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Three-dimensional culture system identifies a new mode of cetuximab resistance and disease-relevant genes in colorectal cancer

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We previously reported that single cells from a human colorectal cancer (CRC) cell line (HCA-7) formed either hollow single-layered polarized cysts or solid spiky masses when plated in 3D in type-I collagen. To begin in-depth analyses into whether clonal cysts and spiky masses possessed divergent properties, individual colonies of each morphology were isolated and expanded. The lines thus derived faithfully retained their parental cystic and spiky morphologies and were termed CC (cystic) and SC (spiky), respectively. Although both CC and SC expressed EGFR (EGFR), the EGFR-neutralizing monoclonal antibody, cetuximab, strongly inhibited growth of CC, whereas SC was resistant to growth inhibition, and this was coupled to increased tyrosine phosphorylation of MET and RON. Addition of the dual MET/RON tyrosine kinase inhibitor, crizotinib, restored cetuximab sensitivity in SC. To further characterize these two lines, we performed comprehensive genomic and transcriptomic analysis of CC and SC in 3D. One of the most up-regulated genes in CC was the tumor suppressor 15-PGD/HPGD, and the most up-regulated gene in SC was versican (VCAN) in 3D and xenografts. Analysis of a CRC tissue microarray showed that epithelial, but not stromal, VCAN staining strongly correlated with reduced survival, and combined epithelial VCAN and absent HPGD staining portended a poorer prognosis. Thus, with this 3D system, we have discovered that increased tyrosine phosphorylation of MET and RON results in cetuximab resistance in the SC cell line that can be overcome by addition of the dual MET/RON tyrosine kinase inhibitor, crizotinib. We have also identified that increased epithelial, but not stromal, versican staining correlates with reduced survival in a clinically annotated CRC tissue microarray.

Significance

By culturing a human colorectal cancer (CRC) cell line (HCA-7) in 3D, we have generated two cell lines (CC and SC) with distinct morphological, genetic, biochemical, and functional properties. Using this 3D system, we have discovered that increased tyrosine phosphorylation of MET and RON results in cetuximab resistance in the SC cell line that can be overcome by addition of the dual MET/RON tyrosine kinase inhibitor, crizotinib. We have also identified that increased epithelial, but not stromal, versican staining correlates with reduced survival in a clinically annotated CRC tissue microarray.


The authors declare no conflict of interest.

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smooth-edged, single-layered cysts with hollow cavities (Fig. 1A and B, Left). Upon closer inspection, less frequent colonies were also observed that appeared to be solid masses with protrusions (Fig. 1A and B, Right). We manually isolated individual cystic and spiky colonies and propagated them on plastic. CC and SC lines thus derived maintained their distinctive cystic and spiky morphologies, respectively, upon repeated passage in 3D. CC and SC were scored for their morphological appearance after 15 d in 3D type-1 collagen; 95% of CC appeared cystic and 78% of SC appeared spiky (Fig. 1A).

We next performed a more detailed morphological characterization of CC and SC. Representative differential interference contrast (DIC) images in Fig. 1A show that CC colonies form hollow unilamellar cysts and that SC colonies form solid masses with protrusions (Fig. 1B). These findings are further confirmed by confocal images through the equatorial planes of CC and SC colonies stained for nuclei and F-actin (Fig. 1C). In addition, apico-basolateral polarity was maintained in CC colonies as determined by subapical ezrin immunoreactivity and EGFR decorating the lateral membranes (Fig. 1D). Transmission electron microscopy of CC colonies showed that apico-basolateral architecture was maintained with rudimentary microvilli detected at the apical surface with tight junctions (TJ) and adherens junctions (AJ) at nearby cell–cell junctions (Fig. 1E, Left panels). Within the protruding regions of SC colonies, TJ and AJ were not observed (Fig. 1E, Right panels). In contrast to their marked morphological differences in 3D, both CC and SC exhibited a similar epithelial cobblestone appearance in 2D (SI Appendix, Fig. S1A), although SC grew faster than CC (SI Appendix, Fig. S1C). Similarly, both CC and SC formed uniform polarized monolayers on Transwell filters with a similar basolateral localization of CDH1 and ITGB1 (SI Appendix, Fig. S1B).

