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

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Abstract

Single cancer stem-like cells (CSCs) from colorectal cancers can be functionally identified by their ability to form large lumen-containing colonies in 3D 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, polarisation, and tight-junctions. RNAi-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 cancer stem-like cells, with additional uses to enable high-throughput screening for novel anticancer compounds.

**Key words:** Colon, intestine, stem, cell, lumen, F-actin, polarisation, brush border, differentiation, primary, gland

**Introduction**

Colorectal tumours originate in the epithelial layer of the large intestine, which is lined with micro-invaginations called crypts. A stem cell population resides at the crypt base and gives rise to the three 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 polarised manner [2; 3; 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 tumours 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 tumours and general mucinous content are used by pathologists to help grade tumours [8]. Tumours 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 tumours 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 three colonic lineages [11]. We have previously shown that a sub-population of single cells derived from certain colorectal cell lines can, when grown for several weeks under 3D conditions, form either large crypt like structures consisting of polarised cells surrounding a cell-free lumen, or instead form small non-lumen colonies [12; 13; 14; 15]. The large lumen colonies express differentiation markers for all three-colon cell lineages, indicating the
multi-potent 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 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 give rise to lumen colonies could be increased by enrichment of cells with high expression of the stem cell markers CD44/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 characterise cancer stem cell differentiation.

Interestingly, some colorectal cell lines such as DLD1 do not differentiate under three-dimensional conditions, and instead form disorganised 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 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 characterised the nature of differentiation of 3D colonies formed by different cell line and primary tumour derived cancer stem cells.

**Methods**

**Cell culture**

Details of cell line origins and validation can be found in supplementary text. All cell lines were cultured in complete 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 two times per week after being pre-warmed and equilibrated overnight in 1% oxygen. For DMOG treatment, culture medium containing 2mM DMOG or DMSO (vehicle control) was overlaid over the gels and this was changed every 24 hours.

For primary culture, six moderate/well differentiated tumour specimens, obtained from fully consenting donors under National Research Ethics study, 07/H0606/120, were mechanically disrupted into small pieces and cultured in serum free medium (Excell-620, Sigma-Aldrich Ltd.) supplemented with 2%
StemPro hESC supplement (Life Technologies Inc.), on non-adherent plastic, until spheroids were formed. The cultures were checked by EpCam immunofluorescence and RT-PCR to consist of CDX1 expressing epithelial cells (not shown). Established colonies were transferred to Matrigel and grown for 7 days, before fixation and immunostaining, as detailed below.

**F-actin Assay of Colonies Grown in Matrigel.**

Single cell suspensions were achieved by FACS or filtration through 30 and 20 μm filters (Celltrics, Partec GmbH). 500 cells were suspended in 40μl of an ice-cold mixture of Matrigel and diluted 1:1 with ice cold DMEM medium. Cell suspensions were seeded in triplicate into 96 well plates pre-coated with a solidified 1:1 Matrigel/DMEM layer (20μl per well). Overlaid Matrigel was allowed to set for 20 minutes at 37°C and culture medium was then added to the wells. Colonies were grown for two weeks, unless otherwise specified, with medium changes every three days. The medium was removed and 100μl of 4% (v/v) paraformaldehyde in phosphate buffered saline (PBS) was added for 20 minutes. Fluid was then removed and 100 μl of PBS containing 1:200 dilution of Triton-x were added for 10 minutes. The fluid was removed and the wells were washed 4 times with 50 mM glycine in PBS. 100μl TRITC-Phalloidin diluted at 1:1000 in PBS was added incubated at 4°C overnight. The colonies were then rinsed three times with PBS as above. 100μl of 10 μg/ml 4'6-
diamidino-2-phenylindole (DAPI, Sigma), in PBS were added to each well and the plate was imaged using a Zeiss LSM510 laser scanning confocal microscope (Carl Ziess Ltd, UK).

