A Strategy to Improve Selectivity and Targeting to Epithelial-Derived Cancer Cells

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ABSTRACT

Examination of genomic and proteomic changes associated with *ras*-driven epithelial to mesenchymal transformation (EMT) of polarized epithelial cells has led to an improved understanding of surface-expressed structures and alterations in components involved in intracellular trafficking events that are altered as normal cells become cancerous. We have identified a mechanism involved in the establishment of cell-cell contracts known as tight junctions (TJs) which involves the orchestrating actions of the protein occludin (Ocln) and its ability to reverse EMT events. A screen for agents to re-activate suppressed occludin (*OCLN*) gene function caused by Ras/Raf/MEK/ERK pathway activation has led to the identification of several small molecules that can reverse cellular changes associated with EMT. Additionally, we had previously observed the up-regulation of a cell-surface expressed import system for small peptides, hPepT1, in pancreatic cancer cells which are known to be to be driven by the Ras/Raf/MEK/ERK pathway. We now describe two approaches to identify agents capable of re-activating Ocln expression which could be modified to be selectively absorbed by cancer cells which over-express functional hPepT1. Using this combined information of a drug that functions only in cancer cells having reduced Ocln expression with an up-regulated uptake mechanism that correlates reduced Ocln expression, we believe that it is possible to identify improved cancer chemotherapies that have reduced off-target drug distribution and thus great functional targeting.

INTRODUCTION

While the actual basis for metastasis is currently unclear (1), the impact of this event is clear: the majority of cancer-related deaths, up to 90%, can be attributed to disseminated disease rather than the impact of the primary tumor (http://www.cancerquest.org/metastasis-overview.html). At least half of the patients diagnosed with cancer present with clinically detectable metastatic disease (2). For these reasons, metastasis is considered the most life-threatening event in patients with cancer (3). Thus, strategies to selectively target disseminated disease have become a primary focus of drug delivery efforts. A standard approach to target these disseminated cancer cells is to use an agent that selectively targets to cancer cells to deliver a toxic payload; primarily antibodies that recognize a cancer-associated structure. It is clear, however, that previous efforts to selectively target cancer cells following systemic administration for the focused delivery of therapeutic agents have resulted in only a small fraction of these materials reaching metastatic sites (4). These approaches, while delivering measurable amounts of potent cytotoxic agents to intended sites typically result in substantial exposure of these agents to non-cancerous tissues as well.

Epithelial to mesenchymal transformation (EMT) is a reversible event, and that reversion is known as mesenchymal to epithelial transformation (MET). We have examined an underlying cellular pathway known to control endogenous mechanisms used by epithelial cells to transition between differentiated and mesenchymal formats in wound repair, replacement of senescent cells, etc (5). Importantly, the Ras/Raf/MEK/ERK pathway selected for our studies not only functions in reversible EMT↔MET process but has also been shown to drive oncogenic conversion following its un-regulated activation (6). We have taken the approach that epithelial cancers are a manifestation of cells driven into EMT, but that, for a variety of reasons, fail to transition back through MET into differentiated epithelial cells. Further, cellular events that "lock" epithelial cells in an oncogenic state might be reversible if the correct cellular "key' was identified. The approach taken in this report describes efforts made to find such a "key" and the equally important issue of finding a means to allow that key to efficiently find the "lock" in an oncogenic epithelial cell. We believe that this concerted approach could lead to novel strategies to selectively

modify cancer cell function, allowing for less cytotoxic methods to control these aberrant cells.

The first step to explore this strategy was to identify a cell-specific target that was critical in the reversible EMT↔MET processes that are also known to be un-regulated in epithelial cancers. It is critical that the EMT↔MET pathway process is understood sufficiently to allow transition of oncogenic epithelial cells back into a differentiated format. We had previously observed that epithelial cells driven to an oncogenic state by constitutively active Raf will down-regulate proteins involved in cell-cell interactions that function to signal the presence of and interaction with an adjacent epithelial cell. While most proteins involved in these cell-cell contacts were suppressed at the protein expression level, only one was observed in our model to be affected at the message level: occludin (Ocln). The significance of this observation is supported by studies showing that reduced levels of Ocln expression correlated with increased cancer progression (7-9). Importantly, forced expression of Ocln protein in the presence of continued expression of activated Raf could reverse the oncogenic phenotype and retrieve a functional epithelial cell (10).

Since that initial demonstration, much has been learned about the Raf/Ocln dynamic and the role played by Ocln as a counter-balance to the Ras/Raf/MEK/ERK pathway. Suppression of the Ras/Raf/MEK/ERK pathway can restore differentiated phenotype and functional TJs in a variety of cells types including transformed canine kidney cells (11), human corneal cells (12), mouse hepatocytes (13), and medullary thyroid carcinoma cells (14). Epigenetic silencing of *OCLN* promotes tumorigenic and metastatic properties of cancer cells involving and re-activation of endogenous Ocln expression makes these cells more susceptible to the induction of apoptosis (15). Further, Ocln functions to control premature senescence events; its forced expression in breast cancer cells induces anoikis and promotes senescence following oxidative stress (16). Overall, these observations support the possibility that inducing Ocln re-expression in cancer cells could lead to increased sensitivity of these cells to agents that could induce cell death via apoptosis and/or senescence pathways (15, 16), leading to a more focused and less toxic therapeutic approach.

The second aspect of our approach requires the identification of a specific target in cancer cells that can be selectively “unlocked” using some pharmacological agent. In our case, we looked for a means to re-activate Ocln expression. Previous studies have

shown that Hela cells treated with retinoic acid receptor α (RARα) agonists or treated with a de-methylating agent followed by a histone deacetylase inhibitor can re-express endogenous Ocln (16). Due to the broad effects produced by activation of RARα-mediated pathways or non-specific de-methylation agents, we have instead attempted to identify *OCLN* expression elements specifically regulated by Ras/Raf/MEK/ERK pathway activation. This pathway inactivates Ocln at the message level through the actions of the suppressor element Slug which interacts with a specific E-box sequence within the *OCLN* promoter; selective suppression of Slug being able to rectify Ocln expression suppressed by activated Raf (17). Thus, rectification of Ocln protein expression in cancer cells might be achieved by selective disruption of Slug function, a suppressor whose increased expression has been associated with epithelial cancers (18).

While chemotherapeutics are typically selected for their ability to enter all cells non-selectively, the final aspect of our strategy to identify a method that might be used to selectively deliver molecules that could affect Slug function to Ras/Raf/MEK/ERK pathway activated cancer cells. While re-activation of Ocln expression by Slug-mediated suppression should have little impact in differentiated epithelial cells with function TJ structures or non-epithelial cells, selective targeting of agents to re-activate Ocln expression should improve the safety to efficacy profile for the drugs being identified. Pancreatic cancer cells, commonly activated by the Ras/Raf/MEK/ERK pathway, strikingly up-regulate the nutrient di/tri-peptide import protein hPepT1 (19). Up-regulated hPepT1 has also been observed on pancreatic, prostatic, and gastric cancer cells and its function appears to be important for cell survival (20, 21), suggesting a critical role for hPepT1 expression in these cancer cells. Previous studies have demonstrated that small molecule can be made into hPepT1 substrates by chemical coupling to certain amino acids (22-24), suggesting that a variety of molecules identified in our studies might be made into a selective hPepT1 substrate.

