Cancer stem cells from colorectal cancer-derived cell lines

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Cancer stem cells (CSCs) are the subpopulation of cells within a tumor that can self-renew, differentiate into multiple lineages, and drive tumor growth. Here we describe a two-pronged approach for the identification and characterization of CSCs from colorectal cancer cell lines, using a Matrigel-based differentiation assay, and cell surface markers CD44 and CD24. About 20 to 30% of cells from the SW1222 cell line form megacolonies in Matrigel that have complex 3D structures resembling colonial crypts. The megacolonies’ capacity to self-renew in vitro is direct evidence that they contain the CSCs. Furthermore, just 200 cells from SW1222 megacolonies initiate tumors in NOD/SCID mice. We also showed that CD44+CD24+ cells enriched for colorectal CSCs in the HT29 and SW1222 cell lines, which can self-renew and reform all four CD44/CD24 subpopulations, are the most clonogenic in vitro and can initiate tumors in vivo. A single SW1222 CD44+CD24+ CSC, when grown in Matrigel, can form large megacolonies that differentiate into enterocyte, enteroendocrine, and goblet cell lineages. The HCT116 line does not differentiate or express CDX1, nor does it contain subpopulations of cells with greater tumor-forming capacity, suggesting that HCT116 line does not differentiate or express CDX1, nor does it contain subpopulations of cells with greater tumor-forming capacity, suggesting that HCT116 line does not differentiate or express CDX1, nor does it contain subpopulations of cells with greater tumor-forming capacity, suggesting that HCT116 line does not differentiate or express CDX1, nor does it contain subpopulations of cells with greater tumor-forming capacity, suggesting that HCT116 line does not differentiate or express CDX1, nor does it contain subpopulations of cells with greater tumor-forming capacity, suggesting that HCT116 line does not differentiate or express CDX1, nor does it contain subpopulations of cells with greater tumor-forming capacity, suggesting that HCT116 line does not differentiate or express CDX1, nor does it contain subpopulations of cells with greater tumor-forming capacity, suggesting that HCT116 line does not differentiate or express CDX1, nor does it contain subpopulations of cells with greater tumor-forming capacity, suggesting that HCT116 line does not differentiate or express CDX1, nor does it contain subpopulations of cells with greater tumor-forming capacity, suggesting that HCT116 line does not differentiate or express CDX1, nor does it contain subpopulations of cells with greater tumor-forming capacity, suggesting that HCT116 line does not differentiate or express CDX1, nor does it contain subpopulations of cells with greater tumor-forming capacity, suggesting that HCT116 line does not differentiate or express CDX1, nor does it contain subpopulations of cells with great...
SW1222 Megacolonies Give Rise to Megacolonies and Small Colonies. Although Small Colonies only Give Rise to More Small Colonies.

SW1222 single cells obtained by filtration were plated in limiting dilutions in 96-well Matrigel-containing plates. Megacolonies and small colonies growing in single wells were pooled separately, disaggregated, and filtered into a single-cell suspension and then replated into Matrigel. Fig. 2A shows that cells from megacolonies were able to reform megacolonies (27.8% on average), as well as small colonies (59.4%), although cells from small colonies could only reform themselves (36.7%) and could not form megacolonies. Cells from both types of colonies were next injected into NOD/SCID mice. As few as 200 cells from megacolonies were able to initiate tumors; however, even 1,000 cells from small colonies were unable to do so (Fig. 2B). These results strongly support the view that the megacolonies are formed from CSCs in the SW1222 cell line. Xenografts derived from megacolonies are able to recapitulate a well-differentiated adenocarcinoma (Fig. S3).

SW1222 FACS-Sorted CD44+CD24+ Cells Are the Most Clonogenic Subpopulation, Form the Greatest Number of Megacolonies, Which Self-Renew and Differentiate, and Are the Most Efficient in Producing Tumors in NOD/SCID Mice. Using the markers CD44 and CD24 to enrich for putative colorectal CSCs from the SW1222 cell line by FACS sorting, we showed that the CD44+CD24+ cells (Fig. 3A, upper 0.5–1%) were the most clonogenic and gave rise to the highest proportion of megacolonies (Fig. 3B). Immunofluorescence analysis of megacolonies derived from single CD44+CD24+ sorted cells expressed markers of all three types of differentiated colonic epithelial cells, namely columnar, goblet, and enteroeudocrine cells (Fig. 3C). The chromogranin-A staining was particularly strong around the lumen-like structures, suggesting that the product was secreted. The overall pattern of staining is as expected on the assumption that these megacolonies, containing multiple lineages, originally arise from single CD44+CD24+ CSCs present in the SW1222 cell line. The sorted CD44+CD24+ cells were also the most efficient at producing tumors in NOD/SCID mice, as would be expected if this subpopulation enriched the most for CSCs from the SW1222 cell line (Fig. 3D).

