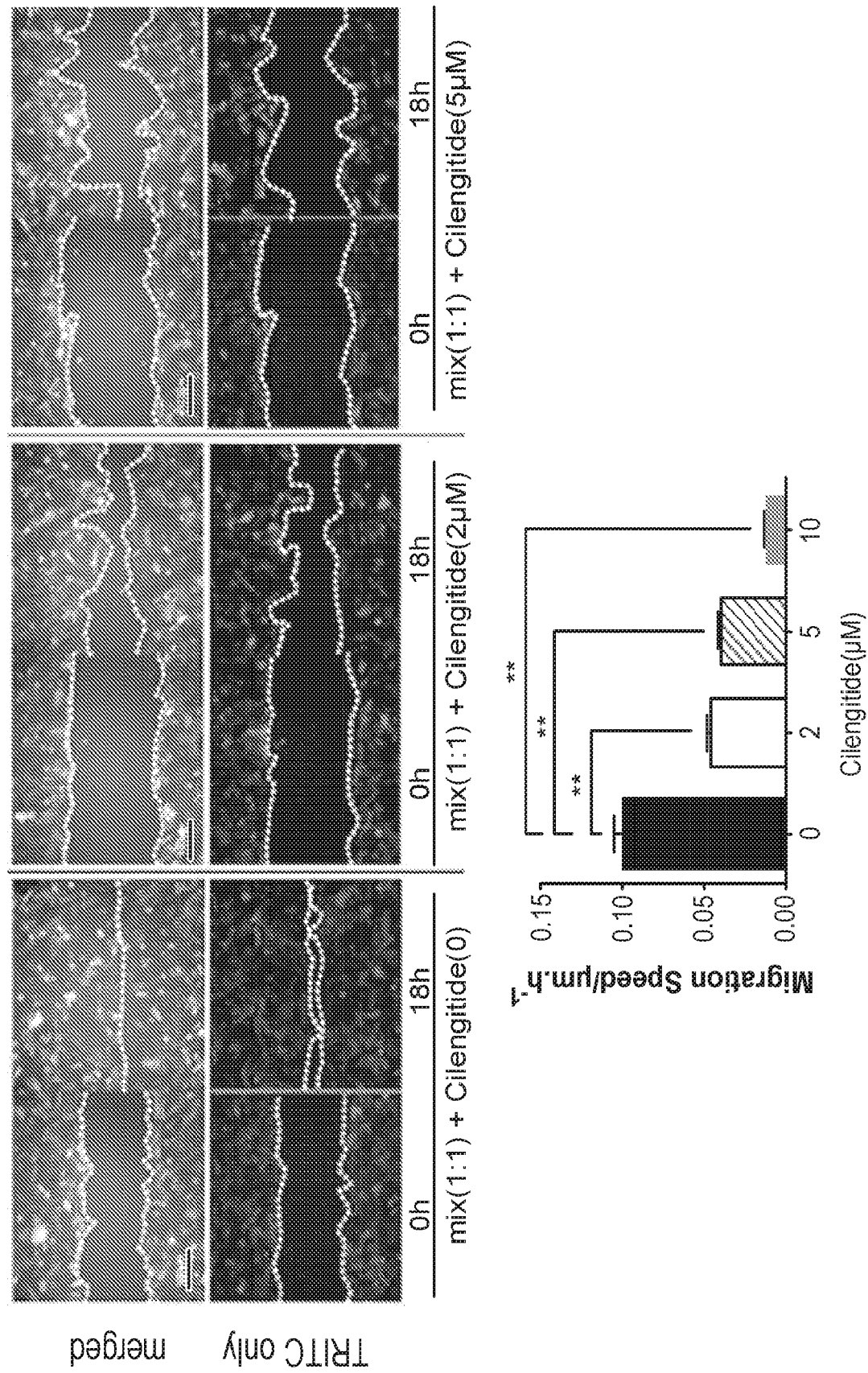


Figure 4J

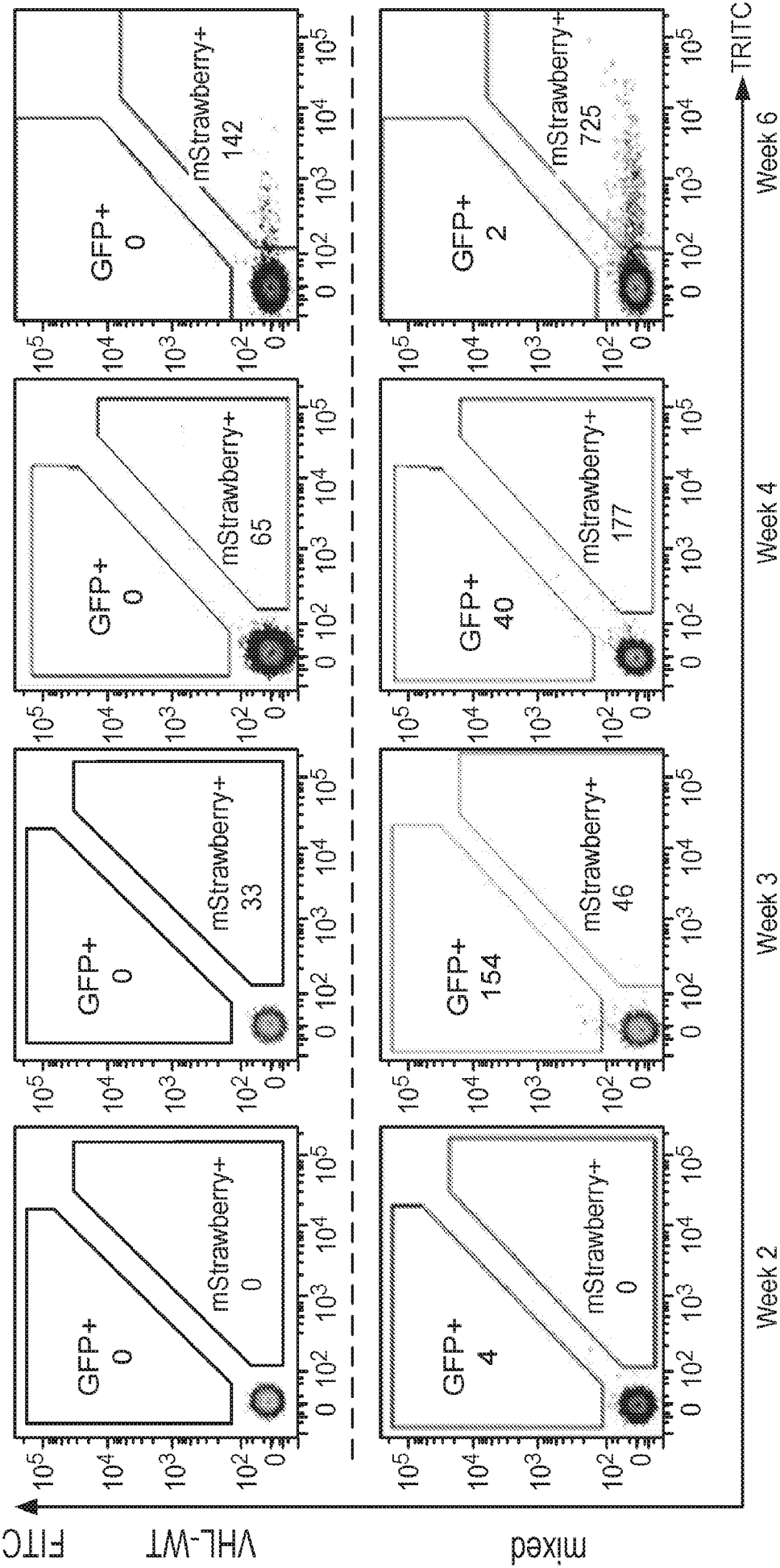


Figure 5A

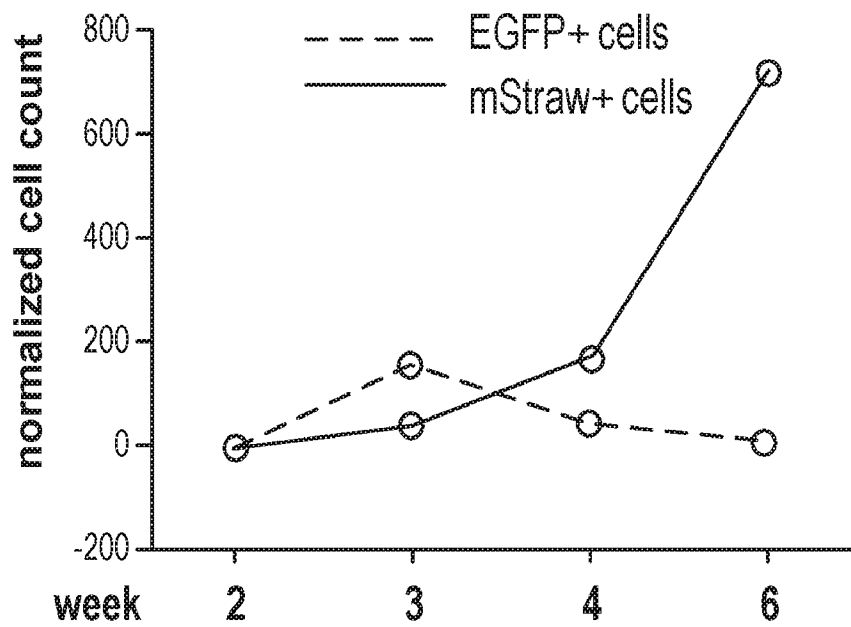
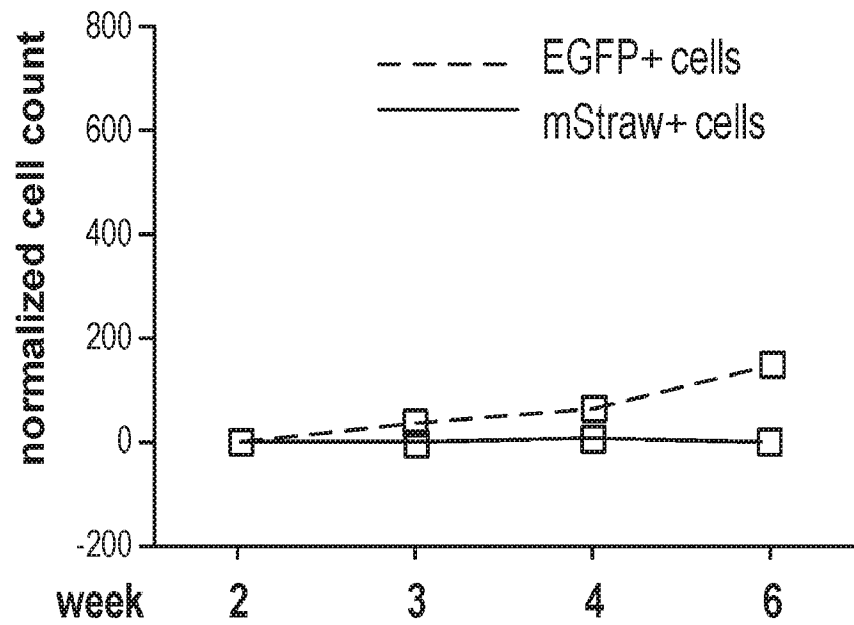


Figure 5A cont.

