Molecular Determinants of Retinoic Acid Sensitivity in Pancreatic Cancer

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Abbreviations:

PDAC: Pancreatic ductal adenocarcinoma
ATRA: All-trans retinoic acid
FABP5: Fatty acid binding protein 5
CRABP2: Cellular retinoic acid binding protein 2

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Clinical trials with retinoids have been generally disappointing in PDAC, underscoring the need for rational patient selection. In this manuscript, we demonstrate that it might be possible to predict the sensitivity of PDAC cells to the exogenous ATRA, based on the relative expression of two intracellular retinoid-binding proteins, FABP5 and CRABP2. Specifically, only PDAC cells with absent FABP5 expression retains ATRA sensitivity in vitro and in xenograft models, while high FABP5 expression (usually accompanied by epigenetic silencing of CRABP2) results in treatment resistance, and a paradoxical increase in cell migration/invasion. ATRA resistance can be partially reversed by combination with chromatin modifying drugs like decitabine and trichostatin A, leading to re-expression of CRABP2. This study provides the impetus for initiating a clinical trial with ATRA in a carefully selected group of PDAC patients, as identified by absent FABP5 expression on immunohistochemistry in their pre-treatment tumor biopsies.
Abstract

**Purpose:** To identify a predictive molecular ‘signature’ for sensitivity to retinoic acid in pancreatic cancer.

**Experimental Design:** Fourteen patient-derived, low-passage pancreatic ductal adenocarcinoma (PDAC) lines with varied expression of fatty acid binding protein 5 (FABP5) and cellular retinoic acid binding protein 2 (CRABP2) were used to evaluate the response to all-trans retinoic acid (ATRA). Cell proliferation, apoptosis, and migration/invasion assays were used to measure the *in vitro* response. Tumor growth was monitored in subcutaneous xenografts in athymic nude mice for 4 weeks.

**Results:** Response to ATRA was observed to be dependent upon differential expression of FABP5 *versus* CRABP2. Thus, elevated FABP5 expression was associated with minimal cytotoxicity and tumor growth inhibition, and a paradoxical increase in migration and invasion. Conversely, CRABP2 expression in the absence of FABP5 was associated with significant tumor growth inhibition with ATRA, even in gemcitabine-resistant tumors. The ATRA-resistant phenotype of FABP5<sup>high</sup>CRABP2<sup>null</sup> cells could be circumvented by ectopic expression of *CRABP2*. Alternatively, re-expression of endogenous CRABP2 could be enabled in FABP5<sup>high</sup>CRABP2<sup>null</sup> PDAC lines by exposure to decitabine and trichostatin A, thereby relieving epigenetic silencing of the *CRABP2* gene promoter. Immunohistochemical staining for FABP5 in archival human tissue microarrays identifies a subset of cases (13/63, ~20%) which are negative for FABP5 expression and might be candidates for ATRA therapy.
Conclusions: The widely used agent ATRA deserves a “second look” in PDAC, but needs to be targeted to patient subsets with biopsy-proven FABP5-negative tumors, or be combined with a chromatin modifying agent in order to re-express endogenous CRABP2.
Introduction

Pancreatic cancer or pancreatic ductal adenocarcinoma (PDAC) is one of the most lethal of human cancers. Out of 44,030 new cases of pancreatic cancer in the United States, 37,660 are estimated to die in 2011 (1). The 5-year overall survival rate of 4% has been steady over the last few decades, despite exhaustive efforts at developing new and improved therapeutic strategies (2). With a better understanding of cellular signaling pathways, it may be possible to salvage abandoned or failed drugs by identifying subgroups of patients which are likely to respond favorably to the targeted therapy.