FACS Analysis

Cells were harvested and 1 x 10^6 cells and labelled with anti-CD44 and anti-CD24. Cell viability was assessed with Sytox Blue. Cells were run on a Dako MoFLow cytometer. Gates were set to exclude dead cells, debris and doublets and the top or bottom 5% of gated CD44/CD24 (see figure 3) labelled cells were collected into separate tubes containing 200μl ice cold DMEM. Cells were then embedded in Matrigel as described above and assayed after 2 weeks growth.

Western Blotting

Western blot was performed on cell lysates as described previously [20].

In Vivo Tumorigenic Assays

Single cells were injected s.c. into the flanks of 6- to 10-week-old female NOD/SCID immunodeficient mice, obtained from the John Radcliffe Hospital Biomedical Services, Oxford, United Kingdom as described in [14]. Animal studies were conducted according to the University of Oxford institutional 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 Characterisation

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 (Figure 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 labelling in single cell derived colonies from a panel of six colon cancer cell lines, grown in a three dimensional Matrigel matrix for 2 weeks. In an SW1222 colony, confocal Z-sectioning showed intense F-actin labelling on the apical cell membranes facing the lumen, which was surrounded by polarised cells expressing cytokeratin 20 on their basolateral membranes (Figure 1B). LS180, SW1222, and C80 formed colonies consisting of polarised cells surrounding central lumens visible by light microscopy (Figure 1C). All phase visible lumens were clearly labelled by intense F-actin staining. HT29, HCT116 and DLD1, did not form observable lumen colonies using phase contrast, and did not exhibit organised bright foci of F-actin
staining, although F-actin was visible in plasma membranes. A gallery of F-actin labelled colonies from various cell lines is shown in supplementary Figure 1.

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 (Figure 2A). HCT116 and DLD1 did not express Villin. In HT29, villin was generally expressed but it was not polarised 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 (Figure 2B). Ezrin was only weakly present at non-apical plasma membranes. Ezrin was strongly expressed in HCT116 in a non-polarised manner.

The brush border enzyme CD26/dipeptidyl peptidase-4 (DPPIV) [7], and the brush border enriched tight junction marker Zo-1[23] were also polarised and enriched in lumen apical membranes (supplementary Figures 2A and B). In contrast non-lumen forming cell lines failed to show organised 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 (Figure 2C) demonstrated 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 (Figure 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 figure 3A/B, the top 5% of CD24/44 (CD24/44 high) expressing SW1222 cells gave rise to significantly more large colonies with F-actin labelled lumens than unsorted/bulk cell populations, and significantly fewer small colonies with no lumens. The bottom 5% CD24/44 expressing cells (CD24/44 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 (Figure 3C/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 stabilisation on differentiation and CDX1 expression**

Lumen formation can be inhibited by long-term hypoxia, accompanied by a down-regulation of CDX1 in a Hif1 dependent manner [15]. To determine whether F-actin was similarly down regulated 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 labelling of the colonies under each condition. Hypoxic colonies were smaller and F-actin focus size and intensity were reduced, compared to 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 labelled lumens, and an increase of colonies without well-defined lumens (Figure 4B). These results show that hypoxia inhibits brush border development in colon cancer stem cells.

To determine if 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 immuno-labelling for Glut1 and Carbonic Anhydrase IX (CAIX) and immunoblotting for Hif1 (supplementary Figure 3A/B/C). We then treated colonies grown from single cells in Matrigel for seven days with DMOG for a further 5 days. The effect on F-actin labelling 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 (Figure 4D).