Identification of a Mode of Cetuximab Resistance. We previously reported that CC form well-differentiated tumors in nude mice, whereas SC form poorly differentiated, locally invasive tumors (8). We next sought to determine if there were additional functional differences between CC and SC. EGFR-neutralizing monoclonal antibodies (cetuximab and paintumumab) are approved by the US Food and Drug Administration for the treatment of advanced wild-type KRAS CRCs (9). Because HCA-7 cell lines are wild type for KRAS, we tested the efficacy of...
cetuximab against CC and SC in 3D. Fig. 1F shows that cetuximab has a greater inhibitory effect on the growth of CC compared with SC, whereas it has only a modest effect on the growth of either CC or SC in 2D (SI Appendix, Fig. S1D).

We set out to determine the mechanism(s) underlying cetuximab resistance in SC. There were no known genetic events that could explain the resistance to cetuximab in SC; KRAS, BRAF, NRAS, PIK3CA, and EGFR were wild type, and MET was not amplified (SI Appendix, Table S1). Nor did we detect any differences between CC and SC at the level of EGFR mRNA expression, total and cell-surface EGFR protein, and EGFR activation following EGF stimulation (SI Appendix, Table S2 and Fig. S2A and B). Based on Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis of gene expression, we found that MAPK signaling was the most up-regulated signaling pathway in SC compared with CC (Fig. 2A and Fig. 3B and C). Bardelli and coworkers recently showed that addition of a MEK inhibitor (pimasertib) to cetuximab could prevent cetuximab resistance (10). Consistent with that result, addition of another MAPK inhibitor (U0126) to cetuximab in SC results in cooperative growth inhibition (Fig. 2B). To determine upstream signaling pathways that might be responsible for increased MAPK signaling in SC, we performed a receptor tyrosine kinase (RTK) array on CC and SC and found that there was a selective increase in phosphorylated MET and RON in SC compared with CC (Fig. 2C). This led us to examine the effect of crizotinib, a dual MET/RON tyrosine kinase inhibitor. Whereas neither cetuximab nor crizotinib alone significantly inhibited the growth of SC, the combination markedly inhibited growth (Fig. 2D). As expected, crizotinib decreased MET tyrosine phosphorylation and cetuximab decreased total and tyrosine-phosphorylated EGFR (Fig. 2E). The combination of cetuximab and crizotinib led to a marked reduction in ERK1/2 and AKT phosphorylation (Fig. 2E).

Genetic Characterization of CC and SC. Based on the morphological and functional differences observed between CC and SC, we decided to perform a more detailed characterization of these lines. CC and SC are derived from microsatellite unstable HCA-7 (11).

Fig. 2. Mechanism of cetuximab resistance in SC. (A) KEGG analysis of gene expression shows that MAPK signaling is the most up-regulated pathway in SC versus CC. (B) Two thousand SC cells were cultured in type-1 collagen for 2 wk. Fresh medium was added with cetuximab (CTX, 3 μg/mL) and/or the MEK inhibitor U0126 (1 μM) every 2–3 d as indicated. Colony count are plotted as mean ± SEM P < 0.05 for the combined treatment compared with control and individual treatments (two-tailed unpaired t test). (C) A portion of the RTK array showing relative levels of phosphorylation of EGFR, MET, and RON between CC and SC. Each signal is spotted in duplicate; reference is spotted on the lower left. (D) Two thousand SC cells were cultured in type-1 collagen for 2 wk. Fresh medium was added with cetuximab (CTX, 3 μg/mL) and/or the MET/RON inhibitor crizotinib (CRIZ, 0.05 μM) every 2–3 d as indicated. Colony counts are plotted as mean ± SEM P < 0.05 for the combined treatment compared with control and individual treatments (two-tailed unpaired t test). (E) One hundred thousand SC cells were cultured in type-1 collagen for 5 d and incubated with cetuximab (CTX, 3 μg/mL) and/or the MET/RON inhibitor crizotinib (CRIZ, 0.05 μM) for 6 or 24 h as indicated. Middle-collagen-layer–containing cells were lysed, resolved on SDS/PAGE, and immunoblotted for the indicated proteins and their phosphorylation state.
Microsatellite unstable tumors are unable to repair base-pair mismatches and have hundreds to thousands of mutations. We performed whole-exome sequencing of parental HCA-7 and their CC and SC derivatives. We identified single nucleotide variants (SNVs) and insertions and deletions (INDELs) for each cell line and calculated pairwise identity-by-state (IBS), in which two individuals are observed to have zero, one, or two alleles in common at a given locus (12). The IBS of all three pairs (CC-SC, CC-HCA-7, and SC-HCA-7) exhibited a mean close to 2 and minor variance, confirming the genetic relatedness of these cell lines (Fig. 3A). Among the three pairs, CC-SC had the lowest IBS mean and largest variance, indicating that CC and SC were more different from each other than parental HCA-7. Nevertheless, there were 152,203 common SNVs and 23,045 INDELs shared between CC and SC, consistent with their common parental origin (Fig. 3A).

