**Supplementary Materials and Methods**

***Immunohistochemistry and Immunofluorescence imaging***

Tissue sections from the resected pancreas of KC and KCM mice, pancreatic tumors of xenografts, tissue microarrays (Biomax), and pancreatic organoid sections from KC and KCM mice were baked overnight at 58°C, deparaffinized with xylene, and rehydrated with alcohols (5 min each). Tissues were incubated with a solution of 3% H2O2 in methanol for quenching endogenous peroxidase activity, followed by heat mediated antigen retrieval with 0.05 M citrate buffer (pH 6.0 for 20 mins). Sections were then blocked with 2.5% horse serum (ImmPRESS Universal antibody kit; Vector Laboratories, Burlingame, CA) (for immunohistochemistry, IHC) and 10% NGS (for immunofluorescence, IF) for 1-2 hrs at room temperature. The tissue samples were incubated overnight at 4°C with specific anti-MUC5AC, Ki67, KLF4, ALDH1A1, EpCAM, and Integrin αvβ5 antibodies **(Supplementary Table 2.2)**. Next, the slides were washed with PBS (4X10 minutes) and incubated with anti-rabbit/anti-mouse secondary antibody (ImmPRESS Universal antibody kit; Vector Laboratories) (for IHC) or fluorescent-tagged secondary antibodies (Themoscientific, 1:300) (for IF) for 30-60 minutes followed by washing with PBS (4X10 minutes).

For IHC, the color was developed by adding substrate chromogen, 3, 3’-diaminobenzidine solution (DAB substrate kit; Vector Laboratories). A brown precipitate indicated positive expression. The slides were counterstained with hematoxylin, followed by dehydration in graded ethanol followed by xylene and mounted with Permount (Vector Laboratories). For IF, the tissues were mounted with coverslips using antifade Vectashield mounting medium containing 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA, and USA). Images were captured under a Zeiss (Carl Zeiss Microimaging, Thornwood, NY) confocal laser scanning microscope. For each IF analysis, 5-6 different fields were captured and the fluorescent intensity was measured using Zen software, followed by statistical analyses using 3-4 biological replicates.

***Generation of MUC5AC knockdown stable cell lines:***

The metastatic fast-growing variant of COLO357 pancreatic cancer cells (FG/COLO357) and SW1990 cells were cultured in DMEM supplemented with 10% fetal bovine serum and antibiotics (100 μg/mL penicillin and 100 μg/mL streptomycin) at 37°C in the presence of 5% carbon dioxide. Endogenous MUC5AC was stably knocked down using three different MUC5AC shRNA constructs cloned in pSUPER.retro.puro-shMUC5AC **(Supplementary Table 1)**. The MUC5AC shRNA and scramble vectors were transfected into phoenix cells using Lipofectamine 2000TM (Invitrogen, Carlsbad, CA, USA) (1). The supernatant from phoenix cells was collected and filtered 48 hours post-transfection and used to transduce the FG/COLO357 and SW1990 cells with the addition of 4 µg/mL polybrene. Clones transduced with MUC5AC shRNA were selected and maintained using the antibiotic puromycin (4 μg/ml) in 10% DMEM medium to obtain stably transfected cells***.***

***Immunoblot Analysis***

For protein analysis, 2×106 of FG/COLO357-Scr/ShMUC5AC and SW1990-Scr/Sh5AC cells were plated on 100 mm cell culture petri dishes in a complete growth medium. After 48 hours of incubation, cells were processed for protein extraction using immunoprecipitation assay buffer (50 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate) containing protease inhibitors (1 mM phenyl–methyl sulphonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin) and phosphatase inhibitors. Protein concentrations were determined using a Bio-Rad D/C protein estimation kit. For high molecular weight MUC5AC, the proteins (40µg) were resolved by electrophoresis on a 2% SDS-agarose gel under reducing conditions while for other molecules, 20-30 ug of proteins were fractionated using 10% SDS-PAGE. Resolved proteins were transferred onto the polyvinylidene difluoride (PVDF) membrane and blocked in 5% non-fat milk in phosphate-buffered saline (PBS) for 1-2 hours before incubating with respective antibodies **(Supplementary Table 2.1)** overnight at 4°C, followed by washes (4 X 10 mins) in PBST (50 mM Tris –HCl, pH 7.4, 150 mM NaCl and 0.05% Tween-20). Further, the membranes were incubated in appropriate Horseradish peroxidase-conjugated secondary antibodies (Amersham Biosciences, Buckinghamshire, UK) (diluted at 1:2000-1:4000 in PBS with 3% non-fat milk) for 1 hour at room temperature, followed by washes in PBST (4 X 10 mins). The blots were then processed with ECL chemiluminescence kit (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA), and the signal was detected by exposing the processed blots to X-ray films (Biomax Films, Kodak, NY, USA) (2).

