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Original Article

Primary cell lines: false representation or model system? a comparison of four human colorectal tumors and their coordinately established cell lines

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Abstract: Cultured cell lines have played an integral role in the study of tumor biology since the early 1900's. The purpose of this study is to evaluate colorectal cancer (CRC) cell lines with respect to progenitor tumors and assess whether these cells accurately and reliably represent the cancers from which they are derived. Primary cancer cell lines were derived from fresh CRC tissue. Tumorigenicity of cell lines was assessed by subcutaneous injection of cells into athymic mice and calculation of tumor volume after 3 weeks. DNA ploidy was evaluated by flow cytometry. Invasive ability of the lines was tested with the MATRIGEL™ invasion assay at 24 or 48 hours. Cells were assessed for the presence of Kirsten-Ras (K-Ras), p-53, deleted in colon cancer (DCC), and Src. Protein profiling of cells and tissue was performed by surface enhanced laser desorption/ionization-time of flight/mass spectroscopy. microRNA expression in cell and tumor tissue samples was evaluated by FlexmiR™ MicroRNA Assays. Four cell lines were generated from tumor tissue from patients with CRC and confirmed to be tumorigenic (mean tumor volume 158.46 mm^3). Two cell lines were noted to be diploid; two were aneuploid. All cell lines invaded the MATRIGEL™ starting as early as 24 hours. K-Ras, p53, DCC, and Src expression were markedly different between cell lines and corresponding tissue. Protein profiling yielded weak-to-moderate correlations between cell samples and respective tissues of origin. Weak-to-moderate tau correlations for levels of expression of human microRNAs were found between cells and respective tissue samples for each of the four pairings. Although our primary CRC cell lines vary in their expression of several tumor markers and known microRNAs from their respective progenitor tumor tissue, they do not statistically differ in overall profiles. Characteristics are retained that deem these cell lines appropriate models of disease; however, data acquired through the use of cell culture may not always be a completely reliable representation of tumor activity *in vivo*.

Keywords: Cell culture, primary cell lines, colorectal cancer

Introduction

Cultured cell lines have played an integral role in the study of tumor biology since the early 1900's. Cell lines provide a living system to investigate cellular metabolism, pharmacokinetics, and anti-cancer therapies. Further, they enable the production of antibodies and vaccines. Although cell lines are widely used to investigate disease processes, established lines can undergo spontaneous mutation and may experience chromosomal, morphologic, and tumorigenic changes. Because of these changes, results from investigation of disease processes using these cell lines may confound, rather than clarify, our understanding of the disease studied. The cause of these molecular and behavioral alterations is multifactorial, with over-passaging beyond "safe" passage numbers and genetic drift implicated as contributory events [1-3]. Though reports of the effects of increasing passage number on key cell line properties have afforded valuable information regarding altered proliferation rates [4], trans-epithelial resistance

[4-5], permeability [5], metabolic activities [6-8], transport properties [9-10], and differentiation marker expression [11-13], there are few studies comparing gene expression of primary cell lines to their tissues of origin and on the subsequent impact on reliability of the cell line to serve as a model system to represent parental tissue [14-15]. In addition, the conclusions deduced from these studies of over-passaged cell lines further reinforce the necessity for the creation and utilization of new primary cell lines.

Colorectal cancer (CRC) arises through a distinct sequence of activation of a number of oncogenes and inactivation of tumor suppressor proteins. Several genes in particular are responsible for colorectal tumorigenesis, including oncogene homolog Kirsten ras (K-Ras), deleted in colon cancer (DCC), and p53 [16]. Dysregulation of the K-Ras proto-oncogene is an early event, while inactivation of the DCC and p53 tumor suppressor genes is a late phenomenon in sporadic CRC development. Similarly, other genes have been shown to play roles in the malignant progression of CRC. Notably, c-Src, the human homologue to avian v-src and member of the proto-oncogenic tyrosine kinases, has been suggested to be linked with the development of the CRC metastatic phenotype, appearing to be a causal factor in reduced homotypic adhesion and increased invasiveness in human CRC and transformed rodent cells [17-18]. The importance of K-Ras, DCC, p53, and c-Src in CRC makes their protein products ideal targets to compare in primary CRC cell lines to parental CRC tissue.

The purpose of the current study is to evaluate the fidelity of CRC cell lines of low passage number with respect to parent tumor tissue and to assess whether these cells are an accurate and reliable representation of the cancers from which they are derived. We have generated 4 primary CRC cell lines from tumor tissue from patients with colorectal adenocarcinoma. We have characterized these cell lines with respect to cell type, tumorigenicity, ploidy, and invasive potential. We have evaluated the expression of K-Ras, DCC, p53, and Src in these cell lines and have compared these levels to those expressed in their tissues of origin. Further, we have compared protein and microRNA (miRNA) profiles in each pair of cell line and respective tissue of origin.

Materials and methods

Patient population and specimen collection

Fresh samples of CRC were collected immediately following operative resection from patients undergoing colectomy at the Penn State Milton S. Hershey Medical Center, as approved by the Institutional Review Board. Surgical specimens were collected in sterile room-temperature cell culture medium, consisting of Minimum Essential Media (MEM) (Hyclone, Logan, UT) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Hyclone) and 1% antibiotic/antimycotic (Sigma, St. Louis, MO). Tumor tissue was used for primary cell line establishment, immunoblot, protein profiling, and miRNA detection.

Creation of cell lines

Tissue was dissected free of fat, blood clots, and necrotic tissue and rinsed three times with a 4°C solution of MEM, 1% antibiotic/antimycotic, and 1% human serum albumin (Sigma). Tissue was minced into approximately 1 mm fragments and transferred to a sterile flask containing MEM (37˚C), DNase I (15 μg/mL) (Sigma), hyaluronidase (0.5 mg/mL) (Sigma), and collagenases IX (0.5 mg/mL) and XI (0.5 mg/mL) (Sigma). The tissue mixture was enzymatically digested by stirring for 20 minutes at 37°C, as previously described [19]. Cells were then centrifuged for 10 minutes at 1250 rpm at 4°C and washed twice with MEM (4°C) and 1% antibiotic/antimycotic. Following repeat centrifugation, cells were resuspended in cell culture medium consisting of MEM, 10% FBS, 1% antibiotic/antimycotic, 1% sodium pyruvate, 1% non-essential amino acids, and 1% vitamin solution (Sigma). Cells were plated onto 100 mm untreated cell culture dishes and maintained at 37°C in a 5% humidified CO_2 incubator to allow for adherence and multiplication. Once cells had adhered and multiplied, they were subcultured with trypsin and 0.25% ethylenediaminetetraacetic acid (EDTA) (Invitrogen, Carlsbad, CA) and used between passages 5 and 20 in the described assays.