We next determined if DMOG treatment induced down-regulation of CDX1 and its target, Cytokeratin 20 [16]. Immuno-labelling of DMOG treated colonies and vehicle treated controls showed a marked down regulation of CDX1 in response to DMOG, though Cytokeratin 20 expression remained virtually unchanged (Figure 5A/B). We also looked at the effect of DMOG on goblet cell lineage differentiation. Immuno-labelling colonies with an anti-goblet cell antibody
indicated that DMOG suppressed goblet cell differentiation in both the C80 and LS180 cell lines (Figure 5C/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 down-regulation could impair F-actin lumen formation we used an LS174T derivative cell line ‘LS174T-CDX1’ with stable expression of a construct encoding a shRNA targeting CDX1, in which CDX1 was down-regulated by RNAi [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 labelling to visualise F-actin. Figure 6A/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 knock down also reduced this number ie CDX1 knockdown inhibited lumens per colony (Figure 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 6Di shows a whole well composite image of an entire well from a 96 well plate that
contained F-actin labelled SW1222 colonies grown in three-dimensional conditions. ImageJ software was then used to automatically recognise and analyse the colonies (Figure 6Dii). 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 (Figures 6D iii and iv, respectively). To validate this approach we used our semi-automatic method to analyse the LS174t-Vector and LS174t-CDX1 colonies manually counted in figure 6C, and found a good correlation between the automated and manual analyses (Figure 6E). Thus our approach enables tissue plate wells to be automatically scanned and analysed for changes in lumen formation.

**Lumen formation is a feature of tumours in vivo**

To determine if lumen formation had relevance to tumours in vivo, we first examined actin and ezrin polarisation in murine xenografts derived from the injection HT29, HCT116 or SW1222 cells into the flanks of NOD/SCID mice. Mice were sacrificed after one month and resulting tumours removed and processed for FFPE tissue sections which were stained by hematoxylin and eosin or immunolabelled with anti-actin/ezrin (Figure 7A). Hematoxylin and eosin staining of these xenograft tumours demonstrated poorly differentiated high grade tumour morphology for HCT116 and HT29 tumours that lacked lumens or actin/ezrin polarisation. SW1222 tumours exhibited a well differentiated phenotype with numerous lumens surrounded by polarised cells.
Actin and ezrin immune-labelling showed that neither HT29 nor HCT116 exhibited polarisation of these markers, whereas SW1222 showed marked luminal polarisation. 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 tumours, we examined if they labelled with the same markers as in vitro lumens. We compared two colorectal tumours 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 tumour confirmed the presence of numerous neoplastic glands, which were absent in the poorly differentiated tumour (Figure 7B). Immunolabelling of sections from the same tumours indicated that lumens in the low grade tumour were composed of polarised cells expressing actin and ezrin on the apical membranes, whereas no polarisation of these markers was observed in the poorly differentiated tumour (Figure 7C). Typical of tumours, cellular debris was present in the lumens. Actin/ezrin positive cells present in the stroma surrounding the glands were probably myofibroblasts (supplementary Figure 4). Lumen glands were strongly positive for polarised CEA, whereas CEA was non-polarised and more cell membrane associated in the high-grade tumour (Figure 7D). Thus at least three 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 tumours.

To determine if lumen formation was a feature of primary tumour cells in vitro, we derived a primary tumour cell culture from the same low grade tumour used in Figure 7B-D. When grown under conditions that promote stem cell populations [25; 26; 27; 28], these primary cells grew as well organised spheroids (Figure 7E, left panel). Following a week-long suspension in Matrigel, the colonies formed large central lumens marked by F-actin, (Figure 7E, right panel). 3 out 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 tumour derived cultures.

**Discussion**

Single stem cells from colorectal cancer lines, when grown under 3D conditions, can differentiate into polarised structures with remarkable structural similarity to normal intestinal crypts. Lumens express many characteristics of normal intestinal brush borders, including microvilli structural proteins
(polarised F-actin, villin, ezrin), enzymatic activity (polarised 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. Nevertheless goblet cells were also often present in colonies with F-actin lumens that had been grown from single cells, illustrating their multi-potent nature. Importantly, we also show that several of these markers of in vitro lumen brush border formation are also expressed in a polarised manner by neoplastic glands present in primary tumours, which are similarly composed of polarised 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 tumour spheroid cultures derived from moderately/well differentiated tumours could also form polarised lumen colonies when transferred into Matrigel.