For detecting Integrin expression upon transfer of enriched supernatant, 1X106 FG/COLO357-Scr/ShMUC5AC cells were seeded in 60 mm dishes. After 72 hours, culture supernatant was collected and centrifuged at 1500 rpm for 2-3 minutes to remove dead cells and debris. The supernatant is then transferred onto a culture of FG/COLO357 Sh-MUC5AC cells after removing the existing media. Before adding the enriched supernatant, 0.5X06 cells were allowed to attach and grow for 12 hours. After 48 hours of culturing the cells in enriched supernatant, the protein lysates were collected and compared using Western blot analysis, as mentioned above.

All the western blots were quantified using ImageJ, and the expression of each protein was normalized with the expression of beta actin in the same lysate. The relative expression of each protein in each group is presented with respect to that of the control group.

***In vitro Limiting Dilution Assay:***

Decreasing concentrations of FG-Scr and FG-Sh5AC cells (1X 104 ,103 ,102) were plated on a 24 well low-attachment plate and cultured for 7 days (3). Spheroid formation was visualized, and representative images were captured using a light microscope. The size of spheroids was measured (for 3-5 fields/group/cell concentration/ replicate) by ImageJ. The spheroids were carefully transferred onto a normal 6 well plate and further cultured for 48 hours. After attachment of the spheroids as colonies, they were stained with 0.2% crystal violet in 10% Ethanol and incubated for 15- 20 minutes at room temperature. Then the stain was dissolved using DMSO, and the absorbance was read at 570 nm using a microplate reader.

***Immunocytochemistry***

Expression and localization of MUC5AC protein was observed using confocal laser scan microscopy (2). For this, 1X104 cells were plated on coverslips and grown for 48h. After washing with Hank’s buffered salt solution (HBSS) (pH=7.4), the cells were fixed in ice-cold methanol at −20°C for 2-3 mins and blocked with 10% goat serum for 1 hour followed by incubation with specific antibodies overnight at 4° C. Cells were then washed and incubated with FITC-conjugated goat antimouse/rabbit secondary antibodies for 1 hour. Thereafter, the cover slips were mounted on glass slides with antifade Vectashield mounting medium containing 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA, and USA). Immunostaining was observed and representative photographs were captured under a Zeiss (Carl Zeiss Microimaging, Thornwood, NY) confocal laser scanning microscope.

***Detection of Actin Filaments Polymerization***

1X104 FG/COLO357-Scr/ShMUC5AC cells were seeded on a sterile coverslip and cultured for 48 hours in 6 well plates and processed as described before (2). Cells were fixed with 3.7% Formaldehyde solution in 5-10 mins PBS, permeablized with 0.1% TritonX100 in PBS for 3-5 mins followed by washing with PBS for 2-3 times. Cells were then stained with 50 ug/ml FITC labeled Phalloidin stain in PBS for 20 minutes at room temperature. After washing with PBS (3 X 5 minutes), the coverslips were mounted with DAPI on slides and were visualized under Fluorescence Microscope.

***Wound Healing Assay***

1×106 of FG/COLO357-Scr/ShMUC5AC cells were seeded on top of coverslips in each well of a 6 well plate and grown till 90% confluency. Then, a scratch was made at 3 independent locations of the coverslip using P200 tip (2). Images of the scratches were captured after 24 and 48 hours of incubation at 10X magnification using confocal microscopy after processing the coverslips for immunofluorescence of MUC5AC (protocol as mentioned above for Immunofluorescence imaging).

***Relative Quantification of Gene Expression***

Total RNA was isolated from cell lines using the QIAGEN RNeasy Mini kit (QIAGEN, Valencia, CA, USA) respectively, according to the manufacturer’s protocol. The mRNA isolated was converted to cDNA using oligo-dT primers. Quantitative real-time PCR was performed on Roche Light Cycler 480 system (Roche Diagnostics, Mannheim, Germany)(2). For qPCR, 10 μl reactions [5 μl 2x SBYR green Master Mix, 3μl of autoclaved nuclease free water, 1 μl diluted RT product (1:5) and 0.5 μl each of forward and reverse primer (primers listed in **Supplementary Table 1**) were performed in triplicate along with non-template controls (NTCs) for each assay under the same conditions. The cycling conditions were comprised of 95°C for 10min, followed by 40 cycles of 95°C for 15 s and 58°C for 1min. Gene expression levels were normalized to the level of β-actin expression used as a control.