The naturally occurring retinoid, all-trans retinoic acid (ATRA) or tretinoin, is approved in the clinic for the treatment of acute promyelocytic leukemia (APL), and is currently being tested in clinical trials in additional tumor types (3). Prior studies in PDAC cell lines and xenografts have reported the ability of ATRA to induce cell cycle arrest, apoptosis and epithelial differentiation, although a closer parsing of the data confirms an highly variable sensitivity to retinoids (4-6). In fact, in some instances, retinoids can induce an increase in PDAC cell migration, through upregulation of intracellular oncogenic pathways (7). Nonetheless, based on the weight of preclinical evidence, two pilot phase II clinical trials have been conducted using the related retinoid, 13-cis RA, in patients with advanced PDAC, but the results were disappointing (8, 9).
ATRA typically causes cellular differentiation, growth arrest and apoptosis in most epithelial cell types through activation of retinoic acid receptors (RARs). But, in cell types like keratinocytes, ATRA can promote cell survival and hyperplasia (10). Recent seminal studies have elucidated the mechanistic bases for these paradoxical effects of RA on cell growth and survival (11, 12). Specifically, it is now established that the “orphan” nuclear receptor peroxisome proliferator-activated receptor β or δ (PPARβ/δ) also binds to, and is activated by, exogenous retinoids. Upon appropriate ligand-dependent activation, PPARβ/δ or RAR bind to their indispensable dimerization partner, retinoid X receptor (RXR), and it is the corresponding heterodimer that targets DNA at PPARδ-response element (DRE) or retinoic acid response element (RARE), respectively, and modulates transcription. While expression of “classical” RAR-dependent genes leads to apoptosis and growth arrest, activation of the PPARβ/δ receptor initiates a transcriptional program that promotes proliferation, cell survival, and tumor growth. The partitioning of ATRA between PPARβ/δ versus RAR receptors is regulated by the relative levels of two critical intracellular ligand binding proteins, fatty acid binding protein 5 (FABP5) and cellular retinoic acid binding protein 2 (CRABP2). Depending on their relative abundance in the cell, FABP5 and CRABP2 deliver exogenous retinoids from the cytosol to either nuclear PPARβ/δ or RARs, respectively, thereby selectively enhancing the transcriptional activity of the cognate nuclear receptors (10).

We undertook this study to evaluate the sensitivity to retinoids in a large panel of genetically characterized patient-derived PDAC cell lines. We hypothesized that
differences in the FABP5 and CRABP2 expression accounts for the variable therapeutic responses to RA in pancreatic cancer, and that altering this ratio to favor partitioning of retinoids from PPARβ/δ to RAR would result in improved therapeutic efficacy. Our studies establish that PDAC cells indeed demonstrate strikingly variable responses to ATRA, based on the relative intracellular levels of FABP5 and CRABP2. Cancer cells that are FABP5highCRABP2null are typically resistant to ATRA-induced growth inhibition in vitro and in vivo, and demonstrate an enhancement in migration and invasion phenotypes upon ATRA exposure. In contrast, PDAC cells that are FABP5nullCRABP2high retain their in vitro and in vivo sensitivity to ATRA. The CRABP2 promoter is epigenetically silenced in PDAC, and enforced expression in cells with otherwise high endogenous FABP5 expression mitigates the paradoxical enhancement in migration and invasion phenotypes observed with ATRA. Finally, using an immunohistochemical assay, we identify that a minor subset of primary PDACs (~20%) completely lack FABP5 expression (i.e., tissue equivalent of FABP5null), and this cohort might represent an enriched ATRA-sensitive population for therapy using retinoids. In light of the recent demonstration that ATRA can induce quiescence of pancreatic stellate cells, and thereby reduce cancer cell proliferation in a paracrine manner (13), our data underscores the need for a “second look” with retinoids in a rationally selected PDAC patient cohort.
Materials and Methods

Cell Lines and Reagents

The patient-derived, low passage cell lines used in this study were generated at our institute (14). All cell lines were maintained in DMEM (Cat no. 11965-092, Invitrogen, CA) supplemented with 10% fetal bovine serum and 1% Pen-Strep. Cultures were routinely tested negative for mycoplasma presence by MycoAlert Mycoplasma detection kit (Lonza, USA). In vitro treatment with ATRA (Sigma Cat. R2625, St. Louis, MO) were done in 0.5% FBS-containing media. CRABP2 expression vector was constructed using Gateway technology (Invitrogen, CA). Pa20C cell line was stably transfected with either pDest26™-CRABP2 (Pa20C-CRABP2) or the empty vector (Pa20C-Vector), and stable clones were maintained in presence of 500μg/ml G418.

In vitro Cell Growth Assay

In vitro cell growth was determined by means of CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI). After 2 days of growth, cells were treated with indicated doses of ATRA in DMSO or DMSO alone for another 3 days before cell growth was measured, as per manufacturer’s instructions.

Apoptosis Assay

Dissipation of mitochondrial transmembrane potential (Δψm) was used to assess apoptosis, as measured by flow cytometry, using Tetramethylrhodamine methyl ester perchlorate (TMRM) dye (Cat# T668, Invitrogen, CA) (15). Briefly, cells in culture were
treated with ATRA for 3 days, before TMRM was added during the last 20 minutes of culture at a final concentration of 100nM. Cells were trypsinized, washed and resuspended in flow cytometry buffer and ran on FACSCalibur (BD Biosciences) instrument. Data was analyzed using the CellQuest Pro (BD Biosciences) software.

**Semi-quantitative Real Time Reverse Transcriptase PCR**

RNA from cultured cells was extracted using RNeasy kit and reverse transcribed with QuantiTect Reverse Transcription Kit, both from Qiagen (Valencia, CA). Real time RT-PCR (qRT-PCR) for human FABP5 and CRABP2 transcripts was performed using the Fast SYBR® Green Master Mix on StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster City, CA). Expression levels were determined using the $2^{-\Delta\Delta Ct}$ method and values normalized to beta-actin as endogenous control. Sequences of the primers used are available upon request.

