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LETTERS

A human colon cancer cell capable of initiating tumour growth in immunodeficient mice

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Colon cancer is one of the best-understood neoplasms from a genetic perspective[1–3], yet it remains the second most common cause of cancer-related death, indicating that some of its cancer cells are not eradicated by current therapies[4,5]. What has yet to be established is whether every colon cancer cell possesses the potential to initiate and sustain tumour growth, or whether the tumour is hierarchically organized so that only a subset of cells—cancer stem cells—possess such potential[6,7]. Here we use renal capsule transplantation in immunodeficient NOD/SCID mice to identify a human colon cancer-initiating cell (CC-IC). Purification experiments established that all CC-ICs were $CD133^+$; the $CD133^-$ cells that comprised the majority of the tumour were unable to initiate tumour growth. We calculated by limiting dilution analysis that there was one CC-IC in 5.7×10^4 unfractionated tumour cells, whereas there was one CC-IC in 262 $CD133^+$ cells, representing >200-fold enrichment. CC-ICs within the $CD133^+$ population were able to maintain themselves as well as differentiate and re-establish tumour heterogeneity upon serial transplantation. The identification of colon cancer stem cells that are distinct from the bulk tumour cells provides strong support for the hierarchical organization of human colon cancer, and their existence suggests that for therapeutic strategies to be effective, they must target the cancer stem cells.

Human tumour biology has long been studied in experimental xenogeneic colon cancer models, typically generated by injecting cell lines or implanting pieces of primary tumours into immunodeficient mice[8–10]. However, cell lines do not recapitulate all aspects of primary tumours and a quantitative assay for single cells is required to determine whether CC-ICs exist in colon cancer. Therefore, we developed a reliable xenograft model through subrenal capsule implantation of human colon cancer cell suspensions into pre-irradiated non-obese diabetic (NOD)/severe-combined immunodeficient (SCID) mice. Tumour formation occurred in 17 out of 17 samples tested, comprising six primary colon cancers, ten liver metastases, and one retroperitoneal metastasis (Table 1 and Supplementary Fig. 1a and b). The histology and degree of differentiation of all xenografts resembled the original tumours from which they were derived (Fig. 1). The tumours were positive for cytokeratin-20 (CK-20) and negative for cytokeratin-7 (CK-7), a pattern seen almost exclusively in colonic adenocarcinoma[11]. Xenografts and parent tumours exhibited similar patterns of expression for multiple mucin antigens and for markers highly associated with colon cancers including carcinoembryonic antigen (CEA)[12] and p53 (ref. 13). The degree of tumour cell proliferation, as revealed by MIB-1 staining[12], was similar in xenografts and parent tumours (Fig. 1). Thus, the xenografts generated in this model matched the phenotypes of the original tumours.

To determine whether this xenotransplant system was quantitative and able to detect single CC-ICs, we performed limiting dilution experiments. Groups of NOD/SCID mice were transplanted with replicate doses of human colon cancer cells over a range from doses unable to initiate tumour growth to doses that always initiated tumour formation (Table 2). The tumour-forming capacity and phenotypic appearance were the same for primary xenografts and tumours passaged into secondary and tertiary recipients. The similar

Table 1 | Patient and tumour characteristics

Patient number	Age/sex	Tumour site	Tumour stage	Tumour differentiation	Xenograft formation	$CD133^+$ in tumour (%)	$CD133^+$ in normal (%)
P1	73/M	Right colon	IIIB	Moderate	Yes	14.0	2.1
P2	80/F	Liver	IV	Moderate	Yes	5.2	
P3	73/M	Liver	IV	Poor	Yes	14.7	
P4	70/F	Liver	IV	Moderate	Yes	1.8	
P5	74/F	Paracolic	IV	Poor	Yes	24.5	
P6	64/M	Liver	IV	Moderate	Yes	6.3	
P7	75/F	Right colon	I	Well	Yes	9.3	1.2
P8	34/F	Right colon	IIIC	Well	Yes	7.5	0.4
P9	63/F	Liver	IV	Well to moderate	Yes	12.1	
P10	66/F	Right colon	IIIC	Well to moderate	Yes	8.9	1.3
P11	74/F	Liver	IV	Moderate	Yes	19.0	
P12	65/F	Right colon	IIIC	Poor	Yes	15.9	0.85
P13	84/F	Sigmoid	I	Moderate	Yes	12.0	1.9
P14	58/M	Liver	IV	Moderate	Yes	17.6	
P15	53/M	Liver	IV	Moderate	Yes	18.2	
P16	75/F	Liver	IV	Not stated	Yes	10.4	
P17	56/M	Liver	IV	Moderate	Yes	3.2	

NOD/SCID mice were injected with bulk colon cancer cells from each tumour. All seventeen tumours generated xenografts in NOD/SCID mice. Ten of the tumours were carried through to tertiary passages in mice (tumours 7–15, and 17). Of the remaining tumours all except three (tumours 1, 2 and 16) were carried through to secondary mice. CD133 expression was determined by flow cytometry for each tumour before implantation. For the six primary colonic tumours, CD133 expression was also determined for normal colonic tissue.