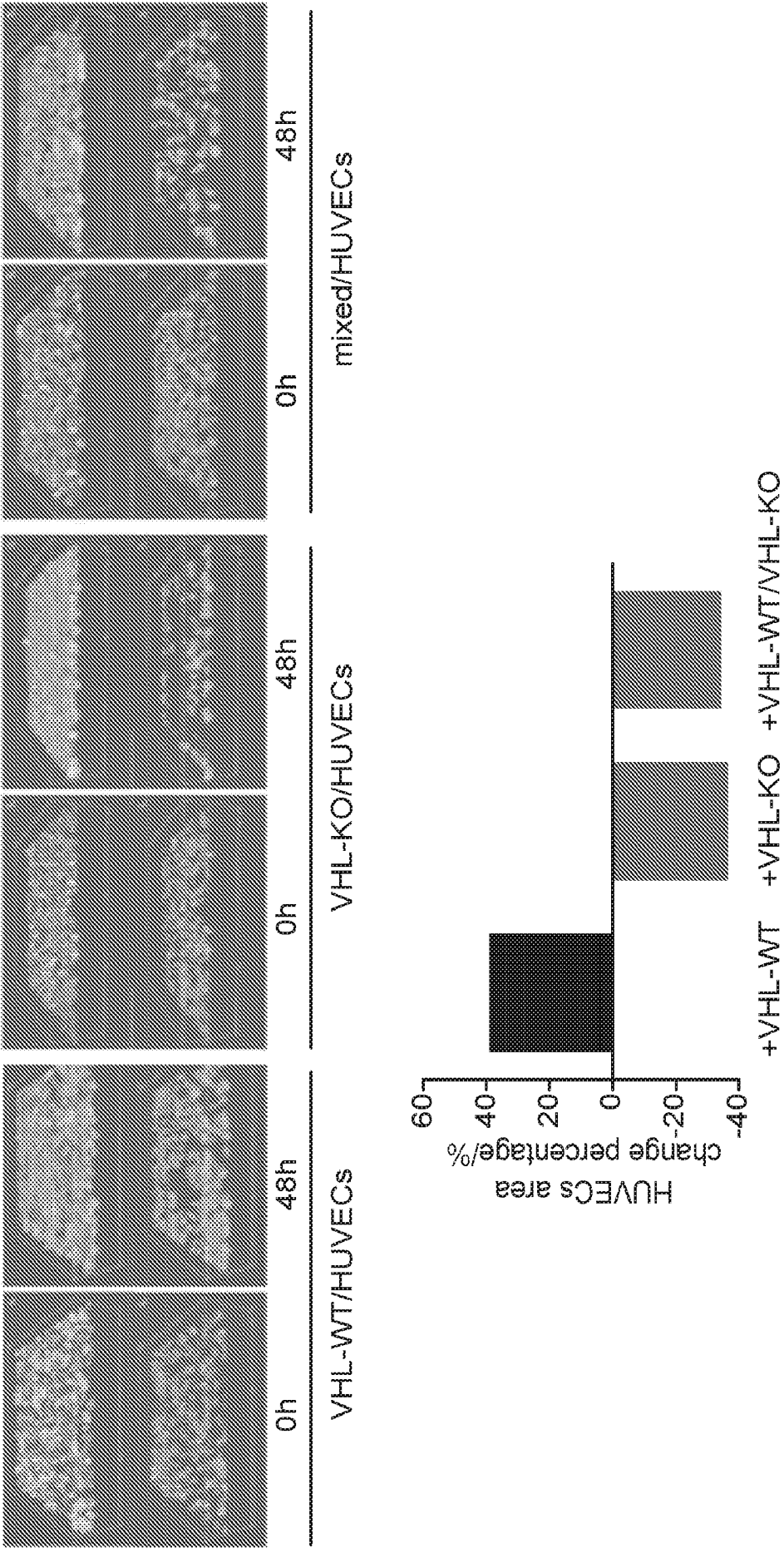


Figure 5B

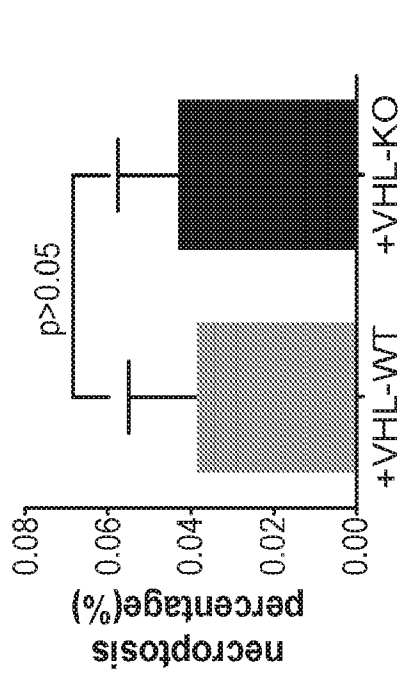


Figure 5D

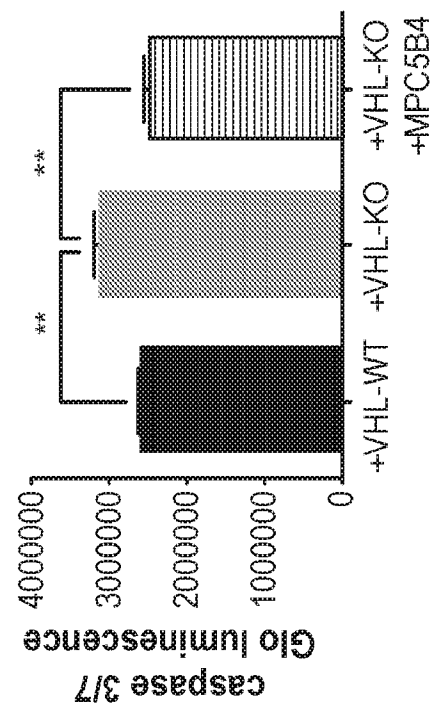


Figure 5E

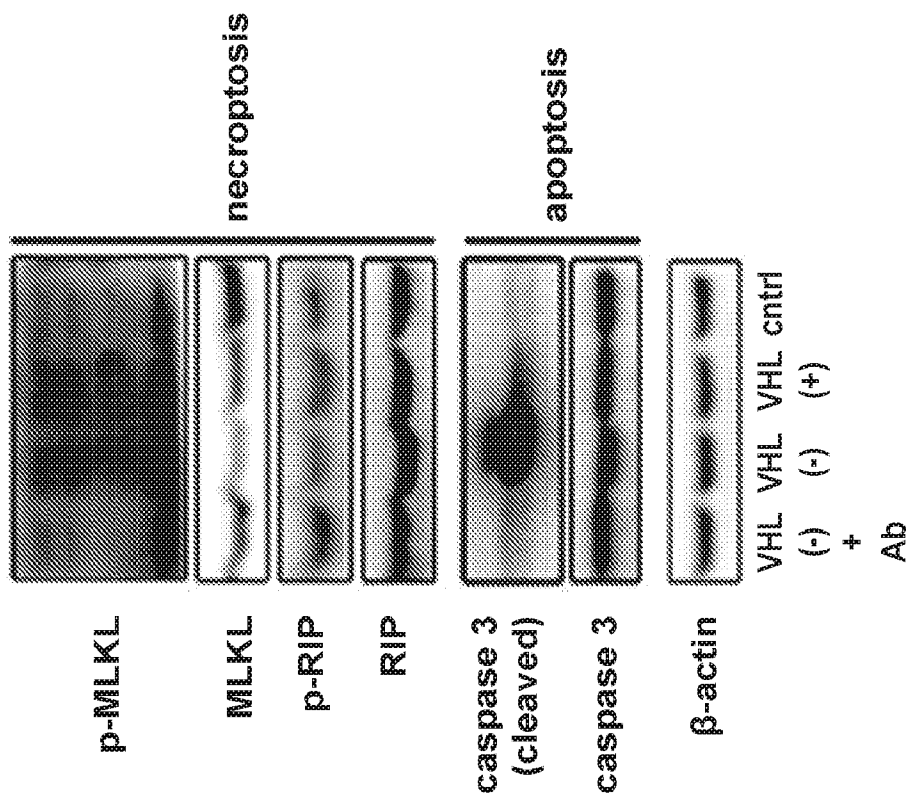


Figure 5C

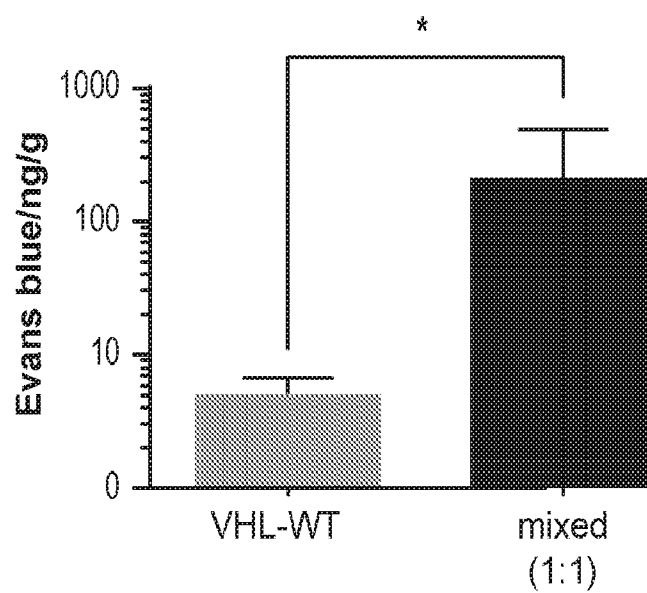
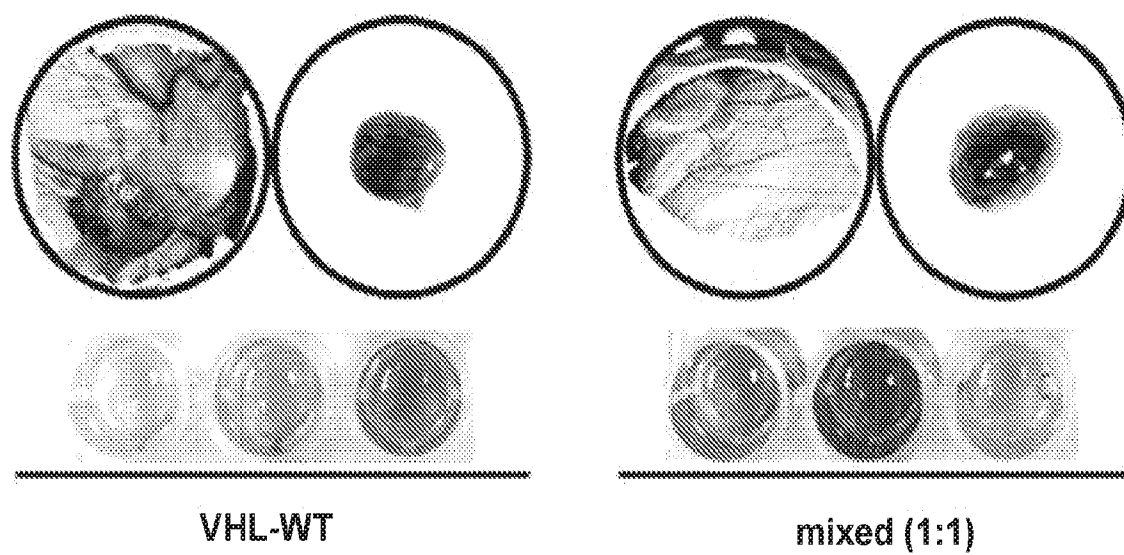
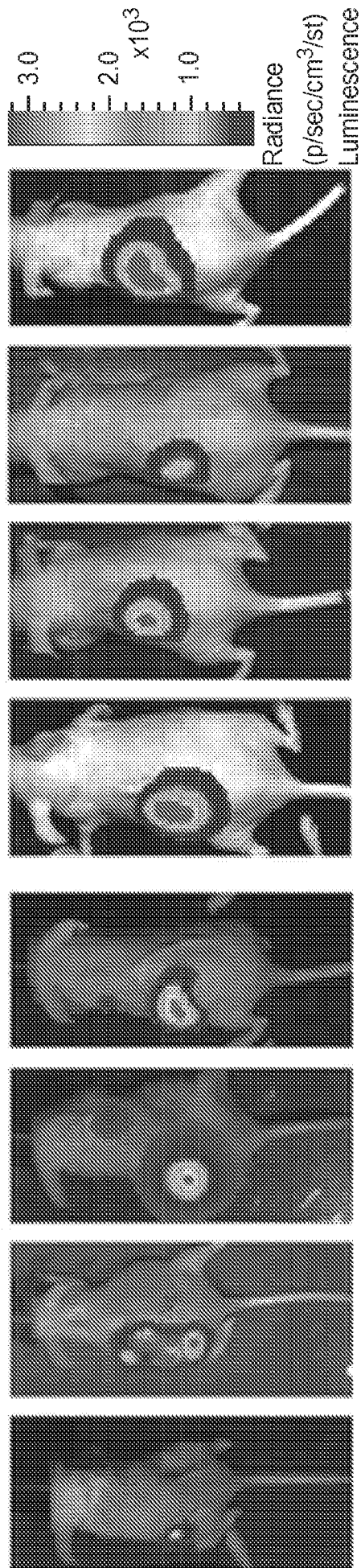


Figure 5F



VHL-WT:VHL/POSTN-KO (1:1)

VHL-WT:VHL-KO (1:1)

Figure 6A

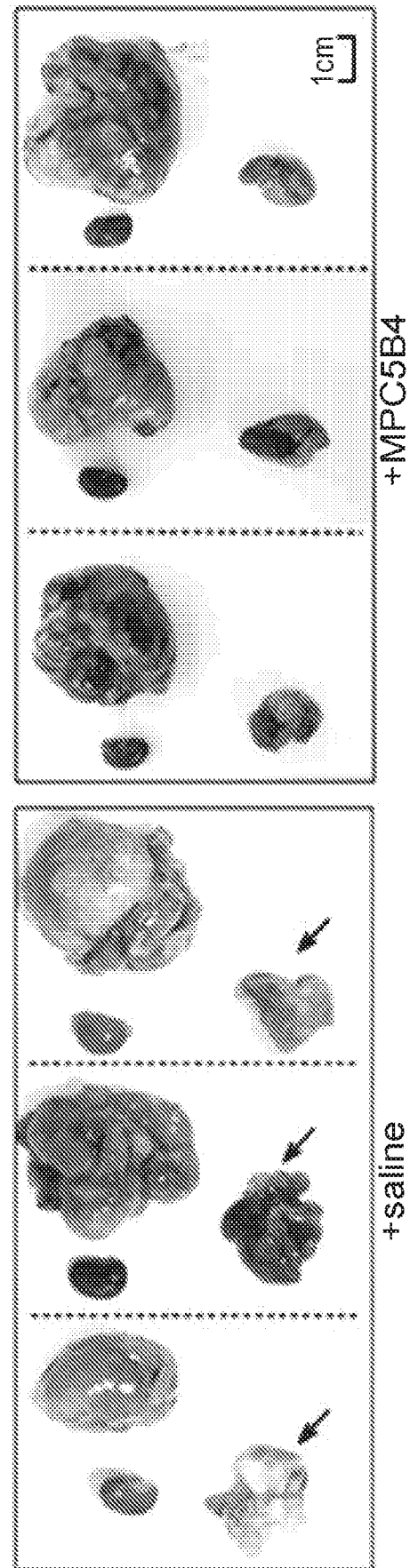


Figure 6B

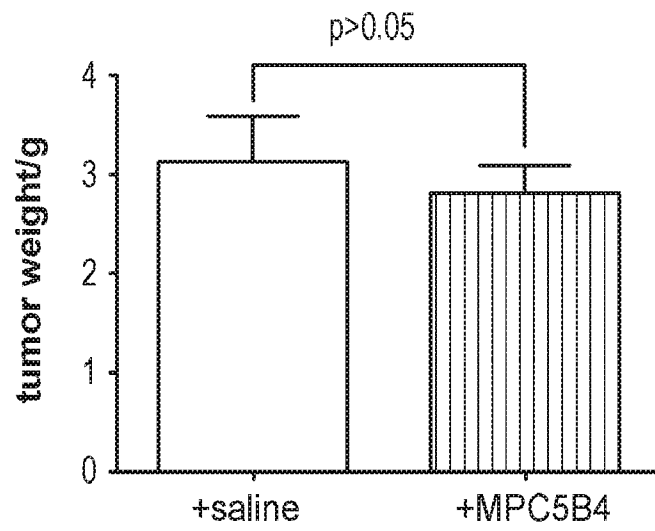


Figure 6B cont.

