Tumor and Stem Cell Biology

Stem Cell Differentiation and Lumen Formation in Colorectal Cancer Cell Lines and Primary Tumors

Neil Ashley, Trevor M. Yeung, and Walter F. Bodmer

Abstract

Single cancer stem–like cells (CSC) from colorectal cancers can be functionally identified by their ability to form large lumen-containing colonies in three-dimensional Matrigel cultures. These colonies contain the three types of differentiated colorectal epithelial cells, and single cells obtained from them can reproduce themselves and form tumors efficiently in immunodeficient mice. In this study, we show how hypoxia affects these CSC-derived lumens to control differentiation of stem-like cells and enterocytes via the homeobox gene CDX1. Lumens were identified by F-actin staining and they expressed many characteristics associated with normal differentiated intestinal epithelium, including brush border enzymes, polarization, and tight junctions. RNA interference–mediated silencing of CDX1 reduced lumen formation. Inhibitory effects of hypoxia on lumen formation and stem cell differentiation, including suppression of CDX1 expression, could be mimicked by inhibiting prolyl-hydroxylases that activate HIF1, suggesting that HIF1 is a critical mediator of the effects of hypoxia in this setting. Cell line–derived lumens were phenotypically indistinguishable from colorectal tumor glandular structures used by pathologists to grade tumor differentiation. Parallel results to those obtained with established cell lines were seen with primary cultures from fresh tumors. This in vitro approach to functional characterization of CSCs and their differentiation offers a valid model to study colorectal tumor differentiation and differentiation of colorectal CSCs, with additional uses to enable high-throughput screening for novel anticancer compounds. Cancer Res; 73(18); 5798–809. ©2013 AACR.

Introduction

Colorectal tumors originate in the epithelial layer of the large intestine, which is lined with microinvaginations called crypts. A stem cell population resides at the crypt base and gives rise to the 3 main differentiated lineages in colon: enterocytes, goblet cells, and entero-endocrine cells (1). Enterocytes lining the crypts of the intestine express a layer of microvilli on their apical membranes known as the brush border, in which many differentiation markers such as villin are segregated in a polarized manner (2–4). Full development of the brush border and associated cytoskeletal and enzyme activities corresponds with intestinal differentiation (5, 6). Although cellular differentiation is ultimately deranged in colonic adenocarcinomas, colorectal tumors usually retain some degree of differentiation, such as glandular structure and expression of differentiation markers, and also frequently express brush border–enriched enzymes such as DPPIV/CD26 and alkaline phosphatase (7).

The glandular form of tumors and general mucinous content are used by pathologists to help grade tumors (8). Tumors are classed as either well/moderately differentiated or "low grade" if they contain many "neoplastic glands" that have a crypt-like structure or as poorly differentiated or "high grade" if they lack such glandular structures (9). Low-grade tumors generally exhibit a better prognosis for the patient (10). Much evidence indicates that adenocarcinomas are driven by a subset of cells with characteristics of stem cells, including the ability to self-renew and to differentiate into all 3 colonic lineages (11). We have previously shown that a subpopulation of single cells derived from certain colorectal cell lines can, when grown for several weeks under 3-dimensional (3D) conditions, form either large crypt-like structures consisting of polarized cells surrounding a cell-free lumen or instead form small non-lumen colonies (12–15). The large lumen colonies express differentiation markers for all 3 colon cell lineages, indicating the multipotent nature of the original cell. Single cells derived from large lumen colonies were capable of forming new large lumen colonies, as well as small non-lumen colonies, whereas cells derived from small non-lumen colonies could only give rise to further small colonies (14). Lumen colony–derived cells were also more tumorigenic in mouse xenografts than cells derived from small non-lumen colonies. The proportion of cells that gives rise to lumen colonies could be increased by enrichment of cells with high expression of both the stem cell markers CD44 and CD24 (14). Collectively, these characteristics indicate that lumen formation is driven by high clonogenic cancer stem cells that can be derived from...
lumen-forming cell lines. Low clonogenic cells are unable to give rise to large lumen colonies and form small non-lumen colonies. Therefore, formation of lumens from single cells can be used to characterize cancer stem cell differentiation.