Cells from megacolonies analyzed by FACS for CD44 and CD24 produced all four combinations of CD44+CD24+ expression. Reanalysis of megacolonies derived from single CD44+CD24+ cells again produced all four combinations of CD44+CD24+ expression, and isolation of CD44+CD24+ cells derived from this second cell sort were capable of reforming megacolonies for a further generation. The ability of megacolonies to self-renew and differentiate is again consistent with their containing the CSC subpopulation (Fig. S4).

FACS analysis of xenografts derived from an initial injection of CD44+CD24+ cells showed that these tumors were able to express all four CD44+CD24+ subpopulations (Fig. S5). The histology of the SW1222-derived xenograft tumors was similar to that of a well-differentiated primary human lumen-forming tumor, whether it was from a SW1222 CD44+CD24+ sorted cell population or from unsorted SW1222 cells (Fig. 3F). The tumors from the SW1222 CD44+CD24+ cells contained differentiated cells, just as did the megacolonies studied in vitro (Fig. 3F).

The overall results both from the clonal analysis of SW1222 cells and from the analysis of the SW1222 CD44+CD24+ sorted cells provide, in our view, unequivocal evidence for the existence and identification of CSCs in the SW1222 cell line.

CSC Analysis in the HCT116 and HT29 Cell Lines. Two additional CRC cell lines were chosen for CSC analysis along the same lines as that described above for SW1222. HCT116 is known to be a highly aggressive cell line with little or no capacity to differentiate, although HT29 has an intermediate capacity to differentiate into enterocytes and mucin-expressing lineages (12–14). The colony-forming abilities of HCT116 and HT29 in Matrigel are shown in Fig. 4A. In this case, colony morphology was classified by whether or not they formed lumens. The colonies that were able to differentiate to form lumens were comparable to those that developed into megacolonies in the SW1222 cell line. The data are consistent with the characterization of HCT116 as highly aggressive and non-differentiating, giving rise to no lumen-forming colonies; however, HT29 has retained at least some capacity to differentiate.

Immunofluorescence analysis of colonies derived from these cell lines, seeded in Matrigel and using the antibodies CDX1 and AU1, is consistent with the above observations and the known lack of CDX1 expression in HCT116 (11) (Fig. 4B). Both cell

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**Fig. 1.** Immunofluorescence of SW1222 cell line colonies. (A) The SW1222 cell line can differentiate and form two distinct colony types: megacolonies with complex 3D lumen-like structures and small colonies. (Scale bar, 400 μm.) (B) Immunofluorescence of SW1222 megacolonies in Matrigel shows low levels of CDX1 and CK20 expression at day 7. (μ) (C) By day 14, megacolonies express much higher levels of CDX1 and CK20. Magnification: 20x.

**Fig. 2.** SW1222 subcloning in Matrigel. (A) Megacolonies and small colonies growing in single wells in a 96-well plate were pooled separately, disaggregated, and filtered into a single-cell suspension and then replated into Matrigel. Fig. 2A shows that cells from megacolonies were able to reform megacolonies (27.8% on average), as well as small colonies (59.4%), although cells from small colonies could only reform themselves (36.7%) and could not form megacolonies. Cells from both types of colonies were next injected into NOD/SCID mice. As few as 200 cells from megacolonies were able to initiate tumors; however, even 1,000 cells from small colonies were unable to do so (Fig. 2B). These results strongly support the view that the megacolonies are formed from CSCs in the SW1222 cell line. Xenografts derived from megacolonies are able to recapitulate a well-differentiated adenocarcinoma (Fig. S3).

**Fig. 3.** (A) Megacolonies and small colonies growing in single wells in a 96-well plate were pooled separately, disaggregated, and filtered into a single-cell suspension and then replated into Matrigel. Fig. 2A shows that cells from megacolonies were able to reform megacolonies (27.8% on average), as well as small colonies (59.4%), although cells from small colonies could only reform themselves (36.7%) and could not form megacolonies. Cells from both types of colonies were next injected into NOD/SCID mice. As few as 200 cells from megacolonies were able to initiate tumors; however, even 1,000 cells from small colonies were unable to do so (Fig. 2B). These results strongly support the view that the megacolonies are formed from CSCs in the SW1222 cell line. Xenografts derived from megacolonies are able to recapitulate a well-differentiated adenocarcinoma (Fig. S3).
lines produced colonies that were negative for PR4D4 staining. A few cells in HT29 colonies expressed CDX1, consistent with HT29’s limited capacity for differentiation.

