**SUPPLEMENTAL TABLES**

**Table S1: OMP-59R5 binding data as assessed by surface plasmon resonance (Biacore).** Binding affinities to human Notch1-4, mouse and rat Notch2 and Notch3 are shown. (nanomolar, nM).

|  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **IgG** | **Human**  **Notch1**  **(nM)** | **Mouse**  **Notch1**  **(nM)** | **Human**  **Notch2**  **(nM)** | **Mouse**  **Notch2**  **(nM)** | **Rat**  **Notch2**  **(nM)** | **Human**  **Notch3**  **(nM)** | **Mouse**  **Notch3**  **(nM)** | **Rat**  **Notch3**  **(nM)** | **Human**  **Notch4**  **(nM)** |
| **OMP-59R5** | >10 | >10 | 0.2 | 1.4 | 1.8 | 0.6 | 1.0 | 1.7 | Not bind |

**Table S2. Histopathology of human pancreatic tumors**

|  |  |
| --- | --- |
| **Tumor** | **Histopathology** |
| OMP-PN4 | Moderately differentiated adenocarcinoma (G2) |
| OMP-PN7 | Adenocarcinoma, stage III (T3N0MX) |
| OMP-PN8 | Adenocarcinoma, stage III (T3N0) |
| OMP-PN11 | Adenocarcinoma, stage III (T3NXMX) |
| OMP-PN13 | Adenocarcinoma, stage III (T3N1MX) |
| OMP-PN16 | Adenocarcinoma, stage III (T3N1) |
| OMP-PN17 | Invasive adenocarcinoma, stage III (T3N0MX) |
| OMP-PN21 | Moderately differentiated adenocarcinoma, stage II (T3N1bMX) |
| OMP-PN23 | Invasive moderately differentiated adenocarcinoma, stage II (T3N1M0) |
| OMP-PN25 | Moderately differentiated (G2) adenocarcinoma, stage 2A (T3N0M0) |

**Table S3. Effect of anti-Notch2/3 antibody OMP-59R5 in combination with gemcitabine on growth of pancreatic xenograft tumors and its association with Kras mutation status and molecular subtypes.** Data were calculated at the end point of the tumor volume shown in Figure S3 and expressed as mean+/-SEM, n=7-10 mice per group. \*: p<0.01 vs. control mAb; \*\*: p<0.05 vs. gemcitabine by two-way ANOVA followed by Bonferroni post-test comparison. R: responders; NR: non-responders; WT: wild type. Classical: tumors with high adhesion-associated and epithelial genes and best survival prognosis; exocrine-like: tumors express high tumor cell-derived digestive enzyme genes; quasimesenchymal: tumors with high expression of mesenchymal associated genes.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
|  | **Tumor Volume Reduction, % of Control mAb** | | |  |  |  |
| **Tumor Type** | **OMP-59R5** | **Gemcitabine** | **Combination** | **Responsiveness to Combination Therapy** | **Kras Mutation Status** | **Molecular Subtype** |
| OMP-PN4 | 55±5\* | 50±3\* | 72±3\*, \*\* | R | WT | Classical |
| OMP-PN7 | 19±11 | 58±4\* | 61±3\* | NR | CAA->CAT(het) [Q61H] | Classical |
| OMP-PN8 | 2±10 | 77±3\* | 94±1\*, \*\* | R | GGT->GAT(het) [G12D] | Quasimesenchymal |
| OMP-PN11 | -7±6 | 26±4 | 25±5 | NR | GGT->GAT(het) [G12D] | Classical |
| OMP-PN13 | 33±5 | 76±2\* | 78±2\* | NR | GGT->GAT(hom) [G12D] | Quasimesenchymal |
| OMP-PN16 | 15±8 | 23±10 | 41±3\*, \*\* | R | GGT->GTT(hom) [G12V] | Quasimesenchymal |
| OMP-PN17 | 15±18 | 64±5\* | 87±1\*, \*\* | R | WT | Quasimesenchymal |
| OMP-PN21 | 25±11 | 41±3\* | 55±8\*, \*\* | R | GGT->CGT(het) [G12R] | Exocrine-like |
| OMP-PN23 | 36±8 | 94±1\* | 93±1\* | NR | WT | Classical |
| OMP-PN25 | 15±12 | 26±6 | 62±3\*, \*\* | R | GGT->GAT(het) [G12D] | Classical |

**Table S4. Gene Set Enrichment Analysis**. Gene Set Enrichment Analysis performed on xenograft tumors OMP-PN4, OMP-PN8, OMP-PN16 and OMP-PN17. FDR<15%. Tumors from control and treated groups at the end of studies were harvested. Tumor RNA was isolated and analyzed for human gene expressions using human-specific microarray gene chips.