**Western Blotting**

Protein extracts were prepared using RIPA buffer (Sigma) and quantified by BCA protein Assay (Thermo Scientific). 50μg protein was resolved on 4-20% Tris Glycine gel (Invitrogen), transferred to nitrocellulose membranes and blotted with primary antibodies against FABP5 (MAB3077, R&D Systems) and CRABP2 (sc-10065, Santa Cruz Biotech) at a dilution of 1:1000. Anti-actin antibody was used as an internal control for protein loading.

**Migration and Invasion Assay**
A modified Boyden chamber was used to perform cell invasion assay (16). Briefly, 20,000 cells in 0.5% FBS media (with DMSO or ATRA) were seeded per BD-Matrigel-coated inserts, placed in a 24-well plate with media containing 10% FBS and cultured for a period of 12-48 hrs, depending on the cell line. Invaded cells were stained with H&E and imaged using a phase-contrast microscope (Olympus BX51). For analysis, cells were counted in 10 randomly selected fields (20X objective lens) and their means plotted after normalizing to values from MTS assay on the same number of cells grown in parallel in the same plate.

For the wound healing assay of cell migration, a scratch was made using a 200μl sterile pipette tip in a confluent layer of cells, washed with media three times before returning them to incubator. Images were taken after a 30 min recovery and after 1-2 days of ATRA treatment, using an inverted phase contrast microscope (Nikon Eclipse TE2000-S) with Spot Advanced software. Six images were taken per wound, at 10X objective lens magnification. The percentage wound area was measured with ImageJ software (version 1.44e, NIH, USA).

**Subcutaneous xenograft model**

Five million of actively growing cells in 1:1 ratio of PBS and BD Matrigel were injected subcutaneously on each flank of 9 athymic nude mice per cohort. When the average tumor volume was approximately 250mm³, mice were randomized to receive either vehicle control or ATRA (15mg/Kg in corn oil, administered, intraperitoneally, from
Monday to Friday for 4 weeks). Mice were routinely inspected physically for any abnormal changes and their body weight taken weekly to monitor any ATRA-related cytotoxicity. Tumor volumes were measured at indicated intervals during the course of treatment and tumor weight taken at the end of the experiment. A portion of each removed tumor was preserved in formalin to be used later in the immunohistochemical analysis. Gemcitabine (NetQem, AP-1025, Research Triangle park,NC), 50mg/kg in saline was given twice a week (Tuesday and Thursday), intraperitoneally. For the combination therapy arm, both treatments were administered concomitantly.

**TUNEL staining**

DeadEnd Fluorometric TUNEL System (Promega Corporation, Madison, WI) was used for TUNEL staining in formalin-fixed paraffin embedded (FFPE) tissue sections, as per manufacturer’s instructions.

**Immunohistochemistry**

Briefly, FFPE tissues sections were deparaffinized using xylenes and hydrated by a graded series of ethanol washes. Antigen retrieval was accomplished by heating the slides in citrate buffer (pH 6.0) at 100°C for 20 minutes. Endogenous peroxidase activity was quenched by 0.3% H₂O₂ in methanol. Nonspecific binding was blocked with 2.5% normal goat serum, before incubation with the primary antibody. Chromogenic detection was enabled using Poly-HRP anti-Rabbit (Leica Biosystems) or ImPRESS Anti-Rat Ig (Vector laboratories) with DAB+ substrate (Dako) as chromogen. Slides were counterstained with Harris-hematoxylin solution. Primary antibodies were: anti-FABP5...
(dilution 1:200, MAB3077, R&D Systems, MN) for staining human tissue microarrays and anti-Ki-67 (dilution 1:200, clone SP6, Cell Marque, CA,) for measuring cell proliferation. Quantification was done by counting Ki-67-positive cells in ten randomly selected fields (40X objective lens magnification) of phase-contrast microscope (Olympus BX51), using five independent xenografts per treatment condition. Only neoplastic cells were evaluated, and stromal cell staining was excluded from the counting process. Fields containing areas of extensive necrosis were also excluded from evaluation.