***MTT Assay***

Cell viability was determined using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide assay, as described previously (4). In brief, FG-Scr/Sh5AC and SW-Scr/Sh5AC cells were seeded into 96-well microplate at a density of 5x103 cells per well, in complete growth medium containing 10% FBS. After 24 h, the culture media was aspirated and replaced with growth medium containing 1% FBS. Every 24 h, cells were incubated with 10 μl of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (5 mg/ml in PBS) solution added to each well for 3 h, and then the absorbance was read at 560 nm with a reference wavelength of 670 nm using microplate reader (2). Readings were taken for 6 days.

***Cell Cycle Analysis***

FG-Scr/ShMUC5AC and SW-Scr/Sh5AC cells were plated at a density of 1x106 cells in 60 mm cell culture Petri dishes. The next day, for in vitro synchronization in the G0 phase, cells were properly washed with PBS and maintained in serum-deprived medium for 48 hours(5). The cells were then released from the block by replacing them with the complete culture medium. Post 48 hours of release, the cells were collected and fixed in 1 ml of 70% ethanol for 45 minutes at 4°C and centrifuged to decant ethanol. The cell pellets were washed once in PBS and then resuspended in 1 ml of Telford’s reagent. After incubating at 4°C for 1 hour, the total DNA content was analyzed using the fluorescence-activated cell sorting method.

***Migration Assay***

For motility assays, 1×106 cells suspended in 2 ml serum-free medium were plated in the top chamber of polyethylene terephthalate membranes inserts (six-well inserts; pore size 8 µm; Becton Dickinson, Franklin Lakes, NJ, USA. To provide chemotactic drive, two ml of 10% serum-containing medium was added to the lower chamber of the well, and the cells were allowed to migrate for 24 h. After removing the non-migrated cells from the upper side of the membrane, the migrated cells on the lower side of the membrane were stained with a Diff-Quick cell stain kit (Dade-Behring Inc, Newark, DE 19714, USA). They were then photographed in 10 random fields of view at 10X magnification. Cell numbers were counted and expressed as the average number of cells/field of view (2).

***Adhesion and Proliferation Assays on Specific ECM Components***

Fibronectin and Vitronectin coated strips (Millipore) were rehydrated with 200 ul PBS for 15 minutes at room temperature. After removing PBS, single-cell suspension of0.5×105 cells was plated in each well of an eight-well strip in triplicates and incubated for 12 hours (for adhesion assay) and 48 hours (for proliferation assay). The wells were washed with 200 ul PBS mildly and stained with 100 ul 0.2% crystal violet in 10% Ethanol. After 10-15 minutes incubation at room temperature, the wells were washed mildly 3-5 times with 300 ul PBS. To dissolve the stain, 100 ul DMSO was added in each well, incubated for approximately 5 minutes with gentle shaking at room temperature. Absorbance from the stain was then measured at 570 nm using a microplate reader.

1×105 FG-Scr and FG-Sh5AC cells/ well were plated on the vitronectin and fibronectin-coated plates. The FG-Sh5AC cells were either left untreated or were treated with enriched supernatant from FG-Scr and FG-Sh5AC cells for 2 hours (for adhesion assay) and 46-48 hours (for proliferation assay). After incubation, the cells were stained with crystal violet, followed by measuring the absorbance, as mentioned before.

***Co-Immunoprecipitation***

2×106 FG/COLO357 and SW1990 cells were grown for 48 hours, and then lysed in non-denaturing lysis buffer [20mM Tris HCl pH 8, 137mM NaCl, 2mM ethylenediaminetetraacetic acid (EDTA), 1% Nonidet P-40 (NP-40), 1mM NaF, 1mM sodium orthovanadate, 1 mM PMSF, aprotinin 5mg/ml, leupeptin 5mg/ml and containing 1% Triton X-100] for 25-35 min at 4°C. For pre-cleaning, the processed lysates were incubated with protein A+G Sepharose beads (Sigma-Aldrich Corp., St Louis, MO, USA) for 8 hours at 4°C on a rotator (6). Total protein concentration in pre-cleared lysates was quantified and equal amounts of total protein (500 µg) in 500µl volumes of non-denaturing lysis buffer were then incubated overnight with anti-MUC5AC antibodies: 45M1 (Abcam) or with respective IgG at 4°C on a rotator. The protein-antibody complexes were incubated with protein A+G Sepharose beads on a rotator overnight at 4°C. The pulled-down immunocomplexes were washed with the lysis buffer (3X) followed by one wash with PBS. The immunoprecipitates and input were electrophoretically resolved and immunoblotted with anti-MUC5AC, anti-integrin β5 antibodies.