Interestingly, some colorectal cell lines such as DLD1 do not differentiate under 3D conditions and instead form disorganized colonies (14, 15). These cell lines retain self-renewal capacity of stem cells but lack the ability to fully differentiate and are correspondingly more aggressive in xenografts and more clonogenic than lumen-forming cell lines (14). One possibility is that differentiation is inhibited in these lines by reduced expression of key transcription factors such as CDX1. CDX1 is a gut transcription factor crucial to colorectal differentiation (16, 17), and it controls the transcription of a number of intestinal differentiation markers including villin (18), cytokeratin 20 (16), and FABP1 (19). In colorectal cancers, CDX1 is frequently transcriptionally silenced by promoter methylation (16, 17).

In this study, we have characterized the nature of differentiation of 3D colonies formed by different cell lines and primary tumor-derived cancer stem cells.

Materials and Methods

Cell culture

Details of cell line origins and validation can be found in the Supplementary Text. All cell lines were cultured in complete Dulbecco's Modified Eagle Media (DMEM) supplemented with 10% FBS and 1% penicillin/streptomycin (Invitrogen). Hypoxic cells were grown in humidified 1% oxygen and 10% CO2 environment using a MiniGalaxy incubator (RS Biotech Ltd.). Medium was replaced 2 times per week after being prewarmed and equilibrated overnight in 1% oxygen. For dimethylxaloylglycine (DMOG) treatment, culture medium containing 2 mmol/L DMOG or dimethyl sulfoxide (DMSO; vehicle control) was overlaid over the gels and this was changed every 24 hours.

For primary culture, 6 moderate/well-differentiated tumor specimens, obtained from fully consenting donors under guidelines and within the limits of the Project License issued by the Home Office, United Kingdom.

Further details of materials and methods can be found in the Supplementary Data.

Results

Lumen characterization

F-actin (filamentous actin) was identified as a useful marker of lumens by staining normal human colon cryosections with fluorescent phalloidin, which is specific for F-actin (Fig. 1A). In normal colon, F-actin is intensely enriched at the apical surface of enterocytes lining the crypt, corresponding to the brush border, presumably due to the presence of microvilli on colonic enterocytes (21, 22). We next examined F-actin labeling in single-cell–derived colonies from a panel of 6 colon cancer cell lines, grown in a 3D Matrigel matrix for 2 weeks. In an SW1222 colony, confocal Z-sectioning showed intense F-actin labeling on the apical cell membranes facing the lumen, which was surrounded by polarized cells expressing cytokeratin 20 on their basolateral membranes (Fig. 1B). LS180, SW1222, and C80 formed colonies consisting of polarized cells surrounding central lumens visible by light microscopy (Fig. 1C). All phase visible lumens were clearly labeled by intense F-actin staining. HT29, HCT116, and DLD1 did not form observable lumen formation.
Figure 1. Polarized F-actin is a marker of colonic brush borders in vivo and labels lumens in vitro. A, confocal images of normal human colon cryosections labeled with TRITC-phalloidin (red) and DAPI (blue) to visualize F-actin and nuclei, respectively. Left image shows longitudinal section; right image shows transverse section. Scale bar, 50 μm. B, z-stack gallery of a SW1222 colony labeled with F-actin (red), DAPI (blue), and cytokeratin 20 (green). Numbers represent distance in z-axis from top. Scale bar, 10 μm. C, single cells from various cell lines were grown in Matrigel for 6 days. Colonies were labeled with TRITC-phalloidin (red) to visualize F-actin and DAPI to visualize nuclei. Bar, 10 μm.
colonies using phase contrast and did not exhibit organized bright foci of F-actin staining, although F-actin was visible in plasma membranes. A gallery of F-actin–labeled colonies from various cell lines is shown in Supplementary Fig. S1.