**Human gene sets significantly down-regulated by OMP-59R5**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **OMP-59R5** | | | | |
| **Gene Set** | **Size** | **p-val** | **FDR** | **Description** |
| OMP\_CSC\_UP | 415 | <0.01 | 0.08 | 464 genes over-expressed in Cd44+ vs. CD44- colon tumor cells |
| MENSE\_HYPOXIA\_UP | 83 | <0.05 | 0.08 | List of hypoxia-induced genes found in both astrocytes and Hela cells |
| ST\_WNT\_CA2\_CYCLIC\_GMA\_PATHWAY | 18 | <0.01 | 0.08 | Some Wnt glycoprotein/Frizzled receptor interactions increase intracellular calcium and decrease cGMP |
| TAKEDA\_NUP8\_HOXA9\_3D\_DN | 29 | <0.01 | 0.09 | Effect of NUP98-HOXA9 on gene transcription at 3 d after transduction down |
| HSA04630\_JAK\_STAT\_SIGNALING\_PATHWAY | 146 | <0.01 | 0.09 | Genes involved in Jak-STST signaling pathway |
| DER\_IFNA\_UP | 62 | <0.01 | 0.09 | Genes up-regulated by interferon-alpha in HT1080 (fibrosacoma) |
| HYPOXIA\_REG\_UP | 33 | <0.05 | 0.11 | Up-regulated by hypoxia in renal cells, and down-regulated with re-oxygenation |
| HARRIS\_HUVECS\_DLL4\_UP | 54 | <0.01 | 0.11 | 68 genes upregulated by DLL4 in Huvec cells (FC>=1.5, p<0.01) |
| TAKEDA\_NUP8\_HOZA9\_UP | 158 | <0.05 | 0.11 | Effect of NUP98-HOXA9 on genes transcription at 3 d after transduction UP |
| HANSON\_NFKAPPB\_IND | 15 | <0.05 | 0.11 | Ras-inducible, NF-kB-regulated genes |
| SRC\_ONCOGENIC\_SIGNATURE | 55 | <0.05 | 0.12 | Genes selected in supervised analyses to discriminate cells expressing c-Src oncogene from control cells expressing GFP |
| BYSTROM\_IL5\_DN | 53 | <0.05 | 0.13 | Genes down-regulated in mouse bone marrow in response to interleukin-5 |
| SANSOM\_APC\_4\_DN | 64 | <0.05 | 0.13 | The top 98 down-regulated genes following Apc loss at day4 |
| SHEPARD\_POS\_REG\_OF\_CELL\_PROLIFERATION | 87 | <0.01 | 0.13 | Positive regulators of cell proliferation in zebra fish |
| STEMPATHWAY | 15 | <0.05 | 0.14 | In the absence of infection, bone marrow stromal cells release hematopoietic cytokines; activated macrophages and Th cells induce hematopoiesis during infection |
| CTNNB1\_ONCOGENIC\_SIGNATURE | 67 | <0.01 | 0.14 | Genes selected in supervised analyses to discriminate cells expressing activated beta-catenin (CTNNB1) oncogene from control cells expressing GFP |
| CLARKE\_IGS\_UP | 72 | <0.01 | 0.14 | 97 over-expressed genes in the invasiveness gene signature (IGS) by comparing CD44+/CD24- breast cancers to normal breast epithelium (NEJM, 2007, 356:217) |
| WANG\_HOXA9\_VS\_MEIS1\_UP | 24 | <0.05 | 0.15 | Genomic signature of progenitors immortalized by Hoxa9 versus Hoza9 plus Meis1 increased expression in Hoxa9-immortalized progenitors |

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Gemcitabine** | | | | |
| EMT-UP | 56 | <0.01 | 0.15 | Genes up-regulated during TGFbeta-induced EMT |
| JECHLINGER\_EMT\_UP | 49 | <0.01 | 0.21 | Genes up-regulated for epithelial plasticity in tumor progression |
| STEMPATHWAY | 15 | <0.05 | 0.22 | Genes involved in hematopoiesis |