Based upon the approach outlined above, we have examined two separate screening strategies to identify agents capable of re-activating *OCLN* expression and which might be made into hPepT1-selective substrates. One strategy used a phage display strategy to identify small peptides capable of disrupting Slug binding to the *OCLN* promoter. The other strategy was cell-based, using a cancer cell line stably expressing a reporter

gene constructed from the open reading frame of luciferase and the *OCLN* promoter. Together, these screening strategies have provided an initial assessment for the feasibility of our approach to identify reversible EMT↔MET process that can be regulated in-synch with agents that affect these processes and which could be selectively targeted based upon concurrent cellular modifications.

MATERIALS AND METHODS

Phage display

DNA and construction of genes.

Oligoduplexes used in affinity selection of phage-displayed peptides and in the Slug protein binding assays were an occludin (*OCLN*) gene promoter fragment containing E-box motif that is underlined. The Oligoduplexes were obtained by annealing the following oligonucleotide pairs: *OCLN* sense, 5'-catccgagtttcaggtgaattggtcaccg-3 and antisense, 5'-cggtgaccaattcacctgaaactcggatg-3; *OCLN* mutant, sense, 5'-catccgagtttcttcagaattggtcaccg-3 and antisense, 5'-cggtgaccaattctgaagaaactcggatg-3. The annealing reaction was carried out in a buffer (10 mM Tris-HCl, pH 7.4, 50 mM NaCl) at 94 ^{o}C for 5 min and then at room temperature for 40 min. Both biotinylated and non-biotinylated oligos were prepared.

A plasmid containing human slug gene was amplified by PCR using primers (sense, 5'-gcggatccccgcgctccttcctggtcaag-3' and antisense, 5'-ggaattcttagtgtgctacacagcagcc -3'), and subcloned into the BamHI/EcoRI site of pProEX HTb (InVitrogen). The resulted plasmid, denoted pSlug, was fused to green fluorescence protein (*gfp*) (Clontech) at the N-terminus of *slug*. *gfp* was amplified by PCR using primers (sense, 5'-gggatccatcgccaccatggtgagc-3' and antisense, 5'-gggatcccttgtacagctcgtccatgc-3'), and subcloned into the BamHI site of pSlug. The resulted plasmid was termed pgfpSlug.

Plasmid constructs containing Renilla luciferase (Rluc) reporter gene under the regulation of human occludin promoter were generated by a multistep process. Briefly, a full-length human *OCLN* promoter genomic DNA (1853 bp fragment containing -1511 to +342 bp from the transcription start site) was generated by PCR using primers (sense, 5"-cgtcgcgaattatgctaagtgaaagaagacag-3' and antisense, 5'-gcggatccgcgctctggacctggctggctctcgg) and OCLN/pGL3 (17) as a template. The PCR products were cut by NruI and BamHI and cloned into the NruI-BamHI site of pcDNA3.1(+) (Invitrogen). Insertion of this OCLN promoter DNA fragment resulted in the deletion of the CMV promoter region in the vector. Rluc gene (Promega) was generated by PCR using primers (sense, 5'-gcggatccgcaccatgacttcgaaagtttatgatcc-3' and antisense, 5'-gatctagagttattgttcatttttgagaactcgctcaacg-3'). The amplified R. luc gene fragment was cut by BamHI and cloned into the BamHI site of pCDNA3.1(+) in which the CMV promoter has been replaced by *OCLN* promoter. The resulted plasmids

were named as pOccRluc. A negative control construct (pRluc) containing Rluc under the control of CMV promoter was also made. All resulting constructs were verified by restriction enzyme digestions and DNA sequencing.

Protein expression and purification.

E.coli BL21 DE3 cells (Stratagene) were transformed with pgfpSlug. Protein expression was induced by addition of 1 mM isopropyl-D-thiogalactopyranoside (IPTG) when the culture OD_{600} reached 0.8.Three hours following IPTG induction, cells were harvested by centrifugation (4000 rpm for 15 min at 4 ^{o}C). The cell pellet was re-suspended in 30 ml of cold lysis buffer (20 mM Tris-HCl, pH 8.0, 20% glycerol, 0.5 M NaCl, 1 mM TCEP, one EDTA-free protease inhibitor tablet). The cell suspension was sonicated on ice, combined with NP-40 (final concentration of 1%) and rocked for 30 min at 4 ^{o}C. The lysate was centrifuged at 14,000 rpm at 4^{o}C for 30 min. The supernatant was incubated with 1 ml of pre-equilibrated (lysis buffer +NP-40) Ni-NTA agarose beads (Qiagen) for 2 hours at 4 ^{o}C with gentle rocking. The suspension was then spun at 2000 rpm and pelleted beads were washed 5 times with 10 ml of cold wash buffer (20 mM Tris-HCl, pH 8.0, 20% glycerol, 0.5 M NaCl, 1 mM TCEP, 15 mM imidazole). Resins were transferred to a poly-prep column (BioRad) and protein was eluted with 5 bed volumes of elution buffer (20 mM Tris-HCl, pH 8.0, 10% glycerol, 0.1 M NaCl, 1 mM TCEP, 250 mM imidazole). The purified protein was analyzed by SDS-PAGE and Western blot.

Assaying for Slug protein binding to E-box DNA.

The procedure for assaying Slug binding to E-box DNA is shown in Figure 1. Briefly, DNA oligoduplexes used in affinity selection of phage-displayed peptides and in Slug binding assays were annealed as described above. 96-well plates were coated with streptavidin (10 µg/ml, 100 µL/well) at 4 ^{o}C overnight. The plates were washed three times with washing buffer (PBS, 0.05% Tween-20), and blocked with blocking buffer (PBS, 0.05% Tween-20, 5% milk). Eight µg of Slug fusion protein was incubated together with 1 µM of biotinylated or unbiotinylated DNA ligands in binding buffer (10 mM Tris-HCl, pH 7.4, 50 mM NaCl, 5% glycerol, 1 mM EDTA, 4 mM DTT) on ice for 30 min in a volume of 200 µL. The mixtures were transferred into plates coated with strepavidin, and incubated at room temperature for 1 h. Captured Slug-DNA complexes were washed 3 times (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween-20), and

detected using a microplate reader (BioTek) for fluorescence, or with HRP-conjugated anti-GFP antibody for luminescence.

Generation of stable cell lines.

The pOccRluc and control constructs pRluc and pcDNA3.1 were transfected into human non-small lung carcinoma cell line H460 and cervix adenocarcinoma cell line Hela (ATCC) using transfection reagent Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Stable cell lines were selected in 500 μg/mL hygromycin, validated for the integration of the chimeric gene into the cell genome by genomic PCR, and maintained in DMEM medium supplemented with 300 μg/mL hygromycin. Integration of plasmid pOccRluc was further functionally verified by treating the cells with MEK inhibitor PD98059 which resulted in increased luciferase expression using a commercial kit (Promega).

Compound screening protocols.