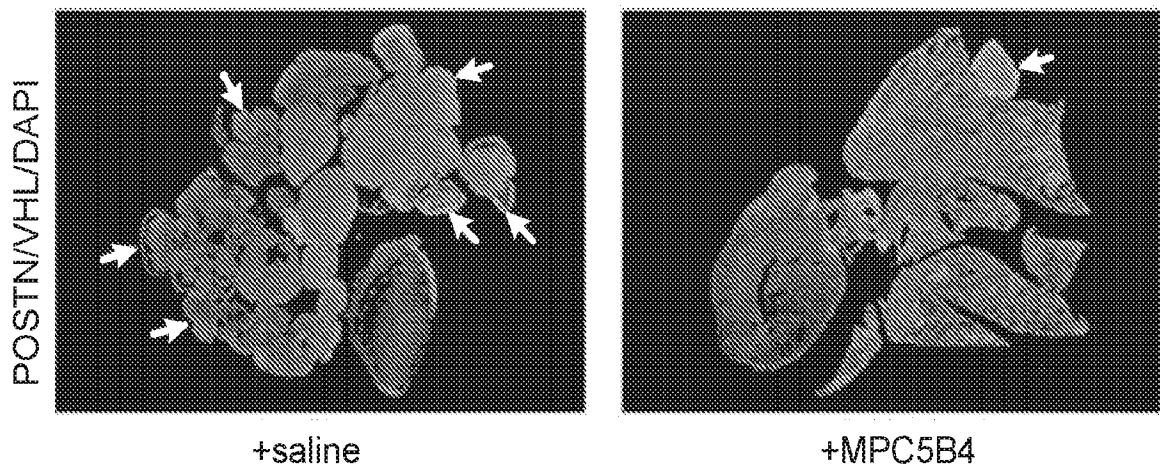


Figure 6C

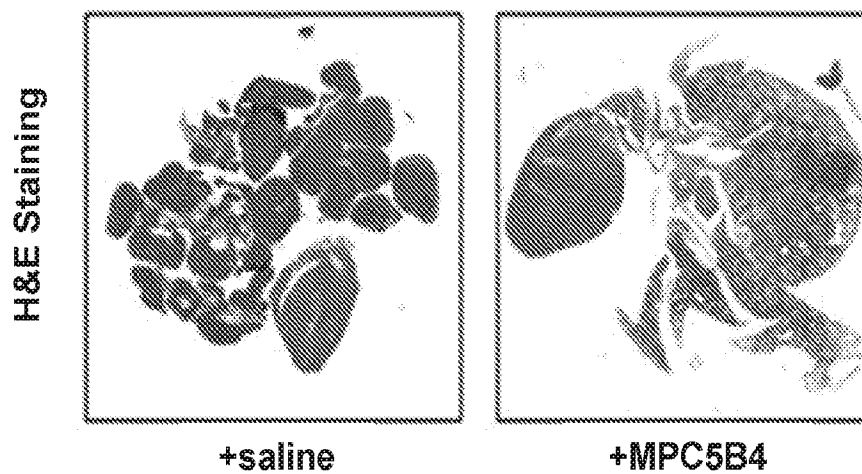


Figure 6D

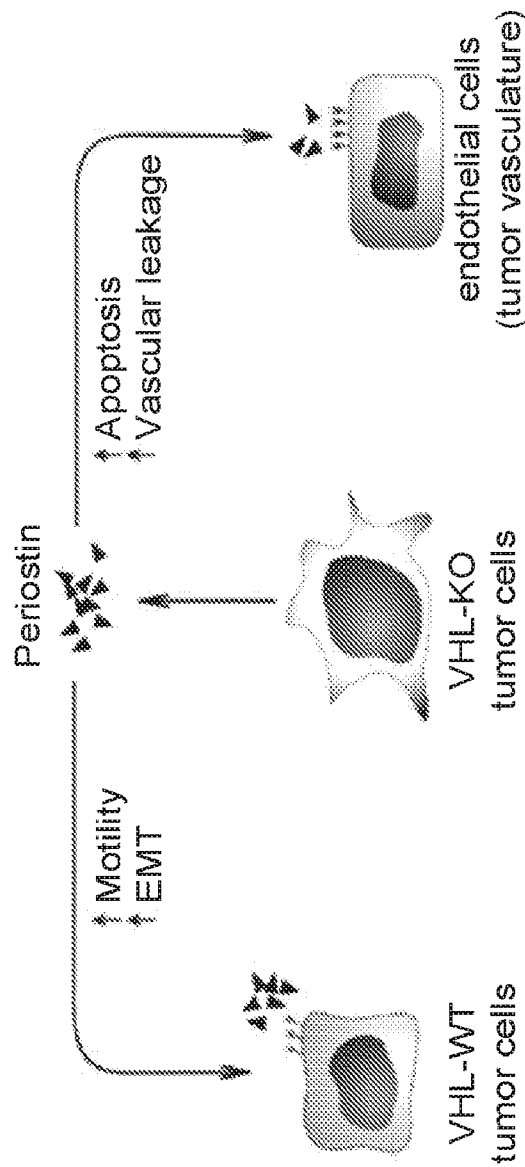


Figure 6F

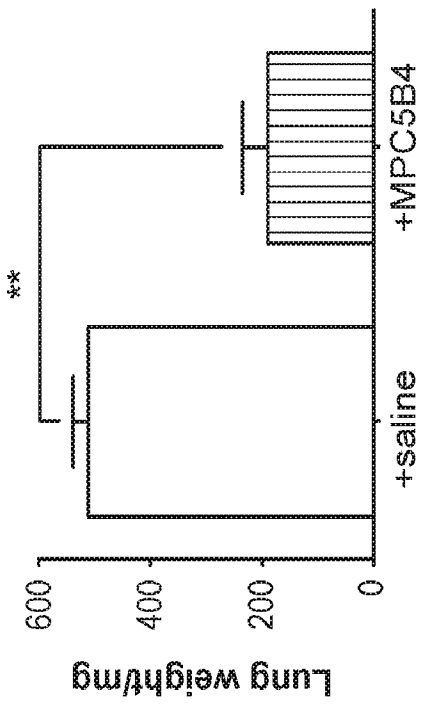
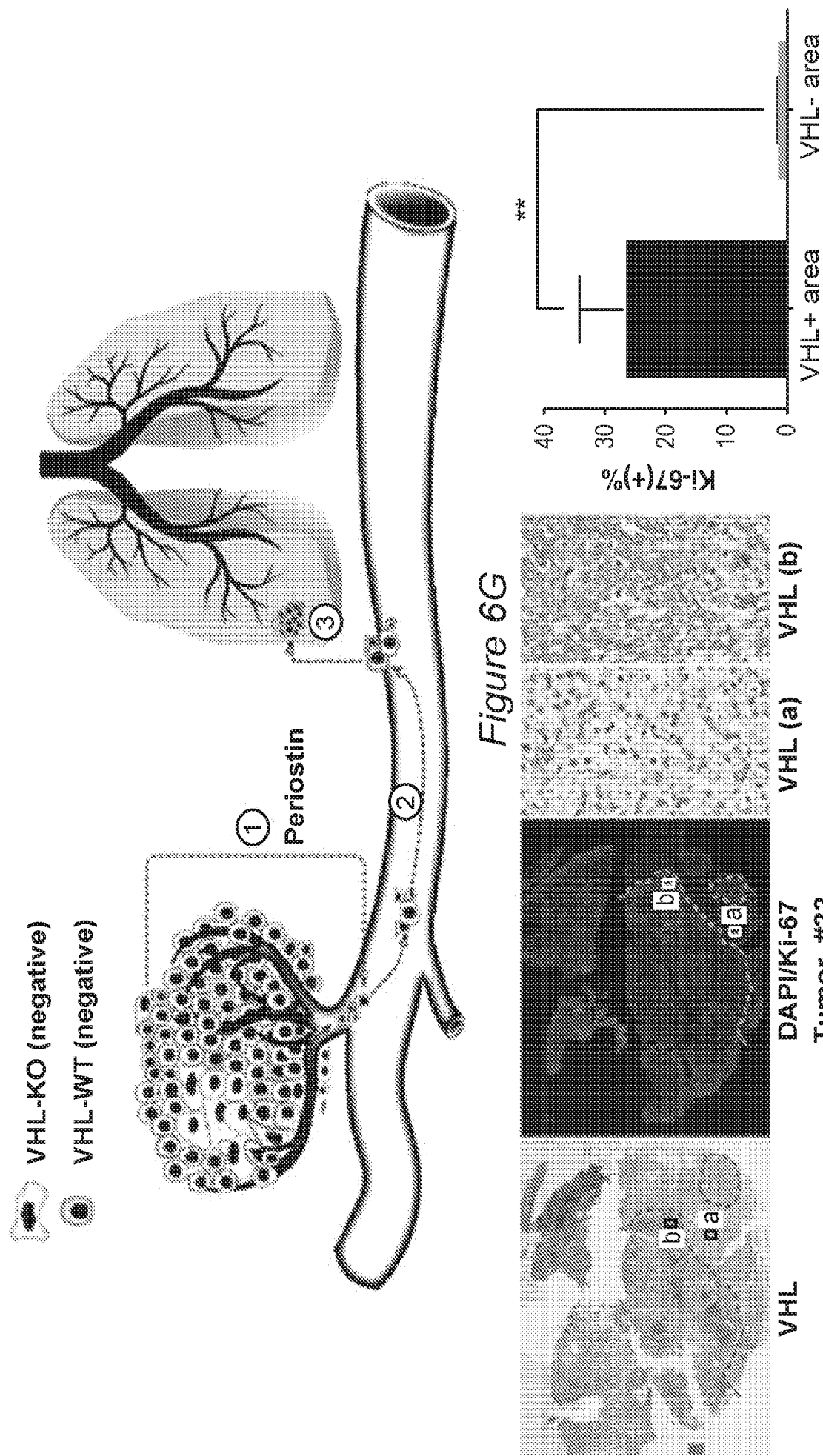