The FACS analyses of the CD44−CD24− subpopulations of cells from these two cell lines, grown in Matrigel, are shown in Fig. 4C. For these two cell lines, the CD44−CD24− population has only slightly less clonogenic capacity than the CD44+CD24+ subpopulation, suggesting that, for these two lines, CD44 is the more important determinant with respect to enriching for CSCs (15).

Immunofluorescence staining with the Ki67 antibody, a well known marker of dividing cells (16, 17), provides a further assessment of clonogenicity, as well as indicating the position of the most rapidly dividing cells in a colony. As shown in Fig. 5, the number of Ki67-positive cells correlates precisely with the aggressiveness of the cell lines and inversely with their propensity to differentiate. The Ki67 cells are in all cases found at the periphery of the colonies. This finding is a priori where the most rapidly dividing cells might be expected to be found. This could be the case even in differentiating colonies, where the peripheral cells are likely to be progenitor cells that divide more rapidly, but to a more limited extent, than the stem cells that might be expected to be found nearer the center of a colony.

CD44+CD24− and CD44−CD24− cells from the HCT116 and HT29 lines were injected into NOD/SCID mice, as before for SW1222. The results show that the selection does not seem to affect the tumor forming capacity of HCT116 cells, as there is no significant difference in the tumor sizes between the CD44+CD24+ and CD44−CD24− subpopulations. For HT29, on the other hand, there is a significant difference in size between these two cell subpopulations, although it is not as great as for the SW1222 cell line (Fig. 6). These results suggest that HCT116 contains a high proportion of CSCs that have lost the capacity to

Fig. 3. Characterization of CD44/CD24 selected SW1222 cells. (A) The extreme 0.5 to 1% of each CD44/CD24 subgroup by FACS analysis was taken for further experiments. (B) CD44+CD24− SW1222 cells are the most clonogenic and form the greatest numbers of megacolonies when grown in Matrigel, compared to other CD44/CD24 subgroups. (C) A single CD44+CD24− cell can give rise to a megacolony forming multiple differentiated cell types: AUA-1 (anti-EpCam pan-epithelial marker), CDX1 (enterocyte), chromogranin A (enteroendocrine), and PR4D4 (mucin, see Fig. S6). Nuclei stained blue with DAPI. Megacolonies grown in Matrigel for 4 weeks. (D) CD44+CD24− sorted cells are more tumorigenic in NOD/SCID mice: 200 and 1,000 cells of CD44+CD24− and CD44−CD24− cells were injected into NOD/SCID mice (Upper) and assessed for tumor forming capacity. The results of the 200 and 1,000 cell groups were combined together in a 2 × 2 contingency table (Lower), and analyzed statistically using Fisher’s exact test (P = 0.007). (E) Both CD44+CD24− sorted and unsorted SW1222 cells are able to form well-differentiated adenocarcinomas that are histologically indistinguishable when injected into NOD/SCID mice (H&E stain, magnification 20×). (F) Xenografts derived from SW1222 CD44−CD24− sorted cells express the differentiation markers chromogranin A (enteroendocrine) and CDX1 (enterocyte).
differentiate. This finding is exactly what might be expected for an aggressive and poorly differentiated tumor and correlates clinically with lymph node metastases (18, 19) and reduced survival (20, 21). HT29, on the other hand, is in general similar to SW1222, with a detectable proportion of stem cells with the capacity to differentiate.

**Increased Expression of CDX1 in HCT116 Reduces Clonogenicity and Induces Lumen Formation Within Colonies.** We have previously shown that CDX1 expression regulates the differentiation of crypt progenitor cells into mature intestinal epithelial cells (22) and, in particular, directly controls the expression of CK20 and probably other markers of differentiation of colonic epithelial cells as well (11). To test whether increasing the expression of CDX1 in a nonexpressing cell line decreases clonogenicity and increases the capacity to differentiate, we compared the clonogenic capacity of the HCT116 cell line stably transfected with a CDX1-expressing vector with that of the HCT116 cell line transfected with a vector control (11) (Fig. 7). The CDX1-expressing HCT116 cells are much less clonogenic, and also induce lumen formation within colonies, which is absent from the vector control. This supports the role of CDX1 in controlling colonic epithelial cell differentiation and shows that CDX1 expression may enable the CSCs in HCT116 to differentiate at least to some extent. The results also help to explain why loss of CDX1 expression (by methylation) is relatively frequently selected for in colorectal cancers (22).