**Statistical Analysis**

All *in vitro* experiments were repeated a minimum of three times to ensure reproducibility. Data presented in figures are from one representative experiment that was qualitatively similar in the replicate experiments. Statistical significance was determined with two-tailed Student’s t-test, one-way ANOVA and Mann-Whitney-U-tests, using Prism (GraphPad Software Inc., San Diego, CA, USA) version 5.01. A value of P<0.05 was regarded as statistically significant.
Results

Expression profile of FABP5 and CRABP2 in PDAC lines

We performed \textit{in silico} analysis of the "Deep Serial Analysis of Gene Expression (Deep SAGE)" data from the recent exomic and transcriptomic analysis of 24 pancreatic adenocarcinoma samples (14), for the expression of various components of retinoic acid signaling pathways. There was a notable pattern of reciprocal differential expression for \textit{FABP5} and \textit{CRABP2} transcripts in these lines (Supplementary Figure S1A). We confirmed this reciprocal expression pattern by qRT-PCR for mRNA (Figure 1A) and western blotting for protein expression (Figure 1B). Notably, only 3 of 13 (23\%) PDAC lines co-expressed the two proteins at a detectable level. Based on the relative expression of FABP5 and CRABP2, we selected a panel of four cell lines which were either FABP5\textsuperscript{high}CRABP2\textsuperscript{null} (Pa04C, Pa14C) or FABP5\textsuperscript{null}CRABP2\textsuperscript{high} (Pa03C, Pa01C), which we predicted to be ATRA-resistant or sensitive, respectively, for further functional studies.

Differential in vitro responsiveness to ATRA

As predicted based upon relative FABP5 and CRABP2 expression, we determined that FABP5\textsuperscript{high}CRABP2\textsuperscript{null} cells (Pa04C and Pa14C) were resistant, while FABP5\textsuperscript{null}CRABP2\textsuperscript{high} cells (Pa01C and Pa03C) were sensitive to ATRA at concentrations up to 10\(\mu\)M on MTS assays (Figure 1C). We then assessed apoptosis in these cells by flow cytometry, using TMRM probe to measure loss of mitochondrial transmembrane potential. Both FABP5\textsuperscript{null}CRABP2\textsuperscript{high} lines (Pa01C, Pa03C) showed an increase in apoptosis even at 1\(\mu\)M ATRA at 72hrs treatment, consistent with their
observed ATRA sensitivity, while FABP5^{high}CRABP2^{null} cells (Pa04C, Pa14C) showed no increase in apoptosis, even up to 10\mu M ATRA (Figure 1D).

PDAC cells were also observed microscopically for morphological changes, upon ATRA treatment at 48hrs. In published studies, ATRA-sensitive PDAC cells were reported to increase in size and develop dendrite-like processes, indicative of cellular differentiation (17). Compared to vehicle-treated cells, only the FABP5^{null}CRABP2^{high} Pa01C demonstrated morphological features consistent with differentiation upon ATRA exposure, while FABP5^{high}CRABP2^{null} Pa04C cells appeared to have reduced dendrite-like processes (Supplementary Figure S1B). Lastly, analysis of DNA content by propidium iodide after 3 days of ATRA treatment confirmed an increase in the sub-G\textsubscript{0} and G\textsubscript{1} populations (indicative of apoptosis and cell cycle arrest, respectively), with a concomitant decrease in both S and G\textsubscript{2} stages of cell cycle that was restricted to FABP5^{null}CRABP2^{high} Pa01C cells, but not in the FABP5^{high}CRABP2^{null} Pa04C cells (Supplementary Figure S1C).

Since metastasis remains the principal underlying cause leading to pancreatic cancer mortality, we examined the effect of ATRA on both migration and invasion, in cells with differential FABP5 and CRABP2 expression. Using a modified Boyden chamber Matrigel invasion assay, we found almost complete abrogation of invasion in the FABP5^{null}CRABP2^{high} Pa01C line (Figure 1E, left). In contrast, significantly more cells invaded through Matrigel upon ATRA exposure of FABP5^{high}CRABP2^{null} Pa04C cells (Figure 1E, right). In a wound healing assay for cell migration,
FABP5nullCRABP2high Pa01C cells failed to exhibit migration upon ATRA treatment, even in the absence of any demonstrable cell death at the endpoint of experiment (Figure 1F, top). Notably, FABP5highCRABP2null Pa04C cells exhibited significantly greater percentage of wound closure (i.e., increased migration) upon ATRA treatment as compared to DMSO control (Figure 1F; bottom).