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behaviour of the primary and passaged tumours made it possible to combine data to calculate the average frequency of CC-ICs in these tumours using the maximum-likelihood estimation method of limiting dilution assay[14,15]. We calculated that on average there was one CC-IC per 5.7×10^4 (95% confidence interval: one per 3.4×10^4 to one per 9.3×10^4) unfractionated colon cancer cells, although the limited number of recipients used for any single sample prevented precise estimation of the patient-to-patient variation. Thus, as has been shown for breast[16] and brain[17] cancer, only a small subset of colon cancer cells are able to initiate tumour growth.

By combining the quantitative assay with cell fractionation, we were able to test whether human colon cancer adheres to the stochastic model, in which every tumour cell has equal tumour initiation potential[6,7,18], or to the cancer stem cell (CSC) model, in which some cell fractions are enriched for CC-IC activity while others are completely devoid of CC-ICs[6,7,18] .We focused on fractionation based on CD133 expression. The phylogenetically conserved protein CD133 was recently identified[19,20] as a potential CSC marker in brain[17] and prostate[19] cancer. CD133 expression ranged from 1.8 to 24.5% in the colon cancer samples described in Table 1 (Fig. 2a). We used immunohistochemistry to show that CD133 was expressed in clusters amid negative cells (Fig. 2b). Normal colon tissue also expressed CD133 but at much lower levels than primary colonic tumours (0.4–2.1% normal versus 8.9–15.9%) (Table 1; Fig. 2a). To determine whether CD133 expression enriches for CC-ICs, colon cancer cells were separated into $CD133^-$ and $CD133^+$ fractions and injected into NOD/SCID mice. Of 47 mice (dose range: 2×10^3 to 2.5×10^5) injected with $CD133^-$ cells, only one mouse transplanted with the highest cell dose (Table 2) generated a tumour. Because the $CD133^-$ fraction was contaminated with 5–15% of cells expressing only low levels of CD133, we conclude that neither $CD133^-$ nor $CD133^{low}$ cells possess CC-IC activity. In contrast, tumours were consistently generated after injection of 1×10^3 colon cancer cells expressing the highest levels of CD133 ($CD133^+$), and injection of 100 $CD133^+$ cells resulted in tumour growth in one of four mice. Thus, while significantly enriched, not every $CD133^+$ cell represents