Figure 6E



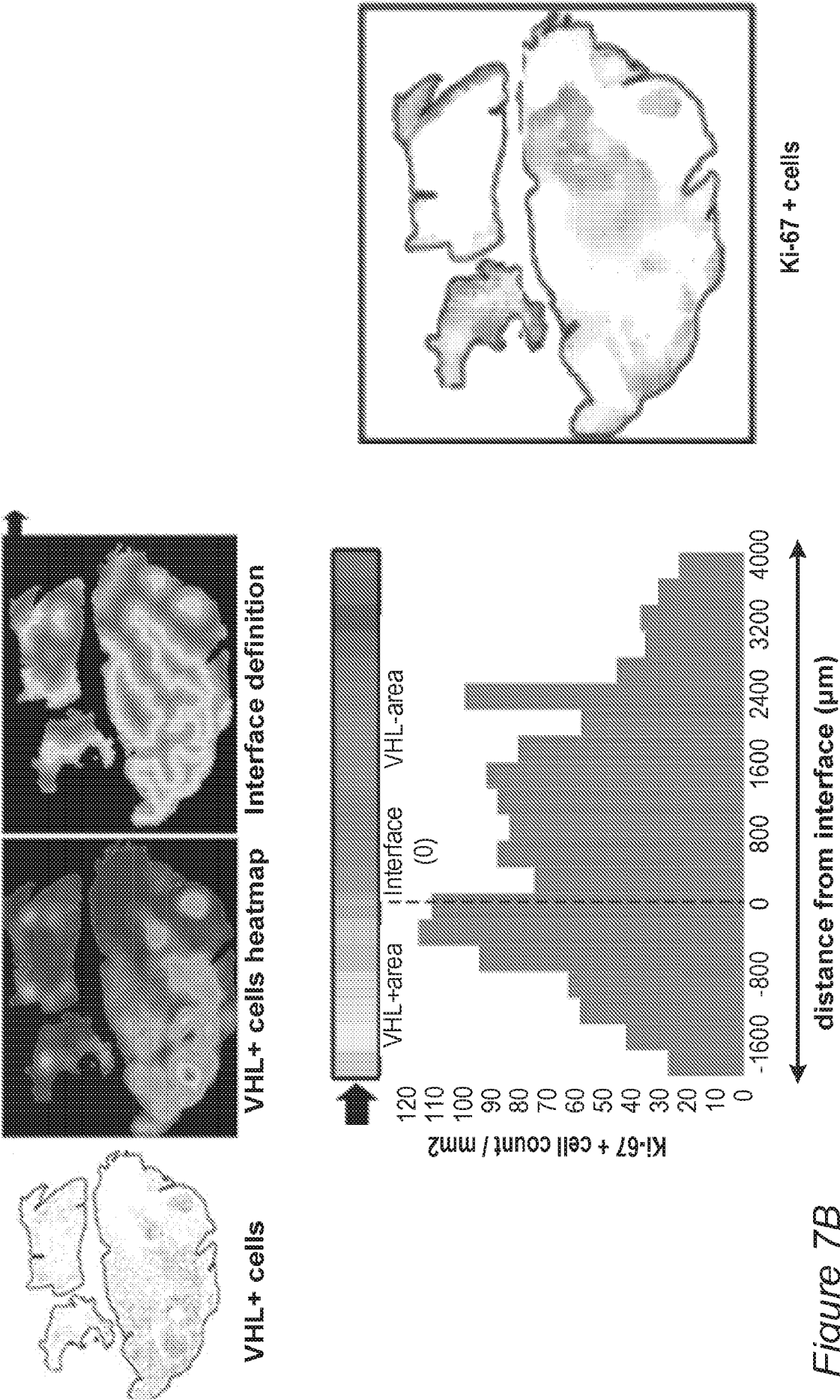


Figure 7B

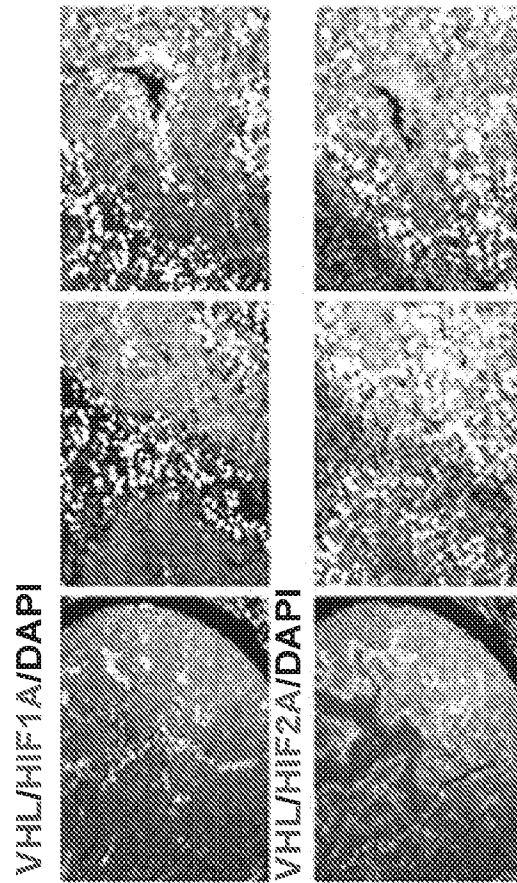


Figure 7D

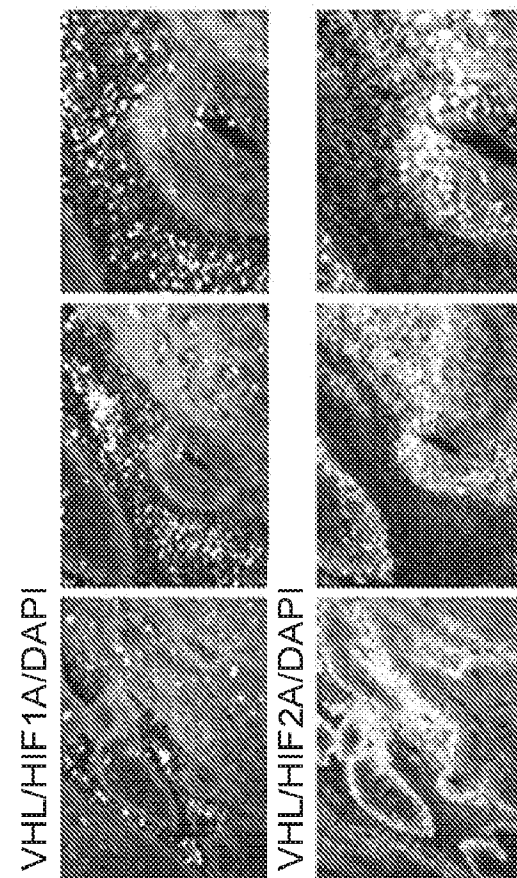


Figure 7C

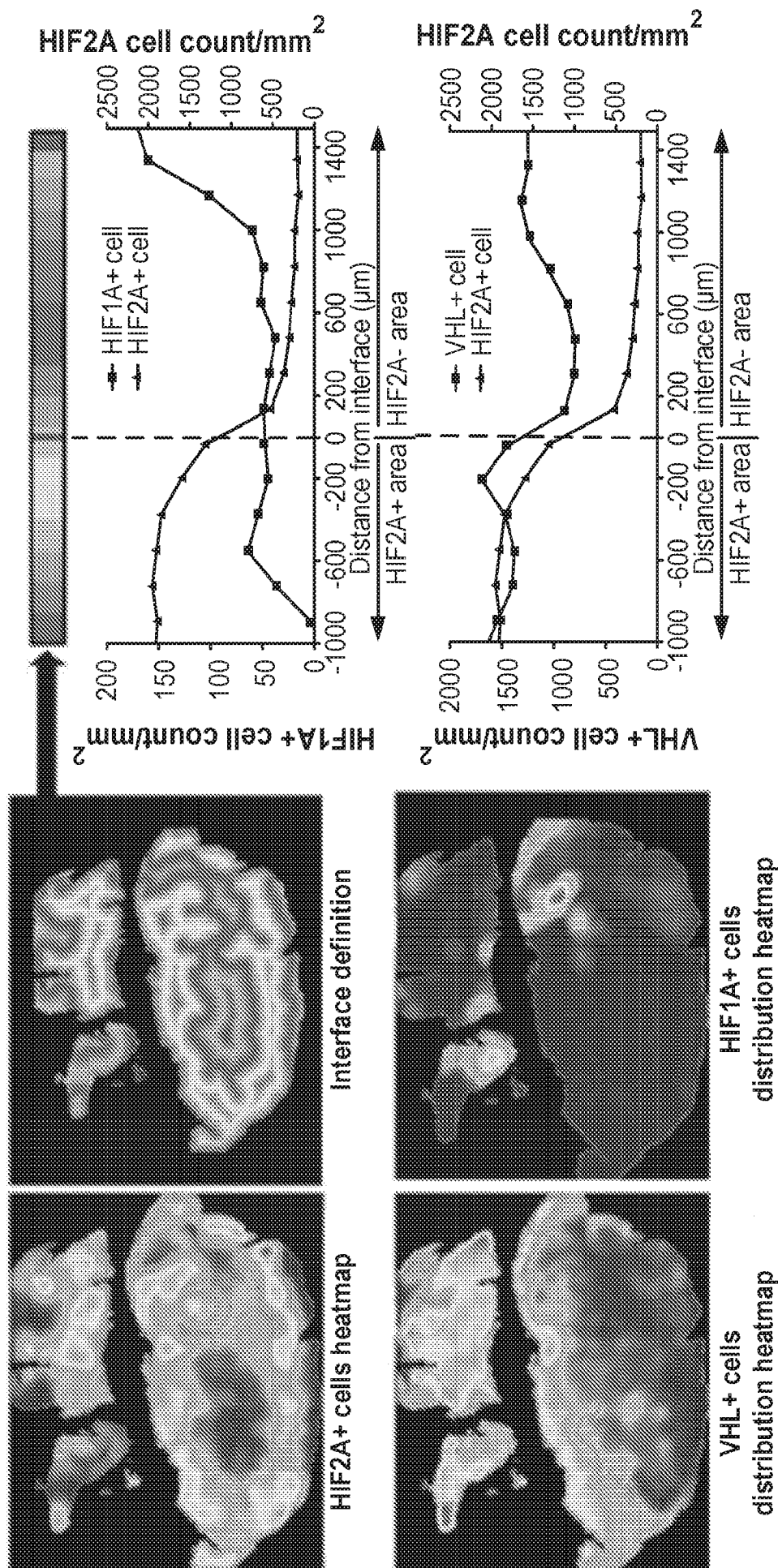
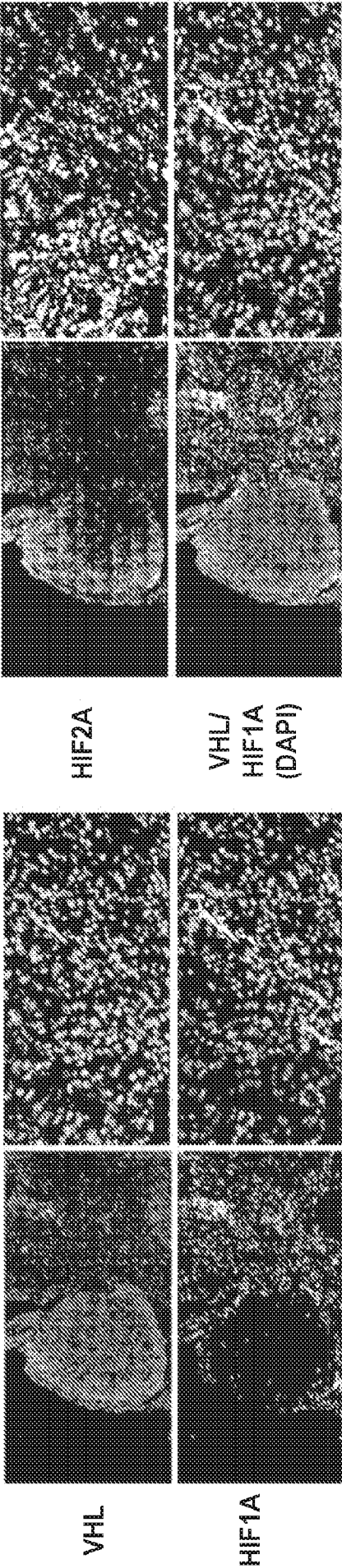


Figure 7E



#22 primary tumor
Figure 7F

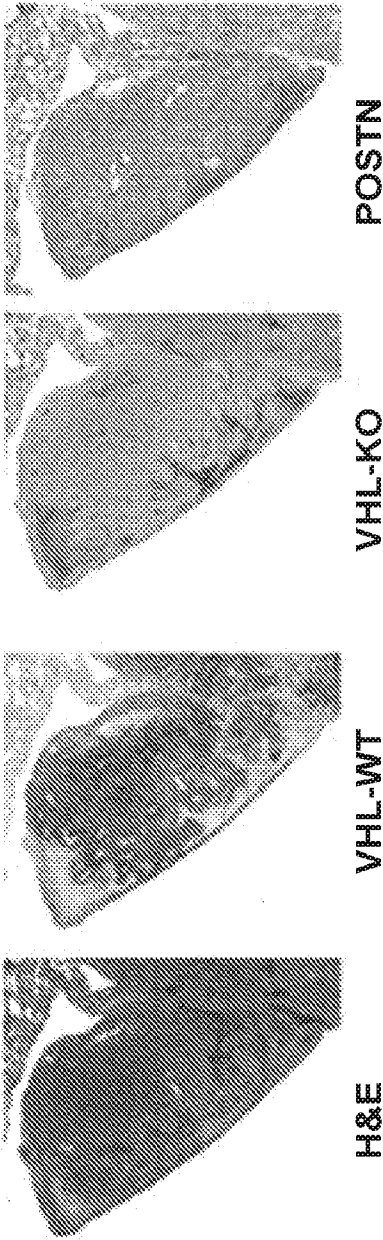


Figure 8A

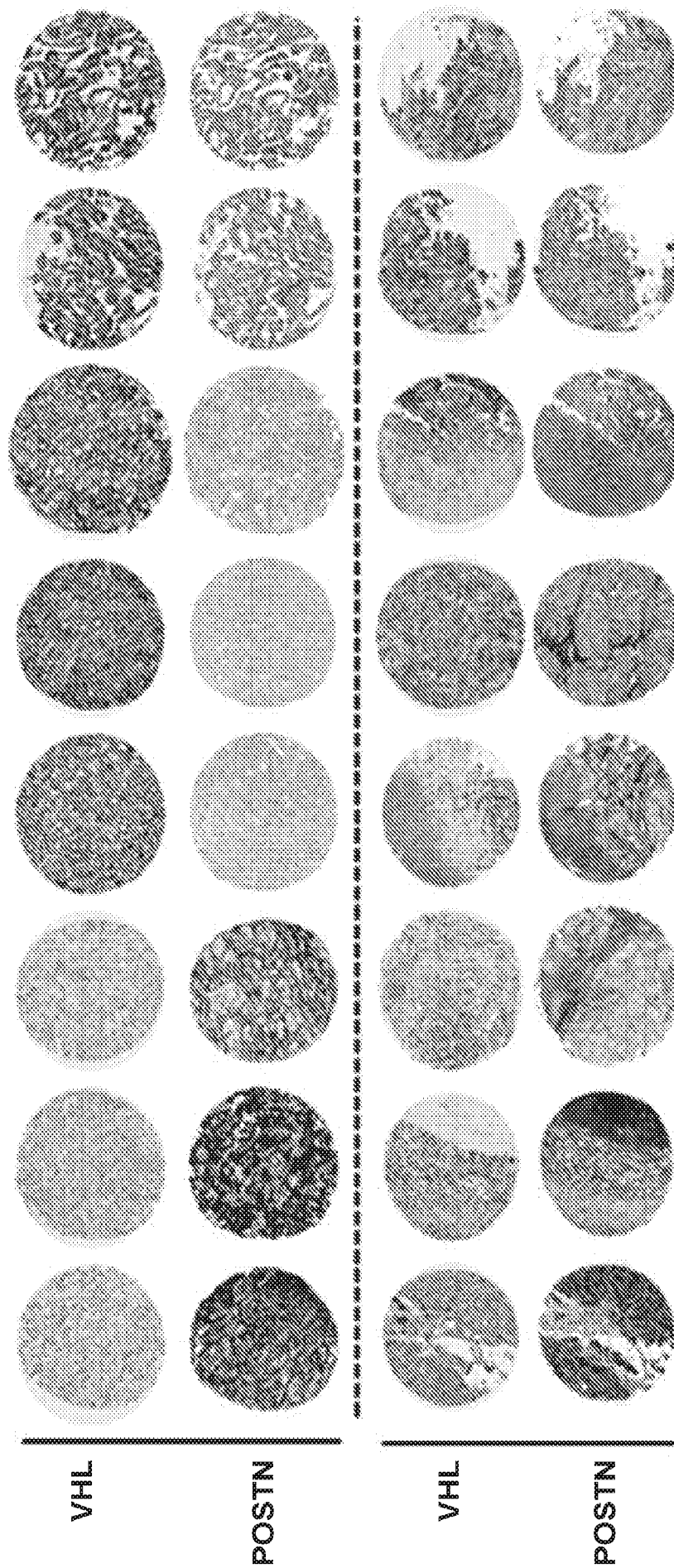


Figure 8B

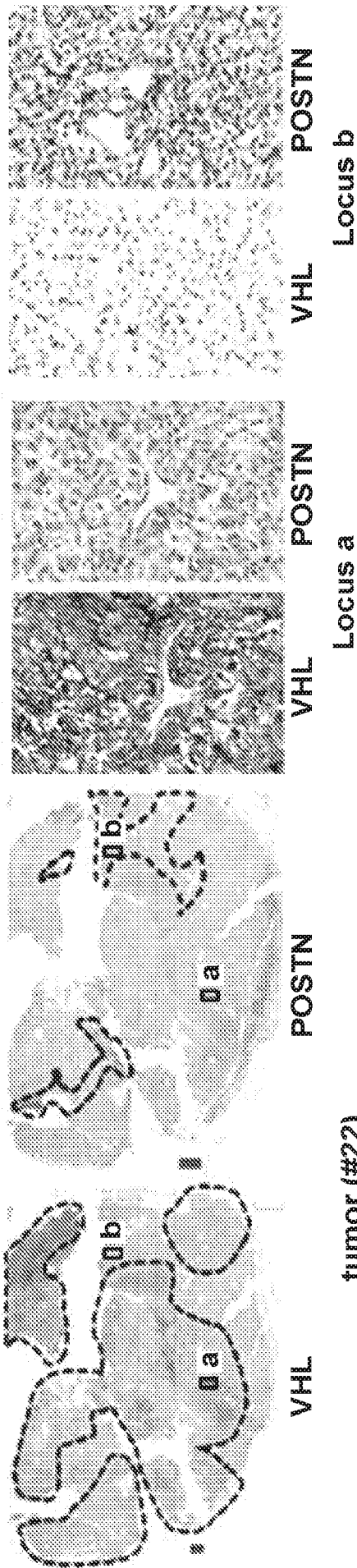


Figure 8C

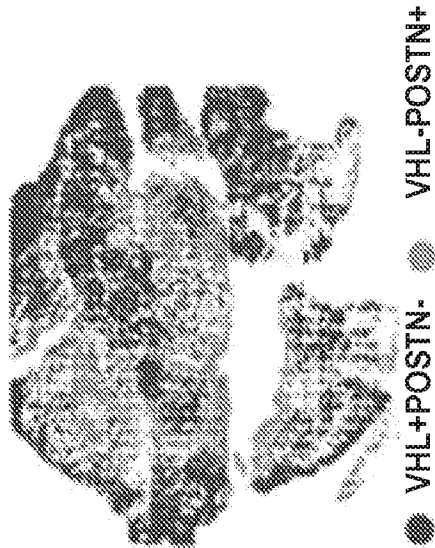
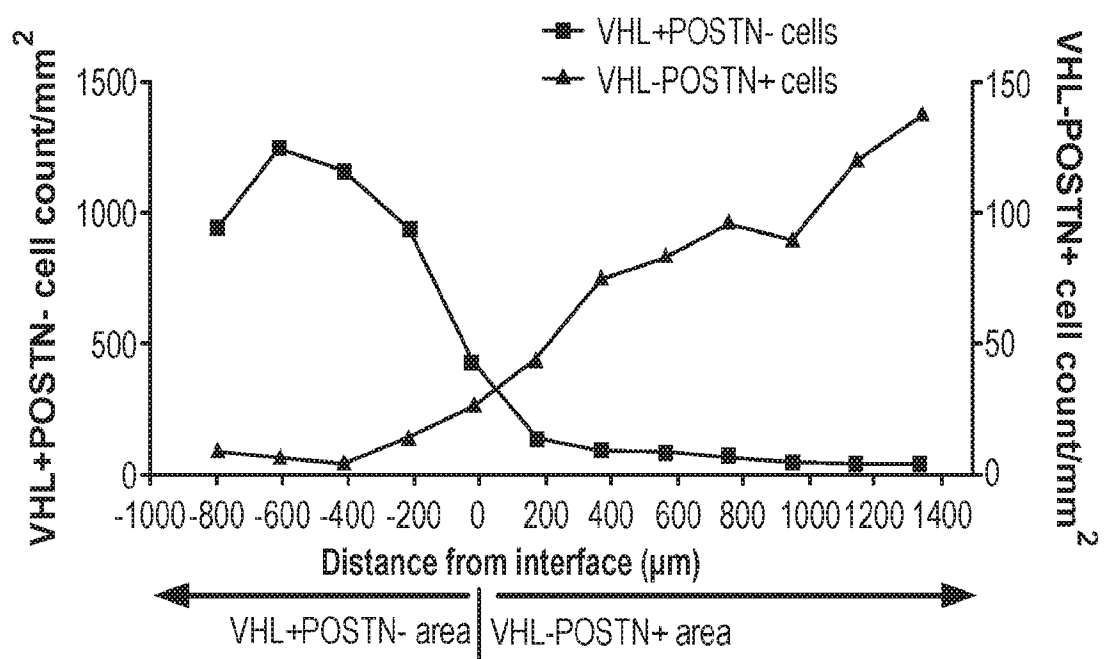
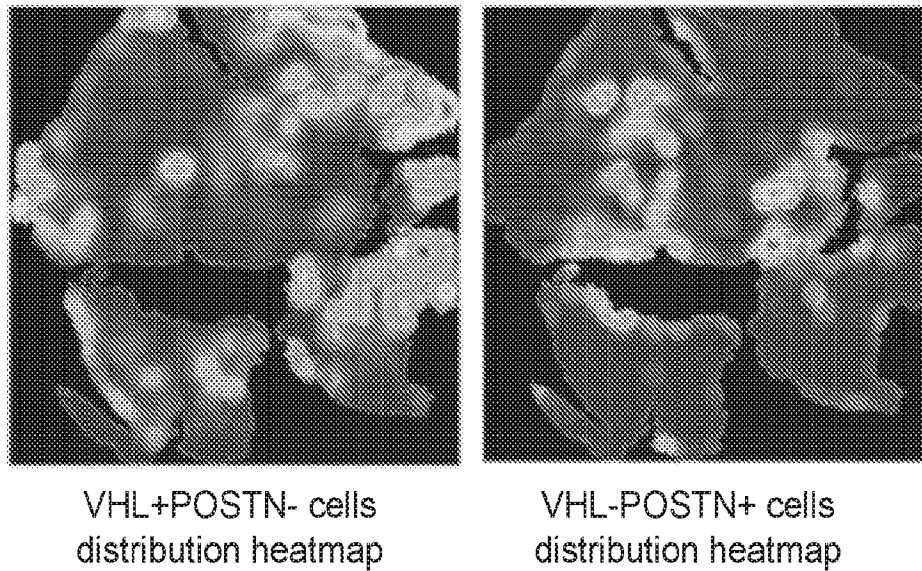