Unlike lumen colonies and well differentiated tumours, non-lumen cell lines, such as HCT116, totally lacked cellular polarisation of several polarity markers such as villin, a situation mirrored in vivo by high grade tumours. Thus cell lines can also be separated into high or low ‘grades’ based on their polarisation in Matrigel.
One possible explanation for this is that these cells lack expression of CDX1 [16], and this may disrupt their ability to efficiently polarise 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 polarisation of intestinal cells [29; 30]. A lack of polarisation and differentiation will lead to an increase in the population of stem cells, and indeed we have found previously that HCT116 does not contain subpopulations of cells with different tumour-forming capacity, unlike the lumen forming lines [14]. Similarly, high grade tumours have a significantly worse clinical prognosis, and exhibit a more ‘stem like’ transcription profile [31; 32].

Tumour 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 polarise 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 down regulation 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 labelled lumens. However we cannot rule out a role for other hypoxia regulated transcription factors differentiation suppression.

Specific cancer stem cell markers remain elusive. Whilst markers such as LGR5 [33], ephrin type-B receptor 2 (EphB2) [34], and Lrig1 [35] appear to mark well defined stem cell populations in normal intestine, their expression in tumours 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 cut off 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 characterised the formation of lumen colonies from cell line derived single cells to represent brush border/enterocyte differentiation and shown the extent of heterogeneity between cell lines and primary tumour 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.

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Figure legends

Figure 1 – Polarised F-Actin is a marker of colonic brush borders in vivo and labels lumens in vitro. (A) Confocal images of normal human colon cryosections labelled with TRITC-phalloidin (red) and DAPI (blue) to visualise 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 labelled with F-actin (red), DAPI (blue) and cytokeratin 20 (green). Numbers represent distance in Z-axis from top to. Scale bar 10 μm. (C)
Single cells from various cell lines were grown in Matrigel for six days. Colonies were labelled with TRITC phalloidin (red) to visualise F-actin and DAPI to visualise nuclei. Bar 10 μm.

**Figure 2 – Lumens represent enterocyte brush border differentiation, but also contain secretory lineages.** Single cells from specified cell lines were embedded in Matrigel and grown for 10 days. Colonies were fixed and labelled with Phalloidin (red), DAPI (blue) and immuno-labelled with (A) Anti-villin or (B) Anti-Ezrin. (C/D) Single SW1222 or C80 cells were grown in Matrigel for 10 days before immunolabelling with either anti-Muc2 antibodies (C) or anti-CEA (D). F-actin was co-labelled with TRITC-phalloidin. Scale bars 20 μm.

**Figure 3 - F-Actin can be used to Identify Lumen Colonies in Stem Cell Enriched Populations.** (A, C) Scatter-gram plots demonstrating the gating of the extreme 5% of CD44/CD24 high and low expressing cells populations of SW1222 or LS180 cells sorted by FACS. The gates were set so that 5% of the total positive populations were selected in both the upper right quadrant (CD44/CD24 high) and the lower left quadrant (CD44/CD24 low). (B, D) Isolated CD24/44 high and low SW1222 or LS180 cells were grown for 2 weeks in Matrigel and stained with TRITC-phalloidin. Colonies were manually characterised into the following categories: large colony = diameter larger than 40 μm. Lumen colony = F-actin foci larger than 10 μm. Bar graphs show total
counts of different colony classifications from three replicate wells for each gating strategy. (B) For SW1222 colonies, Fisher’s exact test (FET) showed that single cells with high CD24/44 expression gave rise to a significantly higher proportion of large lumen colonies at the expense of small non-lumen colonies, when compared to unsorted cells (p = 0.0007). p values represent FET of significance of the change in the proportion of lumen to non-lumen colonies and indicates a significant shift towards lumen colony formation for cells enriched for CD24/44 expression. Chi-square 2x4 table also showed that single cells with high CD24/44 expression gave rise to a significantly higher proportion of lumen colonies to non-lumen colonies irrespective of colony size (p = 0.0003), compared to unsorted cells. The CD24/44 low expressing cells showed a much lower clonogenicity compared to either un-sorted or CD24/44 high cells. Although there was a clear trend towards an increase in the ratio of small non-lumen colonies to large lumen colonies, the very small number of colonies precluded statistical analysis. (D) For LS180, FET showed a similar significant increase in the proportion of large lumen to small non-lumen colonies derived from cells sorted for high CD24/44 expression, compared to CD24/44 low expressing cells (p=0.0002), and a Chi-square 2x4 table showed a significant difference between CD24/44 high and low when comparing all lumen colonies to non-lumen colonies irrespective of colony size (p=0.0001).
Figure 4 - (A) Hypoxia and Hif activation reduces F-actin foci formation.