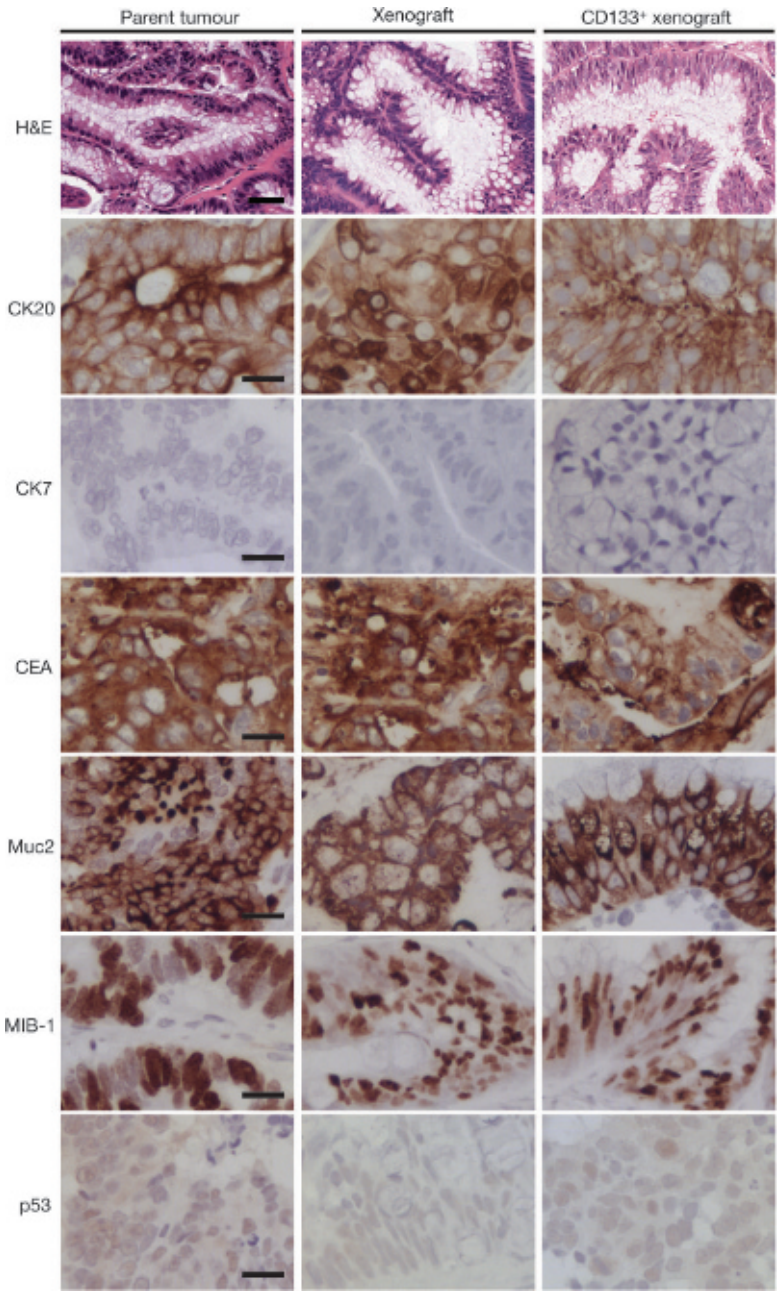

Figure 1 | Xenografts generated from both bulk and $CD133^+$ colon cancer cells resemble the original patient tumour. The parent tumour (tumour 14) is compared with xenografts generated from both primary and secondary passages of the tumour. The initial passage represents a xenograft generated from the injection of 1×10^5 bulk human colon cancer cells. The secondary xenograft was generated from the injection of 500 $CD133^+$ colon cancer cells. The histology of the three tumours, as expressed by H&E (haematoxylin and eosin) staining, shows well to moderately differentiated mucinous adenocarcinomas with intestinal differentiation including numerous goblet cells and intraluminal mucin. The immunohistochemical markers (including CK-20, CK7, CEA, Muc2, MIB-1 and p53) reveal comparable staining patterns in both the bulk and $CD133^+$ xenografts, as compared to the parent tumour. Images for each stain are taken at the same magnification. Scale bar represents 50 μm for H&E and 20 μm for all other stains.

a CC-IC. In total, 45 of 49 mice injected with $CD133^+$ cells developed tumours (Table 2). All tumours generated from $CD133^+$ cells were phenotypically similar to the original tumours (Fig. 1). Moreover, CD133 expression ranged from 1.7% to 22.4% in the xenografts, similar to the range seen in the original tumours. The isolation of tumorigenic and non-tumorigenic fractions, based on CD133 expression, provides strong support for the cellular organization of human colon cancer according to the CSC model.

Another prediction of the CSC model is that CC-ICs should self-renew to generate new CSCs and differentiate to generate non-tumorigenic progeny. Serial transplantation experiments from ten primary xenografts demonstrated that only $CD133^+$ and not $CD133^-$ cells were able to initiate tumour growth in serially transplanted secondary and tertiary mice. Tumours, either primary or passaged, could have been infiltrated with non-malignant cell types, but the high proportion (>98%) of $CD133^-$ cells that co-expressed the human-specific protein epithelial specific antigen from both primary and passaged tumours confirmed they were human colon cancer cells and not infiltrating murine cells or non-epithelial human cells that had somehow been co-passaged (Supplementary Fig. 2). Additionally, we showed that $CD133^-$ cells remained viable and stained positive for epithelial specific antigen under the renal capsule but were unable to regenerate tumours for as long as 15 to 21 weeks post-injection (Supplementary Fig. 3a, b and c). Furthermore, cells positive for epithelial specific antigen were also malignant, staining positive for p53, in cases where the parent tumours were $p53^+$ (Supplementary Fig. 3d). These studies were performed on passaged tumours, so it is highly unlikely the $CD133^-$ cells are pre-malignant cells, rather than malignant cells. Therefore, the $CD133^-$ cells are generated from the $CD133^+$ cells. Thus we can conclude that only $CD133^+$ CC-ICs can be serially passaged, forming xenografts that re-establish tumour heterogeneity, generating both $CD133^+$ and $CD133^-$ progeny in a ratio similar to that in the patient tumour (Fig. 2c).

To determine the frequency of CC-ICs within the $CD133^+$ subset we carried out a limiting dilution assay, using the same principles as described for unfractionated tumour cells[14,15]. The passaged xenografts matched the phenotype and tumour-forming capacity of the parent tumours, enabling us to combine data from passaged and primary cells. The frequency was calculated to average one CC-IC in 262 $CD133^+$ colon cancer cells (95% confidence interval: one in 129 to one in 534), representing a 216-fold enrichment of CC-ICs compared to unfractionated colon cancer cells.