Figure 8D

*Figure 8E*

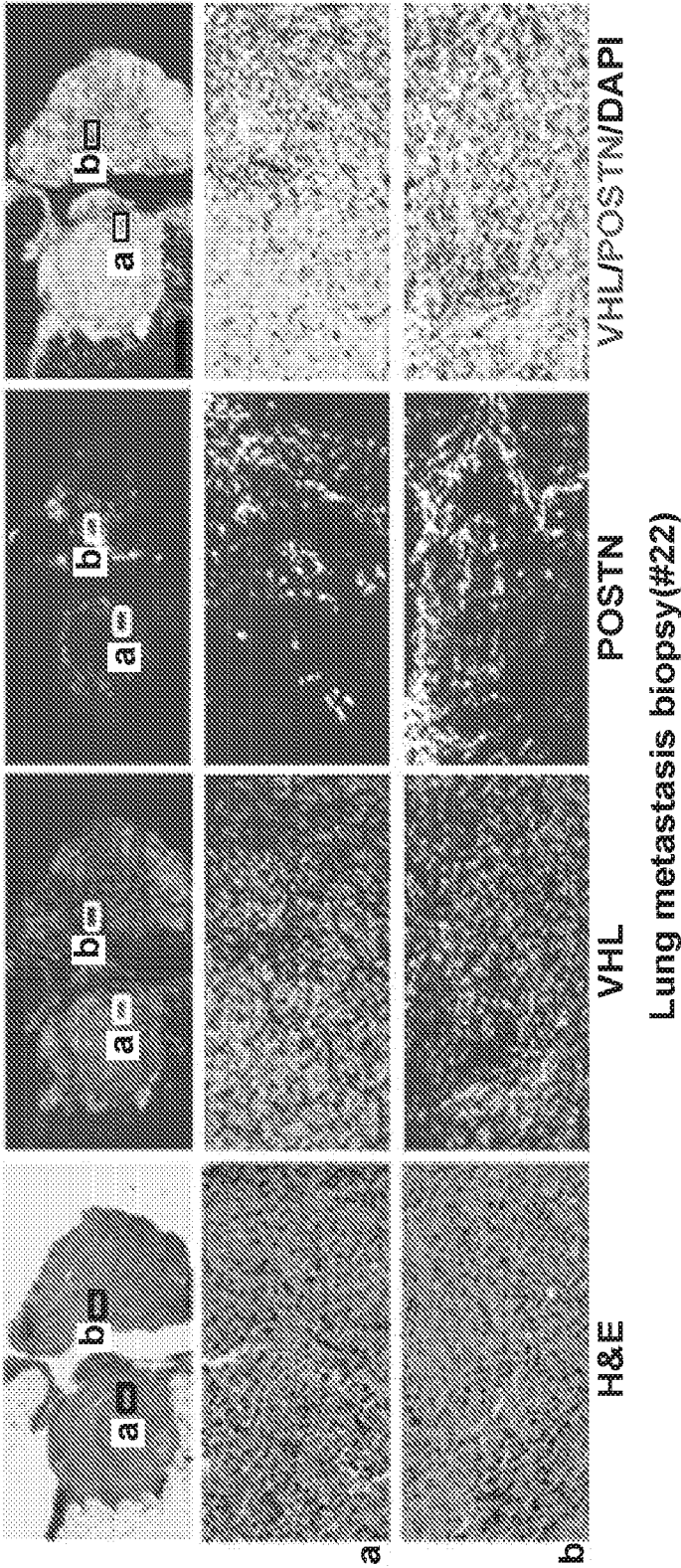


Figure 8F

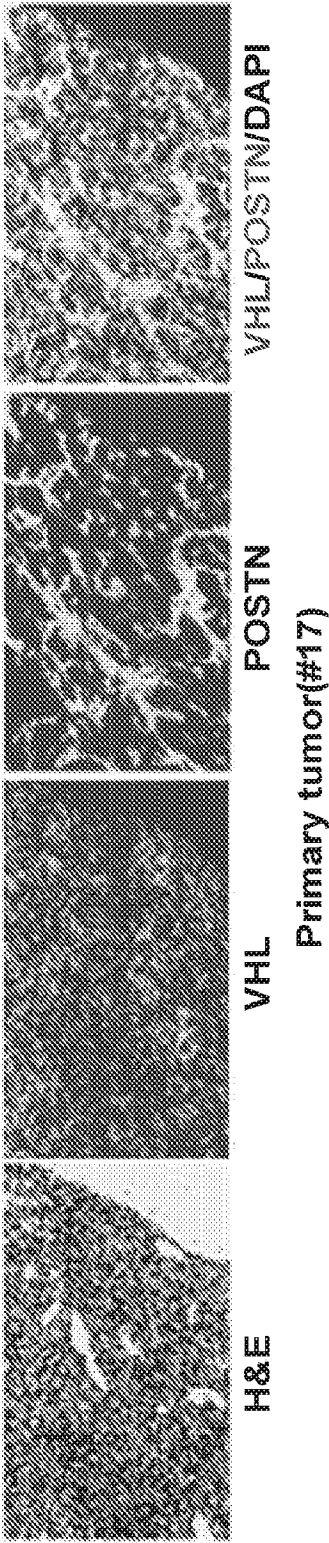


Figure 8G

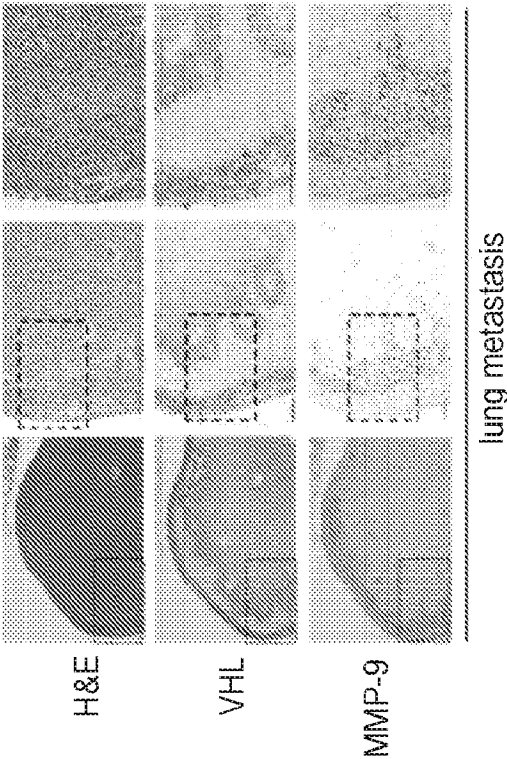


Figure 9A

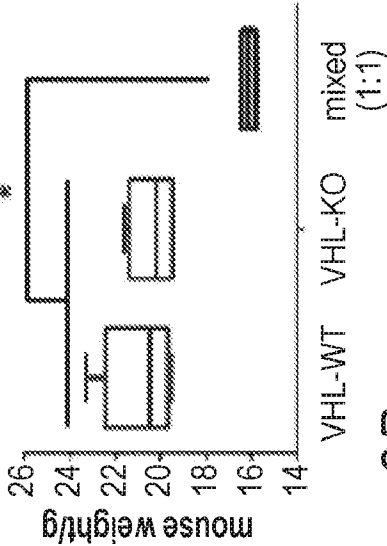


Figure 9B

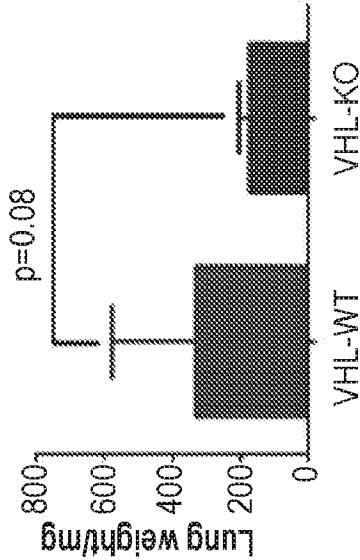
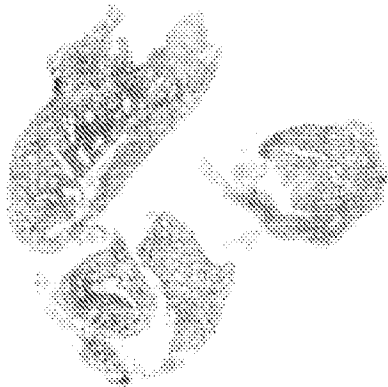


Figure 9C



Lung (H&E, VHL-KO group)