(A) Single cells of either LS180 or SW1222 were grown in Matrigel under normal 20% oxygen or 1% oxygen for 14 days. The colonies were then labelled with phalloidin (red). Scale bar 50 μm. (B) Colony counts of the same experiment showing total number of colonies of LS180 cells in defined categories, from three replicates. Small colony defined as any colony with a radius smaller than 250μm, lumen colony = colony with 1 or more F-actin foci larger than 10 μm. 40 colonies counted from each of three wells for hypoxia and normoxia. Hypoxia induced a significant difference in the proportion of lumen to non-lumen colonies (p = 0.0235, F.E.T. 2x2 table), compared to colonies grown in 20% oxygen. The p value represents a Fisher’s exact test of significance of the change in the proportion of lumen to non-lumen colonies and indicates a significant shift towards non-lumen colony formation in the presence of DMOG. (C) Lumen F-actin formation is inhibited by DMOG. Single cells were grown in Matrigel for 1 week in the presence of 2mM DMOG or vehicle control (DMSO). Colonies were then fixed and F-actin labelled with Phalloidin. (D) Graph showing total colonies with large or small lumens. A total of 40 colonies were counted from each of three wells for each condition. Lumen colony = colony with 1 or more F-actin foci larger than 15 μm. For LS180 and C80 DMOG significantly inhibited lumen formation (p=<0.0001, 2x2 tables F.E.T. of proportion of lumen to non-lumen colonies), compared to colonies...
grown in DMSO control. SW1222 showed a non-statistically significant trend towards reduced lumens by DMOG (p = 0.157, F. E.T.).

**Figure 5 - Prolyl Hydroxylase inhibition down regulates CDX1 and suppresses goblet cell differentiation.** (A, B) 10 day old colonies grown in Matrigel from single cells of either C80 (A) or SW1222 (B) treated with 2 mM DMOG or DMSO for 4 days. Colonies were imaged by phase contrast, and co-immunolabelled with anti-CDX1 and anti-Cytokeratin 20 (CK20). Bars 20 μm. (C) Single LS180 or C80 cells were plated on plastic at 500 cells per well in a 96 well plate and allowed to grow for 1 week in total. On day three 2 mM DMOG or DMSO (vehicle control) was added to the wells. The cells were fixed and labelled with DAPI (blue), Phalloidin (red), and anti-goblet cell mucin (PR5D5) (green). Bars 20 μm. (D) Bar graph showing quantification of goblet cell positive colonies in vehicle or DMOG treated wells. Out of 120 colonies looked at, from a total of three wells for each condition, the number that contained goblet cells and the number that did not are given in the bar graph. DMOG caused a significant drop in the proportion of goblet cell containing colonies compared to DMSO controls for both C80 and LS180 (p = 0.0030, p = 0.0001, respectively, using FET for the relevant 2 x2 table ).

**Figure 6 - CDX1 Knock-Down Reduces F-actin Lumen Formation in LS174T Colonies.** (A) F-actin labelling of LS174T vector and LS174T CDX1
RNAi cultured from single cells in Matrigel for 1.5 weeks. (B) Bar chart showing total counts of 200 colonies in each of three replicate wells, categorised into lumen or non-lumen colonies. Colonies were defined as non-lumen if they contained no F-actin foci with a diameter above 8 μm. Knockdown of CDX1 caused a significant reduction in the proportion of lumen colonies, compared to vector control colonies (p = <0.0001, FET).