***Gelatin Gel Zymography***

2×106 of FG/COLO357-Scr/ShMUC5AC were plated on 100 mm cell culture Petri dishes in the complete growth medium. After 48 hours of culturing the cells either alone or in the presence of enriched supernatant (as mentioned before), the cells were processed for protein extraction. After protein estimation, samples were prepared using a non-reducing sample buffer and loaded onto Gelatin zymogram gel (Invitrogen). The zymogram gel is thereafter renatured using renaturing buffer (2.5% Triton X 100) for 30 minutes at room temperature followed by developing at 37 degrees overnight using developing buffer (Tris Base, Tris HCl, NaCl, CaCl, 0.1% Triton X-100). The gel is then stained with 1% R-250 Coomasie blue at room temperature for 1 hour, followed by a destaining the same with destaining solution (5:4:1 volume of water: methanol: glacial acetic acid) till the clear bands of proteolysis start appearing (7).

**Supplementary Tables**

**Supplementary Table 1: Sequences of primers and shRNAs.**

|  |  |
| --- | --- |
| **Gene** | **Primer** |
| Kras (mouse) genotyping | K004-5’GTC GAC AAG CTC ATG CGG GTG  K006-5’-CCT TTA CAA GCG CAC GCA GAC TGTAGA-3’  K005-5’-AGCTAG CCA CCATGGCTTGAG TAA GTC TGC A-3’ |
| Pdx1;Cre (mouse)  genotyping | F-5’-CTGGACTACATCTTGAGTTGC -3’  R-5’-GGTGTACGGTCAGTAAATTTG -3’ |
| Muc5ac (mouse) genotyping | F- 5’ -GTTTCCACAAAGCACAACCAAAC-3’  R-5’-TAGGGCCAGGCTAAGAGAAACC-3’ |
| MUC5AC (human) qPCR | F: 5’-CTCAGGAATGACGCTTGGACATGG-3’  R: 5’-GGCTGAGGTAGGAGTGAGGTTCTT-3’ |
| MUC5AC (human)  shRNA-11 | F: 5’-PO4- GATCCCCGGACGGTGCTTGACGACATTTCAAGAGAATGTCGTCAA GCACCGTCCTTTTTA-3’  R: 5’-PO4- AGCTTAAAAAGGACGGTGCTTGACGACATTCTCTTGAAATGTCGT CAAGCACCGTCCGGG-3’ |
| MUC5AC (human)  shRNA-2 | F: 5’-PO4- GATCCCCCGTTTGACGGGAAGCAATATTCAAGAGATATTGCTTCC CGTCAAACGTTTTTA-3’  R: 5’-PO4- AGCTTAAAAACGTTTGACGGGAAGCAATATCTCTTGAATATTGCT TCCCGTCAAACGGGG-3’ |
| MUC5AC (human)  shRNA-3 | F: 5’-PO4- GATCCCCGGACAAAGTGGTTCGACGTTTCAAGAGAACGTCGAACC ACTTTGTCCTTTTTA-3’  R: 5’-PO4- AGCTTAAAAAGGACAAAGTGGTTCGACGTTCTCTTGAAACGTCGA ACCACTTTGTCCGGG-3’ |

**Supplementary Table 2.1: Antibodies used in Immunoblotting.**

|  |  |  |  |
| --- | --- | --- | --- |
| **S.no.** | **Protein** | **Source** | **Dilutions** |
| 1. | MUC5AC (CLH2) | Millipore | 1:400 |
| 2. | MUC5AC (45M1) | Abcam | 1:400 |
| 3. | β-Actin | Sigma | 1:10000 |
| 4. | Cyclin-D1 | Cell signaling | 1:1000 |
| 5. | Cyclin-E | Abcam | 1:200 |
| 6. | Klf4 | Thermoscientific | 1:1000 |
| 7. | Integrins | Cell signaling | 1:1000 |
| 8. | Src | Cell signaling | 1:200 |
| 9. | p-Src | Cell signaling | 1:1000 |
| 10. | STAT3 | Cell signaling | 1:1000 |
| 11. | p-STAT3 | Cell signaling | 1:1000 |
| 12. | ALDH1A1 | Santacruz | 1.500 |
| 13. | SOX9 | Cell signaling | 1:1000 |
| 14. | EpCAM | Santacruz | 1.500 |
| 15. | CD133 | Santacruz | 1.500 |
| 16. | CD44 | Cell signaling | 1:1000 |