Commercial cell culture

The colonic carcinoma cell lines HT29 and HCT116 (American Type Culture Collection [ATCC], Manassas, VA), were maintained in cul-

ture at 37°C with 5% CO_2 in RPMI 1640 media (Mediatech, Inc., Herndon, VA) supplemented with 10% FBS and 1% antibiotic/antimycotic. The rat intestinal epithelial cell line IEC-18 (ATCC) was maintained in MEM, 5% FBS, and 0.03% bovine insulin (Sigma). Trypsin with 0.25% EDTA was used to release the above cells from plates for passage and plating for the assays below. Cells were viewed under 20 X using a phase contrast microscope.

Immunofluorescence

5.0 x 10^5 IEC-18 cells and cells from our four primary lines were grown on glass coverslips in 6-well culture plates. Culture media was removed and the cells fixed with 1% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA). They were then permeabilized with 0.2% Triton (Sigma) in phosphate buffered saline (PBS) and blocked with 10% bovine serum albumin in PBS with 0.1% Triton for 1 h. The cells were incubated with polyclonal rabbit anti-pancytokeratin (Santa Cruz, Santa Cruz, CA) for 1 h, washed five times in PBS with 0.1% Triton, and incubated with the secondary antibody Cy 2 -conjugated donkey anti-rabbit (Jackson ImmunoResearch Labs, West Grove, PA) for 1 h. The cells were washed again and coverslips were secured on microscope slides using Aqua-Poly-Mount (PolySciences, Warrington, PA). The slides were viewed at 20X magnification under an upright fluorescence microscope (model 1X51; Olympus, Center Valley, PA), and images captured with a SPOT Insight™ digital camera (Diagnostic Instruments, Inc., Sterling Heights, MI).

Tumorigenicity studies

2.0 x 10^6 cells from our four primary cell lines and the HT29 cell line were suspended in 100 µL PBS and subcutaneously injected into the flanks or shoulders of nude mice (Jackson Laboratory, Bar Harbor, ME). Tumor length (l) and width (w) were measured weekly for three weeks and tumor volume calculated as ½ l x w^2 [19-20]. Tumorigenicity studies were performed in quadruplicate and in accordance with Institutional Animal Care and Use Committee guidelines.

Determination of cell ploidy by flow cytometric cell cycle analysis

Cell DNA content (ploidy) of the four newly established colorectal cancer cell lines was determined by cell cycle analysis. Cell suspensions were prepared by adding 1 mL of hypotonic staining buffer for DNA comprised of 1 mg/mL sodium citrate (Sigma), 0.3% Triton-X 100 (Sigma), 0.1 mg/mL propidium iodide (Calbiochem, San Diego, CA), 0.02 mg/mL ribonuclease A (Sigma) in distilled water to cells from each cell line. Peripheral blood mononuclear cells collected from healthy individuals (as approved by the Penn State Institutional Review Board) were added to each sample as an internal standard to allow for identification of diploid peaks. Suspensions were incubated at 4°C for 30 minutes protected from light, then passed through a cell strainer cap composed of 35 µm nylon mesh (BD Biosciences, San Jose California) to remove debris and aggregates immediately preceeding analysis on a BD FACSCalibur™ flow cytometer (BD Biosciences). A minimum of 20,000 events were acquired for each sample. Data were analyzed using ModFit LT 3.1 software (Verity Software House, Inc., Topsham, ME). DNA content of HT29 and HCT116 cells, known to be aneuploid and diploid, respectively, was also determined and results used for comparison. Cell samples demonstrating one G_0/G_1 peak were classified as diploid; DNA aneuploidy was documented if two discrete G_0/G_1 peaks were present, with the non-reference peak demonstrating greater than 20% total events. The DNA index (DI) was calculated as the ratio of the position (channel number) of the sample G_0/G_1 peak to the position of the diploid reference peak (mixed PBMCs), with a DI of 1.0 indicating the presence of diploid cells, only. Sample acquisition and data analysis were performed in the Penn State Hershey Flow Cytometry Core Facility.

Invasion assays

2.5 x 10^4 cells from our four primary cell lines and from HT29 and HCT116 cell lines were suspended in 200 µL serum-free medium of RPMI and seeded onto MATRIGEL™ (BD Biosciences) inserts. Inserts were transferred into the wells of 24-well plates with 750 µL culture medium containing 10% FBS as a chemoattractant. Cells were allowed to invade through the matrix for 24 or 48 hours. Non-invading cells were removed from the upper surface of the insert membrane with PBS-moistened cotton-tipped swabs. Cells on the underside of the insert were stained with 1% crystal violet in 50% methanol and inserts were allowed to air dry. Counts from all 4 quadrants were obtained and recorded as total number of cells per insert. A minimum of

two inserts at each time point was utilized per primary cell line to assess invasion.

Immunoblot analysis of tumor proteins

Tumor tissue from which our primary cell lines were derived was homogenized in lysis buffer (50 mm HEPES, 100 mm NaCl, 10 mm EDTA, 0.5% NP40, 10% glycerol, supplemented with 0.1 mm PMSF, 0.1 mm NaVO4, 0.5 mm NaF, 5 μg/mL leupeptin, 0.1 mm DTT). Cells from primary cell lines derived from corresponding CRC tissue were grown to 80% confluence, washed in PBS, and lysed in the same buffer. Total protein from lysates and homogenates were quantified according to the BCA protein assay (Pierce, Rockford, IL) and 40 μg loaded onto 10%, 12%, or 15% polyacrylamide gels, depending on the size of the protein of interest. Proteins were separated by SDS-PAGE at 150V for 50 minutes and transferred to nitrocellulose membranes (BioRad, Hercules, CA) at 100 volts for 1 hour at 4˚C. Membranes were blocked with 2% blocking agent (GE Healthcare Life Sciences, Piscataway, NJ) for 2 hours at room temperature and incubated with the primary antibody anti-cytokeratin (BD Biosciences, Franklin Lakes, NJ), -K-Ras (Millipore, Billerica, MA), -p53 (Millipore), -DCC (Abcam, Cambridge, MA), or -Src (clone GD11, Upstate, Charlottesville, VA) antibodies overnight at 4˚C. Blots were washed 3 times in Tris-buffered saline-Tween 20 and incubated for 1 hour with appropriate horseradish peroxide-conjugated secondary antibodies (Invitrogen) at room temperature. Blots were washed, developed using the Western Lightening chemiluminescent kit (PerkinElmer, Waltham, MA), and exposed to Hyblot CL autoradiography film (Denville Scientific, Metuchen, NJ). All membranes were stripped using 1M Tris -HCl (pH 6.8), 10% SDS, and mercaptoethanol and reprobed for actin (Santa Cruz) as a loading control. Densitometry analysis was performed on a Dell computer using the public domain NIH Image program (U.S. National Institutes of Health; http://rsb.info.nih.gov/nih-image/). A ratio of the protein of interest to actin was calculated for all cell lines and corresponding tumor tissue. Assays were performed in triplicate.