**EMT and stem cell gene sets significantly up-regulated by Gemcitabine**

**Stem cell gene sets significantly down-regulated by OMP-59R5+Gemcitabine**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **OMP59R5+Gemcitabine** | | | | |
| **Gene set** | **Size** | **p-val** | **FDR** | **Description** |
| OMP\_CSC\_UP | 415 | <0.01 | 0.03 | Genes over-expressed in CD44+ vs CD44- colon tumor cells |
| WEINBERG\_ESC\_EXP1 | 325 | <0.01 | 0.17 | Genes over-expressed in hES cells |
| ESC\_SELF\_RENEWAL | 30 | <0.05 | 0.19 | Genes when down-regulated are involved in mESC differentiation |
| STEMCELL\_COMMON\_UP | 159 | <0.05 | 0.19 | Genes enriched in mouse embryonic, neural and hematopoietic stem cells |
| WEINBERG\_NOS\_TARGETS | 153 | <0.01 | 0.20 | Genes actived by Nanog, Oct4 and Sox2 in hES cells |
| MILANO\_GSI\_RAT\_DN | 49 | <0.01 | 0.21 | Genes down-regulated by GSI-treated rats |

Table S5. Effects of OMP-59R5 on Mouse Gene Expression in the Stroma of Xenograft Tumors. Mean differential expression values for OMP-59R5 treated vs. control mAb treated tumors from OMP-PN4, OMP-PN8, OMP-PN16, and OMP-PN17: 1.5-fold threshold and p<0.05; n=3 per group/tumor type.

|  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Probeset** | **PN4**  **Fold** | **PN4**  **pVal** | **PN8**  **Fold** | **PN8**  **pVal** | **PN16**  **Fold** | **PN16**  **pVal** | **PN17**  **Fold** | **PN17**  **pVal** | **Symbol** | **Gene Title** |
| 1418106\_at | -6.90 | 1.41E-06 | -2.18 | 2.03E-04 | -2.98 | 4.85E-06 | -4.36 | 2.81E-04 | HeyL | Hairy/enhance-of-split related with YRPW |
| 1419302\_at | -34.40 | 0.00E+00 | -5.75 | 1.30E-09 | -8.38 | 0.00E+00 | -23.16 | 0.00E+00 | HeyL | Hairy/enhance-of-split related with YRPW |
| 1438886\_at | -17.85 | 0.00E+00 | -2.18 | 1.69E-03 | -3.64 | 6.40E-05 | -7.75 | 2.43E-08 | HeyL | Hairy/enhance-of-split related with YRPW |
| 1421964\_at | -2.52 | 1.95E-07 | -2.64 | 7.34E-04 | -1.58 | 2.71E-03 | -4.43 | 1.45E-05 | Notch3 | Notch gene homolog 3 (Drosophila) |
| 1421965\_s\_at | -2.81 | 1.86E-08 | -3.23 | 3.57E-05 | -2.05 | 3.24E-07 | -3.87 | 9.44E-06 | Notch3 | Notch gene homolog 3 (Drosophila) |
| 1417466\_at | -2.75 | 1.00E-10 | -3.55 | 1.07E-06 | -2.69 | 7.27E-08 | -7.88 | 3.00E-10 | Rgs5 | Regulator of G-protein signaling 5 |
| 1420942\_s\_at | -3.28 | 2.80E-09 | -8.05 | 1.22E-08 | -2.97 | 1.13E-06 | -10.55 | 7.12E-08 | Rgs5 | Regulator of G-protein signaling 5 |

**SUPPLEMENTAL MATERIALS AND METHODS**

**Chemicals and Reagents.** Gemcitabine was obtained from Eli Lily Pharmaceuticals Co. Hank’s balanced salt solution (HBSS), Dulbecco’s Modified Eagle Medium (DMEM) and Medium 199 were from Gibco-Invitrogen. Fetal bovine serum was obtained from Hyclone. Fluorescence activated cell sorting buffer, (FACS buffer), consisted of HBSS medium supplemented with 2% heat-inactivated fetal bovine serum and 20mM Hepes.

**Characterization of OMP-59R5 antibody.**

For antibody binding, human 293 HEK cells were transfected with cDNA expression vector encoding the human Notch2 (EGF1–15), human Notch3 (EGF9–14), mouse Notch2 (full length), mouse Notch3 (EGF9–14), as well as a second vector encoding green fluorescent protein to mark the transiently transfected cells. Twenty-four to 48 hours post transfection, cells were incubated with OMP-59R5 (10 μg/mL) for 1 hour at 37°C. Thereafter, cells were incubated with fluorescent secondary antibody, Goat-anti-human IgG H/L-phycoerythrin (PE) and analyzed by flow cytometry using a FACS Caliber instrument (BD Bioscience). Specific OMP-59R5 binding was assessed by determining the presence of cells positive for GFP signal and PE signal.