**Differential in vivo responsiveness to ATRA**

Having confirmed the contrasting *in vitro* effects of ATRA in PDAC cells with differential expression of FABP5 and CRABP2, we assessed therapeutic efficacy for this compound *in vivo*, using xenograft models. Mirroring the *in vitro* data, ATRA significantly inhibited the tumor growth of FABP5nullCRABP2high Pa01C xenografts (Figure 2A, left, P<0.0001), while the FABP5highCRABP2null Pa04C xenografts were completely resistant (Figure 2A, right) (both tumor volume and tumor weights were measured as parameters of growth, with equivalent results). The excised xenografts from the control and treatment arms were stained and analyzed for the cell proliferation marker Ki-67, and for apoptosis using TUNEL. There was a significant reduction in the numbers of Ki-67 positive nuclei, in conjunction with a massive increase in the numbers of TUNEL-positive nuclei, in ATRA-sensitive FABP5nullCRABP2high Pa01 xenografts, as compared to the vehicle control (Figures 2B and 2C). In striking contrast, there was a modest, but statistically significant increase in Ki-67-positive nuclei upon ATRA treatment in the ATRA-resistant FABP5highCRABP2null Pa04C xenografts; minimal TUNEL-positive nuclei were observed in the treated xenografts. We observed a comparable outcome with ATRA therapy in an independent pair of xenografts predicted to be either ATRA-sensitive (FABP5nullCRABP2high Pa03C), or ATRA-resistant (FABP5highCRABP2null Pa04C).
Next, we tested the ATRA-sensitive line Pa01C in a subcutaneous xenograft model using the combination of ATRA and gemcitabine. The parental xenograft is known to be resistant to gemcitabine, and as expected, no significant tumor growth inhibition was observed with monotherapy (Figure 2D, both tumor weight and tumor volume are depicted). In contrast, the combination of gemcitabine and ATRA resulted in significant tumor growth inhibition, although the efficacy was only marginally improved over the considerable efficacy of ATRA alone. Nonetheless, these results underscored that, in the appropriate biomarker profile predicting retinoid responsiveness, ATRA might be useful as a second line treatment in gemcitabine resistant tumors.

**Modulation of CRABP2/FABP5 ratio in ATRA resistant PDAC**

To check if altering the relative endogenous levels of CRABP2 and FABP5 proteins within PDAC cells will alter the effects of ATRA exposure, we ectopically expressed the CRABP2 gene in the Pa20C cell line, which harbors an endogenous FABP5\textsuperscript{high}CRABP2\textsuperscript{null} profile (Figure 1B). Several stable clones were tested, and one clone that expressed higher CRABP2 than endogenous FABP5 was selected for further experiments. Relative expression of FABP5 and CRABP2 was confirmed by both qRT-PCR (Figure 3A) and western blotting (Figure 3B). We then examined the subcellular localization of endogenous FABP5 in these cells by immunofluorescence, upon ATRA treatment. In the vector-transfected Pa20C line, nearly all of the cytoplasmic FABP5
translocated to the nucleus upon ATRA treatment (Supplementary Figure S3A, Vector). In contrast, ectopic expression of CRABP2 in the Pa20C cell line blocked the ATRA-induced nuclear translocation of FABP5 (Supplementary Figure S3A, CRABP2). Thus, in the presence of high levels of intracellular CRABP2 compared to FABP5, ATRA preferentially binds to CRABP2 and gets shuttled to its cognate nuclear receptors. In agreement with this observation, Pa20C-CRABP2 cells displayed diminished in vitro growth (Figure 3C, P<0.01) and significantly greater apoptosis (Figure 3D, P<0.001) upon ATRA treatment as compared to the vector-transfected cells. Cell cycle analysis also showed an increase in the sub-G₀ population, indicative of apoptosis, in the Pa20C-CRABP2 clone upon ATRA treatment (Supplementary Figure S3B and S3C).

We then examined the effects of modulating the FABP5 to CRABP2 ratio on the invasion and migration phenotypes. As expected, vector-transfected Pa20C cells showed higher invasion and migration in a Boyden chamber assay upon ATRA treatment (Figure 3E and Supplementary Figure S3D), likely due to high endogenous FABP5 expression. In contrast, ectopic overexpression of CRABP2 significantly abrogated the ATRA-induced increase of invasion and migration (Figure 3E and Supplementary Figure S3D). Comparable results were observed in wound healing assays, wherein CRABP2 overexpression mitigated the enhanced migration of Pa20C cells upon ATRA treatment that is observed with the vector-transfected cells (Figure 3F). This suggests that an endogenous FABP5^{high}CRABP2^{null} ATRA-resistant cell line can potentially be rendered sensitive to ATRA by increasing the intracellular expression of CRABP2.
CRABP2 Expression is silenced by promoter methylation in PDAC

Analysis of promoter region of the human CRABP2 revealed a conserved CpG island comprised of 129 CpG sites, and 1.79 kb in length (Supplementary Figure S4A, Ratio of observed to expected CpG is 0.68). We grew a panel of FABP5\textsuperscript{high}CRABP2\textsuperscript{null} cell lines Pa04C, Pa20C and Pa08C in the presence of decitabine (DAC), an inhibitor of DNA methylation, and Trichostatin A (TSA), an HDAC inhibitor. We observed a massive increase in the levels of CRABP2 transcripts (20-40-fold elevation), mainly upon DAC exposure, which was augmented by TSA, suggesting epigenetic silencing of the CRABP2 promoter in PDAC cells (Supplementary Figure S4B), a finding that was confirmed by performing methylation-specific PCR (MSP) targeted at the CRABP2 promoter CpG island (Supplementary Figure S4C).