Protein profiling by Surface Enhanced Laser Desorption/Ionization-time of Flight/Mass Spectroscopy (SELDI-TOF/MS)

Lysates and homogenates (see above) were incubated with chip-specific binding buffer (100 mM Na acetate pH 4.0 for CM10 ProteinChip™ and 10% acetonitrile, 250 mmol/L NaCl in PBS for H50 ProteinChip™) on ice for 30 minutes before application to each chip surface. Prior to loading, the H50 ProteinChip™ was pre-washed with 50% methanol samples and then all chips were washed once with 200 μL of appropriate binding buffer. Three replicates from each sample were spotted on each of the following types of ProteinChips™: H50, "reversed phase" or hydrophobic-binding, and CM10, for weak cation exchange. Following 30 minutes of incubation with vigorous shaking, samples were removed from the chip surfaces by washing each spot with appropriate binding buffer three times, followed by a rinse with deionized water. Chip surfaces were allowed to air-dry and 1 μL of sinipinic acid was applied to each spot, as an energy absorbing molecule. Following air-dry of the surfaces, an additional 1 μL of sinipinic acid was applied to each spot and again allowed to dry. SELDI-TOF MS was then performed using a PBS IIC ProteinChip™ reader (Ciphergen Biosystems, Inc., Fremont, CA). Both chip types were used for analysis. Instrument settings were: deflector mass = 10 kDa, laser shots per spot = 91, detector voltage = 2900V, maximum molecular mass = 200 kDa, optimization range = 15 kDa to 50 kDa. Detector sensitivities were 9 for H50 chips and 8 for CM10 chips. Laser intensities were 240 for CM10 and 260 for H50. Calibration was performed using all-in-one protein standards (Ciphergen). One pooled reference sample comprised of equal amounts of all 8 human CRC samples was used per chip for spectral quality control. Samples were batch analyzed using filter settings of 0.2 times the expected peak width, peak signal of ≥ 2 times the noise, baseline smoothing before fitting the baseline, and automatic minimal mass detection of 1000 Da. All data were normalized to total ion current. For each primary CRC cell line sample and each tissue sample, mass values (representing proteins) appearing a minimum of two of three times across the three replicates and within 2% value of each other for each chip surface were taken for comparison against the mass measurements generated for respective tissue samples. Corresponding intensities (representing expression levels) of these protein peaks were also compared between primary cells and parent tumor tissue. Following analysis of the adjusted means, levels of agreement were assigned according to the following desig-

nations (defined prior to analysis): concordance correlation coefficients of 0.0 - 0.4: weak agreement; 0.4 - 0.8: moderate agreement; > 0.8: strong agreement. The value $p < 0.05$ was considered statistically significant.

miRNA detection and comparison by FlexmiR™ MicroRNA Assay

miRNA profiles for each cell line and its parent tissue were obtained utilizing a multiplex liquid bead array format. The FlexmiR miRNA array kit (Luminex Corporation, Austin, TX) was utilized according to the manufacturer's protocol to obtain expression information for 320 known human miRNAs. Briefly, RNA was obtained from the 4 primary CRC cell lines and their respective tumor tissues of origin through a modified TRIzol® (Invitrogen, Carlsbad, CA) method. All steps were followed per manufacturer's instructions with the exception of substitution of bromochloropropane for chloroform for aqueous and organic phase separation [21]. Total RNA was de-phosphorylated utilizing 2.5 μL of Calf Intestinal Phosphatase per sample, and biotinylated with Biotinylation Buffer (15 μL/sample) and Biotinylation Enzyme (7.5μL/sample). Biotin-labeled samples were then hybridized at 60˚C for 60 min to a panel containing fluorescently tagged bead-coupled locked nucleic acid (LNA™)-modified capture probes. Three hundred nineteen probes (microspheres containing complementary sequences to miRNA targets based on specific known sequences listed in the Sanger miRBase Database, version 8.0), comprising 5 human microsphere pools, were utilized to assess human miRNA expression in each cell or tissue sample. Five microspheres containing a unique capture probe specific to synthetic RNA oligonucleotides and having no biologic equivalents were used as controls in each pool. Streptavidin-phycoerythrin (75 μL/sample) was then added to the sample-probe mixture, labeling the bound biotinylated miRNA. Tagged samples were then analyzed on a Luminex 200™ instrument with IS 2.3 software (Luminex) and microsphere fluorescence and SAPE intensity identified and measured. Beads were gated to exclude those that did not contain both fluorescent tags that denote bead address. Those that passed the gate were grouped into discrete clusters based on fluorescence levels of each of the two tags. A simultaneous assessment of phycoerythrin signal allowed signal intensity of bound material to be associated with probe information. A background control of water, treated in the identical manner as RNA samples throughout the assay, was used to obtain a median fluorescent intensity that was subtracted from all sample median fluorescent intensities in the microsphere pools. A minimum of 100 bead events for each probe from each pool was recorded for each sample and its median fluorescent intensity exported for further data analysis. Intra-sample normalization between pools was performed according to baseline-subtracted intensities of four supplied normalization microspheres that represent known human small nucleolar RNAs.

Statistical analysis

SELDI TOF/MS protein profiling - The intensity and mass measurements were transformed via the natural logarithm in order to better approximate normality. The first analysis consisted of constructing a difference between the logarithm -transformed intensities of the paired cell and tissue measurements. A repeated measurements analysis of covariance was applied to these differences, in which the statistical model assumed an autoregressive structure based on cluster distance for the correlations within a pair. The second analysis consisted of quantifying the level of agreement between the cell and tissue measurements via the concordance correlation coefficient [22].

FlexmiR™ MicroRNA Assay - Correlations between levels of human microRNA expression for each pairing of cell sample and respective tissue sample were expressed by the Kendall tau coefficient of the correlation of the ranks. Correlation, or level of agreement, was defined as the following: tau coefficient values of 0.0 – 4.0: weak agreement; 0.41 – 0.8: moderate agreement; >0.8: strong agreement. The value $p < 0.05$ was considered statistically significant.