For epitope mapping, C-terminal truncations of the selected antigen were generated as human Fc fusions from the 3rd EGF domain to the 12th EGF domain in a mammalian expression vector (pcDNA3.1). The constructs were transfected into HEK 293T cells and supernatant was isolated after several days. The supernatants were captured on an anti-human Fc ELISA plate and the purified Fabs (10 µg/ml) were incubated with the captured Fc fusions. Fab binding was detected using an anti-human Fab specific antibody conjugated to HRP.

To determine the effect of OMP-59R5 on blocking Notch2 and Notch3 signaling, PC3 cells were transfected with an expression vector encoding a full-length Notch receptor as well as plasmids encoding a Notch-dependent luciferase reporter construct (8xCBF luciferase) and a transfection control reporter (renilla luciferase). Fusion proteins containing the extracellular domain of human DLL4, a Notch ligand, fused to human Fc were coated onto 96-well plates. The serially diluted antibodies were added to the appropriate wells. Notch receptor–expressing PC3 cells were added to the wells and incubated overnight. Luciferase levels were measured 18 hours later using a dual luciferase assay kit (Promega, Madison, WI) with firefly luciferase activity normalized to Renilla luciferase activity.

To determine OMP-59R5 binding affinities, recombinant Notch proteins were immobilized on a CM5 carboxyl chip using standard amine chemistry (NHS/EDC). Each surface was blocked with ethanolamine. OMP-59R5 was 2-fold serially diluted from 25 nM to 0.20 nM. For each immobilized surface, all 8 dilutions were sequentially injected and the binding assessed. The binding affinities were determined using a Biacore™ 2000. The data was fit using the simultaneous global fit equation to yield affinity constants (KD) for each protein.

***In Vivo* Animal Studies.** NOD/SCID mice were purchased from Harlan Laboratories (Indianapolis, Indiana) and maintained under specific pathogen-free conditions and provided with sterile food and water *ad libitum*. The animals used in this study were housed in a U.S. Department of Agriculture-registered facility in accordance with NIH guidelines for the care and use of laboratory animals. The mice were allowed to acclimate for several days prior to the studies. Freshly dissociated single cell suspensions or frozen cells were used for efficacy studies. Briefly, xenograft tumors were removed, cut into small pieces with a scalpel and minced with a sterile razor blade after removing necrotic portion and connective tissues. A digestion solution containing Collagenase III (10X) in MEM medium (Cambrex, East Rutherford, NJ) with a 1:100 dilution of DNAseI (Worthington, Lakewood, NJ) was mixed with the tumor fragments and incubated for 1.5-2 hour at 37oC for enzymatic dissociation with mechanical agitation with a pipette every 15 min. The reaction was inactivated by adding equal volume of FACS buffer and filtered through a 40μm mesh to remove aggregates and undigested tissues. Cells were centrifuged and resuspended in 1ml of ACK medium (0.15M NH4Cl, 10mM KHCO3, 0.1mM Na2EDTA in distilled water) on ice for 2 min to remove red blood cells. The reaction was terminated by adding FACS buffer and cells were centrifuged and resuspended in 1:1 (v/v) mixture of FACS buffer and Matrigel containing bFGF and VEGF (BD Bioscience, San Jose, CA).

For subcutaneous xenograft models, single cell suspension ranging from 5,000 to 100,000 cells/100 μL were implanted into the right flank region of 6-8 week old NOD/SCID mice with a 25-gauge needle. To determine the effect of antibody on established xenograft tumor growth, tumor-bearing animals were randomized and treatment started when the mean tumor volumes reached about 100 mm3. For pancreatic tumors, gemcitabine was given once a week between 10 mg/kg to 40 mg/kg depending on the sensitivity of the tumor. Taxol was given at 15 mg/kg weekly for breast and ovarian tumors. The antibody was given once every other week at 40 mg/kg throughout the course of study. Both antibodies and chemotherapeutic agents were administered intraperitoneally. To evaluate the effect of the antibody on tumor recurrence, animals were treated with gemcitabine at 70 mg/kg once a week in OMP-PN8 and 2.5 mg/kg cisplatin twice a week in OMP-Lu68 until the tumor regressed (about 3-4 weeks). Thereafter, animals were given either the control antibody or OMP-59R5 once every 2 week for additional 6-8 weeks. Subcutaneous tumor growth was measured with an electronic caliper (Coast Tools Company, San Leandro, CA). Tumor volumes were calculated with the formula (L xW2)/2, where L was the longest and W was the shortest axis of the tumor. Animal weights were recorded once a week. Mice were examined frequently for overt signs of any adverse drug-related side effects. Animals were weighed every day if they showed more than 15% body weight loss and euthanized if they showed 20% body weight. The mice were euthanized using a CO2 chamber followed by cervical dislocation. Tumors were harvested and stored for further analysis.