Figure 9D



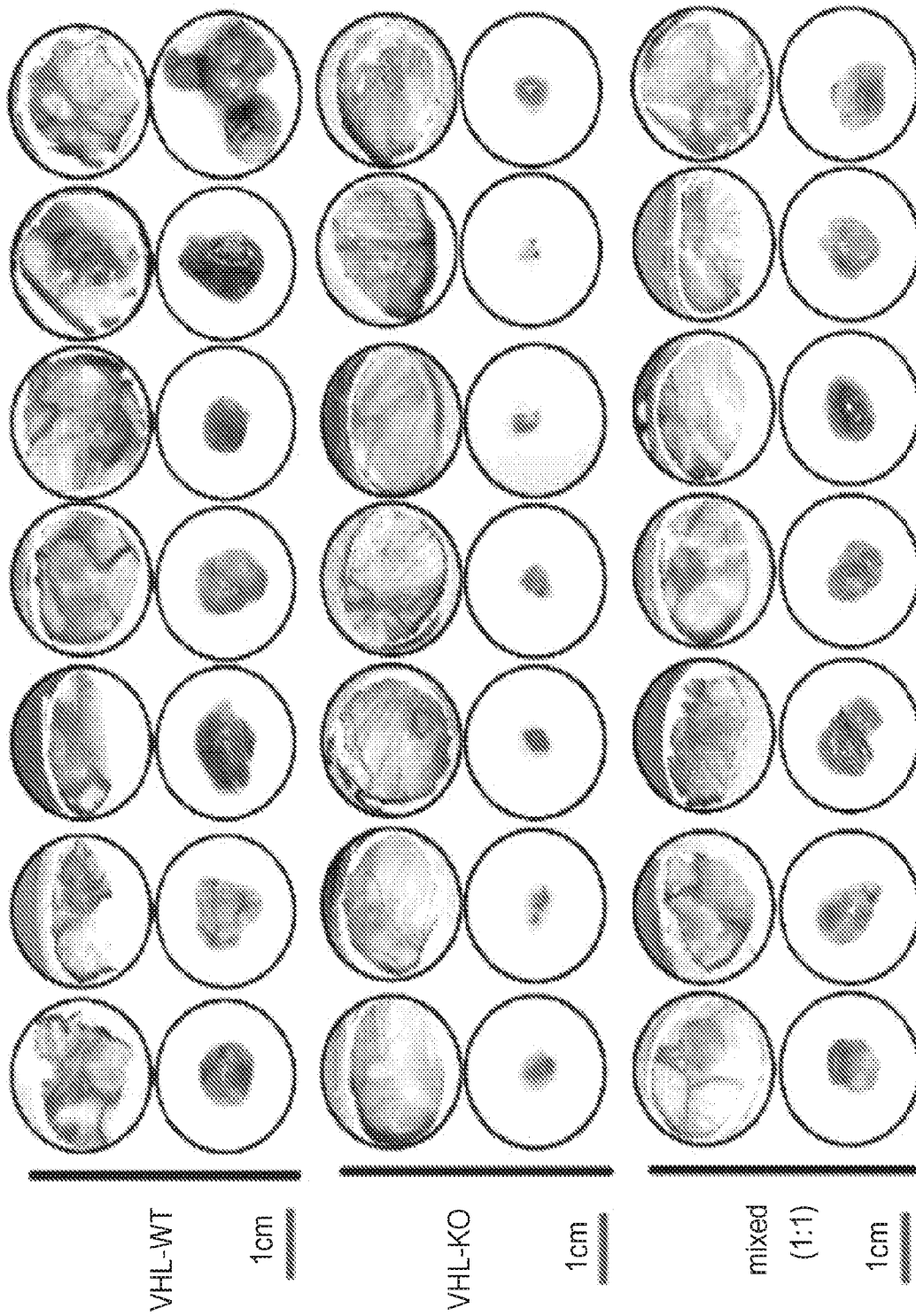


Figure 9G

Figure 9H

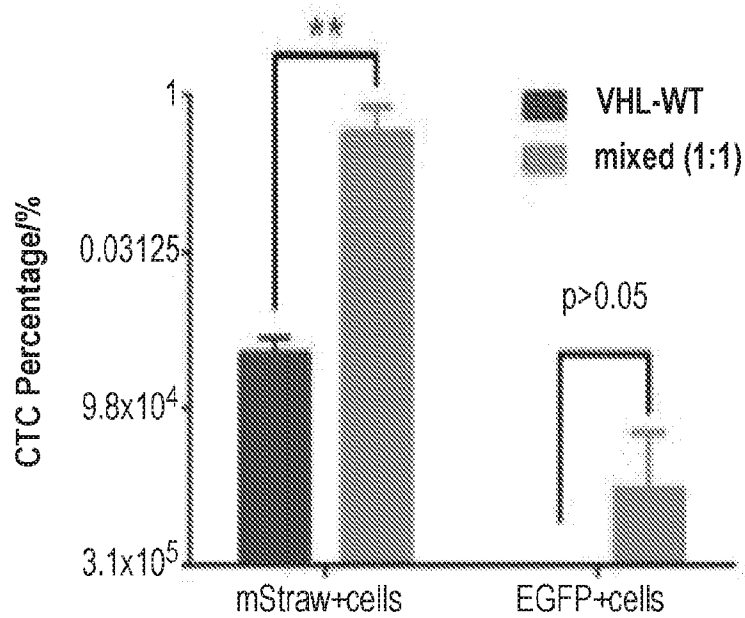
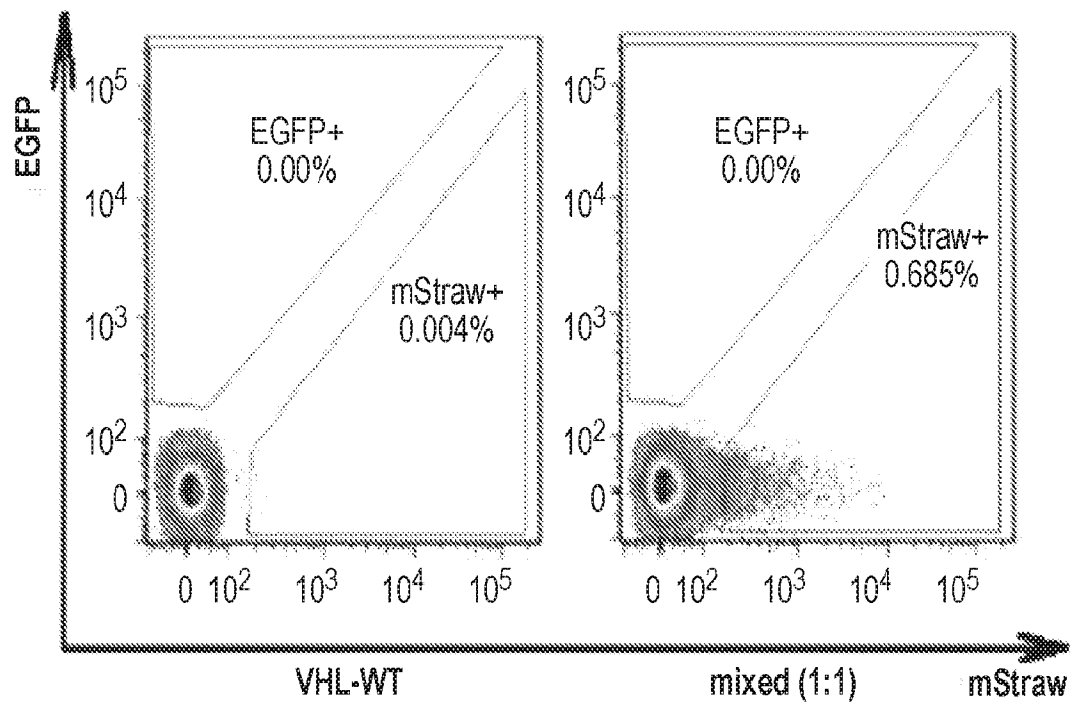