Results

Generation and confirmation of primary colonic adenocarcinoma cell lines

Twenty-six tumor specimens were collected. Enzymatic digestion of CRC tumor tissue yielded successful culture of four primary cell lines, designated CRS4, CRS30, CRS61, and CRS63. All four primary cell lines were derived from histopathologically confirmed CRC tissue. **Table 1**

Table 1. Clinical characterization of colorectal tumor samples

Patient/ Sample	Tumor Location	T	N	M	Stage	Histologic Grade
CRS4	Rectal	T3	N1	M0	IIIB	Moderately differentiated
CRS30	Ascending colon	T3	N2	M0	IIIC	Poorly differentiated
CRS61	Descending colon	T3	N0	M0	IIA	Moderately differentiated
CRS63*	Liver	T4	N0	M1	IV	Well differentiated

T: tumor invasion; N: nodal involvement; M: distant metastasis.
**CRS63 was generated from metastatic hepatic tumor. The primary site of the CRC was rectal.*

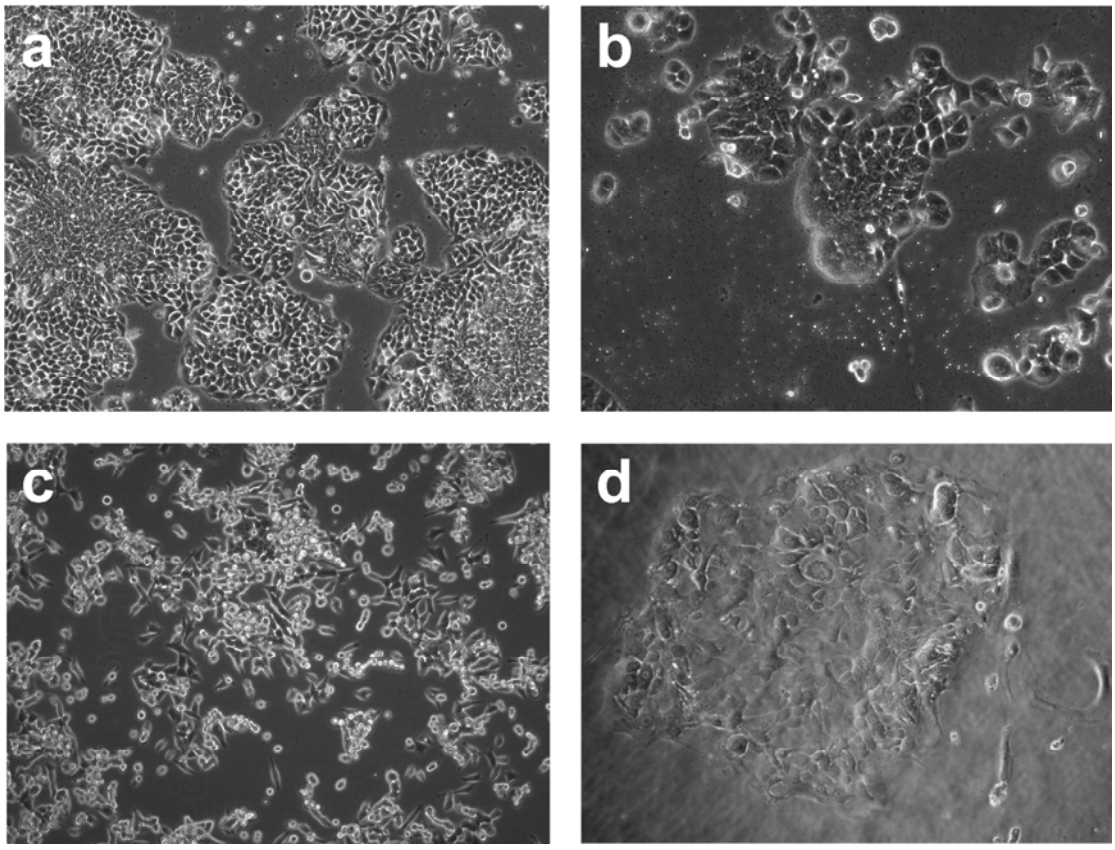

Figure 1. Morphology of primary CRC cell lines. Phase contrast images of primary cell lines CRS4 (a), CRS30 (b), CRS61 (c), and CRS63 (d) are shown.

outlines location, stage, and histologic grade for each patient. Cells grew in colonies rather than sheets (**Figure 1A-D**) and did not terminally differentiate upon confluence. To confirm that cell lines were epithelial, all lines were subjected to immunofluorescence and immunoblot for cytokeratin, an epithelial cell marker (**Figure 2A-E**).

Tumorigenicity of primary cell lines

To confirm that the cell lines were able to grow as tumors *in-vivo*, 2.0 x 10^6 cells were subcutaneously injected into nude mice. All four primary cell lines showed tumor growth within 7 days of injection. At three weeks, cell lines produced