**Discussion**

Through a combination of colony formation and surface marker selection, we have shown how CSCs can be recognized in the colorectal cancer-derived cell line SW1222. Thus, between 20 and 30% of SW1222 cells can form megacolonies containing crypt-like structures with multiple markers of differentiation when grown in Matrigel. The 3D structure of these megacolonies is strikingly similar to the morphology of organoids that can be generated from normal intestinal stem cells (23), suggesting that normal and cancer stem cells have similar mechanisms for self-renewal and differentiation. The expression of the differentiation marker, CDX1, increases with time in these colonies and cells from these colonies can self-renew, differentiate, and initiate tumors in immunocompromised NOD/SCID mice. Putative CSC markers, such as CD44, CD24, and, notably CD133, have been used to enrich for CSCs in colorectal and other tumors, although mainly in fresh tumor

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**Fig. 4.** Cell line clonogenicity. (A) Clonogenicity of HCT116, HT29, and SW1222 in Matrigel. Numbers of lumen-forming and total colonies counted per well. One thousand cells plated in Matrigel per well in 96-well plates, grown for 2 weeks. (B) HCT116 and HT29 colonies grown in Matrigel for 2 weeks stain positive for AUA1. HCT116 colonies are CDX1-negative, and HT29 colonies weakly express CDX1. Nuclei stained blue with DAPI. Magnification, 20x. (C) The CD44+CD24+ and CD44+CD24− subpopulations are of comparable clonogenicity for HCT116 and HT29 cells grown in Matrigel. Three hundred cells seeded per well in 24-well plates.

**Fig. 5.** Ki-67 staining for HCT116, HT29, and SW1222 colonies grown in Matrigel. Magnification, 20x.
However, not all CD44+CD24+ cells are clonogenic, and a few are reduced levels of CDX1, chromogranin A, and mucin. This finding suggests that the center of the megacolony may contain the most differentiated cells, as well as be the most efficient subset of SW1222 CD44+CD24+ cells in forming tumors in NOD/SCID mice. This finding is consistent with selection for CD44+CD24+ cells in SW1222 enriching significantly for CSCs in the SW1222 cell line. However, not all CD44+CD24+ cells are clonogenic, and a few CD44+CD24− cells are also able to form colonies. More specific markers, including perhaps aldehyde dehydrogenase 1 (27), are needed for enriching for CSCs. The more differentiated cells, including goblet and enteroendocrine cells, appear to be located toward the periphery of the megacolonies, and the less differentiated cells more toward the center of the colonies, where there are reduced levels of CDX1, chromogranin A, and mucin. This finding suggests that the center of the megacolony may contain the major part of the CSC population.

Our results with SW1222 are consistent with earlier work on the HRA-19 colorectal carcinoma cell line, which was shown to be able to differentiate into multiple lineages, including neural, goblet, and enteroendocrine cells, from a single original clone (28). Both sets of observations are consistent with evidence that normal colonic crypts in both mice (29) and humans (30) are clonal, and so have normal stem cells that can give rise to all of the three types of differentiated cells found in colorectal crypts. These tumor-derived cell lines are therefore recapitulating, in a deranged manner, the situation in a normal crypt.

The results for SW1222 have been extended to two other cell lines, HCT116 and HT29, which represent different extents of differentiation, or the lack of it. HCT116 is a highly aggressive cell line that shows no ability to differentiate and does not express CDX1; HT29 has an intermediate capacity to differentiate. Compared with HCT116, SW1222 retains the ability to differentiate and express high levels of CDX1, consistent with the hypothesis that SW1222 contains a lower proportion of undifferentiated cancer stem cells. This result confers a less aggressive phenotype, as demonstrated by a lower clonogenic and tumorigenic potential than HCT116. HCT116 cells do not significantly separate into different types of colony-forming cells, nor into different categories with respect to the ability to form tumors in NOD/SCID mice. This finding is consistent with the HCT116 cell line containing almost only CSCs, as also found by Kai et al. (31). HT29, however, behaves in an analogous way to SW1222, although with a higher proportion of clonogenic cells and a lesser propensity to differentiate. The relative numbers of dividing cells in the three lines are proportional both to their clonogenicity and tumor-formation capacity.