Cell-based reporter screening system. H460-pOccRluc, along with positive control H460-pRluc, and negative control H460-pcDNA3.1 cells were seeded at 8000 cells/well in 100 μL DMEM complete medium in 96-well white with clear bottom plates. Plates were made up in triplicate, seeded on day 1, treated on day 2 with test compounds, and assayed on day 4. Test compounds were tested at 5 μM, being added in DMSO (final concentration of 0.4%); with control wells receiving an equivalent amount of DMSO. The addition of DMSO to some wells was also used to serve as a negative control. After 48 hr of test compound exposure, the medium was aspirated, and cells were lysed with 30 μL/well fresh-prepared lysis buffer (150 mM HEPES, pH 8.0, 0.05% Triton X-100, 1 mg/ml Porcine Gelatin, pH 7.6, 10% Glycerol) at room temperature for 10 min with shaking. 30 μL of 1X Renilla luciferase assay substrate working solution (0.01 mg/ml Renilla luciferase assay substrate Coelenterazine, 5M NaCl, 0.5M KH_2PO_4, 0.1M EDTA, pH 8.0, 1mg/ml Porcine Gelatin, pH7.6) was then added to each well and luminescence was read immediately using a luminometer (BioTek).

Protein-based screening system. Test compounds were first incubated in binding solution with E-box DNA or control biotin-labeled oligoduplexes. After addition to steptavidin-coated plates, these oligoduplexes were exposed to eight ug of GFP-Slug. After washing with washing buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween-20), the presence of materials capable of competing for the Slug binding site on the

occludin promoter E-box was monitored by the lack of or reduced fluorescent signal for bound GFP-Slug using a fluorimeter. Biotin-labeled E-box oligoduplexes without test compounds served as a positive control, while a mutant E-box sequence lacking the ability to bind Slug (17) and non-biotinylated E-box oligoduplex served as negative controls.

Phage display. Two phage display peptide libraries, Ph.D.-7 and -12 (New England Biolabs), were used to generate random 7-mer or 12-mer peptide sequences in the N-terminal of pVIII coat protein of M13 phage. The phage selection procedure, based on affinity purification, was performed as described in the manufacturer's manual and with modifications (25, 26). The target, 1 μM biotin-labeled occludin promoter E-box oligoduplex, was incubated with $2x10^{11}$ phage in a TBST solution (50 mM Tris-HCl pH7.5, 150 mM NaCl, 0.1% (v/v) Tween-20) in a final volume of 400 μL for 1 h at room temperature. Phage/DNA mixtures were then added to 100 μL of 1 mg of streptavidin-coated paramagnetic beads (Dynabeads M-280 streptavidin, Invitrogen) previously washed three times in 500 μL of TBST and incubated for additional 30 min before the beads were collected and washed five times with 500 μL of ice-cold TBST. In the last wash, beads were transferred to a clean tube and eluted with 100 ul of elution buffer (0.2 M Glycine-HCl, pH 2.2, 1 mg/ml BSA) for 10 min and neutralised with 15 μL of 1M Tris-HCl (pH 9.1). An aliquot (1 ul) of eluted phage was used for micro-titration, with the remaining phage being amplified for next rounds of bio-panning. The selection procedure was repeated for two additional rounds. In the first round, binding and washing reactions were in TBS with 0.1% (v/v) Tween-20, but in the second and third rounds TBS with 0.5% Tween-20 were used. Single-stranded DNA was then prepared to detect consensus binding sequences.

Validation of selected peptide phages for target binding by ELISA. One clone from each group of identified target binding peptides containing a consensus sequence was selected and assayed for DNA target binding by ELISA. Briefly, the phage-containing supernatants, saved when carrying out the plaque amplification for DNA sequencing, were amplified and purified. Biotinylated E-box DNA was incubated with the phage in solution. The mixtures were transferred into a streptavidin-coated ELISA plate. Bound phage-DNA complexes were detected with HRP-conjugated anti-M13 antibody (Pharmacia) and a microplate reader set at 410 nm.

Assay of selected peptides for competitive inhibition of Slug binding to E-box DNA. After the validation of selected peptide phages for target binding by ELISA, identified E-box-binding peptide variants were synthesized by American Peptide Co., and tested for their ability to compete with Slug for binding to the E-box DNA. Specifically, biotinylated E-box DNA, each peptide to be characterized, and Slug protein were incubated in binding buffer on ice for 30 min. The mixtures were transferred into plates coated with strepavidin and incubated at room temperature for 1 h. The captured peptide-DNA or Slug-DNA complexes were washed 3 times and detected as described above. In another experiment, Slug and E-box DNA were first incubated on ice for 30 min, the testing peptides were added, and then the ability of the peptides to remove Slug from the E-box DNA was determined as described above.

HYPOTHESIS AND RATIONALE OF STUDIES

***Hypothesis behind strategy to find agents to rectify cell-cell contacts*.** Normal epithelia undergo dynamic changes that involve transient periods of de-differentiation that allows for mitosis and cell migration followed by differentiation to re-establish functional barriers (Fig 1A). Such events are essential for development, wound repair, and replacement of senescent cells. Our hypothesis centers on the idea that epithelial cancer cells have entered into the de-differentiation pathway but have lost the ability to re-establish functional barriers required for re-establishment of a differentiated phenotype (Fig 1B). A variety of factors could block the re-establishment of functional barriers that drive differentiation (27). We have focused on identifying ways to reverse EMT events with the outcome of potentially overcoming drug resistance to various anti-cancer agents (28) or making cancer cells significantly more susceptible to these agents.