FABP5 expression in resected PDAC tissues

In order to identify PDAC subsets that might be predicted to be sensitive to retinoids based on low or absent FABP5 expression, we performed immunohistochemical staining for FABP5 in an archival human pancreatic adenocarcinoma tissue microarray. Both normal and cancer tissue sections from 63 cases were analyzed. Normal ductal epithelial cells were essentially negative for FABP5 expression (Figure 4A, left). The expression of intracellular FABP5 was variable in the neoplastic epithelial cells, with 50 of 63 cases (~80%) showing moderate to strongly positive staining (Figure 4A, right). In contrast, 13 cases (~20%) were completely negative for FABP5 labeling in the neoplastic epithelial cells (Figure 4A, middle), and the patients from whom these samples were derived might be candidates for retinoid
therapy in a first or second line setting. Thus, an immunohistochemical assay in tissue biopsies or resection samples might facilitate the identification of a minority of PDAC patients (~20%, Figure 4B) who could benefit from a molecularly-targeted differentiation therapy.
Discussion

The vitamin A metabolite retinoic acid (RA) regulates multiple biological processes and plays key roles in embryonic development and in tissue remodeling in the adult. RA displays distinct anticancer activities in certain malignancies like acute promyelocytic leukemia and head and neck tumors, which forms the basis for its therapeutic application in the clinic. Notably, some carcinomas not only fail to become growth inhibited upon treatment with RA, but instead respond to treatment with enhanced proliferation (7, 11, 18). A striking example of the pro-carcinogenic effect of RA was observed in the β-carotene and Retinol Efficacy Trial (CARET), a lung cancer chemoprevention study that was terminated prematurely due to increased cancer incidence in the treatment cohort (19).

The recent comprehensive genomic and transcriptomic analysis of 24 pancreatic cancer samples from our group provides us with a unique resource to investigate the presence of a predictive molecular ‘signature’ of retinoid-sensitivity in pancreatic cancer (14). Except for one missense mutation in RARβ, no other somatic mutation, gene amplification or duplication of any of the RA signaling components was found. However, in silico analysis of the Deep SAGE data on these samples showed a differential expression pattern of FABP5 and CRABP2 tags between individual PDAC cell lines. The corresponding retinoid binding proteins were recently shown to be critical intracellular partitioning factors for either anti-survival or pro-survival effects of RA (10). Thus, we could test the cell lines with either high FABP5 or high CRABP2 for their responsiveness to ATRA. True to our hypothesis, we found FABP5highCRABP2null PDAC
lines to be resistant to ATRA-mediated growth inhibition and apoptosis with increased migration and invasion phenotypes), while FABP5<sup>null</sup>CRABP2<sup>high</sup> cell lines retained exquisite ATRA sensitivity. Although not included in our panel, higher FABP5 levels could also explain the previously reported paradoxical increase in migration/invasion upon ATRA treatment observed in the CAPAN-1 PDAC cells (7). We then confirmed our results in vivo using xenograft models, generated using four independent PDAC lines, two with a FABP5<sup>high</sup>CRABP2<sup>null</sup> profile and two with FABP5<sup>null</sup>CRABP2<sup>high</sup> expression. An important caveat in this paradigm is the status of the downstream RAR receptors. Thus, even if CRABP2 is the prominent intracellular carrier protein for exogenous retinoids, the tumor suppressive effects will not be observed unless RARs are expressed. In this regard, we confirmed the expression of RAR<sub>α</sub> and RAR<sub>β</sub> transcripts in all cell lines in our panel (data not shown). This is consistent with the low frequency of methylation of the corresponding gene promoters in PDAC (20), in contrast to other solid tumors like lung cancer (21).