***In vivo* limiting dilution study.** Single cell suspensions from control and treated tumors were incubated with biotinylated mouse antibodies (α-mouse CD45-biotin 1:100 dilution and rat α-mouse H2Kd-biotin 1:50 dilution (BioLegend) on ice for 30 min followed by addition of streptavidin-labeled magnetic beads (Invitrogen, Carlsbad, CA) to remove mouse cells. The remaining human cells in the suspension were collected, counted and diluted to appropriate cell doses, mixed in the mixture of 1:1 (v/v) FACS buffer and Matrigel and injected subcutaneously in NOD/SCID mice. Mice were followed for up to 3 months.

**Vascular perfusion.** Mice received sixty mg/kg pimonidazole (Hypoxyprobe) via intraperitoneal injection and 100ug of Biotinylated Lycopersicon Esculentum (Tomato) Lectin (Vector Laboratories, B-1175) via intravenous tail vein injection, thirty and five minutes respectively, prior to necropsy. Two minutes preceding the lectin injection, mice were anesthetized with ketamine and xylazine, and following the five minute incubation with lectin, mice were perfused via direct injection into the left ventricle with two mL saline. Whole tumors were excised and processed into formalin-fixed, paraffin-embedded (FFPE) slides or as non-fixed Optimal Cutting Temperature (O.C.T., Tissue-Tek, Sakura)-embedded slides.

**Histology.** Formalin-fixed, paraffin imbedded sections were dewaxed and Heat Induced Epitope Retrieval (HIER) was performed in a pressure cooker (Biocare) using Citrate Buffer (Dako), blocked with 1% hydrogen peroxide in water and then treated for 30 minutes with CAS Block (Invitrogen) before primary antibody incubation. The primary antibody was anti-Ki-67 (Vector, VP-RM04) and was detected with an HRP conjugated antibody (Dako, EnVision+ System). Immunohistochemistry slides were scanned using Imagescope (ScanScope AT, Aperio) and analyzed for DAB positive nuclei vs. total hematoxylin positive nuclei, after excluding necrotic regions. For immuno-fluorescence, OCT blocks were processed to 10um slides and fixed for 15 minutes in 4% paraformaldehyde in PBS and blocked with CAS block. Primary antibodies included anti-CD31 (BD Biosciences, 550274), anti-Desmin clone D93F5 (Cell Signaling Technologies) and anti-pimonidazole (Hypoxyprobe) and were detected utilizing Alexa Fluor conjugated antibodies and preserved with Prolong Gold (Molecular Probes). Lectin was visualized with DyLight 488 Streptavidin (Vector, SA-5488). Desmin and CD31 co-localization was imaged on an Olympus FV10i confocal microscope. CD31, Desmin, pimonidazole, and lectin were imaged together using an Olympus BX51 microscope with a SensiCam Camera (Cooke). Monochromatic images were pseudocolored to produce the merged images (IPLabs, BD Biosciences). Lectin positive perfused vessel structures were manually counted using four random fields per tumor and three to four tumors per treatment group at 20x magnification.

**Western Blot Analysis.** Nuclear and cytoplasmic fractions of xenograft tumors were prepared using NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo Scientific). Whole tumor lysates were prepared by homogenizing tissues in Tissue Extraction Reagent I (Invitrogen) with lysing matrix D (MP Biomedicals). BCA assay was performed to quantify the protein. Seven to 10µg total protein was loaded per lane. Samples were separated on 4-12% NuPAGE Bis-Tris Midi gels, and transferred to PVDF membranes (Invitrogen).  Antibodies against the cleaved form of Notch3 intracellular domain (ICD) were generated by immunizing rabbits with a Notch3 ICD cleaved peptide. Western Blots were probed with rabbit anti-Notch3.ICD, anti-Notch2 rabbit mAb and anti-Notch3 rabbit mAb (1:1000, Cell Signaling Technology), and anti-beta-Actin mAb (1:10,000) (Sigma-Aldrich). Secondary antibodies were HRP-conjugated anti-rabbit (1:2000) or HRP-conjugated anti-mouse secondary antibodies (1:3000) (Cell Signaling Technology). Images were quantified using ImageJ software (NIH) and values expressed as relative to beta-Actin.