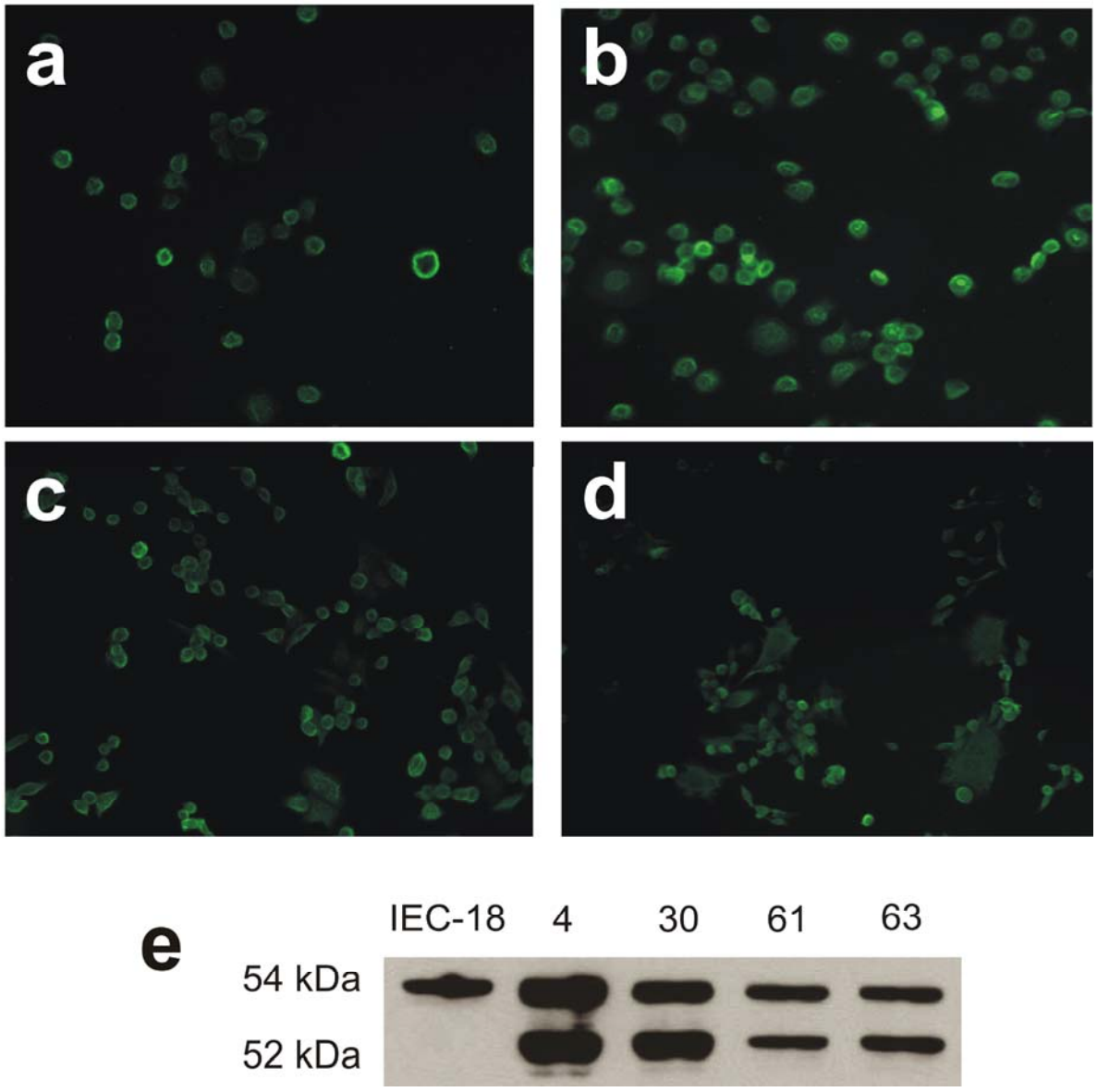

Figure 2. Cytokeratin expression of primary CRC cell lines. Immunofluorescent staining of CRS4 (a), CRS30 (b), CRS61 (c), and CRS63 (d) for cytokeratin is shown. Immunoblot of cytokeratin in all 4 primary CRC cell lines and control IEC-18 cells, is shown in (e).

tumor volumes of 144 mm^3, 294 mm^3, 64 mm^3, 131 mm^3 for cell lines CRS4, CRS30, CRS61, and CRS63, respectively (**Figure 3**).

DNA ploidy of CRC lines

Cell DNA ploidy of the four newly established colorectal cancer cell lines was determined by flow cytometric cell cycle analysis. Two of the cell lines, CRS4 and CRS30, were shown to be aneuploid, with each demonstrating a DI of 1.60. CRS61 and CRS63 were determined to be diploid, with DIs of 1.13 and 1.01, respectively. As expected, control cell lines HT29 and HCT116 were noted to be aneuploid and diploid, respectively, with a DI of 1.59 for HT29 and a DI of 1.18 for HCT116. All coefficients of variation were < 5.0% for all samples analyzed, including the two control lines.

Primary CRC cell invasion

Invasive ability of the primary CRC cell lines was tested using the MATRIGEL™ Basement Mem-

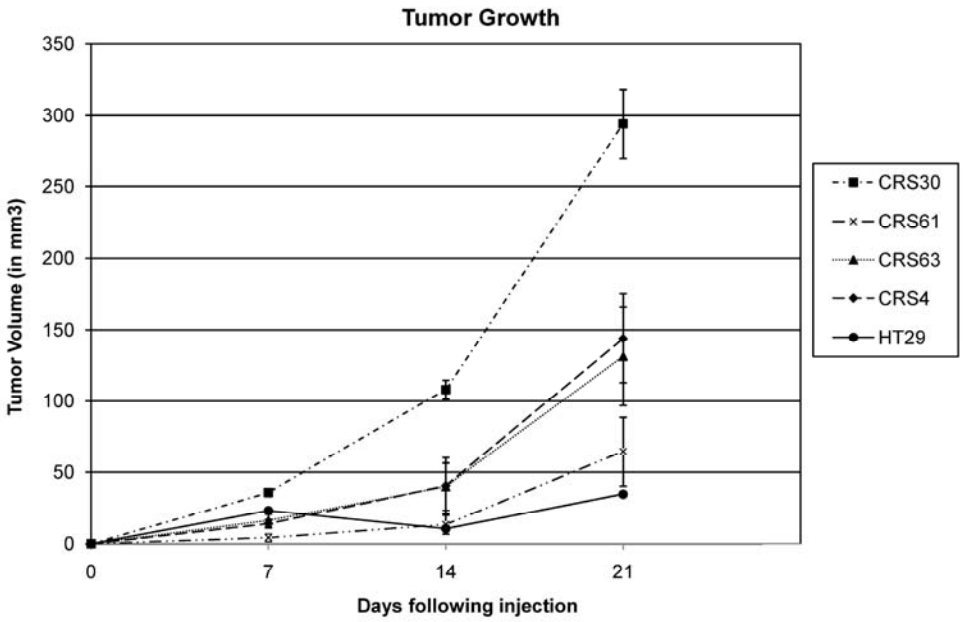

Figure 3. Tumor growth of primary CRC cell lines. All primary cell lines demonstrated tumor growth by 7 days. Growth rate was variable among cell lines. N= 4 for primary CRC cell lines; HT29 was used a reference CRC line.

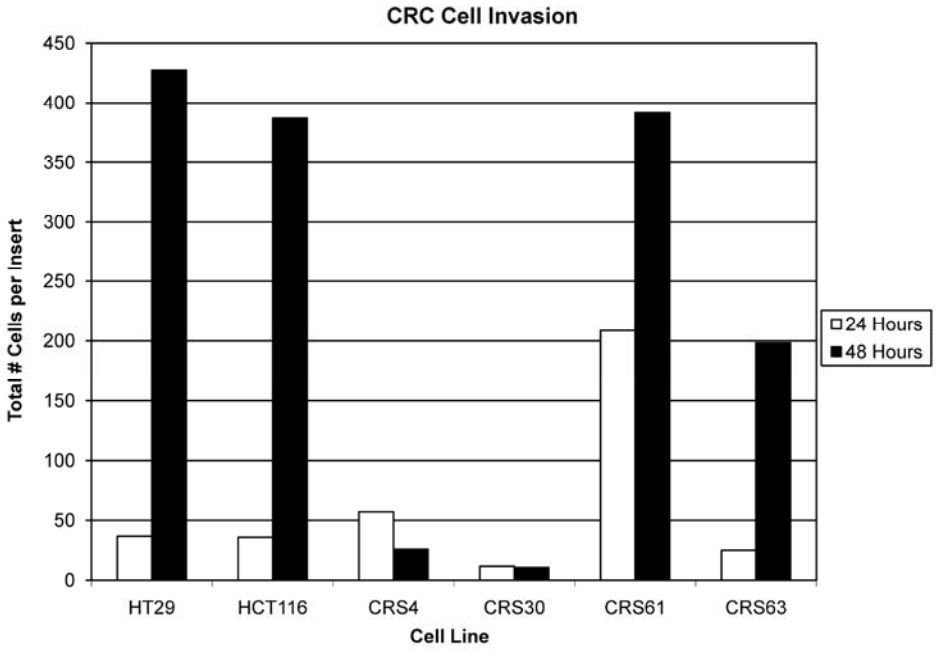

Figure 4. Cell invasion of primary CRC cell lines. All cell lines invaded at 24 h, with CRS61 and CRS63 being more invasive at 48 hours, with invasion by CRS61 at 48 h comparable to rates of invasion of cells of reference, HT29 and HCT116, at 48 h. N ≥ 2 for all primary cell lines.

brane Matrix. Cells from our four primary cell lines were applied to MATRIGEL™ matrices and allowed to invade through the membrane. HT29 and HCT116 cells were used as controls.