In our series of PDAC lines, only a minority (3 of 13, 23%) co-expressed both FABP5 and CRABP2. The binding affinity of the CRABP2-RAR pathway for RA (K<sub>d</sub> of 0.1–0.2nM) exceeds that of the FABP5-PPAR<sub>β</sub>/δ pathway (K<sub>d</sub> of 10–50nM) (22-24). Thus, in tumors with comparable expression of FABP5 and CRABP2, one might expect to see at least partial tumor suppressive effects of ATRA, based on the kinetics of partitioning between nuclear receptors. We simulated this scenario by enforced expression of CRABP2 in a cell line (Pa20C) with an endogenous FABP5<sup>high</sup>CRABP2<sup>null</sup> profile. High CRABP2 levels resulted in cytoplasmic sequestration of FABP5,
presumably by the preferential binding of ATRA to CRABP2 and subsequent nuclear channeling to RAR receptors. This led to increased apoptosis and mitigation of the paradoxical enhanced migration/invasion phenotype upon ATRA treatment, as compared to the resistant parental cell line. This serves as a proof-of-principle to show that increasing the expression of CRABP2 can make an otherwise resistant cell line, sensitive to ATRA treatment. Owing to the absent CRABP2 expression in several PDAC lines and the presence of a 1.7kb CpG island in the putative promoter region of the CRABP2 gene, we suspected the epigenetic silencing of the CRABP2 promoter. Interestingly, the CRABP2 promoter was recently shown to be methylated in head and neck tumors (25). Besides promoter methylation, histone deacetylation also causes lower expression of many genes. Such epigenetically silenced genes are shown to be re-expressed upon treatment with decitabine (DAC) and trichostatin A (TSA) in pancreatic cancer cell lines (26). DAC (5-aza-2'-deoxycytidine), a cytosine nucleoside analog which hypomethylates DNA by inhibiting DNA methyltransferases, is already approved by the Food and Drug Administration (FDA) to treat myelodysplastic syndromes (MDS) (27). TSA, an inhibitor of class I and II mammalian histone deacetylase enzymes (HDACs), has also been successfully used in various preclinical cancer models, including pancreatic cancer. Exposure to DAC and TSA in low CRABP2-expressing cell lines resulted in significant re-expression (20-40 fold elevation) of CRABP2. This opens up the possibility of combinatorial use of ATRA with decitabine alone, or in combination with HDAC inhibitors, in the treatment of ATRA-resistant tumors. Of note, there is an ongoing phase II clinical trial of decitabine alone or in combination with valproic acid (HDAC inhibitor) and ATRA for treatment of acute
myeloid leukemia, and a phase I clinical trial of liposomal-ATRA and valproic acid for various solid tumors (28). Moreover, the combination of ATRA with TSA showed promising results in a preclinical study on otherwise ATRA-resistant renal cell carcinoma cell lines (29). These recent developments points towards the need of clinical trials involving ATRA with epigenetic modifiers like DAC and TSA in the treatment of pancreatic cancer.

Although re-expression of epigenetically silenced CRABP2 in tumors with endogenous FABP5 expression is likely to improve the therapeutic efficacy of ATRA, in our series the most unequivocal effects of retinoids were seen in PDAC cells that lacked FABP5 expression altogether. Even a relatively gemcitabine resistant cell line (Pa01C) with a predicted “sensitive” profile (FABP5nullCRABP2high) showed significant growth inhibition with single-agent ATRA, and only minimal additive effect of combination with gemcitabine. In a prospective clinical trial, the identification of appropriate candidates for molecularly-based therapies is a challenge, especially in patients with advanced PDAC who do not undergo surgical resection, and only limited biopsy material might be available for testing. In this context, immunohistochemical staining for FABP5 provides a relatively simple approach for identifying neoplasms that lack protein expression, and is amenable to limited formalin-fixed tissues.

In conclusion, we found retinoid sensitivity of PDAC to be dependent on the relative intracellular expression of FABP5 and CRABP2 proteins. It is possible to identify a subset of tumors that are negative for FABP5, and these are likely to represent an
enriched subset of patients that might benefit from retinoid therapy. Such “personalized” patient selection will hopefully ensure a more favorable clinical response using retinoids than an “all-comers” approach.
Disclosure of Potential Conflict of Interest

No potential conflicts of interest were disclosed.

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Figure Legends

Figure 1. Expression Profile of FABP5 and CRABP2 in PDAC cell lines and *in vitro* responsiveness to ATRA.

(A) Real time qRT-PCR with 14 patient-derived, low passage PDAC cell lines showing relative expression levels of *FABP5* and *CRABP2* normalized to β-actin. Mean of three experiments done in triplicates plotted with error bar denoting ± SEM.

(B) Western blot analysis showing protein expression of FABP5 and CRABP2 in a panel of 13 PDAC lines, with actin as protein loading control. Three of 13 (23%) co-expressed both proteins, while 10 of 13 (76%) express one or the other binding protein.

(C) ATRA treatment reduces *in vitro* growth of FABP5*null*CRABP2*high* (Pa01C, Pa03C) cells, while FABP5*high*CRABP2*null* (Pa04C, Pa14C) are completely resistant even at the highest dose. Values are plotted relative to the DMSO treated control and represent mean of three independent experiments, done in triplicates.

(D) Apoptosis assay measuring mitochondrial transmembrane depolarization using TMRM dye and measured by flow cytometry, plotted as mean of three independent experiments. Results from two FABP5*high*CRABP2*null* ATRA-resistant (Pa04C, Pa14C) and two FABP5*null*CRABP2*high* ATRA-sensitive (Pa01C, Pa03C) lines are shown.