**Supplementary Table 2.2: Antibodies used in Immunostaining.**

|  |  |  |  |
| --- | --- | --- | --- |
| **S.no.** | **Protein** | **Source** | **Dilutions** |
| 1. | MUC5AC (45M1) | Abcam | 1:400 |
| 2. | Ki67 | Abcam | 1:300 |
| 3. | Klf4 | Abgenta | 1.100 |
| 4. | Integrin αvβ5 | LSBio | 1.50 |
| 5. | ALDH1A1 | Santacruz | 1:300 |
| 6. | pSTAT3 | Cell Signaling | 1:100 |
| 7. | EpCAM | Santacruz | 1:300 |

**References**

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**Supplementary Figure Legends**

**Supplementary Figure 1: (A)** Breeding strategy for generating KrasG12D; Pdx1-Cre (KC) and KrasG12D; Pdx1-Cre; Muc5ac-/- (KCM) animals. KC and KCM animals were sacrificed at 10, 20, 30, 40, and 50 weeks of age. **(B)** Quantitative analysis showing a decrease in pancreas weight (in mg) in the KCM mice group; the difference was significant at the 50 weeks of age. **(C)** Genotype strategy and representative gel image from the tail and organoid DNA from KC and KCM animals for recombination event at KRasG12D locus.Immunofluorescence analysis showing decrease Aldh1a1 **(D, E)** and EpCAM **(F, G)** expressing cells in the KCM mice pancreatic sections.

**Supplementary Figure 2: (A)** Immunoblot showing expression of MUC5AC in a panel of PC cell lines at protein level (A549: lung cancer cell line used as a positive control, HPNE: normal transformed pancreatic ductal epithelial cell). **(B)** ELISA assay showed the availability of secreted MUC5AC in the cell supernatant. **(C)** Localization of MUC5AC on the cell surface and intercellular junctions was determined by immunofluorescence. **(D, E, F, G)** MUC5AC expression was silenced in FG-COLO357 and SW1990 cells via retroviruses carrying short hairpin RNAs (shRNAs) against MUC5AC at the mRNA level using quantitative RT PCR and protein level (Sh5AC-1 and 2 are two different Sh-transfected clones). **(H)** Quantitative representation of the cell cycle analysis reveals G1 to S phase arrest in SW-Sh5AC cells. **(I)** Immunoblot shows a significant reduction in Cyclins D1 and E, the key cyclins controlling G1 to S transition, upon MUC5AC KD in SW1990 cells. **(J)** FG/COLO357 and **(K)** SW1990 cells were examined for the impact of MUC5AC knockdown on cell viability via MTT assay (Day 1-6) under low serum conditions (1%). Sh5AC cells show reduced viability as compared to control cells. **(L)** Representative picture showing MUC5AC and Ki67 staining in the serial sections of FG-Scr and FG-Sh5AC orthotopic tumor tissues. **(M)** Representative images of Ki67-stained pancreatic tissues from KC and KCM mice at early (10 weeks) and late (50 weeks) time-points of progression.

**Supplementary Figure 3: (A)** Pictorial representation of colony-forming units in FG-Scr and FG-Sh5AC group at serial dilutions (1X 104 ,103 ,102) in normal attachment 6 well plate**. (B)** Wound healing assay showing lesser motility of FG-Sh5AC cells as compared to FG-Scr cells with MUC5AC-expressing cells in the migratory front (white arrows). **(C)** Phalloidin staining suggests a decrease in actin polymerization in FG-Sh5AC cells. **(D)** Pictorial representation of metastatic lesions in spleen, stomach, diaphragm, mesentery, and liver of mice injected with FG-Scr cells. **(E)** Quantitative representation indicating the metastatic incidences in mice injected with FG-Scr and FG-Sh5AC cells. **(F)** Quantitative analysis of colorimetric assay reveals that MUC5AC-KD (FG-Sh5AC) cells adhere significantly better on **(i)** vitronectin- and **(ii)** fibronectin-coated plates upon treatment with conditioned media from FG-Scr cells as compared to the untreated FG-Sh5AC group. **(G)** Pictorial representation of migrated cells in FG-COLO357 cells upon MUC5AC KD and Integrin β5 KD either alone or in combination. MUC5AC-expressing cells lost their migratory potential significantly after integrin β5 KD. **(H)** Quantitative and pictorial representation of migrated cells in the presence or absence of C1889, an inhibitor of pSTAT3. Images demonstrate that pSTAT3 inhibition in MUC5AC expressing cells decreases their migration. **(I)** Representative demonstration of SP in FG-Scr and FG-Sh5AC cells upon Klf4 KD and in the presence of MUC5AC-enriched culture supernatant.