Figure 9I

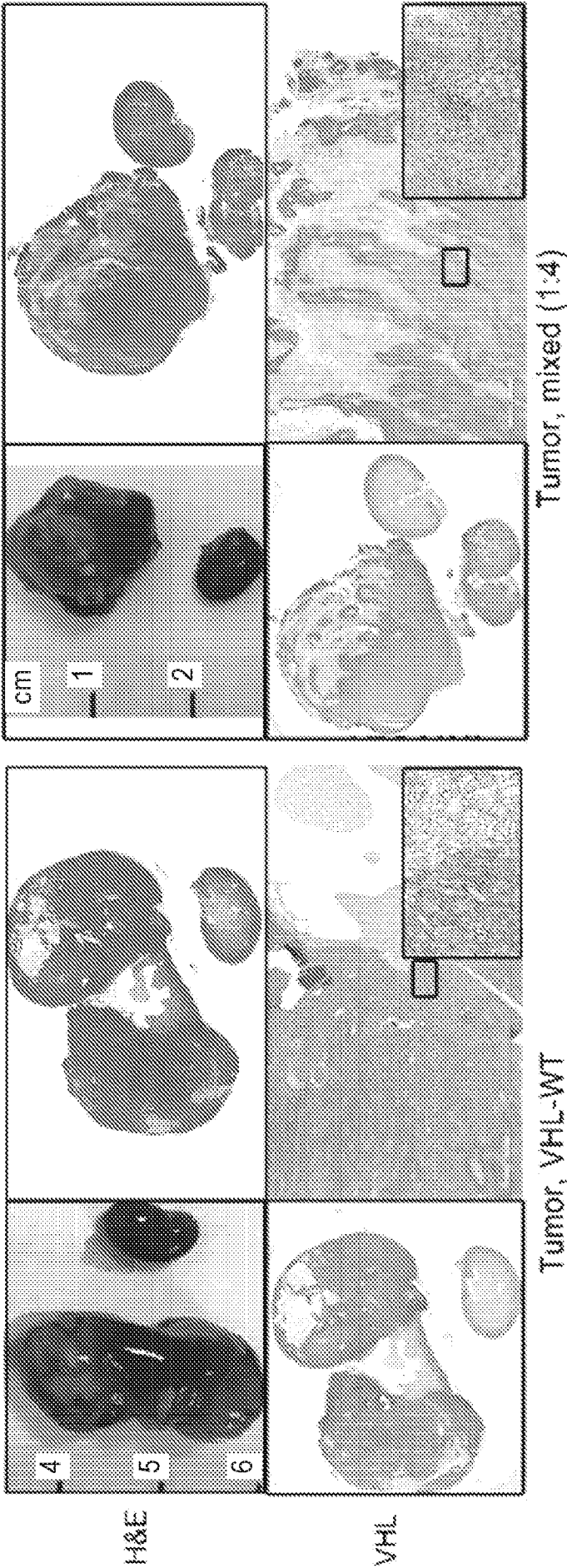


Figure 9J

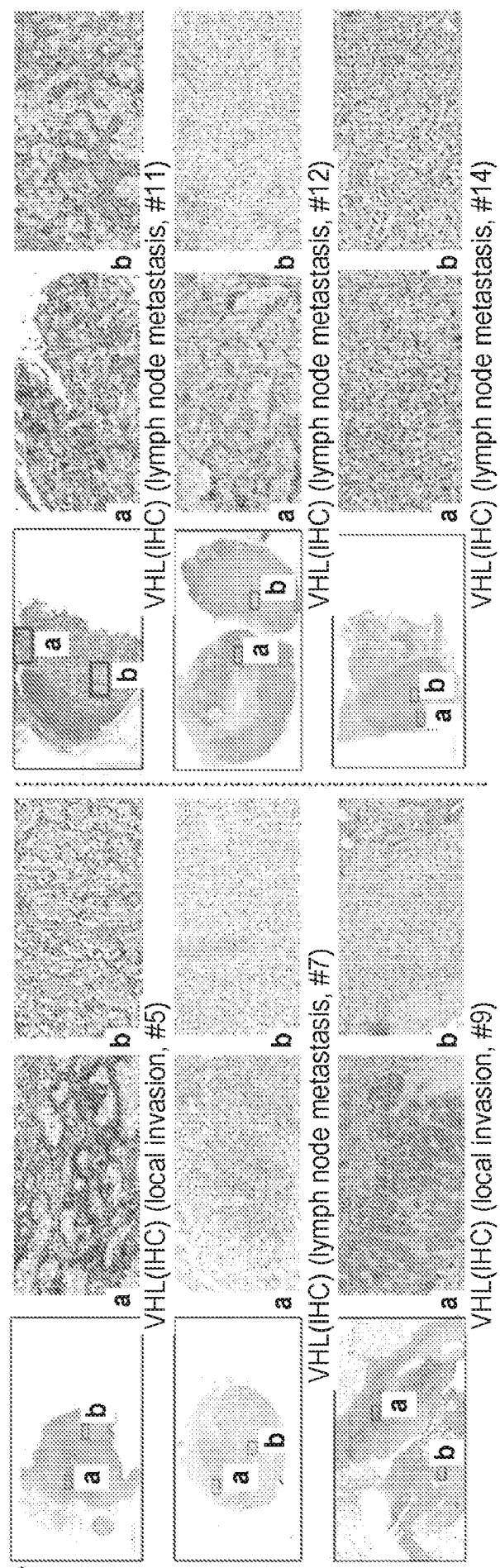


Figure 10A

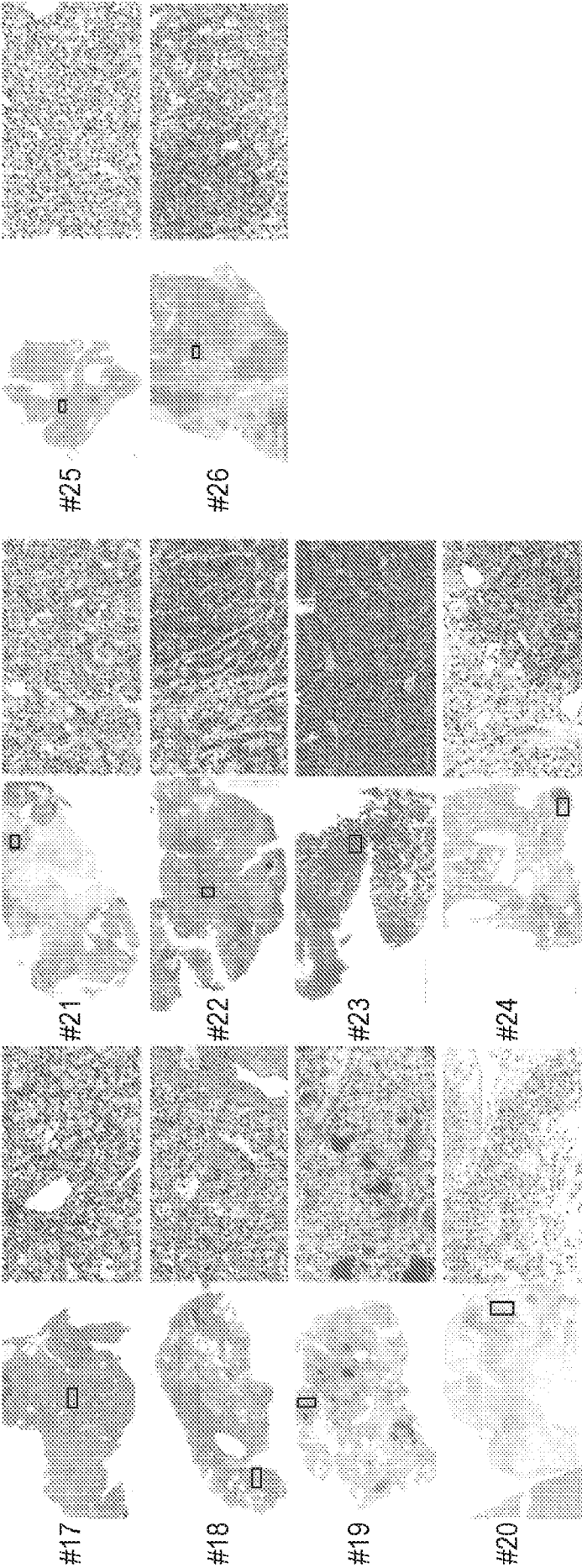


Figure 10B

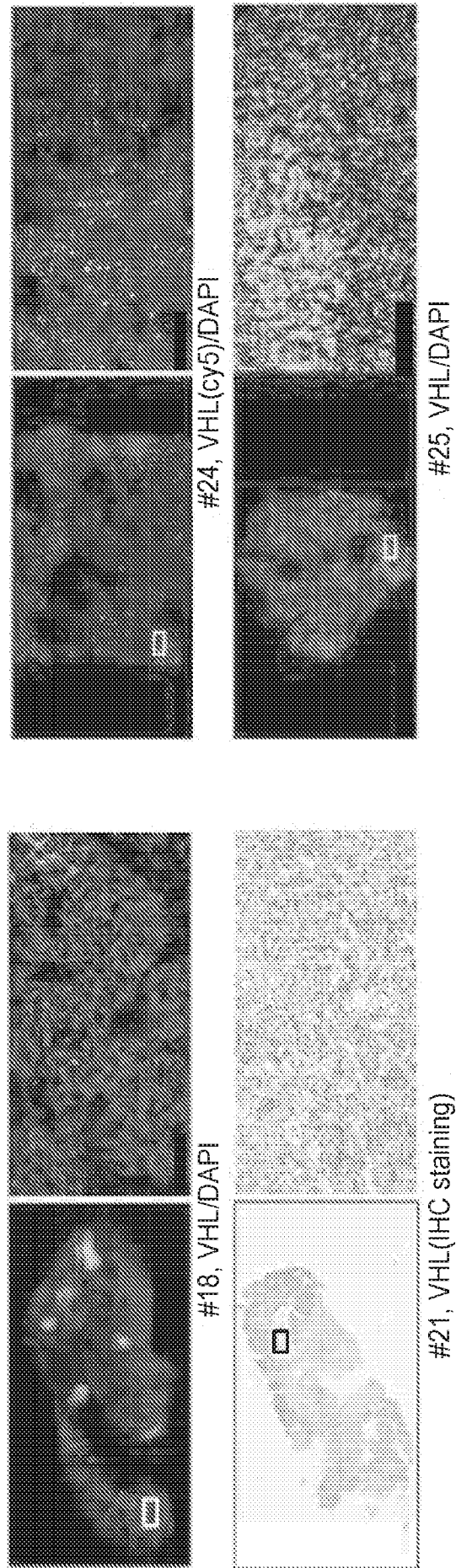


Figure 10C

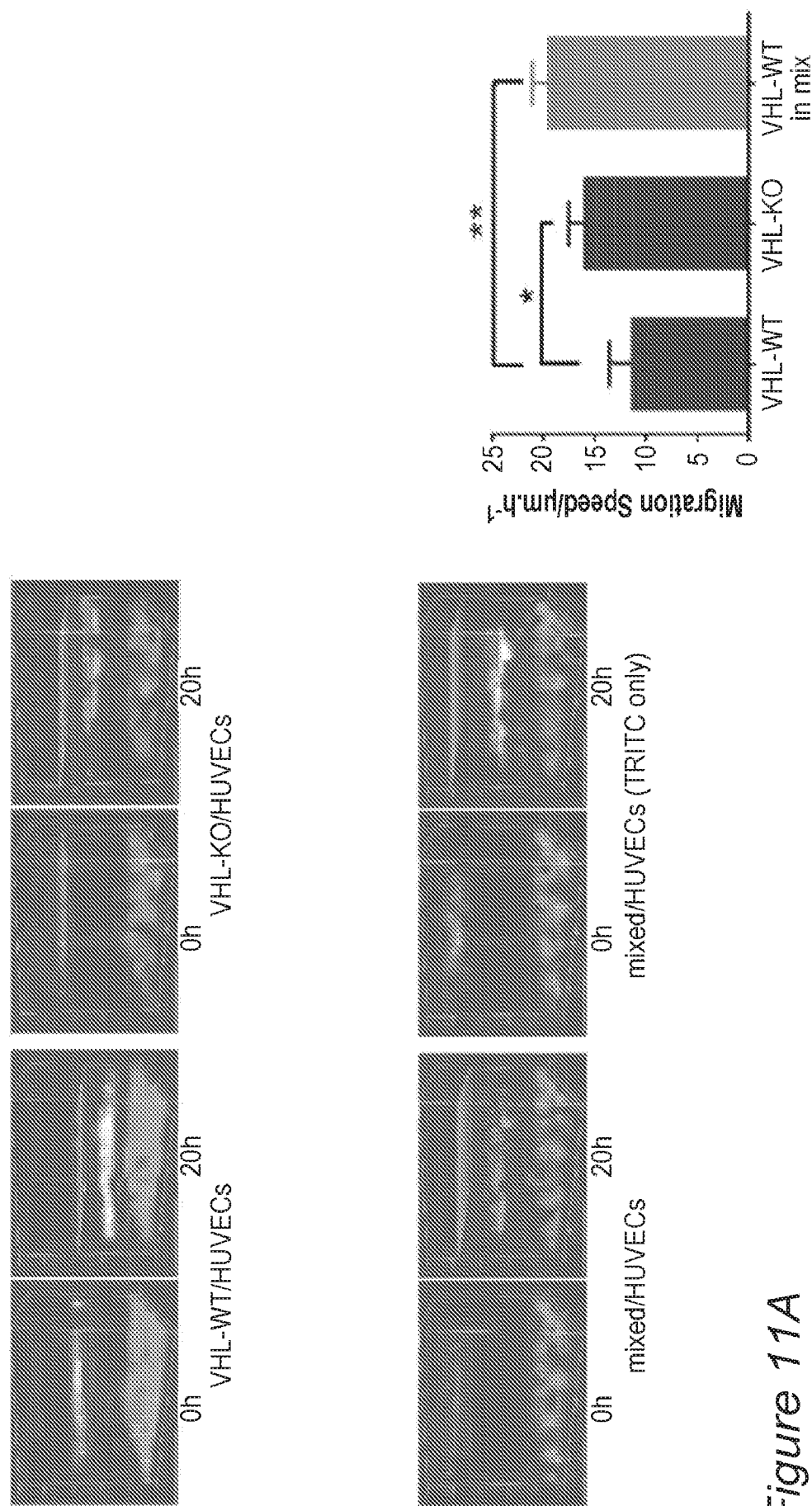


Figure 11A

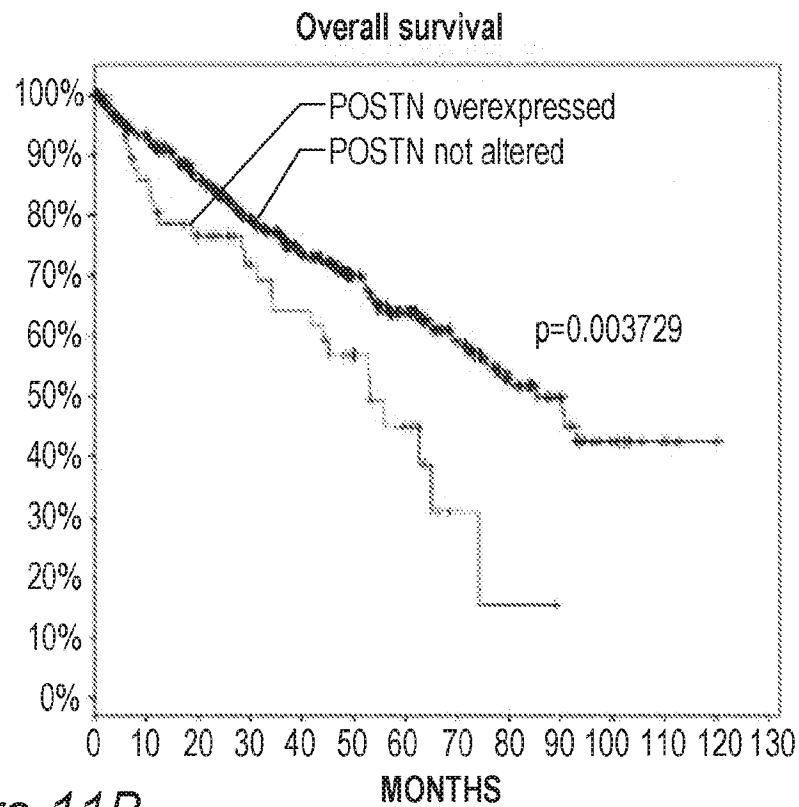


Figure 11B

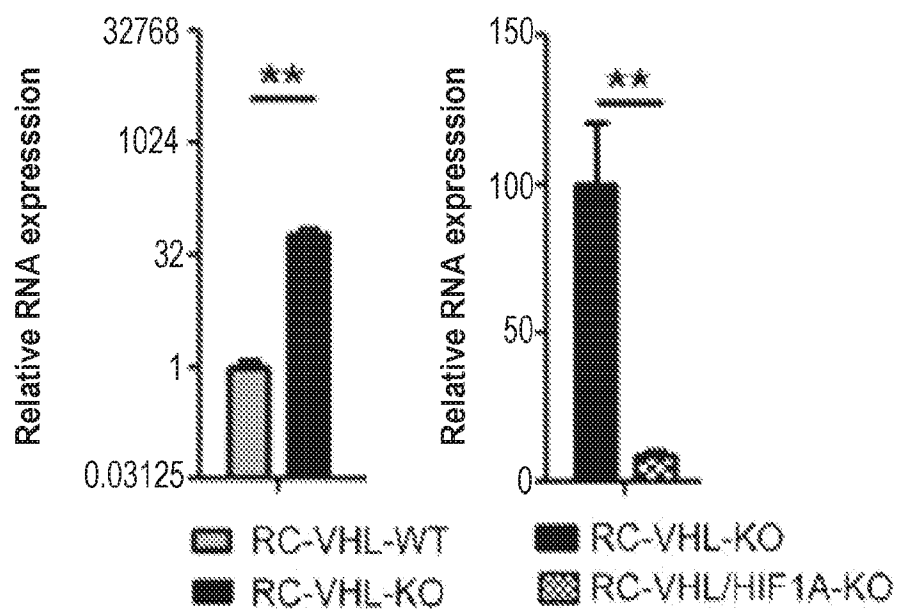
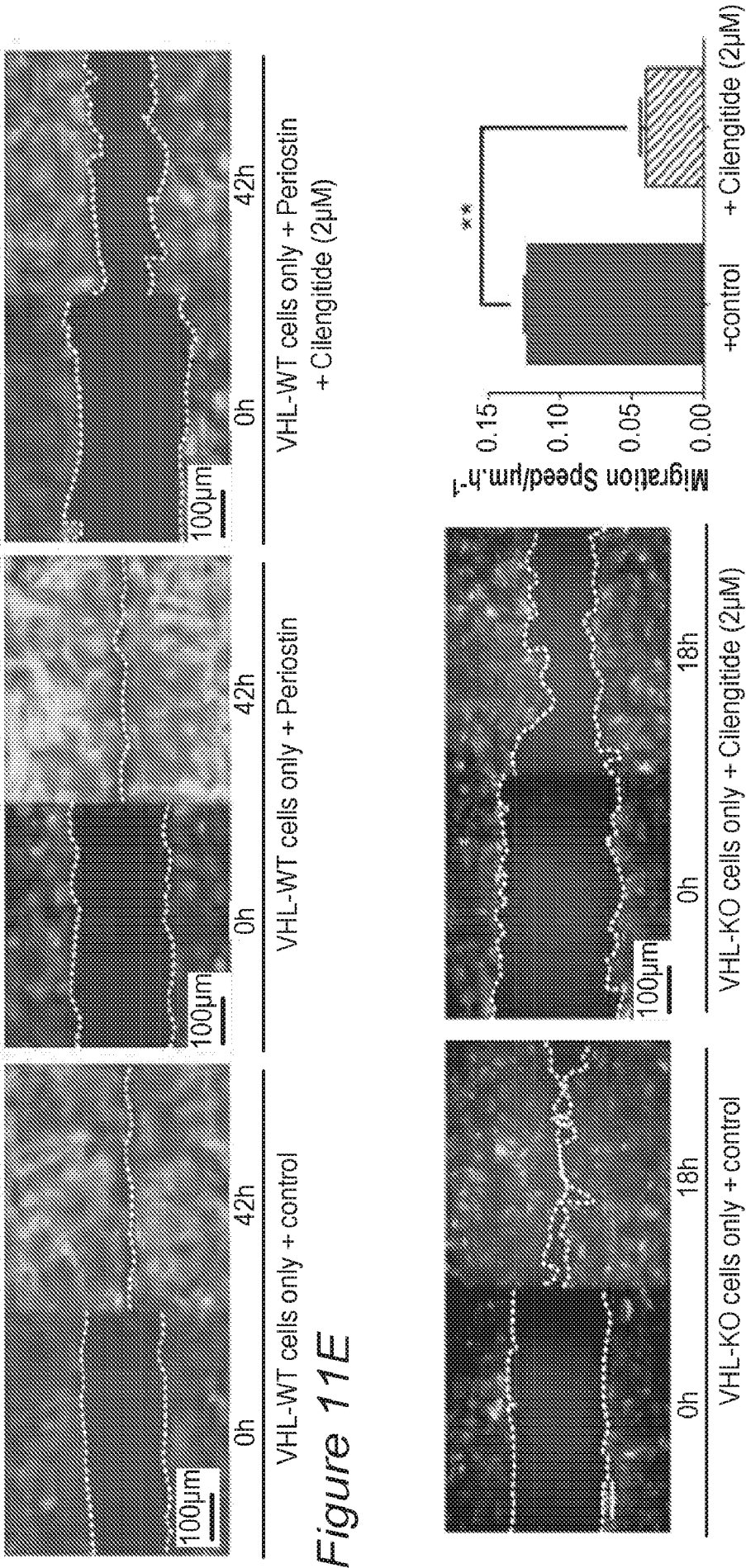