We next examined whether other intestinal brush border markers were also present in lumens. Villin, an F-actin–interacting protein associated with intestinal differentiation, was strongly enriched in lumens and its staining overlapped that for F-actin (Fig. 2A). HCT116 and DLD1 did not express villin. In HT29, villin was generally expressed but it was not polarized and did not overlap F-actin. Ezrin, another F-actin–interacting protein enriched in the brush border, was also strongly enriched in lumens where it was clearly located at the apical cell membrane (Fig. 2B). Ezrin was only weakly present at non-apical plasma membranes. Ezrin was strongly expressed in HCT116 in a nonpolarized manner.

The brush border enzyme CD26/dipeptidyl peptidase-4 (DPPIV; ref. 7) and the brush border–enriched tight junction marker ZO-1(23) were also polarized and enriched in luminal apical membranes (Supplementary Fig. S2A and S2B). In contrast, non-lumen–forming cell lines failed to show organized expression of either marker.

We next examined whether formation of F-actin–enriched lumens in colonies derived from a single-cell precluded the presence of secretory lineages. Co-staining of SW1222 colonies with phalloidin and anti-MUC2, a goblet cell–specific mucin (Fig. 2C), showed that goblet cells were present in F-actin colonies grown from single cells. We also examined expression of carcinoembryonic antigen (CEA), a membrane-associated and secreted glycoprotein commonly used as a cancer marker. CEA was present almost exclusively in the lumens of C80 and SW1222 colonies (Fig. 2D).

The above evidence indicates that lumen formation in colonies derived from single colorectal cells represents brush border differentiation but does not preclude differentiation along secretory lineages.

Cancer stem cell enrichment

To determine whether stem cell–enriched cell fractions gave rise to increased frequencies of lumens with F-actin/brush border differentiation, we FACS-sorted SW1222 and LS180 cells according to expression of cancer stem cell markers CD24 and CD44, as described previously (14). Single sorted cells were then plated in Matrigel and grown into colonies for 2 weeks. In Fig. 3A and B, the top 5% of CD24/44 (CD24 and CD44 high)-expressing SW1222 cells gave rise to significantly more large colonies with F-actin–labeled lumens than unsorted/bulk cell populations and significantly fewer small colonies with no lumens. The bottom 5% CD24/44-expressing cells (CD24 and CD44 low) cells showed very low clonogenicity, and resulting colonies were virtually all-small with absent or poorly developed F-actin foci. Similar results...
were obtained with LS180 cells (Fig. 3C and D). Thus, stem cell–enriched populations give rise to a larger proportion of larger colonies with increased brush border/enterocyte differentiation and a much smaller proportion of small non-lumen–forming colonies.

Effect of hypoxia and Hif1α stabilization on differentiation and CDX1 expression

Lumen formation can be inhibited by long-term hypoxia, accompanied by a downregulation of CDX1 in an Hif1α-dependent manner (15). To determine whether F-actin was similarly downregulated by hypoxia, we compared LS180 and SW1222 cells grown in Matrigel for 14 days in either normoxia or hypoxia (1% O₂). Figure 4A shows representative images of F-actin labeling of the colonies under each condition. Hypoxic colonies were smaller and F-actin focus size and intensity were reduced, compared with normoxia. The number of F-actin foci appeared to be similar. Quantification of colonies indicated a significant drop in the proportion of colonies with F-actin–labeled lumens and an increase of colonies without well-defined lumens (Fig. 4B). These results show that hypoxia inhibits brush border development in colon cancer stem cells.
To determine whether inhibition of lumen formation was dependent on Hif transcription factors, we cultured colonies in the presence or absence of the prolyl-hydroxylase inhibitor DMOG. Prolyl-hydroxylases hydroxylate Hif transcription factors, marking them for degradation and so DMOG interferes with this process leading to accumulation of Hif1α (24). We first confirmed that DMOG treatment induced expression of Hif1 and Hif1-responsive genes in cell lines by immunolabeling for Glut1 and carbonic anhydrase IX (CAIX) and immunoblotting for Hif1 (Supplementary Fig. S3A–S3C). We then treated colonies grown from single cells in Matrigel for 7 days and with DMOG for a further 5 days. The effect on F-actin labeling was determined with TRITC-phalloidin. Figure 4C shows that DMOG inhibited F-actin lumen formation in C80 and LS180 and SW1222. Large F-actin lumens were inhibited and, instead, many colonies exhibited small F-actin foci. There was no obvious effect on colony size. Quantitation of the colony morphologies showed a significant reduction in the proportion of colonies with large, well-defined F-actin lumens and an increase in the proportion of colonies with small F-actin foci (Fig. 4D).