It is now becoming quite clear that the proportion of CSCs in a tumor can vary from very high and with little capacity to differentiate, as in HCT116, to much lower with much more differentiation, as in SW1222. The possibility that CSCs may in some cases constitute a relatively high proportion of the cells in a cancer is supported by recent data on highly efficient tumor formation from primary cells from melanoma patients in NOD/SCID IL2rg−/− mice (32). Melanomas are, however, notoriously aggressive tumors.

Theoretical models (33) can readily explain substantial variation in the proportion of CSCs versus differentiated cells in a cancer. The ratio between CSCs and differentiated cells can be shown, on reasonable assumptions, to depend on the rate of differentiation relative to the turnover rate of the CSCs. This is exactly as might be expected, namely that the less the propensity to differentiate, in general, the more aggressive the tumor and the higher the proportion of CSCs in it. The CSC field has been dogged by the assumption that CSCs are necessarily a small proportion of the cells in a cancer, which is clearly not the case.

It was suggested by Wong et al. (22) that selection for reduced expression of CDX1 in colorectal cancers (by methylation) is likely to be associated with a reduced capacity of the CSCs to differentiate and so contributing to an increased rate of growth. Following this suggestion, Chan et al. (11) provided evidence that CDX1 expression is a key switch for intestinal epithelial differentiation, probably mainly of columnar cells, so that studying the patterns of expression of CDX1 should enable better characterization of CSCs both in colorectal cancer cell lines and in primary colorectal cancer. The data presented in this article corroborate that suggestion, especially in showing that forced expression of CDX1 in HCT116 leads to reduced clonogenicity and, most significantly, a substantial proportion of crypt-forming colonies. This finding is consistent with the control of columnar cell differentiation by CDX1 and directly explains the advantage of selection for CDX1 down-regulation.

In preliminary further studies we have extended the classification of our cell-line panel into crypt colony-forming versus not, and are using this classification for the search for further markers for stem cell characterization and enrichment. We will also explore developing our colony-forming assays using the more sophisticated cell culture conditions described by Ootani et al. (34) and for the application to fresh tumor tissue.
We have obtained preliminary evidence, using our CSCs from the cell lines, that hypoxia inhibits differentiation and so maintains stemness, as has been shown in neuroblastoma tumors (35). There are clearly many advantages to working with CSCs from cell lines, both for basic investigation of the properties of CSCs and through those findings, identification of novel targets for drug development, as well as for the possible development of relatively high-throughput drug-response assays for CSCs. We suggest that the data presented in this article provide a basis for these important developments.

Materials and Methods

Detailed discussion can be found in the SI Materials and Methods.

Cell Lines. HCT116 and HT29 colorectal cancer cell lines were originally obtained from the American Type Culture Collection (ATCC). The SW1222 cell line was a generous gift from Meenhard Herlyn, Wistar Institute, Philadelphia. All three cell lines were cultured in complete DMEM supplemented with 10% FBS and 1% penicillin/streptomycin (Invitrogen). Cells were incubated at 37°C in a humidified 10% CO2 environment and were grown to 50 to 80% confluence before the next passage or further experiments. Cellular suspensions were obtained through trypsinization (0.5% trypsin for 2–5 min) and single cells were obtained by passing cells through a 20-μm filter (Celltrics; Partec). The stably transfected HCT116-CDX1 and vector-control cell lines were previously generated in our laboratory and maintained with genetin selection pressure (11). All colony counts are based on the counts from three wells.

Immunohistochemistry and Confocal Immunofluorescence. Immunohistochemistry was performed according to standard procedures. Dilutions of antibody used were: anti-CDX1 mAb (in-house; 1:100), anti-KRT20 mAb (Dako; 1:100), AUA1 anti-EpCam mAb (in-house; 1:100), PR4D4 mAb (in-house; 1:10), and then visualized under fluorescent microscope. Peter Thomas for his help on the confocal microscope, Professor Zhanfeng Cui for the use of the multiphoton microscope, and Sally Hill, Karla Watson, Osama Al-assi, and Thomas Brunner for their help with animal work. This work was mainly funded by a Cancer Research U.K. program grant (to W.F.B. as Principal Investigator), by a Rhodes Scholarship (S.C.G.), and by support from the Jacqueline Seroussi Memorial Foundation for Cancer Research. T.M.Y. is a Royal College of Surgeons of England Research Fellow.