All three lines shared a homozygous frameshift mutation in type 2 TGF-β receptor (TGFBR2), resulting in receptor truncation, a homozygous mutation in APC resulting in a premature stop at codon 1554 and a heterozygous R150W mutation in TP53. There were no nonsynonymous mutations in mismatch repair genes (PMS2, MLH1, MSH2, and MSH6). However, HCA-7, CC, and SC all exhibited hypermethylation of MLH1, and MLH1 protein was not detected by immunohistochemistry (IHC) (SI Appendix, Fig. S3). All three lines were wild type for KRAS, BRAF, PIK3CA, and EGFR.

**Fig. 3.** Genetic and transcriptional characterization of CC and SC. (A) Genetic relatedness among CC, SC, and parental HCA-7 by pairwise IBS distance plot. A comparison of SNVs and INDELs between CC and SC is shown within the IBS plot. (B) Principal component analysis of transcriptional profiles for CC (blue) and SC (red) cultured in 3D for 5, 10, and 15 d, represented by small, medium, and large spheres, respectively. (C) Circos plot of copy number variation (CNV) and differential expression between CC and SC. Chromosomal location is marked on the outer circle. The chromosomal location of the top 100 up-regulated genes in SC (red) and CC (blue) is depicted. The top 10 up-regulated genes in CC and SC are displayed in the center table in decreasing order of expression. The innermost circle shows relative CNV between CC and SC with gains in CC and SC depicted as blue and red, respectively.
The genetic differences between CC and SC accounted for less than 2% of total SNVs and less than 1% of total INDELs (Venn diagrams in Fig. 3A). There were heterozygous missense mutations in 156 genes in CC and 172 genes in SC (SI Appendix, Fig. S4A). Of note, there were 14 genes with homozygous mutations unique to SC (SI Appendix, Table S1 and Fig. S4A). In CC, six genes had unique homozygous missense mutations (SI Appendix, Table S1 and Fig. S4A). In addition, CC and SC had five and four unique insertions or deletions, respectively (SI Appendix, Table S1).

We next examined gene expression in CC and SC in 3D culture. RNA was isolated from CC and SC cultured in 3D for 5, 10, and 15 d for microarray analysis. Principal component analysis showed that both cell type and time contributed to the transcriptional landscape (Fig. 3B). To focus on the contribution of cell type rather than time in culture, we focused on genes with consistent differential expression between CC and SC at all three time points. The top 100 genes (blue for CC, red for SC) from that list are aligned along their respective chromosomes in the Circos plot in Fig. 3C (complete list in SI Appendix, Table S2). The top 10 genes for each line are listed in the center of the Circos plot in decreasing order of fold-change.

The inner circle of the Circos plot depicts copy-number gains and losses as determined by exome sequencing. Based on CC and SC genetic comparison, we found copy-number gain in chromosome 9p24.3–9p21.3 in SC that may underlie the consistent overexpression of a cohort of genes in this region. Similarly, there were gene clusters showing copy number gains and losses in the inner circle with corresponding changes in gene expression.