We next determined whether DMOG treatment induced downregulation of CDX1 and its target cytokeratin 20 (16). Immunolabeling of DMOG-treated colonies and vehicle-treated controls showed a marked downregulation of CDX1 in response to DMOG, although cytokeratin 20 expression remained virtually unchanged (Fig. 5A and B). We also looked at the effect of DMOG on goblet cell lineage differentiation.
Immunolabeling colonies with an anti-goblet cell antibody indicated that DMOG suppressed goblet cell differentiation in both the C80 and LS180 cell lines (Fig. 5C and D). Thus, DMOG treatment leads to rapid loss of CDX1 expression but not cytokeratin 20 and inhibits both brush border/enterocyte and secretory goblet cell differentiation.

To confirm that CDX1 downregulation could impair F-actin lumen formation, we used an LS174T-derivative cell line LS174T-CDX1 with stable expression of a construct encoding a short hairpin RNA targeting CDX1, in which CDX1 was downregulated by RNA interference (RNAi: ref. 16). An LS174T line, LS174T-vector, expressing an empty vector was used as a control, as these cells maintain normal CDX1 levels. Single cells from either LS174T siCDX1 or LS174T-vector were embedded in Matrigel and allowed to grow into colonies for 10 days, before fixation and phalloidin labeling to visualize F-actin. Figure 6A and B shows that low CDX1 significantly reduced the proportion of colonies with well-defined F-actin lumens. We also manually quantified the total number of lumens per colony in the same data and found that CDX1 knockdown also reduced this number, that is, CDX1 knockdown inhibited lumens per colony (Fig. 6C). We next took advantage of the CDX1 RNAi F-actin data to validate a semi-automatic approach to lumen formation that would facilitate high-throughput analysis of stem cell differentiation using public ImageJ software. Figure 6D, i, shows a whole well-composite image of an entire well from a 96-well plate that contained F-actin–labeled SW1222 colonies grown in 3D conditions. ImageJ software was then used to automatically recognize and analyze the colonies (Fig. 6D, ii). The software was able to detect lumens accurately in a well-differentiated colony and was able to distinguish that colony from a poorly differentiated colony lacking F-actin foci (Fig. 6D, iii and iv, respectively). To validate this approach, we used our
semiautomatic method to analyze the LS174T-vector and LS174T-CDX1 colonies manually counted in Fig. 6C and found a good correlation between the automated and manual analyses (Fig. 6E). Thus, our approach enables tissue plate wells to be automatically scanned and analyzed for changes in lumen formation.