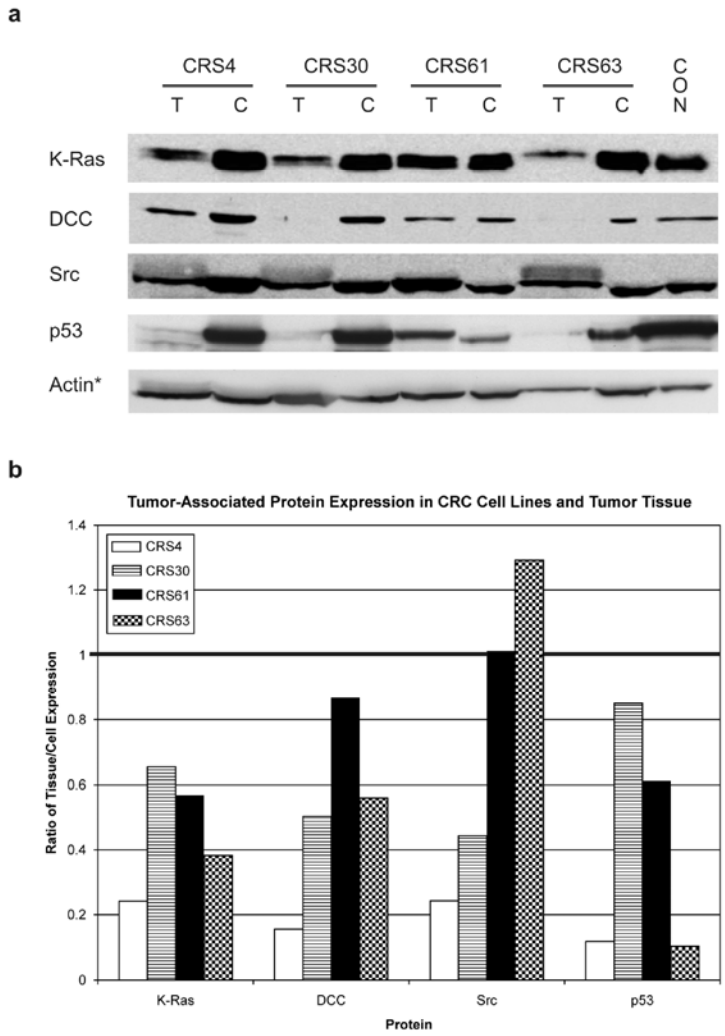

Figure 5. Tumor-associated protein expression in cell lines and parental tumor tissue. a) Representative immunoblots demonstrating varied protein expression between different cell lines (C) and also between cell lines and corresponding tissue (T). CON = control CRC cell line: A431 for K-Ras and Src, SW480 for p53, and HCT116 for DCC. *Representative actin blot. b) Comparisons of protein expression between cell lysates and tissue homogenates were made by use of the ratio of tissue expression/cell expression, where ratio >1 = stronger tissue expression and ratio <1 = stronger cell expression.

Invading cells were counted at 24 and 48 hours. As seen in **Figure 4**, measurements of the number of invading cells per insert at these time points revealed that all primary cell lines invade a biologically active matrix material resembling mammalian cellular basement. Further, they invaded at different rates, suggesting that generated CRC cell lines possess varying degrees of metastatic potential.

Tumor-associated protein expression in cell lines and parental tumor tissue

Expression of several CRC-associated proteins was evaluated in the tumor tissue and their respective cell lines to assess how comparable the colon cancer cell lines were to the cancers from which they were derived. Immunoblot analysis of cell lysates and tissue homogenates from the tumors of origin demonstrated varied expression of K-Ras, DCC, Src, and p53 between different cell lines and also between cell lines and corresponding tissue (**Figure 5**). Two of the 4 pairings demonstrated greater Src expression in the cells as compared to tissue. However, in CRS61, Src expression was similar in both cells and tissue while in CRS63, Src expression was greater in tissue. On the other hand, in all 4 cell-tissue pairings, K-Ras and DCC were more highly expressed in cell lines as compared to tissue. The expression of p53 was highly variable across the pairs. Expression of the tumor-associated proteins evaluated would be expected to be increased in the epithelial cell lines, as compared to their tissue counterparts because the cell samples represent a purified epithelial population, while the homogenized samples generated from the cancer tissue contain several cell types.

Table 2. Similarities in protein expression levels by intensity between cells and tumor tissue

Cell-Tissue Pair	Chip Surface	Correlation Coefficient	Correlation Strength	95% CI	P-Value
CRS4	CM10	0.284	Weak	(0.081, 0.464)	0.0116
	H50	0.591	Moderate	(0.064, 0.860)	0.0363
CRS30	CM10	0.663	Moderate	(0.355, 0.841)	0.0022
	H50	0.861	Strong	(0.725, 0.932)	<0.0001
CRS61	CM10	0.988	Strong	(0.947, 0.997)	<0.0001
	H50	0.487	Moderate	(0.147, 0.724)	0.0127
CRS63	CM10	0.692	Moderate	(0.394, 0.858)	0.0017
	H50	0.817	Strong	(0.633, 0.914)	<0.0001

Table 3. Similarities in proteins by masses between cells and tumor tissue

Cell-Tissue Pair	Chip Surface	Correlation Coefficient	Correlation Strength	95% CI	P-Value
CRS4	CM10	0.465	Moderate	(0.116, 0.711)	0.0158
	H50	0.370	Weak	(-0.398, 0.834)	0.2722
CRS30	CM10	0.328	Weak	(-0.123, 0.667)	0.1310
	H50	0.510	Moderate	(0.119, 0.764)	0.0165
CRS61	CM10	0.658	Moderate	(0.070, 0.907)	0.0348
	H50	0.436	Moderate	(-0.023, 0.743)	0.0594
CRS63	CM10	0.665	Moderate	(0.319, 0.855)	0.0039
	H50	0.545	Moderate	(0.036, 0.829)	0.0391