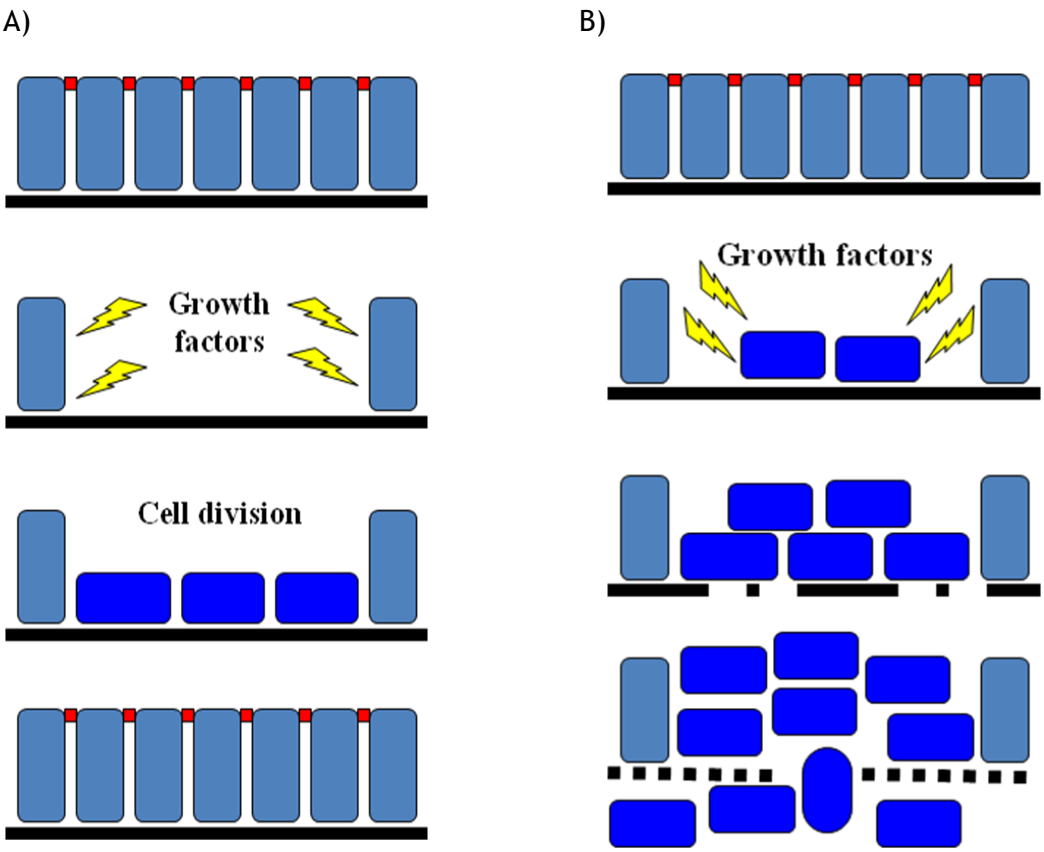


Figure 1. Hypothesis for epithelial oncogenesis based upon an inability to reverse genotypic and phenotypic events driving epithelial to mesenchymal transformation (EMT). A) Normal sequence of events at an epithelium where lesions or senescence leads to a loss of differentiated epithelial cells resulting in compromised barrier function. Note the role of growth factors to drive cell division of un-differentiated cells prior to their re-differentiation following the establishment of functional cell-cell contacts. B) In the presence of constitutive growth drivers (or lack of growth suppressors), epithelial cells fail to re-differentiate and continue to grow, possibly with the capability to migrate to distant sites in the body following a breach of the basement membrane. Tight junction complexes (red box); Basement membrane (solid black bar); Basement membrane penetrated by oncogenic epithelial cells (hatched black bar).

In the present studies we have focused on the mechanism used by active *raf* to down-regulation of tight junction (TJ) structures, with particular attention on the TJ protein Ocln which appears to have orchestrating capability in the formation of nascent TJs

(10). Using an epithelial cell model, we have determined that activation of the Ras/Raf/MEK/ERK pathway results in an increase in function of the repressor protein Slug which directly blocks *OCLN* transcription to maintain epithelial cells in a de-differentiated form (17). As a result of this de-differentiation, epithelial cells should express a distinct set of surface elements, some of which might be used to target pro-drug agents to these non-polarized cells following systemic administration. We had examined potential uptake mechanisms in pancreatic cancer cell lines with the intent of identifying such an uptake mechanism and made the observation that a low-affinity, high-capacity, poorly-selective uptake protein, known as PepT1, was over-expressed on these cells (19). Thus, a rationale exists to search for either small peptide-like agents that might enter cancer cells through PepT1 and block slug-mediated suppression of the *OCLN* promoter or for small molecules that could re-activate *OCLN* expression. Once reaching epithelial cancer cells, the agent would induce functional TJ structures to be re-established, leading to suppression of cancer cell growth and potentially an increased susceptibility to chemotherapeutic agents (Fig. 2).

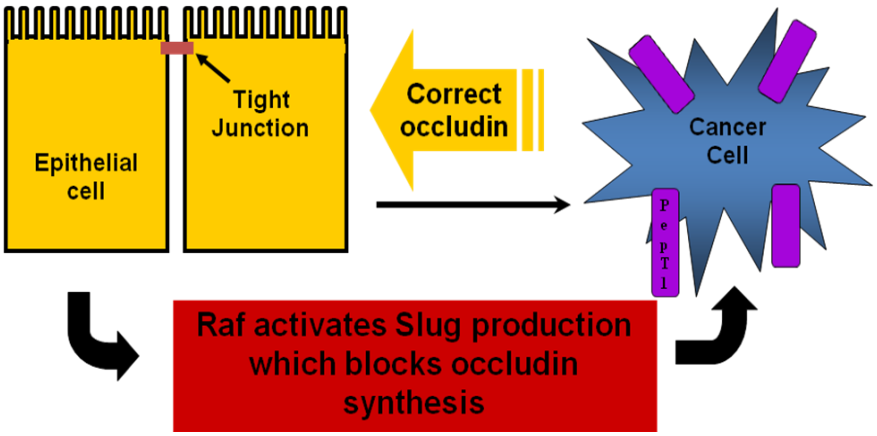


Figure 2. Conceptual cartoon depicting how disorganization of tight junction function leads to oncogenic conversion and how restitution of function tight junctions through the orchestrating actions of occludin (Ocln) can lead to rectification of differentiated epithelial cells.

RESULTS

Activity of hPepT1 required for functional targeting. While hPepT1 is strikingly up-regulated at the protein level in pancreatic cancer cell lines (19), we wished to better understand the nature of hPepT1 function in response to Ras/Raf/MEK/ERK pathway activation. We used a non-cancerous rat salivary gland epithelial (Pa4) cells induced to undergo EMT by the introduction of constitutively active Raf (Pa4-Raf1) that were then induced to revert through MET program following in the forced expression of Ocln (Pa4-Raf1-Ocln) as previously described (10). We compared the gene expression profiles of Pa4, Pa4-Raf1, and Pa4-raf1-Ocln cells using a commercial array from Paradigm Genetics Inc (Research Triangle Park, NC), focusing on PepT1 and elements known to regulate its function. Although PepT1 was not significantly altered in this array comparison (data not shown), two genes known to regulate Pept1 function were tightly regulated in the Raf/Ocln dynamic: Slc9a5 and Prkaa2. Scl9a5 is an isoform of the Na^+/H^+-exchanger whose activity is critical to maintain the proton gradient required for optimal Pept1 function (29). The mRNA level of Scl9a5 was observed to increase 1.5-fold following EMT induced by constitutively active Raf1 (Pa4→Pa4-Raf1) and to decrease in a comparable fashion (1.7-fold) in Raf1-active cells following the forced expression of Ocln Pa4-Raf1→Pa4-Raf-1-Ocln). Prkaa2 is an AMP-activated protein kinase that suppresses the activity of hPept1 (22). The mRNA level of Prkaa2 decreased 2.1-fold following EMT induced by constitutively active Raf1 and increased 2.7-fold in Raf1-active cells following in the forced expression of Ocln. Together, these results suggest that cellular processes that control hPepT1 activity are positioned to ensure its increased activity following EMT driven by Ras/Raf/MEK/ERK pathway activation and more tightly regulated function when expressed in differentiated epithelial cells.

Validation of Slug-E-box Binding Assay. A method to monitor Slug binding to the E-box domain promoter region of *OCLN* was validated through a series of positive and negative control studies (Table 1). As a positive control, biotin-labeled E-box oligoduplex of *OCLN* promoter DNA was incubated with a Slug-green fluorescent proteins (GFP) protein chimera (Fig. 3). Complexes formed in solution were captured on surfaces conjugated with streptavidin and detected using a horseradish peroxidase (HRP) conjugated antibody that recognized GFP; a signal that could be reduced by the inclusion of an equal amount of E-box DNA lacking biotin (Table 1). In addition, assay

output signals were independent of addition sequence for biotin-labeled or unlabeled forms of *OCLN* E-box DNA when mixing with Slug-GFP, suggesting that the interaction between *OCLN* E-box DNA and Slug is dynamic and thus reversible (data not shown). To verify specificity, Slug-GFP was incubated with two different mutant forms of biotin-labeled occludin E-box oligos (Table 1). For a constant amount of *OCLN* E-box sequence DNA, Slug-GFP bound in a dose-dependent manner; saturated binding occurred at ~8 µg, a ratio that was used in all subsequent studies (Table 2).