Figure 11C

Figure 11D



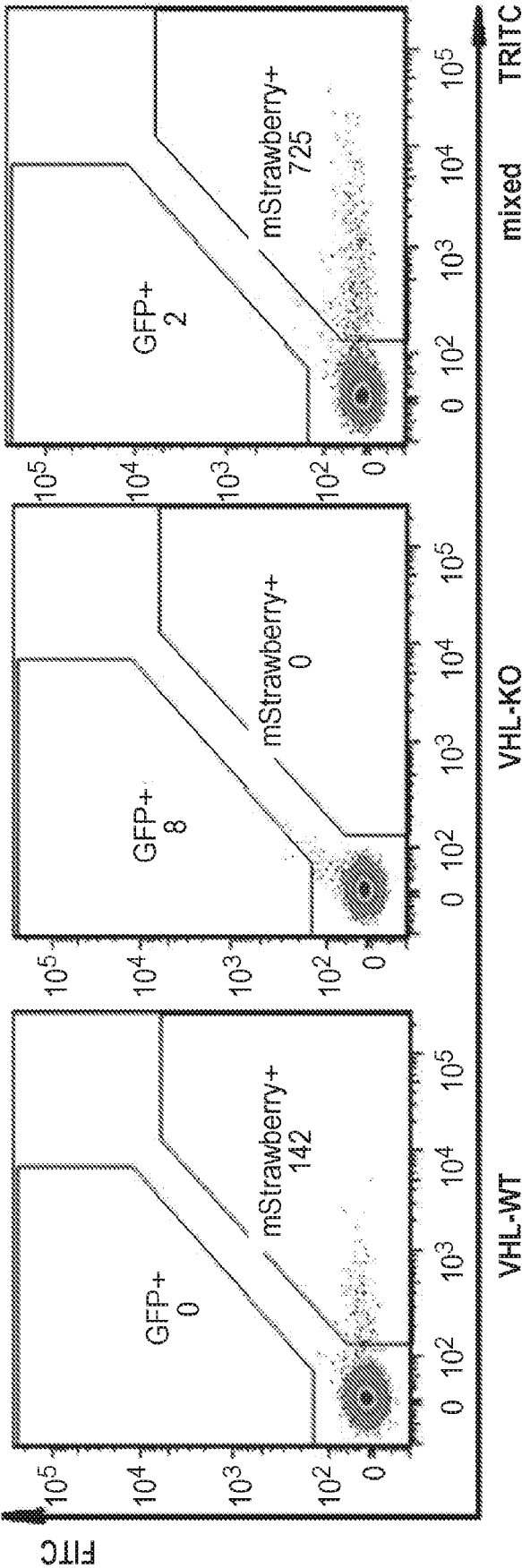


Figure 11G

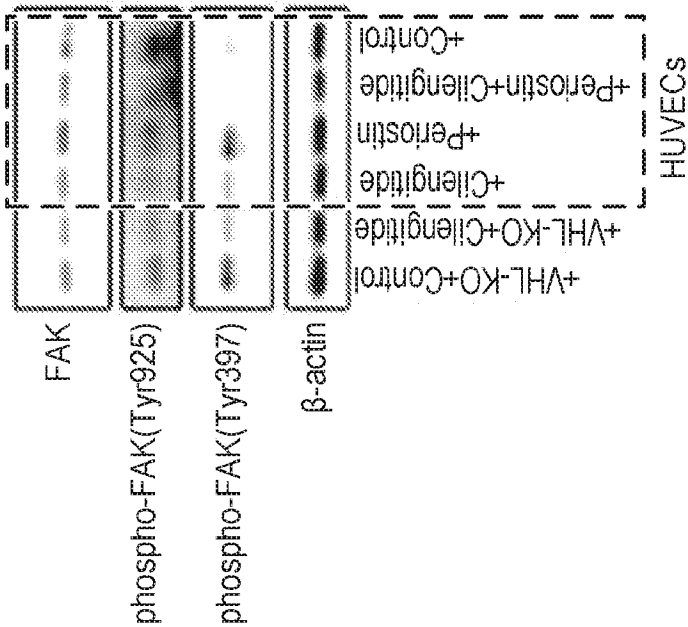


Figure 11H

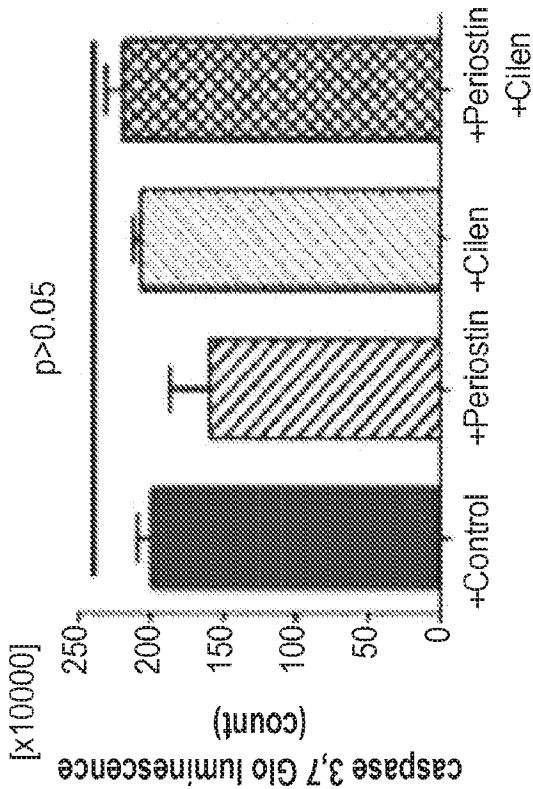
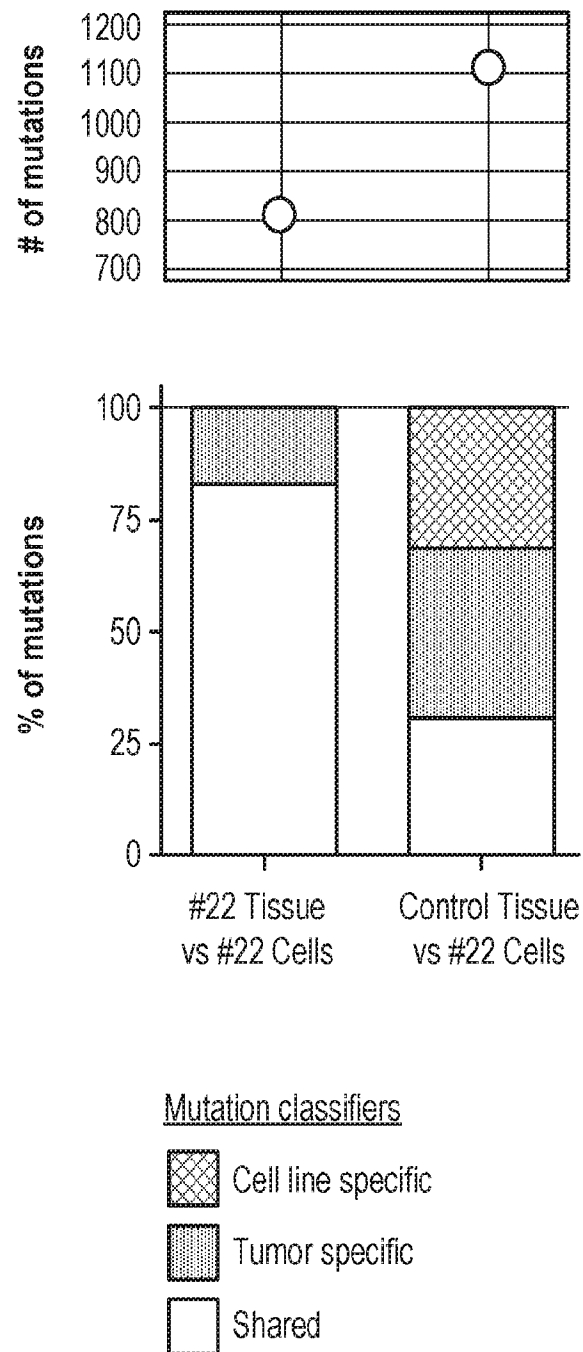


Figure 11I

*Figure 12A*

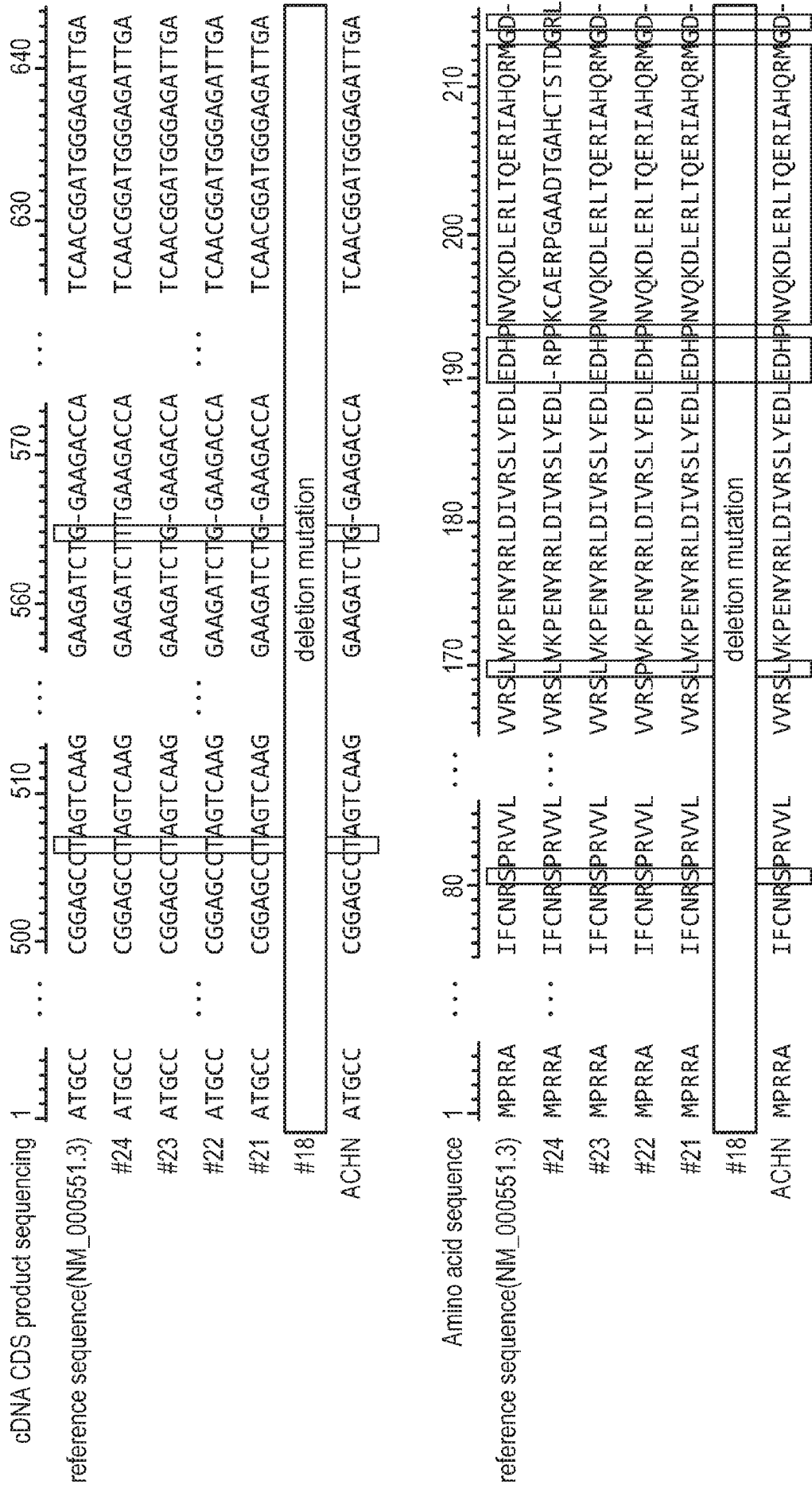


Figure 12B

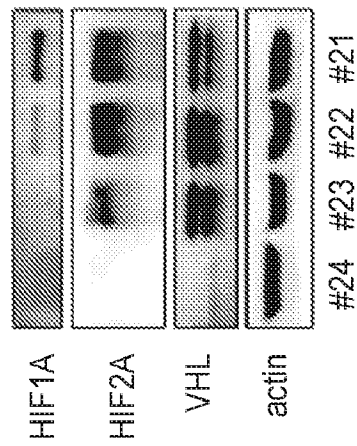


Figure 12E

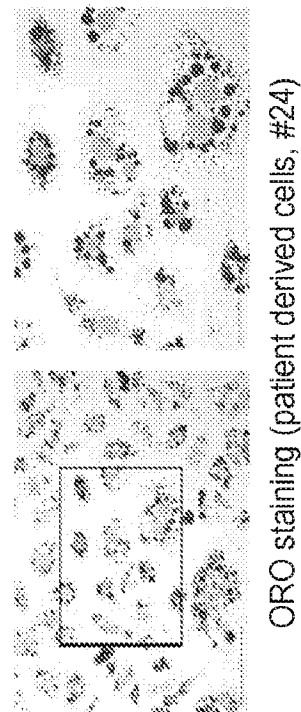


Figure 12D

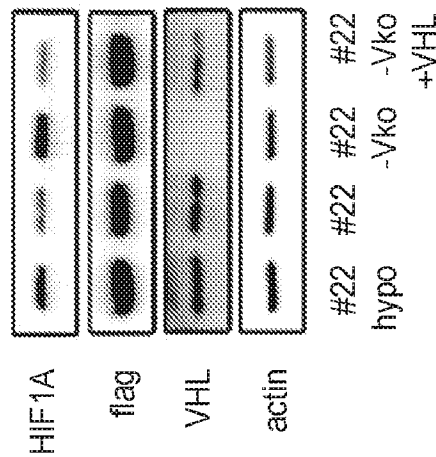


Figure 12C

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 20/19601

A. CLASSIFICATION OF SUBJECT MATTER

IPC - A61K 38/46, A61K 48/00, A61P 35/00 (2020.01)

CPC - A61K 48/00, C12N 2310/20, C12N 15/11, C12N 15/113

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

See Search History document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

See Search History document

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CHUANYU et al., Periostin promotes migration and invasion of renal cell carcinoma through the integrin/focal adhesion kinase/c-Jun N-terminal kinase pathway, Tumor Biology, April 2017, Vol 39, No 4, pg 1-8; abstract; pg 2, col 1, para 2-3	1-3, 11, 19
Y		4-5, 10, 12-18
Y	US 2017/0145405 A1 (THE BOARD OF TRUSTEES OF THE LELAND STANFORD JUNIOR UNIVERSITY) 25 May 2017 (25.05.2017) abstract; para [0007], [0008], [0029]	4-5, 13-18
Y	SCHOKRPUR et al., CRISPR-Mediated VHL Knockout Generates an Improved Model for Metastatic Renal Cell Carcinoma, Scientific Reports, 30 June 2016, Vol. 6, page 29032 (pp 1-13); abstract	10
Y	US 2008/0025946 A1 (SIVAKUMAR et al.) 31 January 2008 (31.01.2008); abstract	12
Y	US 2007/0122379 A1 (NIELSEN et al.) 31 May 2007 (31.05.2007); abstract; para [0010], [0127], [0132]	18

☐ Further documents are listed in the continuation of Box C.☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"D" document cited by the applicant in the international application

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

9 June 2020

Date of mailing of the international search report

06 JUL 2020

Name and mailing address of the ISA/US

Mail Stop PCT, Attn: ISA/US, Commissioner for Patents
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INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 20/19601

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

*****Continued in Supplemental Box*****

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-5 and 10-19

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.

PCT/US 20/19601

Continuation of Box No. III (Observations where unity of invention is lacking):

Group I+, claims 1-28, directed to a method for inhibiting metastasis of renal cell carcinoma (RCC) in a subject having RCC by reducing the presence or biological activity of periostin. The method will be searched to the extent that the reduction of periostin encompasses reducing expression of periostin by genetically modifying a cell to delete a nucleic acid encoding periostin. It is believed that claims 1-5 and 10-19 encompass this first named invention, and thus these claims will be searched without fee to the extent that the method/agent of reducing periostin encompasses genetically modifying an RCC tumor cell to delete a nucleic acid encoding periostin. Additional method(s)/agent(s) of reducing periostin will be searched upon the payment of additional fees. Applicants must specify the claims that encompass any additionally elected method(s)/agent(s) of reducing periostin. Applicants must further indicate, if applicable, the claims which encompass the first named invention, if different than what was indicated above for this group. Failure to clearly identify how any paid additional invention fees are to be applied to the "+" group(s) will result in only the first claimed invention to be searched. An exemplary election would be wherein the reduction of periostin comprises an anti-periostin (POSTN) monoclonal antibody, in addition to genetically modifying an RCC tumor cell to delete a nucleic acid encoding periostin (claims 1-6, 10-28).

Group II, claims 29-32, directed to a pharmaceutical composition.

The inventions listed as Groups I+ and II do not relate to a single special technical feature under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Special technical features

Group I+ has the special technical feature of a method of treating a subject, that is not required by Group II.

Group II has the special technical feature of a pharmaceutical composition, that is not required by Group I+.

The inventions of Group I+ each include the special technical feature of a different method of/agent for reducing periostin, and is considered a distinct technical feature.

Common technical features

The inventions of Group I+ share the common technical feature of a method for inhibiting, treating or preventing metastasis of RCC in a subject having RCC, the method comprising administering to a primary RCC tumor site an agent that reduces presence or biological activity of periostin, wherein reducing the presence or biological activity of periostin comprises reducing, silencing or eliminating the expression of periostin, wherein reducing the presence or biological activity of periostin inhibits metastasis. However, these shared technical features are previously made obvious by the article "Periostin promotes migration and invasion of renal cell carcinoma through the integrin/focal adhesion kinase/c-Jun N-terminal kinase pathway" by Chuanyu et al. (Tumor Biology. April 2017, Vol 39, No 4, page 1010428317694549), (hereinafter "Chuanyu").

Chuanyu teaches inhibiting metastasis of RCC in a subject having RCC by reducing presence or biological activity of periostin, wherein reducing the presence or biological activity of periostin inhibits metastasis (Abstract "Periostin (POSTN) is an extracellular matrix protein which is overexpressed in a variety of cancers and has been related to tumorigenesis of renal cell carcinoma...Our research thus indicates that POSTN promotes renal cell carcinoma cell migration and invasion through interaction with integrins alpha-v/beta-3 and alpha-v/beta-5 and subsequent activation of the focal adhesion kinase/c-Jun N-terminal kinase pathway. These results suggest that POSTN plays a critical role in renal cell carcinoma metastasis and may represent a potential target for novel therapeutic approaches against renal cell carcinoma."), yet does not specifically teach a method comprising administering to a primary RCC tumor site in a subject having RCC an agent that reduces presence or biological activity of periostin, wherein reducing the presence of biological activity of periostin comprises reducing, silencing or eliminating the expression of periostin. Given that Chuanyu suggests inhibiting periostin in order to inhibit metastasis in renal cell carcinoma may be a way to treat renal cell carcinoma (abstract), it would have been obvious to an artisan of ordinary skill in the art to experiment with administration of agents that inhibit expression of periostin in RCC patients using known techniques such as directed gene editing and antisense based silencing, in order to treat RCC by reducing periostin activity.

As the technical features were known in the art at the time of the invention, they cannot be considered special technical features that would otherwise unify the groups.

Therefore, Group I+ and II inventions lack unity under PCT Rule 13 because they do not share the same or corresponding special technical feature.