**Fig. 4.** In vitro and in vivo validation of HPGD and VCAN expression in CC and SC. (A) CC and SC grown in 3D in type-1 collagen for 15 d were lysed and resolved on 10% SDS/PAGE and immunoblotted for HPGD and β-actin. (B) Formalin-fixed, paraffin-embedded (FFPE) sections from CC and SC tumors established as subcutaneous xenografts in athymic nude mice were immunostained for HPGD (red), CDH1 (green), and DAPI (blue). (Scale bars: 50 μm.) (C) LC/MS/MS analysis of PGE$_2$ and the HPGD metabolite (13,14-dihydro-15-keto-PGE$_2$) from the conditioned medium of CC and SC cultured in 3D for 14 d. Results are plotted as mean concentration (ng/mL) ± SEM. n.d., not detected. (D) Conditioned media from CC and SC grown in 3D and VCAN-overexpressing HEK293Ts cultured on plastic were treated with chondroitinase, resolved on 7% SDS/PAGE, and immunoblotted for VCAN. (E) FFPE sections from CC and SC xenografts were immunostained for VCAN (red), CDH1 (green), and DAPI (blue). (Scale bars: 50 μm.) (F) ChIP with antibodies against the indicated histone modifications was performed on chromatin isolated from CC (blue) and SC (red) colonies grown in 3D in type-1 collagen for 15 d (n = 6). The immunoprecipitated DNA was amplified with 16 real-time PCR primers spaced across the VCAN locus as indicated at the bottom of the panels. Results represent ChIP fold-enrichment over input as mean ± SD. Top panel represents the matched RNA-Seq signal for VCAN gene expression in CC and SC.
expression in the outer circle. The most striking examples were chromosomes 13 and 21 where nearly all of the chromosomes showed high-copy-number variation (CNV) and expression in CC. Chromosome and digital karyotyping established that most of the CNV and overexpression clusters could be explained by gains or losses of whole chromosomes or parts of chromosomes, for example, loss of one chromosome 13 in SC and gain of two additional chromosome 21’s in CC (SI Appendix, Fig. S5 and Table S3). Beyond that, we found little evidence suggesting a contribution of copy-number changes to the expression differences between CC and SC. Although there were no global differences in DNA methylation between CC and SC (SI Appendix, Fig. S7), there were differences in methylation of specific histone H3 residues visible by immunostaining in both 3D cultures and tumors in nude mice (SI Appendix, Fig. S7).

In three recent CRC gene expression studies (CCS, CRCA, and CCMS), each study identified a subtype associated with a low degree of differentiation, epithelial-to-mesenchymal transition, and poor prognosis; these subtypes were 3 (48 genes), 5 (185 genes), and 4 (288 genes), respectively (13–15). We found that these gene signatures are selectively up-regulated in SC, consistent with their less differentiated and more invasive features compared with CC (SI Appendix, Fig. S4B).

In Vivo and In Vitro Validation of HPGD and VCAN Expression in CC and SC Cells. We decided to focus on selected genes up-regulated in CC and SC. The top four up-regulated genes in CC are UBD, GALNT12, NUDT11, and HPGD. Based on our previous work in parental HCA-7 cells linking EGFR and eicosanoid metabolism, we elected to study HPGD, a key enzyme in prostaglandin metabolism (16). HPGD metabolizes PGE\_2 to 13,14-dihydro-15-keto-PGE\_2, thereby acting as a tumor suppressor (17, 18). We detected HPGD protein in cell lysates from CC, but not SC (Fig. 4A). In addition, the HPGD metabolite 13,14-dihydro-15-keto-PGE\_2 was measured in conditioned medium from CC, but not SC (Fig. 4C). Moreover, HPGD immunofluorescence was observed in the epithelial component of CC xenografts in athymic nude mice, but not in their SC counterparts (Fig. 4B). Thus, CC overexpress the tumor suppressor HPGD, which is consistent with their more differentiated and less invasive features. However, incubation with the HPGD inhibitor, SW033291, did not result in increased growth or spiky morphologic conversion of CC colonies. Instead, at higher concentrations (100 μM) CC colonies showed reduced size, indicating arrest of colony growth (SI Appendix, Fig. S8 A and B). VCAN was the most up-regulated gene in SC compared with CC when placed in a 3D type-1 collagen environment (Fig. 3C). VCAN is a large chondroitin sulfate proteoglycan that is produced by both cancer cells and tumor stroma. It is part of a stromal gene signature that recently has been linked to poor clinical outcome in CRC (29). Indeed, we noted that the \textit{VCAN} locus was present within a large heterochromatin domain that is DNA hypomethylated in CRC (29). We next performed ChIP assays of SC and CC cultured in 3D to test whether reprogrammed chromatin modifications were specifically targeted to \textit{VCAN} chromatin. We examined multiple chromatin modifications using a set of 16 pairs of quantitative PCR primers spaced at ~10-kb intervals across the 160-kb \textit{VCAN} locus, including regions upstream and downstream from the genic portion. As expected, the interior of this region was depleted for H3K9Me3 and enriched for H3K9Me2 in both CC and SC, consistent with a CRC facultative heterochromatin domain that is common to both (Fig. 4F, CC in blue, SC in red). We further observed enrichment of H3K27Me3 at specific regions within this domain in CC (Fig. 4F). This modification is strongly linked to transcriptional repression and gene silencing, consistent with low VCAN expression in CC. In contrast, these same regions were depleted of H3K27Me3 and highly enriched with H2K27Ac in SC. H3K27Ac is typically enriched at gene regulatory elements, including enhancers, to encode a chromatin state permissive for transcriptional activation. Indeed, we observed strong enrichment of the transcription-coupled modification H3K36Me3 over the \textit{VCAN} gene in SC, but not in CC, consistent with transcriptional up-regulation (30). Thus, the \textit{VCAN} locus resides in a large chromatin domain that acquired reciprocal activating and repressive chromatin modifications in SC versus CC, suggesting an epigenetic basis for overexpression of VCAN in SC.