Lumen formation is a feature of tumors in vivo

To determine whether lumen formation had relevance to tumors in vivo, we first examined actin and ezrin polarization in murine xenografts derived from the injection of HT29, HCT116, or SW1222 cells into the flanks of NOD/SCID mice. Mice were sacrificed after 1 month and resulting tumors removed and processed for formalin-fixed, paraffin-embedded (FFPE) tissue sections, which were stained by hematoxylin and eosin or immunolabeled with anti-actin/ezrin (Fig. 7A). Hematoxylin and eosin staining of these xenograft tumors showed poorly differentiated high-grade tumor morphology for HCT116 and HT29 tumors that lacked lumens or actin/ezrin polarization. SW1222 tumors exhibited a well-differentiated phenotype with numerous lumens surrounded by polarized cells. Actin and ezrin immunolabeling showed that neither HT29 nor HCT116 exhibited polarization of these markers, whereas SW1222 showed marked luminal polarization. Thus in vivo, cell lines grow in a manner similar to in vitro growth in Matrigel. Because similar glandular structures (neoplastic glands) are a feature of human colorectal adenocarcinomas and are used by pathologists to grade tumors, we examined whether they labeled with the same markers as in vitro lumens. We compared 2 colorectal tumors that had been graded previously as either moderately differentiated (low-grade) or poorly differentiated (high-grade). Hematoxylin and eosin staining of tissue sections from the low-grade tumor confirmed the presence of numerous neoplastic glands, which were absent in the poorly differentiated tumors (Fig. 7B). Immunolabeling of
Figure 7. Lumen formation is a feature of tumors in vivo, and human primary low- and high-grade tumors show differences in ezrin/actin and CEA polarization. A, lumen-forming and non-lumen-forming cell lines form well-differentiated (low-grade) and poorly differentiated (high-grade) tumors, respectively, as xenografts in vivo. Hematoxylin and eosin or anti-actin/ezrin immunostaining of FFPE sections from murine xenograft tumors derived from SW1222, HCT116, or HT29 cells injected into NOD/SCID. Inset shows magnification. B, typical hematoxylin and eosin staining of FFPE tissue sections derived from a moderately differentiated (low-grade) primary colorectal tumor from a patient, with numerous neoplastic glands visible (left), and a poorly differentiated (high-grade) human colorectal tumor, with no glands (right). C and D, anti-actin/anti-ezrin or anti-CEA/villin labeling of FFPE tissue sections derived from the same patient derived high- and low-grade tumors shown in B. E, characterization of lumen formation in primary cultures derived from a low-grade tumor. Left, phase contrast image of typical primary cultured spheroid cancer cell colony derived from the same low-grade human colorectal tumor shown in B-D. Right, F-actin labeling of these primary colonies following growth in Matrigel for 7 days. F, summary of lumen formation efficiency in Matrigel of a panel of 6 primary colorectal cancer cultures derived from 6 separate patients.
sections from the same tumors indicated that lumens in the low-grade tumor were composed of polarized cells expressing actin and ezrin on the apical membranes, whereas no polarization of these markers was observed in the poorly differentiated tumors (Fig. 7C). Typical of tumors, cellular debris was present in the lumens. Actin/ezrin-positive cells present in the stromal surrounding the glands were probably myofibroblasts (Supplementary Fig. S4). Lumen glands were strongly positive for polarized CEA, whereas CEA was nonpolarized and more cell membrane associated in the high-grade tumor (Fig. 7D).

Thus, at least 3 markers of in vitro lumens also mark in vivo glands, indicating that in vitro lumens from cell lines are virtually identical in nature to lumens in primary tumors.

To determine whether lumen formation was a feature of primary tumor cells in vitro, we derived a primary tumor cell culture from the same low-grade tumor used in Fig. 7B–D. When grown under conditions that promote stem cell populations (25–28), these primary cells grew as well-organized spheroids (Fig. 7E, left). Following a week-long suspension in Matrigel, the colonies formed large central lumens marked by F-actin, (Fig. 7E, right). Three of 6 primary cultures derived from separate patients were capable of lumen formation (Table 1). Thus, we conclude that lumen formation represents the same process in vivo as it does in vitro and can therefore be used to identify stem cell populations in primary tumor–derived cultures.

Discussion

Single stem cells from colorectal cancer lines, when grown under 3D conditions, can differentiate into polarized structures with remarkable structural similarity to normal intestinal crypts. Lumens express many characteristics of normal intestinal brush borders, including microvilli structural proteins (polarized F-actin, villin, ezrin), enzymatic activity (polarized DPPIV), cell surface and secreted glycoprotein (CEA), and tight junctions (TJP1/Zo-1). Thus, colorectal cancer stem cells that produce lumen colonies can be considered to be differentiating predominantly along the enterocyte lineage, the most common colonic lineage. Neverthelless, goblet cells were also often present in colonies with F-actin lumens that had been grown from single cells, illustrating their multipotent nature. Importantly, we also show that several of these markers of in vitro lumen brush border formation are also expressed in a polarized manner by neoplastic glands present in primary tumors, which are similarly composed of polarized cells surrounding a lumen. Thus, it is most likely that these neoplastic lumens also indicate stem cell differentiation in the same way as their in vitro counterparts. This is supported by our observation that primary tumor spheroid cultures derived from moderately well-differentiated tumors could also form polarized lumen colonies when transferred into Matrigel.