Protein profiling in cells and tissue

Protein "footprints" for each of the cell and tissue samples were generated by SELDI-TOF/MS. Repeated measurements analysis of covariance followed by analysis via the concordance correlation coefficient indicated that while each cell sample and its progenitor tissue were not significantly different from each other based on the overall combined presence (p-values all <0.05 for each cell-tissue pair; data not shown) and levels of expression (p-values all <0.05 for each pair; data not shown) of their protein constituents, they displayed levels of weak to moderate agreement based on the proteins comprising the profiles, indicating that few to a moderate number of proteins were expressed in both the cell and tissue sample for each pair. Intensity of the expressed proteins, however, was very similar between cell and progenitor tissue, exhibiting an overall moderate to strong correlation, with one exception of weak agreement of expression from the CM10 chip for the CRS4 cell-tissue pairing. **Table 2** lists the concordance correlations for protein intensity for each chip surface for all four cell-tissue pairings. **Table 3** lists the correlation coefficients for comparisons of proteins by mass.

miRNA expression in cells and tissue

Application of the Kendall tau correlation coefficient was used to assess the intensity of known human miRNA expression between cell samples and their respective parental tissue samples. Moderate correlations were assigned to the

Table 4. Similarities in microRNA expression between cells and tumor tissue and across cell lines

Cell Line	Tumor Tissue	Correlation Coefficient	95% CI	Cell Line	Cell Line	Correlation Coefficient	95% CI
CRS4	CRS4	0.49	(0.44, 0.55)	CRS4	---	---	---
	CRS30	0.55	(0.50, 0.59)		CRS30	0.76	(0.73, 0.80)
	CRS61	0.67	(0.62, 0.71)		CRS61	0.72	(0.69, 0.76)
	CRS63	0.35	(0.27, 0.42)		CRS63	0.73	(0.70, 0.76)
CRS30	CRS30	0.52	(0.47, 0.57)	CRS30	---	---	---
	CRS61	0.64	(0.59, 0.69)		CRS61	0.72	(0.69, 0.76)
	CRS63	0.33	(0.26, 0.41)		CRS63	0.72	(0.69, 0.75)
	CRS4	0.46	(0.40, 0.52)		CRS4	0.76	(0.73, 0.80)
CRS61	CRS61	0.66	(0.62, 0.71)	CRS61	---	---	---
	CRS63	0.34	(0.26, 0.41)		CRS63	0.81	(0.79, 0.84)
	CRS4	0.49	(0.43, 0.54)		CRS4	0.72	(0.69, 0.76)
	CRS30	0.58	(0.53, 0.62)		CRS30	0.72	(0.69, 0.76)
CRS63	CRS63	0.34	(0.26, 0.41)	CRS63	---	---	---
	CRS4	0.51	(0.45, 0.56)		CRS4	0.73	(0.70, 0.76)
	CRS30	0.59	(0.54, 0.63)		CRS30	0.72	(0.69, 0.75)
	CRS61	0.67	(0.62, 0.71)		CRS61	0.81	(0.79, 0.84)

Table 5. Total unchanged and increased miRNA expression by fold-change in CRC primary cells compared to parental tissue (expression values of cell miRNAs divided by tissue miRNA expression) and progenitor tissue compared to its derived cell line (expression values of tissues divided by cell values)

Sample	No Change	>2 Fold	> 10 Fold	> 50 Fold	> 100 Fold
CRS4 Cells	7 (2%)	123 (39%)	16 (5%)	3 (<1%)	0
CRS4 Tissue	7 (2%)	38 (12%)	8 (2%)	2 (<1%)	0
CRS30 Cells	12 (4%)	34 (11%)	3 (<1%)	0	0
CRS30 Tissue	12 (4%)	118 (37%)	11 (3%)	1 (<1%)	0
CRS61 Cells	10 (3%)	105 (33%)	10 (3%)	2 (<1%)	0
CRS61 Tissue	10 (3%)	20 (6%)	3 (<1%)	0	0
CRS63 Cells	1 (<1%)	188 (59%)	74 (23%)	10 (3%)	5 (2%)
CRS63 Tissue	1 (<1%)	4 (1%)	0	0	0

CRS4 pairing, the CRS30 pairing, and the CRS61 pairing, indicating that the cells and respective tumor tissues in each of these pairs expressed a number of similar miRNAs at comparable levels of expression. A weak correlation was demonstrated for the CRS63 pair, in which only a small number of similarly expressed miRNAs was exhibited in both the cell and respective tissue. **Table 4** (left side) lists the Kendall's tau correlation coefficients for each

cell-tissue combination, showing weak to moderate strengths of correlation between primary cell lines and respective tumor tissue of origin for all pairs (all p values <0.0001). miRNA expression was further assessed by fold-change increase in cell and tissue samples for each pair, as shown in **Table 5**.

miRNA expression was also compared amongst the four primary cell lines and amongst the four tumor tissues. The CRS4 and CRS30 cell lines exhibited a moderate degree of similarity, and therefore, agreement, between themselves and to the CRS61 and CRS63 cell lines. The CRS61 and CRS63 cell lines were shown to strongly correlate to each other, indicating that these two cell lines are very similar in their miRNA expression profiles. **Table 4** (right side) outlines the tau correlation coefficients for each cell to cell comparison and shows that expression was moderate to strong in strength of correlation from cell line to cell line (all p values <0.0001).

When compared to each other, the tumor tissues CRS4, CRS30, and CRS63 revealed multiple similarities earning an assignment of moderate agreement between them, much like their derived cell lines. The tissues CRS61 and CRS63 were found to weakly correlate to each other, unlike their strongly correlating derived cell lines. The strong correlation between cell lines argues in favor of generalizing cell culture data for CRC.

Discussion

Cell culture, a technique that celebrated its 100th anniversary in 2007, is recognized as a major tool for the study of cellular signaling pathways, tissue engineering, the production of monoclonal antibodies, generation of vaccines, and testing of pharmaceuticals. Ross Granville Harrison's first successful establishment of frog nerve fibers *in vitro* from neurons of explanted tissue and his optimization of culture conditions established an instrumental biological system to study tumor biology and cancer cell behavior [23]. Although the use of cell culture has yielded an invaluable amount of data in the field of cancer biology, it is with caution that we relate what is learned regarding cell behavior *in vitro* to *in vivo* processes.

Our reason for the development of novel colon cancer cell lines is two-fold. First, a number of established CRC cell lines available through repositories such as the ATCC, such as SW480, SW620, HT29, and HCT116, have been in use for several decades. Derivation of SW480 and HCT116 cells, for example, was reported in 1976 and 1981, respectively [24-25]. Further, these cell lines, once made available for public use, are typically of high passage number. Observations of spontaneous mutation and changes in cell characteristics are evident in cell lines maintained in culture for long periods of time and reports relating variability of key cell properties to increasing passage numbers have received much attention [4-12]. These investigations provide compelling evidence for the need to increase the number of characterized cell lines.