Fluorescent-Activated Cell Sorting. Fluorescent-activated cell sorting was performed using standard protocols. Cells were labeled with mouse anti-human CD44-PE and mouse anti-human CD24-FITC (Caltag; Invitrogen). Cells were analyzed using a DakoCytometry Cytamark machine, or sorted using a MoFlo cell sorter (Beckman Coulter). The top or bottom 0.5 to 1% of each CD44/CD24 subpopulation was collected for further experiments.

Matrigel Colony Morphology Assay. Single cell suspensions were obtained using filtration and resuspended in a 1:1 mixture of Matrigel (BD Biosciences) and medium, and plated in 96-well plates in a limiting dilution. After 4 weeks of incubation, wells with single colonies were identified and colonies were retrieved using Matrigel Recovery Solution (BD Biosciences) as per manufacturer’s protocol. Viability of the cells was checked using Trypan Blue before being used for further experiments.

Live and Dead Cell Staining in Matrigel. Cells were grown in a 1:1 mixture of Matrigel and serum-free medium (DMEM with 1% penicillin/streptomycin). A 100-μl suspension containing between 200 and 1,000 cells was 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. 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.

Statistical Analysis. All data are shown as mean ± SEM. For 2 × 2 contingency tables, Fisher’s exact test was used.

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Supporting Information

Yeung et al. 10.1073/pnas.0915135107

SI Materials and Methods

Antibodies. Mouse anti-human CD44-PE (Clone MEM-85, isotype IgG2b) and mouse anti-human CD24-FITC (Clone SN3, isotype IgG1) were obtained from Caltag. Invitrogen. Mouse isotype controls IgG2b-PE and IgG1-FITC were obtained from Invitrogen.

AUA-I was obtained from the monoclonal antibody service, Cancer Research U.K. It is a mouse anti-human EPCAM antibody (1). The mouse anti-human mucin antibody PR4D4 (2) was acquired from Dako (M7019). Rat anti-human chromogranin A pAb was purchased from Dako. Mouse anti-human K-67 mAb clone MIB-1 was obtained from Dako.

Fluorescent-Activated Cell Analysis and Sorting. Analysis was performed on 1 × 10^6 cells that were washed with FACS buffer (1% FBS and 1% sodium azide in PBS). The resulting pellet was resuspended in the residual volume of FACS buffer and incubated on ice in the dark for 15 min with 5 μL of CD44-PE, 5 μL of CD24-FITC, or 5 μL of isotype antibody control. The cells were then washed twice with FACS buffer. The resulting labeled cells were then resuspended in 500 μL of 2% formaldehyde in PBS. The cells were analyzed using a DakoCytomation Cyan machine.

For a live cell sort, 1 × 10^5 cells were incubated with 20 μL of CD44-PE and 20 μL of CD24-FITC antibodies on ice in the dark for 15 min, and washed twice in serum-free DMEM. The cells were then resuspended in 1,000 μL of serum-free DMEM. Propidium iodide (PI) was added to the cellular suspension. Cells were sorted using a MoFlo cell sorter (Beckman Coulter). Propidium iodide-stained cells were then analyzed using a DakoCytomation Cyan machine.

Matrigel Colony Morphology Assay. Single-cell suspensions were obtained as described above and were resuspended in a 1:1 mixture of growth factor reduced phenol red free Matrigel (BD Biosciences) and complete DMEM. For limiting dilution assay and subcloning experiments, between two and five cells per well were deposited in a 96-well plate. Next, 200 μL of complete DMEM was placed in each well and changed twice weekly. After 4 weeks of incubation, wells with single colonies were identified and colonies were retrieved using ice-cold Matrigel Recovery Solution (BD Biosciences) and incubated on ice for 1 h as per manufacturer’s protocol. Colonies were then washed twice with ice-cold PBS and then trypsinized briefly to make a single cell suspension. Viability of the cells was checked using Trypan Blue before being replated back in Matrigel or used for further experiments.

Immunohistochemistry. Xenograft tumors obtained from the subcutaneous flanks of NOD/SCID mice were harvested once they exceeded 10 mm in any dimension. Tumors were carefully excised and excess adjacent stromal tissue was removed. Tumors were fixed in 2% formaldehyde in PBS for 5 min, washed twice in PBS, and then embedded in paraffin blocks according to standard pathological laboratory practice.