Analysis of VCAN and HPGD Immunoreactivity in a CRC Tissue Microarray. We next examined VCAN and HPGD immunoreactivity in a clinically annotated tissue microarray consisting of 174 CRCs from 174 individuals (8). There was no difference in overall survival for individuals with high and low staining for VCAN in the stroma (Fig. 5A). However, individuals with high staining for VCAN in the epithelium had significantly worse overall survival than those with low epithelial VCAN staining (Fig. 5B and SI Appendix, Fig. S9A). HPGD immunoreactivity was detected only in the epithelium (SI Appendix, Fig. S9B). Loss of HPGD staining was frequently observed in CRCs, with only eight tumors displaying appreciable staining (Fig. 5C). There was a trend for individuals with HPGD-positive tumors to have a better outcome. Individuals with tumors lacking HPGD and high VCAN staining had a worse survival than those with only high epithelial VCAN immunoreactivity (Fig. 5D). Thus, this 3D system has allowed us to identify that epithelial, and not stromal, VCAN staining portends a poor prognosis in CRC. In separate analyses we found that increased HPGD mRNA expression correlated with better survival and that increased VCAN expression was detected in the poor-prognosis, subtype C CRC samples (SI Appendix, Fig. S10 A and B) (31–33).

Discussion

Here, we provide a detailed genomic and transcriptomic characterization of a 3D model system to study CRC. By placing single HCA-7 cells into a well-defined extracellular matrix (type-1 collagen), we have generated two cell lines (CC and SC) with contrasting morphologic and functional properties. Of note, CC and SC appear indistinguishable when cultured on plastic and on Transwell filters. A number of other CRC lines also were cultured in type-1 collagen, but only HCA-7 exhibited a uniform cystic morphology. A striking functional difference between CC and SC was the fact that CC were markedly growth-inhibited by cetuximab in 3D, whereas SC were refractory to its growth inhibitory effects. Neither line responded to cetuximab in 2D. We excluded all known genetic events that could explain the resistance of SC to
cetuximab. Both CC and SC cells were wild type for KRAS, BRAF, PIK3CA, EGFR, and MET. Moreover, cetuximab resistance in SC could not be explained by differential EGFR cell-surface availability; EGF led to equivalent EGFR tyrosine phosphorylation, and the extracellular domain antibody, C225, showed equivalent surface staining in CC and SC (SI Appendix, Fig. S2 A and B). When comparing the gene expression profile of CC and SC cells by KEGG analysis, we observed increased MAPK signaling in SC cells (Fig. 2A). Moreover, a small-molecule MAPK inhibitor, U0126, in combination with cetuximab, led to cooperative growth inhibition of SC in 3D (Fig. 2B).