Unlike lumen colonies and well-differentiated tumors, non-lumen cell lines, such as HCT116, totally lacked cellular polarization of several polarity markers such as villin, a situation mirrored in vivo by high-grade tumors. Thus, cell lines can also be separated into high or low “grades” based on their polarization in Matrigel.

One possible explanation for this is that these cells lack expression of CDX1 (16), and this may disrupt their ability to efficiently polarize and differentiate. Ectopic re-expression of CDX1 in HCT116 and DLD1 can lead to the formation of primitive lumens in Matrigel (14, 15). Correspondingly, CDX1 and the related transcription factor CDX2 have been shown to be important for polarization of intestinal cells (29, 30). A lack of polarization and differentiation will lead to an increase in the population of stem cell and indeed we have found previously that HCT116 does not contain subpopulations of cells with different tumor-forming capacity, unlike the lumen-forming lines (14). Similarly, high-grade tumors have a significantly worse clinical prognosis and exhibit a more “stem-like” transcription profile (31, 32).

Tumor formation is frequently associated with hypoxia, and our previous studies have shown that prolonged hypoxia inhibits lumen formation (15). Correspondingly, hypoxia leads to a failure to polarize F-actin correctly, as did treatment of colonies with the Hif1α activator DMOG. DMOG also inhibited CDX1 expression and goblet cell differentiation, although cytokeratin 20 expression was not affected, probably due to a difference in turnover rate between CDX1 and cytokeratin 20. As the CDX1 promoter contains a number of putative Hif-binding sites (unpublished data), it is possible that downregulation of CDX1 is directly induced by Hif-mediated transcriptional inhibition. It seems likely that the loss of F-actin lumen expression in response to hypoxia/DMOG treatment is triggered by a loss of CDX1. This is supported by the observation that targeted knockdown of CDX1 in LS174T cells leads to a significant reduction in the size and intensity of F-actin–labeled lumens. However, we cannot rule out a role for other hypoxia-regulated transcription factors in differentiation suppression.

Specific cancer stem cell markers remain elusive. While markers such as LGR5 (33), ephrin type-B receptor 2 (EphB2; ref. 34), and Lrig1 (35) appear to mark well-defined stem cell populations in normal intestine, their expression in tumors does not appear to be entirely specific to cancer stem cells as low clonogenic cells are also marked, at least for EphB2 (31), and LGR5 (36, 37). F-actin lumen formation should therefore be useful for defining the cutoff in expression of markers such as LGR5 and EphB2 that define cancer stem cell populations from non-stem cells. The ability of single cancer stem cells to grow into differentiated lumen colonies provides an unambiguous tool to estimate stem cell populations. Our approach therefore allows for the functional characterization of CSCs and their differentiation, independent of expression of stem cell surface markers. Furthermore, the F-actin lumen assay can be adapted for high-throughput applications, which should be useful for identifying drugs that can target stem cells.

In summary, we have characterized the formation of lumen colonies from cell line–derived single cells to represent brush border/enterocyte differentiation and show the extent of heterogeneity between cell lines and primary tumor cultures. Thus, the cancer stem cells from the cell lines, in their ability to form lumen colonies, have a striking similarity to normal...
intestinal stem cells, which also divide to form the colonic crypt.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: N. Ashley, T. Yeung, W.F. Bodmer
Development of methodology: N. Ashley
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): N. Ashley, T. Yeung
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): N. Ashley, W.F. Bodmer
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