Alternatively, after megacolonies were grown for 4 weeks in Matrigel, complete DMEM was removed and the colonies were washed twice with ice-cold PBS. Two-percent formaldehyde was added to each well, and with gentle pipetting, the colonies were retrieved and fixed for 5 min, then washed twice in PBS. The colonies were then spun down, and resuspended in a preheated mixture (45 °C) of 1.2% (final concentration) agar in serum-free media. This mixture with colonies was then pipetted into an open-top 1-mL syringe, let to set, and then expelled into a paraffin embedding cassette. Specimens were sectioned into 4-μm thick slices and mounted onto microscope glass slides (Menzel-Glaser Superfrost Plus). Sections were then deparaffinised in xylene twice for 3 min, and hydrated in 100% ethanol, 100% Industrial Methylated Spirit (IMS), and 70% IMS for 2 min each. Slides were then rinsed in distilled water. Some slides were stained with H&E to assess cellular morphology, and others stained for immunofluorescence. For the latter, antigen retrieval was done using the Dako Target Retrieval solution at 100 °C for 20 min, followed by cooling to room temperature for 1 h. Slides were then washed twice in PBS and once in 0.5% PBS-Tween for 5 min each. Tissue specimens were then encircled using the hydrophobic ImmEdge pen (Vector Labs) and blocked for 30 min using Invitrogen blocking reagent (0.1 g/10 mL PBS-Tween). Slides were then incubated with a primary antibody at room temperature for 1 h and washed 2 × 5 min with PBS-Tween. The primary antibodies used included AUA1 (anti-EPCAM) 1:100, CDX1 1:100, CK20 1:100, chromogranin A 1:500, Ki-67 1:100 and PR4D4 1:100. Slides were next incubated with a secondary antibody (either goat anti-mouse or goat anti-rabbit) conjugated to HRP at a dilution of 1:100 at room temperature for 1 h, followed by 2 × 5 min washes with PBS-Tween. The visual signal was amplified using tyramide 488 Alexa Fluor kit (Invitrogen) for 10 min in the dark at room temperature, followed by 2 × 5 min washes with PBS-Tween. Coverslips were mounted onto the tissue specimens using Vectorshield containing DAPI. Immunofluorescent slides were examined using a Zeiss Axioskop 2 Plus microscope and representative images were captured with Axioscope software. Some slides were examined with a Zeiss LSM confocal microscope.

Confocal Immunofluorescence: in Situ Staining of Colonies Within Matrigel. Cells were plated in 50 μL of Matrigel in 8-well chamber slides (Nunc) and overlaid with complete DMEM. Colonies were observed for up to 14 days, when they were fixed in situ for 5 min each with –20 °C 100% methanol and 2% formaldehyde in PBS. The chambers were then gently washed 2 × 5 min with PBS and then 1 × 5 min with PBS-Tween. The slides were blocked for 30 min using Invitrogen blocking reagent (0.1 g/10 mL PBS-Tween) and incubated with primary antibody at 4 °C overnight and then gently washed 3 × 5 min with PBS-Tween. The slides were incubated with secondary antibody conjugated to HRP overnight at 4 °C, followed by 3 × 5 min washes with PBS-Tween. The visual signal was amplified by incubation with tyramide 488 Alexa Fluor kit (Invitrogen) for 10 min in the dark at room temperature, followed by 3 × 5 min washes with PBS-Tween. Nuclei were stained with DAPI and serial images were obtained using a Zeiss LSM confocal microscope.

Multiphoton Microscopy. Multiphoton microscopy achieves optical sectioning of tissue by restricting fluorophore excitation and emission to the focal plane of interest. It prevents leakage of fluorescence from planes not in focus and obviates the need for pinholes, unlike confocal microscopy. By reducing the degree of

Yeung et al. www.pnas.org/cgi/content/short/0915135107

1 of 5
photodamage and cytotoxicity, multiphoton microscopy allows the visualization of live cells in situ (4).

Individual cells were seeded at a concentration of $1 \times 10^4$ cells per well in 24-well plates in Matrigel. Complete DMEM was changed twice weekly. After cells had grown for 7 days, acridine orange was added at a concentration of $2 \mu$/mL for 30 min, and serial images were acquired immediately afterward using a multiphoton microscope, courtesy of Z. F. Cui, Department of Engineering Science, University of Oxford, United Kingdom.