To find upstream kinases mediating MAPK activation, we performed an RTK array and found a selective increase in MET and RON tyrosine phosphorylation (Fig. 2C). Although MET amplification has been reported as a mechanism of acquired resistance to EGFR inhibition (34, 35), increased tyrosine phosphorylation of MET (independent of its amplification) has not. Crizotinib is a broadly acting, small-molecule tyrosine kinase inhibitor that is approved for clinical use for individuals with non–small-cell lung carcinoma (36). Although crizotinib is particularly effective in tumors with ALK and ROS1 mutations, it has the highest affinity for MET with RON being an additional target (37–39). Crizotinib, in combination with cetuximab, led to marked growth inhibition of SC cells in 3D (Fig. 2D) Consistent with the reduction in colony number, the cetuximab/crizotinib combination also worked cooperatively to reduce downstream signaling as monitored by levels of ERK1/2 and AKT phosphorylation (Fig. 2E). Finally, as to the likely source of increased MET/RON tyrosine phosphorylation, among other possibilities, up-regulation of MET/RON ligands (HGF/HGFL) and/or inactivation of MET/RON tyrosine phosphatases may play a regulatory role (40, 41).

We performed whole-exome sequencing of HCA-7, CC, and SC. Pairwise analysis among the three revealed that the CC–SC pair was the most divergent. Nevertheless, there was more than 98% similarity between CC and SC as scored by SNVs and INDELs (Fig. 3A). The SNVs and INDELs unique to CC and SC are listed in SI Appendix, Table S1. Although the majority of clustered changes in gene expression could be explained by losses or gains in chromosomal regions as determined by karyotyping, the overexpression of VCAN and HPGD was not due to copy-number gains or losses or chromosomal rearrangements (SI Appendix, Fig. S5 and Table S3). Future studies will be needed to determine if any of these genetic events in SC contribute to cetuximab resistance.

We compared CC and SC gene expression over time in 3D by microarray. Two of the top four genes overexpressed in CC are bona fide tumor suppressor genes—GALNT12 and HPGD—whereas the role of the other two—UBD and NUDT11—in CRC is uncertain (42–44). Because we previously showed EGFR-induced COX-2 expression and basolateral release of one of the COX-2 products, PGE₂, in HCA-7 cells, we decided to focus on HPGD because HPGD metabolizes PGE₂ to 13,14-dihydro-15-keto-PGE₂ (16, 45, 46). HPGD thus reduces the levels of PGE₂, which is thought to be the major tumor-promoting eicosanoid (47). There was a marked reduction in intestinal tumors when Apcem mice were crossed to Cox-2 null mice and a marked increase in tumor burden when Apcem mice were crossed to Hpgd null mice, supporting the notion that tumor suppression in intestinal neoplasia is achieved by inhibiting PGE₂ production (via Cox-2 loss) or assuring its degradation (via Hpgd) (48). We now report that the less aggressive HCA-7–derived CC show up-regulation of HPGD compared with the more aggressive SC and also report selective up-regulation of the HPGD metabolite, 13,14-dihydro-15-keto-PGE₂, in CC. Among the EGFR ligands, AREG potently induces COX-2, so it will be of interest to examine the effect of the EGFR-signaling axis on HPGD expression (49). However, when directly tested, HPGD inhibition did not confer a transformed phenotype to CC colonies as assessed by colony growth and morphologic conversion, indicating that HPGD may not be functionally linked to the benign CC phenotype (SI Appendix, Fig. S8 A and B). On the other hand, HPGD expression remained strongly associated with better survival in CRC (Fig. 5 C and D).
VCAN was the most up-regulated gene in SC. Of interest, VCAN has also been shown to interact with and influence EGFR signaling (50, 51). VCAN is a major component of the extracellular matrix, being produced by both tumor cells and the surrounding stroma (52). Recent work has identified VCAN as part of a poor prognosis stromal gene signature (19, 20). However, Michael Karin and coworkers demonstrated that VCAN was produced by epithelial-derived Lewis lung cancer cells and that it was a potent activator of macrophages, resulting in proinflammatory tumor progression (53). We show that VCAN is selectively overexpressed in the less differentiated, more invasive SC compared with their CC counterparts. In a clinically well-annotated CRC tissue microarray, we show that epithelial, but not stromal, VCAN immunoreactivity is linked to poor prognosis in CRC. Another group, using a different antibody to VCAN, reached a different conclusion as to the prognostic significance of epithelial versus stromal VCAN staining (54). Future studies are needed to determine whether VCAN produced by epithelial or stromal cells differs in form or function. Our current studies do not address the biological function of VCAN. It is a large protein with a number of splice and proteolytic isoforms that bind to cell-surface and extracellular proteins (55, 56). Our work identifies epithelial regulation of VCAN expression (21, 22).