Table 1. Validation of specificity for Slug interaction with human *OCLN* E-box.

Treatment	Slug only	E-Box only	Slug + E-Box	Slug + E-Box mutant 1	Slug + E-Box mutant 2	Slug + E-Box*	Slug + E Box +E-Box*
Luminescence	4	0	340	4	7	3	145

E-box: Biotin-catccgagtttcaggtgaattggtcaccg

E-box*: -catccgagtttcaggtgaattggtcaccg

E-box mutant 1: Biotin-catccgagtttcttcagaattggtcaccg

E-box mutant 2: Biotin-catccgagtttaaggtaaattggtcaccg

Table 2. Slug-GFP binds to the *OCLN* E-box in a dose-dependent manner.

Slug (µg/well)	0.1	0.2	0.4	0.8	1	2	4	6	8	10	15	20
Luminescence	45	45	90	130	145	200	240	265	280	280	290	260

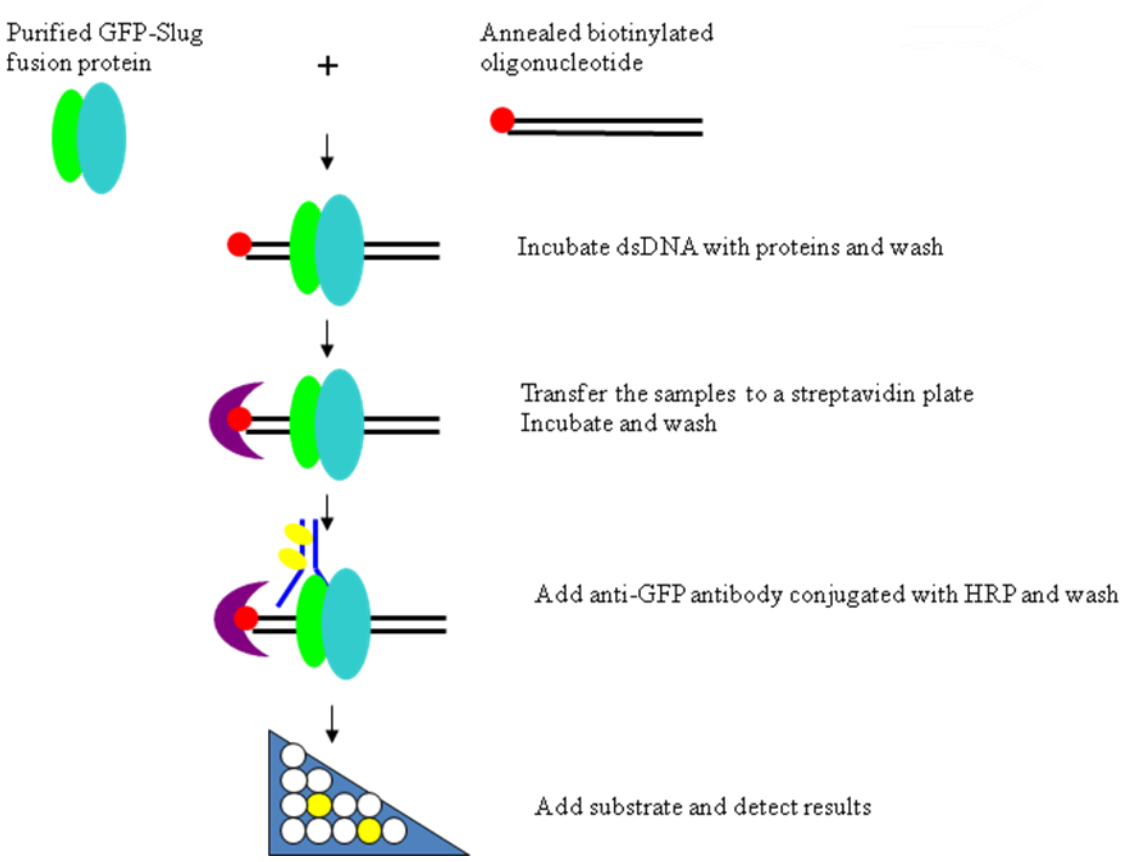


Figure 3. Diagram of screening method to identify molecules and short peptides capable of blocking interactions between Slug and the E-box region of the promoter region of *OCLN*. The gene for human Slug was genetically fused to green fluorescent protein (GFP) and the resulting chimera was used in a 96-well plate format to evaluate small molecules or short peptides identified through panning with phage that displayed random formats of either 7 or 12 amino acid peptide sequences.

Phage panning with OCLN E-box DNA. Ph.D.-7 and Ph.D.-12 phage peptide libraries were panned using biotin-labeled *OCLN* E-box DNA (Table 1). Three rounds of panning were carried out in each screen with the Ph.D.-12 library being screened once and the Ph.D.-7 library being screened twice. Subtraction for E-box specificity was performed by a binding step using biotin-labeled DNA sequence containing a mutant E-box sequence shown not to interact with Slug (17). Ten individual phages from the third round of each panning were propagated with DNA encoding the insert in the fusion protein (pVIII) being sequenced and translated into amino acid sequences (Table 3). There were significant variations in the amino acid sequences that selectively bound

to *OCLN* E-box DNA, although some sequences were enriched with a frequency as high as 30%. Closer inspection, however, was used to identify short peptide sequences of 2-5 amino acids that repeated within the 7- or 12- amino acid phage libraries as well as between these libraries. These similarities are highlighted with underlining (Table 3).

Table 3. Peptide sequences identified through Phage panning that bind to the *OCLN* E-box sequence.

Library	Peptide sequence	Frequency
Ph.D.-7	YSLRLDY	0.25
	KCCFPIS	0.15
	KCCFINA	0.1
	KCCYVPL	0.1
	KCCYATH	0.05
	QAPTRVQ	0.05
	APTAVSK	0.05
	KVWQIVT	0.05
	KVWLLPP	0.05
	RPPVFHM	0.05
	NKTIMAL	0.05
	IPKHTYR	0.05
Ph.D.-12	KHTFIDYLVSQA	0.3
	FNDRPVLSYMNL	0.1
	KCCFHSTENPHP	0.1
	HMDYYNWRRLSL	0.1
	LRSKIRIPLNRR	0.1

By combining data from the Ph.D-7 and -12 phage studies, the sequence (Iso/Leu)-Asp-Tyr was found to be present in 55% of all the phage sequenced. Further examination of the Ph.D-12 phage studies suggested that this sequence could be extended with a preceding phenylalanine and a following valine: Phe-(Iso/Leu)-Asp-Tyr-X-Val. Another sequence observed in both the Ph.D.-7 and -12 phage panning was Lys-Cys-Cys-(Phe/Tyr) which occurred with an overall frequency of 50% (Table 3). Other amino acids sequences repeated within sequences phage were Arg-Arg (20%), Ala-Pro-Thr-X-Val (10%), Lys-Val-Trp (10%), and Pro-Pro (10%). Together, this data set is consistent

with the idea that a defined set of structures exists within the *OCLN* E-box DNA that can be selectively targeted through distinct peptide sequences.