**Live and Dead Cell Staining in Matrigel.** Cells were grown in a 1:1 mixture of Matrigel and DMEM supplemented with FCS at a density of 500 cells per 50 $\mu$/mL in each chamber of the Nunc 8-well slides. Colonies were grown for up to 4 weeks. Before analysis, complete DMEM was removed, and the wells were rinsed with 400 $\mu$/L PBS. Next, 250 $\mu$/L of fresh PBS was placed in each well. A PI stock solution was prepared to 0.02 mg/mL (BD Pharmingen). A fluorescein diacetate (FDA, Fluka) stock solution was prepared (5 mg/mL in acetone). A working solution of FDA was made by adding 40 $\mu$/L of stock FDA to 10 mL PBS. Next, 750 $\mu$/L of working FDA were added to 225 $\mu$/L stock PI. Forty microliters of this mixture were added to each Nunc well. After incubation for 3 min, this was removed and the chambers were kept on ice. FDA and PI fluorescence were visualized using the green and red channels on a Zeiss Axioscope 2 Plus microscope.

**In Vivo Tumorigenic Assays.** Single cells were resuspended in a 1:1 mixture of Matrigel and serum-free medium (DMEM with 1% penicillin/streptomycin). A 100-$\mu$/L suspension containing between 200 and 1,000 cells was 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. 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. Mice from different experimental subgroups were mixed to avoid bias from cage effects and were housed in a specific pathogen-free environment. Food and water were given ad libitum. Hair around the injection site was removed regularly using razor clippers. The mice were observed daily for tumor growth and general health. Once a tumor was palpable, the size was measured externally in three dimensions using calipers, and the tumor volume calculated using the formula $V = \left(\frac{\pi}{6}\right) \times (a \times b \times c)$ where $a$, $b$, and $c$ represent the length, width, and depth of the tumor. When the tumors reached 10 mm in any one dimension, or if there was any sign of skin ulceration or animal suffering, the mice were killed by cervical dislocation according to protocol. Tumors were excised and fixed in 2% formaldehyde in PBS for 5 min and embedded in paraffin for histological analysis. Some xenograft specimens were also finely minced, washed four times with tumor wash (DMEM with penicillin 200 IU/mL, streptomycin 200 $\mu$/g/mL, nystatin 200 IU/mL, and gentamycin 100 $\mu$/g/mL), digested with collagenase (Worthington Biochemical Corporation, 300 units/mL) at room temperature for 1 h and passed through a 20-$\mu$/m filter to obtain a cellular suspension for further experiments or recultured in medium for cell line expansion.


**Fig. S1.** Visualization of live SW1222 megacolonies in Matrigel using a multiphoton microscope, staining with acridine orange (DNA, green; acidic and secretory compartments, orange (1, 2). Megacolonies are complex 3D structures containing multiple lumens and secretory vesicles. Outlines of goblet cells are highlighted (arrow). See also Movie S1 and Movie S2 for stacked images.

Fig. S2. Most of the cells within SW1222 megacolonies grown in Matrigel after (A) 2 weeks and (B) 4 weeks are still viable, with live cells staining green (fluorescein diacetate) and dead cells red (propidium iodide).

Fig. S3. Xenografts derived from megacolony cells are able to recapitulate a well-differentiated adenocarcinoma (H&E stain, magnification, 20x).

Fig. S4. Successive subcloning of megacolonies using CD44$^+$CD24$^+$ selection can give rise to further megacolonies for at least two generations. Megacolonies derived from CD44$^+$CD24$^+$ cells are able to reform all four CD44/CD24 subpopulations.
Fig. S5. Xenografts derived from CD44^+CD24^+ SW1222 cells are able to reform all four CD44^+ CD24^+ subpopulations, similar to xenografts derived from unsorted SW1222 cells.

Fig. S6. PR4D4 staining (green) in normal colonic tissue. Magnification, 20x. Nuclei stained blue with DAPI. PR4D4 is an in-house monoclonal antibody that is specific for mucin produced by goblet cells [Richman PI, Bodmer WF (1988) Control of differentiation in human colorectal carcinoma cell lines: epithelial-mesenchymal interactions. J Pathol 156:197–211.].
Movie S1. Stacked images of live SW1222 megacolonies grown in Matrigel stained with Acridine Orange (DNA, green; acidic and secretory compartments, orange.) Megacolonies are complex 3D structures containing multiple lumens and secretory vesicles, resembling the normal colonic epithelium.

Movie S2. Stacked images of live SW1222 megacolonies grown in Matrigel stained with Acridine Orange (DNA, green; acidic and secretory compartments, orange.) Megacolonies are complex 3D structures containing multiple lumens and secretory vesicles, resembling the normal colonic epithelium.