(E) Invasion Assay using modified Boyden chamber and (F) wound healing assay for Pa01C and Pa04C. ATRA exposure significantly blocks invasion (E) and migration (F) in the FABP5*null*CRABP2*high* Pa01C cells, while actually enhancing both parameters in the FABP5*high*CRABP2*null* Pa04C line. Scale bar is 100μm and 250μm in Figs E and F, respectively. Lower panels summarize mean of three independent experiments done in
triplicates (after normalizing to growth in MTS assay). Error bar denotes ± SEM. ** $P<0.01$, *** $P<0.001$

**Figure 2. In vivo therapeutic response to ATRA is dependent on relative expression of FABP5 and CRABP2.**

Athymic nude mice were injected subcutaneously with either Pa01C (FABP5$^{\text{null}}$CRABP2$^{\text{high}}$) or Pa04C (FABP5$^{\text{high}}$CRABP2$^{\text{null}}$) and treated with either ATRA (Red line) or oil as control (Black line).

**(A)** Mean of tumor volumes measured at indicated time intervals plotted for Pa01C (left) and Pa04C (right). Bar denotes mean of tumor weight (in mg) at the end of study. Significant tumor growth inhibition is observed in the FABP5$^{\text{null}}$CRABP2$^{\text{high}}$ Pa01C xenografts, while the FABP5$^{\text{high}}$CRABP2$^{\text{null}}$ Pa04C xenografts are completely resistant.

**(B)** Ki-67 staining on excised xenografts confirms significant reduction in proliferation in FABP5$^{\text{null}}$CRABP2$^{\text{high}}$ Pa01C xenografts, and a modest but significant increase in Ki-67 labeling in the FABP5$^{\text{high}}$CRABP2$^{\text{null}}$ Pa04C xenografts. Representative light microscopic images shown at 40X objective lens magnification. Scale bar is 40μm.

**Right** Bar denotes values plotted as mean of number of positive nuclei from 10 fields per section (5 sections per group). Error bar denotes ± SEM. * $P<0.05$, ** $P<0.001$.

**(C)** TUNEL staining on excised xenografts demonstrates pronounced apoptotic nuclei in the ATRA-treated FABP5$^{\text{null}}$CRABP2$^{\text{high}}$ Pa01C xenografts, and minimal staining in the ATRA-treated FABP5$^{\text{high}}$CRABP2$^{\text{null}}$ Pa04C xenografts. Representative images shown at 20X objective. Scale bar is 100μm.
(D) Efficacy of single agent ATRA and combination therapy in Pa01C, a gemcitabine-resistant xenograft. *Left* Mean of tumor volumes measured at various time intervals plotted, treated with indicated drug combinations. Gemcitabine (50mg/kg) in saline was given twice a week. *Middle* Representative images of tumor-bearing mice from each group. *Right* Mean of tumor weight (in mg) at the culmination of study. Error bar denotes ± SEM.

Figure 3. CRABP2 overexpression in FABP5^{high}CRABP2^{null} Pa20C cell line

(A) Quantitative RT-PCR for FABP5 and CRABP2 expression normalized to β-actin. (B) Western Blotting for FABP5 and CRABP2 in parental Pa20C ("Vector") and CRABP2 over-expressing cell line Pa20C-CRABP2 ("CRABP2").

(C) Pa20C-CRABP2 cells demonstrate significantly reduced growth by MTS assay compared to Pa20C-vector cells (*P*<0.01) with ATRA treatment.

(D) Pa20C-CRABP2 cells demonstrate significantly higher apoptosis by TMRM-based assay, compared to Pa20C-vector cells (*P*<0.001) with ATRA treatment.

(E) Invasion and migration assays using modified Boyden chamber. Ectopic expression of CRABP2 significantly mitigates the paradoxical increase in invasion and migration observed with ATRA treatment in Pa20C cells. Bar denotes mean of three independent experiments done in triplicates (after normalizing to growth in MTS assay). Error bar denotes ± SEM.

(F) Wound healing assay. Pa20C with or without CRABP2 overexpression treated with ATRA for 24hrs, before a wound was introduced and monitored for another 24 hrs. Scale bar is 250μm.
Figure 4. Immunohistochemical staining for FABP5 in a human PDAC tissue microarray.

(A) Representative images of FABP5 staining representing 63 surgically resected PDAC cases are shown, including negatively-stained normal pancreatic ductal epithelium (left) and pancreatic ductal adenocarcinoma cells (middle, negatively-stained and right, positively-stained).

(B) Summary of FABP5 staining pattern in the cases analyzed and the possible predicted outcome to ATRA treatment.
References


18. Morgan E, Kannan-Thulasiraman P, Noy N. Involvement of Fatty Acid Binding Protein 5 and PPARbeta/delta in Prostate Cancer Cell Growth. *PPAR Res* 2010;2010:


Figure 1
Figure 2
Figure 3
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