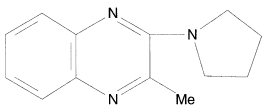
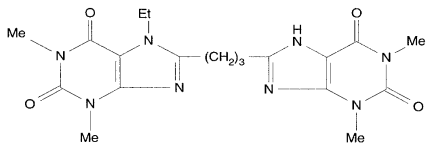
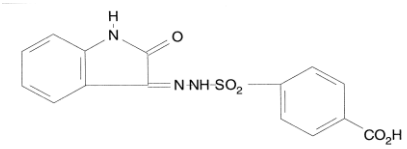
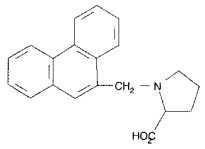
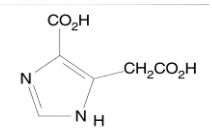
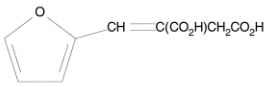
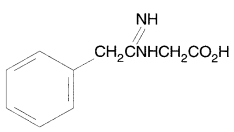
Inhibition of Slug binding to E-box oligoduplex. To determine whether peptides selected by phage display outcomes could inhibit Slug binding to the *OCLN* E-box, three peptides with the sequences Thr-Phe-Iso-Asp-Tyr-Pro-Val-Leu-Ser (TFIDYPVLS), Lys-Cys-Cys-Phe-Pro-Iso-Ser-Thr-Glu (KCCFPISTE), and Gln-Pro-Pro-Thr-Val-Ser-Val-Ser-Lys (QPPTVSVSK) were synthesized (Table 3). The peptide Iso-Glu-Asp-Gly-Ser-Val-Gly-Leu-Ala (IEDGSVGLA) was used as a negative control (Fig. 3). About 65% of Slug binding to target DNA was blocked by TFIDYPVLS or KCCFPISTE but only a 42 % reduction in Slug binding resulted in the presence of QPPTVSVSK (Fig. 3). Interestingly, others peptides synthesized that emulated the zinc finger domains of Slug {Stegmann, 1999 #60} only reduced GPF-Slug binding by ~30% (data not shown).

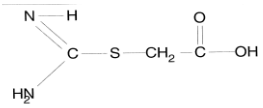
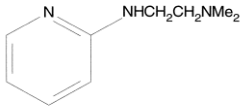
Identification of Ocln re-activating molecules. Additional agents capable of re-activating Ocln expression were identified using cancer cells modified to contain a chimeric gene composed of the Ocln promoter and the luciferase open reading frame. De-repression of the Ocln promoter suppressed by active Ras/Raf/MEK/ERK function in these cells resulted in luciferase-induced light as a high-throughput screening (HTS) readout. This HTS method was use to screen a chemical library obtained from the National Cancer Institute (NCI). After two screening rounds, 15 hits were identified. These hits included the compounds PD98059, U0126, and Triciribine. Both PD98059 and UO126 inhibit MEK1 and MEK2 kinases to represses anchorage-independent growth (30). Triciribine, an Akt kinase inhibitor, can induce apoptosis and inhibit cell growth (31). De-repression of *OCLN* promoter function by these kinase inhibitors functioned as positive controls, validating the HTS protocol used. Increased Ocln protein levels, corresponding to increased luciferase activity, were confirmed by Western blotting for all hits (data not shown).

Identification of Ocln re-activating molecules. A number of compounds identified from the NCI chemical library were effective at 10 μM with the ability to out-perform the positive control of 20 μM PD98059 for luciferase induction. These compounds induced Ocln expression, a MET morphology change, and growth suppression (Table 4). Several other compounds were identified in the screen out-performed 20 μM PD98059 when tested at 100 μM but failed to have an effect when tested at 10 μM. Another cadre of molecules induced MET morphology change and growth suppression without

producing an effect on luciferase expression greater than that observed for PD98059. This screening approach was unbiased toward mechanism of action. Most compounds identified at hits had the ability to induce morphology changes consistent with MET and growth suppression which correlated well with re-activation *OCLN* promoter function. A few molecules with growth suppressive function did not correlate with *OCLN* activation; this group of compounds was not pursued further.

Table 4. High throughput screening hits. Compound code name, molecular formula, molecular weight, and compound structure information were provided by the library source: the National Cancer Institute. Hits listed out-performed the MEK inhibitor PD98059 at 20 μM for induction of luciferase expression. Morphology change consistent with MET, and extent of growth suppression are listed.

Code Name Mol. Formula Mol. Weight	Compound Structure	MET; Growth inhibition at effective conc.
42846-F $C_{13}H_{15}N_3$ 213 Da		Yes ~ 40% at 10 μM
23583-Q $C_{19}H_{24}N_8O_4$ 428 Da		Yes ~ 60% at 10 μM
133488-F $C_{15}H_{11}N_3O_5S$ 345 Da		ND Effective at 10 μM
102728-T $C_{20}H_{19}NO_2$ 342 Da		ND Effective at 10 μM
40384-C $C_6H_6N_2O_4$ 170 Da		Yes ~ 45% at 100 μM
61735-L $C_9H_8O_5$ 196 Da		Yes ~ 40% at 100 μM
27895-C $C_{10}H_{12}N_2O_2$ 192 Da		Yes ~ 55% at 100 μM

403734-Y **$C_3H_6N_2O_2S$** **134 Da**		**Yes** **~ 55% at 100 µM**
30154-J **$C_9H_{15}N_3$** **165 Da**		**Yes** **~ 55% at 100 µM**

Examination of hits identified from the NCI chemical library led to the recognition of a few recurring structural elements. Compounds capable of affecting MET and cancer cell growth at 10 µM contained several aromatic ring moieties that were modified by either methyl or carboxyl groups. Interestingly, compounds that failed to affect MET and cancer cell growth at 10 µM but did so when tested at 100 µM were smaller, simpler compounds than the first group. Except in one case, these smaller, less active compounds also contained an aromatic structure and either a methyl and carboxyl group. Importantly, the size and nature of compounds identified in this screen were amenable for modification to become selective substrates for hPepT1.

DISCUSSION

Herein we describe the rationale behind a novel approach to improve the selectivity and targeting of molecules that could induce an MET event in epithelial cancer cells and take initial steps toward the validation of that approach. In some ways, this approach is similar to that proposed by others who have examined genes associated with EMT events as a strategy to identify possible therapeutic targets for treating ductal pancreatic adenocarcinoma (28). Our strategy extends previous approaches by taking advantage of two important observations: MET events driven by Ras/Raf/MEK/ERK pathway activation can be reversed by the expression of the TJ protein Ocln (10) and pancreatic epithelial cancer cells can up-regulate expression of the nutrient di/tri-peptide import protein hPepT1 (19). At the intersection of these observations rests the idea that compounds resembling hPepT1 substrates could be selectively taken up by this transporter over-expressed on certain types of epithelial cancer cells. In the present studies, we have presented evidence suggesting that hPepT1 is not only up-regulated at the protein level but that the cellular conditions following EMT provide an environment for increased functional expression of this

uptake pathway. Further, we describe strategies to identify compounds which might specifically affect EMT↔MET events by re-activating Ocln expression suppressed due to the activation of the Ras/Raf/MEK/ERK pathway and which could directly or be modified to function as hPepT1 substrates.