C) Manual counts of averaged numbers of F-actin lumens per colony of LS174T-vector or LS174T-CDX1 colonies from the same experiment as used for B. A total of 120 colonies were analysed (40 each from three replicates). The number of lumens in LS174T-CDX1 was significant reduced compared to the vector control (Student t-test, p =< 0.0001). Error bars = SD. (D) Semi-Automated Analysis of F-actin Lumen Formation using ImageJ. (D i) Composite image of an entire well from a 96 well plate containing F-actin labelled SW1222 colonies grown in Matrigel from single cells for 10 days. (D ii) Masked (outlined) colonies automatically identified by the ImageJ algorithm. (D iii) A well differentiated lumen colony is shown with 7 automatically detected F-actin foci. (D iv) A small poorly differentiated colony with only 1 F-actin focus automatically detected. E) Automatic F-actin lumen counting was applied to the LS174T data used for (C), and detected a significantly reduced number of F-actin lumens per colony in the LS174T-CDX1 knock-down colonies (Student t-test, p = 0.001).
Figure 7 – Lumen formation is a feature of tumours in vivo, and human primary low and high grade tumours show differences in ezrin/actin and CEA polarisation. (A) Lumen forming and non-lumen forming cell lines form well differentiated (low grade) and poorly differentiated (high grade) tumours respectively as xenografts in vivo. Hematoxylin/eosin or anti-actin/ezrin immunostaining staining of FFPE sections from murine xenograft tumours derived SW1222, HCT116 or HT29 cells injected into NOD/SCID. Inset shows magnification. (B) Typical hematoxylin and eosin staining of FFPE tissue sections derived from (left) a moderately differentiated (low grade) primary colorectal tumour from a patient, with numerous neoplastic glands visible, and (right) a poorly differentiated (high grade) human colorectal tumour, with no glands . (C, D) Anti-actin/anti-ezrin or anti-CEA/villin labelling of FFPE tissue sections derived from the same patient derived high and low grade tumours shown in B. (E) Characterisation of lumen formation in primary cultures derived from a low grade tumour. Left panel shows phase contrast image of typical primary cultured spheroid cancer cell colony derived from the same low grade human colorectal tumour shown in panels B, C D. Right hand panel shows F-actin labelling 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.


Figure 2
Figure 3
Figure 4
Figure 5

A Phase CDX1 Cytokeratin 20 Merge

DMSO
LS180
DMOG

B

DMSO
DMOG
SW1222

C DAPI/Goblet Cell

DMSO DMOG

LS180
C80

D

Number of Goblet Cell Positive Colonies

Control DMOG

p = 0.0001

p = 0.003

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Figure 6

(A) LS174T-Vector  LS174T-CDX1

(B) 

![F-actin Lumen vs No Lumen Graph](image)

<table>
<thead>
<tr>
<th>LS174t Vector</th>
<th>LS174t CDX1 RNAi</th>
</tr>
</thead>
<tbody>
<tr>
<td>F-actin Lumen</td>
<td>No Lumen</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>p-value</th>
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<tr>
<td>&lt; 0.0001</td>
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</table>

(C) 

![Average Number of F-actin Lumen per Colony Graph](image)

- Vector Control
- CDX1 RNAi

Average Number of F-actin Lumens per Colony

(D) 

- Raw Image
- Numbered Colony Outlines
- Lumen Colony
- Non-Lumen Colony

(E) 

![Average Number of F-actin Lumen per Colony Graph](image)

- Vector Control
- CDX1 RNAi

Average Number of F-actin Lumen per Colony

p-value < 0.0001
Stem Cell Differentiation and Lumen Formation in Colorectal Cancer Cell Lines and Primary Tumours

Neil Ashley, Trevor Yeung and Walter F Bodmer

Cancer Res  Published OnlineFirst July 18, 2013.

Updated version  Access the most recent version of this article at: doi:10.1158/0008-5472.CAN-13-0454

Supplementary Material  Access the most recent supplemental material at: http://cancerres.aacrjournals.org/content/suppl/2013/08/06/0008-5472.CAN-13-0454.DC1

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