Second, we wished to assess the reliability of primary cell lines in representing their tumor tissues of origin. Though studies at the subcellular level unquestionably provide a better understanding of the molecular basis of disease and provide a springboard for the development of anti-cancer drugs, these conclusions are derived from tightly controlled experimental environments, without the evaluation of tumor tissue itself. In addition, tissue specimens are often difficult to obtain and integrity can be severely compromised secondary to the manner in which they are procured, rendering valuable tools, like mRNA microarray, difficult to apply as a result of tissue ischemia and RNA degradation.

Subsequent to their isolation and use in the described assays, each cell line has been subcultured through greater than 50 passages, with each passage a population doubling, and with no decrease in proliferative capacity (data not shown). The experiments described in the current work were performed on cells at low passage number to most closely reflect characteristics of the isolated cells, and therefore, their tumors of origin. We have used the term "primary cell lines" as a descriptive term to denote that these cells were isolated from tumor tissue and were not artificially transformed or immortalized. These cell lines exhibit characteristics that are ascribed to tumor cells, specifically tumorigenicity, invasion, and expression of tumor-associated proteins, even at the low passage numbers at which these cells were evaluated. In addition, two of the four cell lines demonstrate DNA aneuploidy, a feature commonly

noted in cancer cells, particularly those derived from solid tumors.

We have established 4 new CRC cell lines that exhibit comparable tumorigenicity and invasiveness to other established CRC cell lines. All 4 cell lines demonstrated tumorigenicity and the ability to invade a biologically active matrix similar to basement membrane, a property that is integral to the process of metastasis. Our cell lines invaded at different rates. One might expect that cell lines derived from higher-staged cancers would be more invasive, and invade more rapidly. However, CRS61, a Stage II cell line, was more invasive than the two Stage III lines, CRS4 and CRS30, again suggesting that what occurs *in vitro* does not necessarily mimic *in vivo* behavior.

All four cell lines and their respective tumor tissue of origin demonstrated varying expression of K-Ras, DCC, Src, and p53. Intuitively, one would expect the CRC cell lines to have larger amounts of these proteins since they are pure epithelial cell populations. This, however, was not a consistent finding across our cell and tissue samples. Differences in tumor-associated protein expression noted in this study may not be simply explained by the fact that the cell lines are purified populations, suggesting that *in vitro* growth alters protein expression.

Sandberg and Ernberg have designed a tissue similarity index (TSI) as a means to assess tumor characteristic gene expression in cell lines [26]. Through their scoring algorithm, they have identified that 34 of 60 NCI60 cell lines tested had highest TSI scores, indicating that gene expression levels characteristic for their tumors were similarly expressed in the cell lines, reflecting their presumed origin. The remaining 26 cell lines received low TSI scores for their presumed tumor origin, widely differing in their expression of tumor characteristic genes, and thus demonstrating that cell lines can lose tissue-specific up-regulation of genes. Moreover, they have identified several lines as being of other origin than those originally presumed [26]. Similarly, Virtanen *et al*. compared gene-expression profiles of 38 lung cancer cell lines with 44 tumors from lung cancer through cDNA microarray [27]. Twenty-four of the 38 lines resembled their tissue counterparts sufficiently. However, small groups of cell lines, differing by subtype of lung cancer, were significantly distinct [27].

To further evaluate the differences between our cell lines and the tissues from which they were derived, we used SELDI-TOF/MS for the purpose of protein profiling and the FlexmiRä MicroRNA Assay to compare expression levels of known human miRNAs between our sample groups. SELDI-TOF/MS affords the ability to generate a general protein fingerprint of cells and tissue, enabling an overall and broad comparison of protein expression between cells and coordinate tumor tissue. The FlexmiRä assay enables the evaluation of differences in levels of expression of known human miRNAs between the two sample groups.

Protein profiling of each cell line and its progenitor tissue yielded protein "footprints" that, based on the combination of protein constituents and intensity of the expressed proteins, overall, were similar for each cell-tissue pair. Although not statistically different from one another in this respect, cells and tissue in each pair, however, exhibited varying levels of correlation based on their proteins expressed and intensities thereof. Weaker correlations between samples in each pair may be attributed to the heterogeneity of the cancer tissues and, again, the likelihood that in vitro growth alters protein expression. Despite these differences, cell lines are largely representative of the cancer types they are derived from and can prove useful in the study of these diseases.

A more focused evaluation was achieved through the comparison of microRNAs in our cell and tumor tissue samples. MicroRNAs are small, non-coding, non-messenger RNAs that regulate gene expression through binding of complementary messenger RNAs. Only 18-24 nucleotides in length, miRNAs facilitate either mRNA degradation or translational inhibition, based on precision, or degree, of complementarity. Small in size, miRNAs have been shown to be stable, ideal markers to assess expression in various preparations of archived tissue [28-31]. To date, several hundred human microRNA sequences [32] have been reported, though detailed mechanisms of function have only been demonstrated for a small fraction.

While not significantly different from their respective parental tumors, the primary cell lines exhibit a weak to modest, at most, similarity in overall microRNA expression pattern to these tissues. In addition, a weak to moderate correla-

tion was seen between in each cell line and all 4 tissue samples. This is not surprising, as each miRNA has the ability to target hundreds of mRNAs, and shared expression between cell lines of similar type should be expected. Further, as evidenced by Sandberg *et al*., cell lines with high TSI scores retain the characteristics of the type of tissue from which they are derived, even though they may not necessarily be directly derived from the same tumor specimens [26].

Conclusions

Cell culture is a valuable tool to study the mechanisms of cancer development and progression, particularly invasion and metastasis. Though utilization of tumor tissue would be ideal, multiple factors exist that deem this approach problematic. We have established 4 new primary CRC cell lines with which to study the process of colorectal carcinogenesis and metastasis. Our findings demonstrate that although expression of some proteins and miRNAs in our primary cell lines vary as compared to the tumors from which they have been derived, they do not statistically differ in their overall profiles and do retain certain characteristics as their parental tissue. Thus, cell lines, although without ideal fidelity, remain a close representation of *in vivo* processes. Until alternative methodologies are discovered, cell culture will continue to be a mainstay in the elucidation of details governing tumor biology and cancer behavior. We must remain cognizant, however, that *in vivo* processes are indeed more complex than *in vitro* studies allude to and that these events are regulated by a delicate balance of physiological, environmental, and biochemical influences.

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Abbreviations: CRC = colorectal cancer, DCC = deleted in colon cancer, DI = DNA index, EDTA=ethylenediaminetetraacetic acid, FBS = fetal bovine serum, K-Ras = Kirsten ras, MEM = Minimum, Essential Media, miRNA = microRNA, PBS = phosphate buffered saline, SELDI-TOF/MS = surface enhanced laser desorption/ionization-time of flight/mass, TSI = tissue similarity index.

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