Two types of screens were established to identify Ocln re-activating molecules; both were based upon recent findings that defined the mechanism used by cells to suppress Ocln expression following Ras/Raf/MEK/ERK pathway activation. One screening format was based upon selectively disrupting the interaction between Slug and the E-box motif of the *OCLN* promoter. Phage panning was used to identify short peptide sequences capable of inhibiting Slug/E-box motif interactions and the ability of peptides identified in this way were verified to block Slug binding to the E-box motif of the *OCLN* promoter. In this next stage of this program, minimal peptide structures that retain the ability to block Slug/E-box motif interactions will be identified through a rational design approach based upon these peptide sequences identified in this screen.

A second screen employed a Raf1-driven epithelial cell with an inserted reporter chimera gene that allowed for the expression of luminescence signal following re-activation of *OCLN* promoter function. Not being focused to a particular element of the dynamic established between the Ras/Raf/MEK/ERK pathway and the Ocln protein, this screen led to the identification of compounds from a chemical library whose mechanism of action would be unknown. The unbiased outcomes from this screen could be grouped into two categories: those that affected MET and cancer cell growth at 10 μM and those that produced these same outcomes but only at higher concentrations, in this case 100 μM. Subsequent studies will involve making a series of modifications to these hits to indentify critical elements for function in order to develop potential lead candidates.

Overall, these studies have described not only a strategy to identify a novel class of anti-cancer agents but provided *in vitro* evidence that cancer cells can be affected by molecules identified through these screening strategies. These screens were performed with the intent of identifying hits that could be developed into potential lead candidates. To identify these leads, we will combine information obtained from peptide sequences capable of blocking Slug binding to the OCLN promoter E-box with that of small molecule compounds identified from an NCI chemical library. The

approach described represents a unique strategy where a selective uptake pathway up-regulated in cancer cells driven by a particular oncogene pathway is integrated into the lead development as a means of targeting the delivery of compounds that functionally block an MET suppression system. In this case, we have used the specific relationship between the Ras/Raf/MEK/ERK pathway and the TJ protein Ocln to allow for the selective targeting and reversal of EMT processes. Once cells revert via MET phenotype changes, we believe they may 1) stop growing aggressively with a reduced potential for metastasis (1, 14), and 2) be more susceptible to stress agents currently being tested as chemotherapeutics. The dynamic used in our studies should be useful to treat epithelial cancers derived from the pancreas, stomach, and prostate where up-regulation of the Ras/Raf/MEK/ERK pathway can be confirmed. It is possible that other cancer cells may be similarly targeted and treated via other dynamics established to control EMT↔MET events, such as those involving the PI3K/PTEN/Akt/mTOR pathway (6, 31).

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REFERENCES

1. Hunter KW, Crawford NP, Alsarraj J. Mechanisms of metastasis. Breast Cancer Res. 2008;10 Suppl 1:S2.
2. DeVita VT, Jr., Young RC, Canellos GP. Combination versus single agent chemotherapy: a review of the basis for selection of drug treatment of cancer. Cancer. 1975 Jan;35(1):98-110.
3. Martin TA, Jiang WG. Loss of tight junction barrier function and its role in cancer metastasis. Biochim Biophys Acta. 2009 Apr;1788(4):872-91.
4. Bae YH, Park K. Targeted drug delivery to tumors: myths, reality and possibility. J Control Release. 2011 Aug 10;153(3):198-205.
5. Micalizzi DS, Farabaugh SM, Ford HL. Epithelial-mesenchymal transition in cancer: parallels between normal development and tumor progression. J Mammary Gland Biol Neoplasia. 2010 Jun;15(2):117-34.
6. Chappell WH, Steelman LS, Long JM, Kempf RC, Abrams SL, Franklin RA, et al. Ras/Raf/MEK/ERK and PI3K/PTEN/Akt/mTOR inhibitors: rationale and importance to inhibiting these pathways in human health. Oncotarget. 2011 Mar;2(3):135-64.
7. Tobioka H, Tokunaga Y, Isomura H, Kokai Y, Yamaguchi J, Sawada N. Expression of occludin, a tight-junction-associated protein, in human lung carcinomas. Virchows Arch. 2004 Nov;445(5):472-6.
8. Tobioka H, Isomura H, Kokai Y, Tokunaga Y, Yamaguchi J, Sawada N. Occludin expression decreases with the progression of human endometrial carcinoma. Hum Pathol. 2004 Feb;35(2):159-64.
9. Tokunaga Y, Tobioka H, Isomura H, Kokai Y, Sawada N. Expression of occludin in human rectal carcinoid tumours as a possible marker for glandular differentiation. Histopathology. 2004 Mar;44(3):247-50.
10. Li D, Mrsny RJ. Oncogenic Raf-1 disrupts epithelial tight junctions via downregulation of occludin. J Cell Biol. 2000 Feb 21;148(4):791-800.
11. Chen Y, Lu Q, Schneeberger EE, Goodenough DA. Restoration of tight junction structure and barrier function by down-regulation of the mitogen-activated protein kinase pathway in ras-transformed Madin-Darby canine kidney cells. Mol Biol Cell. 2000 Mar;11(3):849-62.
12. Wang Y, Zhang J, Yi XJ, Yu FS. Activation of ERK1/2 MAP kinase pathway induces tight junction disruption in human corneal epithelial cells. Exp Eye Res. 2004 Jan;78(1):125-36.
13. Lan M, Kojima T, Osanai M, Chiba H, Sawada N. Oncogenic Raf-1 regulates epithelial to mesenchymal transition via distinct signal transduction pathways in an immortalized mouse hepatic cell line. Carcinogenesis. 2004 Dec;25(12):2385-95.
14. Ning L, Kunnimalaiyaan M, Chen H. Regulation of cell-cell contact molecules and the metastatic phenotype of medullary thyroid carcinoma by the Raf-1/MEK/ERK pathway. Surgery. 2008 Dec;144(6):920-4; discussion 4-5.
15. Osanai M, Murata M, Nishikiori N, Chiba H, Kojima T, Sawada N. Epigenetic silencing of occludin promotes tumorigenic and metastatic properties of cancer cells via modulations of unique sets of apoptosis-associated genes. Cancer Res. 2006 Sep 15;66(18):9125-33.
16. Osanai M, Murata M, Nishikiori N, Chiba H, Kojima T, Sawada N. Occludin-mediated premature senescence is a fail-safe mechanism against tumorigenesis in breast carcinoma cells. Cancer Sci. 2007 Jul;98(7):1027-34.