The present studies do not provide an explanation for the persistent morphological and functional differences between CC and SC in 3D. When we isolate individual spiky colonies from SC type-1 collagen cultures and embed them as single cells in type-1 collagen, we consistently observe a predominant spiky morphology in the resulting colonies. A similar experiment with the infrequent CC-derived spiky colonies results in colonies with predominantly cystic morphology. This Luria–Delbrück fluctuation-like analysis is insufficient to elucidate the genetic and/or epigenetic versus environmental basis of the phenotypic differences between CC and SC (57). However, because both CC and SC form predominantly cystic colonies in Matrigel, we favor the notion that exposure to specific environments, in this case type-1 collagen, provides cues that magnify the underlying genetic and epigenetic differences between the two lines. Whatever the ultimate mechanism(s) underlying differences between CC and SC in 3D, this system provides a useful tool to identify epithelial-derived, disease-relevant genes, as well as additional therapeutic strategies in CRC.

Materials and Methods
Reagents. PureCol bovine type-1 collagen was purchased from Advanced Biomatrix. All cell culture components were purchased from HyClone Laboratories. Protein G agarose and rhodamine-phalloidin were purchased from Life Technologies. Anti-ezrin antibody was purchased from Cell Laboratories (AlexaFluor-linked) were used. Slides were washed and mounted in Prolong (Life Technologies). Confocal microscopy was performed using a Nikon A1R.

Immunohistochemistry. Tumor xenografts were fixed in neutralized formalin and embedded in paraffin. Slices were deparaffinized with serial histoclear and ethanol. Antigen retrieval was performed in citrate buffer (pH 6) with high pressure for 30 min. Primary antibodies used were the following: anti-VCAN (DSHB, 12C5; 1:100); anti-HPGD (LS-BIO, 2C10; 1:100); and anti-E-cadherin (Abcam, EP7007; 1:500). Secondary antibodies from Life Technologies (AlexaFluor-linked) were used. Slides were washed and mounted in Prolong (Life Technologies). Confocal microscopy was performed using a Nikon A1R.

Transmission Electron Microscopy. Collagen cultures were rinsed with 0.1 M sodium cacodylate buffer, fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4 at RT for 1 h and stored at 4 °C overnight. Samples were polymerized at 60 °C for 48 h. Thick sections (0.5–1 μm) were collected using a Leica Ultracut microtome, contrast-stained with 1% toluidine blue, and imaged with a Nikon AZ100 microscope. Ultra-thin sections (70–80 nm) were cut, collected on 300-mesh copper grids, and poststained with 2% uranyl acetate.
acetate and then with Reynold’s lead citrate. Samples were subsequently imaged on the Philips/FEI Tecnai T12 electron microscope at various magnifications.

**Human RTK Array.** Cell lysates from 3D culture were collected and protein concentration in each sample was measured by the BCA assay (Thermo Scientific). Three hundred milligrams of protein were analyzed using the Human Phospho-Receptor Tyrosine Kinase Array Kit (R&D Systems) according to the manufacturer’s protocol. After applying chemiluminescence detection solution, membranes were exposed to imaging film and developed using a Kodak D-19omat processor (Kodak). **Statistical Analyses.** Two-tailed, two-sample t tests were used to determine statistical significance. P values of less than 0.05 were considered significant. Calculations were performed using GraphPad and R-2.15. DNA methylation data were analyzed with Lumi package for R on the original intensity data (idat files) (60). Microarray data were normalized with RMA, followed by analyses in R and Excel. Functional enrichment analysis on the up-regulated and down-regulated genes was implemented separately in a Gene Ontology biological process as well as in KEGG pathways by WebGestalt (61, 62). Enrichment P values were generated by a hypergeometric test and adjusted by Benjamini and Hochberg’s multiple test (63). Gene set enrichment analysis was performed using software provided by software.broadinstitute.org/gsea/index.jsp.

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