17. Wang Z, Wade P, Mandell KJ, Akyildiz A, Parkos CA, Mrsny RJ, et al. Raf 1 represses expression of the tight junction protein occludin via activation of the zinc-finger transcription factor slug. Oncogene. 2007 Feb 22;26(8):1222-30.
18. Shih JY, Yang PC. The EMT regulator slug and lung carcinogenesis. Carcinogenesis. 2011 Sep;32(9):1299-304.
19. Gonzalez DE, Covitz KM, Sadee W, Mrsny RJ. An oligopeptide transporter is expressed at high levels in the pancreatic carcinoma cell lines AsPc-1 and Capan-2. Cancer Res. 1998 Feb 1;58(3):519-25.
20. Mitsuoka K, Kato Y, Miyoshi S, Murakami Y, Hiraiwa M, Kubo Y, et al. Inhibition of oligopeptide transporter suppress growth of human pancreatic cancer cells. Eur J Pharm Sci. 2010 Jun 14;40(3):202-8.
21. Mitsuoka K, Miyoshi S, Kato Y, Murakami Y, Utsumi R, Kubo Y, et al. Cancer detection using a PET tracer, 11C-glycylsarcosine, targeted to H+/peptide transporter. J Nucl Med. 2008 Apr;49(4):615-22.
22. Pieri M, Christian HC, Wilkins RJ, Boyd CA, Meredith D. The apical (hPepT1) and basolateral peptide transport systems of Caco-2 cells are regulated by AMP-activated protein kinase. Am J Physiol Gastrointest Liver Physiol. 2010 Jul;299(1):G136-43.
23. Landowski CP, Vig BS, Song X, Amidon GL. Targeted delivery to PEPT1-overexpressing cells: acidic, basic, and secondary floxuridine amino acid ester prodrugs. Mol Cancer Ther. 2005 Apr;4(4):659-67.
24. Tsume Y, Hilfinger JM, Amidon GL. Enhanced cancer cell growth inhibition by dipeptide prodrugs of floxuridine: increased transporter affinity and metabolic stability. Mol Pharm. 2008 Sep-Oct;5(5):717-27.
25. Marks JD, Hoogenboom HR, Griffiths AD, Winter G. Molecular evolution of proteins on filamentous phage. Mimicking the strategy of the immune system. J Biol Chem. 1992 Aug 15;267(23):16007-10.
26. Choo Y, Klug A. Designing DNA-binding proteins on the surface of filamentous phage. Curr Opin Biotechnol. 1995 Aug;6(4):431-6.
27. Royer C, Lu X. Epithelial cell polarity: a major gatekeeper against cancer? Cell Death Differ. 2011 Sep;18(9):1470-7.
28. Hotz HG, Hotz B, Buhr HJ. Genes associated with epithelial-mesenchymal transition: possible therapeutic targets in ductal pancreatic adenocarcinoma? Anticancer Agents Med Chem. 2011 Jun;11(5):448-54.
29. Thwaites DT, Anderson CM. H+-coupled nutrient, micronutrient and drug transporters in the mammalian small intestine. Exp Physiol. 2007 Jul;92(4):603-19.
30. Crews CM, Alessandrini A, Erikson RL. The primary structure of MEK, a protein kinase that phosphorylates the ERK gene product. Science. 1992 Oct 16;258(5081):478-80.
31. Yang L, Dan HC, Sun M, Liu Q, Sun XM, Feldman RI, et al. Akt/protein kinase B signaling inhibitor-2, a selective small molecule inhibitor of Akt signaling with antitumor activity in cancer cells overexpressing Akt. Cancer Res. 2004 Jul 1;64(13):4394-9.

TABLES

Table 1. Validation of specificity for Slug interaction with human *OCLN* E-box.

Treatment	Slug only	E-Box only	Slug + E-Box	Slug + E-Box mutant 1	Slug + E-Box mutant 2	Slug + E-Box*	Slug + E Box +E-Box*
Luminescence	4	0	340	4	7	3	145

E-box: Biotin-catccgagtttcaggtgaattggtcaccg

E-box*: -catccgagtttcaggtgaattggtcaccg

E-box mutant 1: Biotin-catccgagtttcttcagaattggtcaccg

E-box mutant 2: Biotin-catccgagtttaaggtaaattggtcaccg

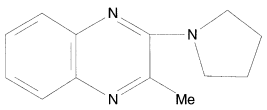
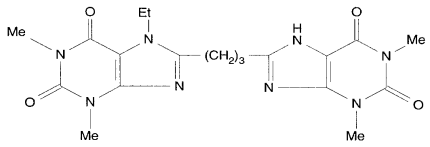
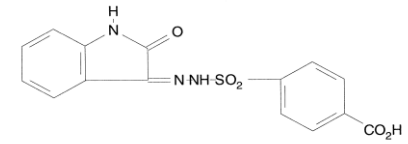
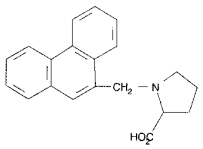
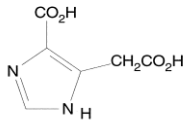
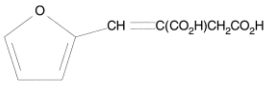
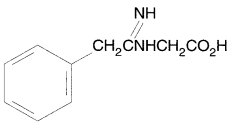
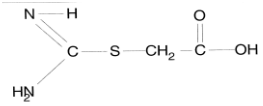
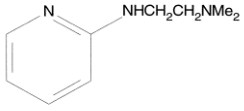
Table 2. Slug-GFP binds to the *OCLN* E-box in a dose-dependent manner.

Slug (µg/well)	**0.1**	**0.2**	**0.4**	**0.8**	**1**	**2**	**4**	**6**	**8**	**10**	**15**	**20**
Luminescence	**45**	**45**	**90**	**130**	**145**	**200**	**240**	**265**	**280**	**280**	**290**	**260**

Table 3. Peptide sequences identified through Phage panning that bind to the *OCLN* E-box sequence.

Library	Peptide sequence	Frequency
Ph.D.-7	YSLRLDY	0.25
	KCCFPIS	0.15
	KCCFINA	0.1
	KCCYVPL	0.1
	KCCYATH	0.05
	QAPTRVQ	0.05
	APTAVSK	0.05
	KVWQIVT	0.05
	KVWLLPP	0.05
	RPPVFHM	0.05
	NKTIMAL	0.05
	IPKHTYR	0.05
Ph.D.-12	KHTFIDYLVSQA	0.3
	FNDRPVLSYMNL	0.1
	KCCFHSTENPHP	0.1
	HMDYYNWRRLSL	0.1
	LRSKIRIPLNRR	0.1

Table 4. High throughput screening hits. Compound code name, molecular formula, molecular weight, and compound structure information were provided by the library source: the National Cancer Institute. Hits listed out-performed the MEK inhibitor PD98059 at 20 μM for induction of luciferase expression. Morphology change consistent with MET, and extent of growth suppression are listed.

Code Name Mol. Formula Mol. Weight	Compound Structure	MET; Growth inhibition at effective conc.
42846-F $C_{13}H_{15}N_3$ 213 Da		Yes ~ 40% at 10 μM
23583-Q $C_{19}H_{24}N_8O_4$ 428 Da		Yes ~ 60% at 10 μM
133488-F $C_{15}H_{11}N_3O_5S$ 345 Da		ND Effective at 10 μM
102728-T $C_{20}H_{19}NO_2$ 342 Da		ND Effective at 10 μM
40384-C $C_6H_6N_2O_4$ 170 Da		Yes ~ 45% at 100 μM
61735-L $C_9H_8O_5$ 196 Da		Yes ~ 40% at 100 μM
27895-C $C_{10}H_{12}N_2O_2$ 192 Da		Yes ~ 55% at 100 μM
403734-Y $C_3H_6N_2O_2S$ 134 Da		Yes ~ 55% at 100 μM
30154-J $C_9H_{15}N_3$ 165 Da		Yes ~ 55% at 100 μM