Interestingly, the estimate of CC-IC frequency when back-calculated to take into account the proportion of $CD133^+$ cells within the unfractionated tumour is ~20-fold higher than when unfractionated cell suspensions were assayed. For example, multiplication of the CC-IC frequency (one in 262) by the mean level of CD133 expression for all samples (12%) yields an estimate of 20 CC-ICs per 57,000 unfractionated tumour cells, instead of the one in 57,000 measured in the initial limiting dilution assay. One possible explanation for this finding is that the $CD133^-$ progeny are negatively regulating the growth of the $CD133^+$ CC-IC fraction, thereby requiring a greater overall number of $CD133^+$ cells to give rise to a tumour, as has been observed in human haematopoietic stem cells[21].

Here we have identified and characterized CC-ICs from human colon tumour samples on the basis of their ability to initiate human colon cancer after transplantation into NOD/SCID mice. CC-ICs possessed two key criteria that define stem cells: after transplantation at limit dilution, single CC-ICs proliferated extensively and differentiated to produce tumours that were phenotypically similar to the original patient tumours, and as a population they self-renewed, enabling re-establishment of colon cancer in secondary and tertiary recipient mice. CC-ICs were almost exclusively $CD133^+$, while the $CD133^-$ fraction that comprised 81–98% of the tumour mass had no CC-IC activity. Thus colon cancer, like acute myelogenous leukaemia[22], breast[16] and brain[17] cancer is organized as a hierarchy in which a small population of CSCs sustain the tumour. The calculated frequency of CC-ICs was, on average, one in 262 $CD133^+$ cells, so clearly the majority of $CD133^+$ cells are not CC-ICs. As described for CD34 expression on acute myelogenous leukaemic stem cells, this result suggests there may be a hierarchy of CC-ICs and progenitors[23]. Thus, future studies using additional cell surface markers in combination with CD133 are necessary to purify the CC-IC fraction further. Finally, clonal tracking studies need to be carried out to establish self-renewal at the single cell level and determine whether different subclasses of CC-ICs exist[23].

Although we found $CD133^+$ CC-ICs in primary and metastatic tumours, most primary colon cancers tested were derived from right-sided tumours and may not be representative of all forms of colon cancer. Nevertheless, our findings should stimulate future studies directed towards increasing the range of colon cancer samples tested and addressing whether qualitative or quantitative CC-IC differences have prognostic value. Analysis of CC-ICs using molecular genetic techniques should further our understanding of the genetic abnormalities commonly associated with colon cancer, such as microsatellite status.

Table 2 | Limiting dilution analysis of the human colon cancer initiating cell

Colon cancer cell source	Cell dose	Number of samples tested	Identification numbers of samples tested	(Number of primary mice with tumours)/ (total number injected)	(Number of secondary mice with tumours)/ (total number injected)	Total number of mice with tumours (%)
Bulk	1×10^4	8	3–5,10–14	0/4	0/4	0/8 (0)
	2.5×10^4	8	4,6,10–14,17	1/6	0/2	1/8 (12.5)
	5×10^4	10	3–11,15	2/5	2/5	4/10 (40)
	7.5×10^4	8	3,5,7–9,11–13	4/8		4/8 (50)
	1×10^5	10	3–5,7–13	6/6	4/4	10/10 (100)
	1×10^6	17	1–17	17/17		17/17 (100)
	2×10^6	8	6–9,12–17	8/8		8/8 (100)
$CD133^+$	1×10^2	4	7,8,14,15		1/4	1/4 (25)
	5×10^2	6	5,6,11,13,14,17	1/1	4/5	5/6 (83.33)
	1×10^3	7	5–8,10,12,17	1/1	6/6	7/7 (100)
	5×10^3	8	8–13,15,17	1/1	7/7	8/8 (100)
	1×10^4	10	5,7–14,17	1/1	9/9	10/10 (100)
	2×10^4	9	5–7,9–11,13–15		9/9	9/9 (100)
$CD133^-$	5×10^3	5	6,9,11,12,17		0/5	0/5 (0)
	1×10^4	6	8,10,12,14,15,17	0/1	0/5	0/6 (0)
	2×10^4	6	5–7,9,10,13	0/1	0/6	0/6 (0)
	5×10^4	8	5,7–9,11,12,14,15	0/1	0/7	0/8 (0)
	1×10^5	8	6,8,10,12–15,17	0/1	0/7	0/8 (0)
	2.5×10^5	9	5,7–9,11,13–15,17		1/9	1/9 (11.1)

NOD/SCID mice were transplanted with: bulk ($n = 61$), $CD133^+$ ($n = 49$), and $CD133^-$ ($n = 47$) human colon cancer cells. All doses are displayed with the exception of 2×10^3 for $CD133^+$ ($n = 5$) and $CD133^-$ ($n = 5$), in which tumour-formation rates were 100% and 0%, respectively. Mice were killed at 6–21 weeks post-injection. Mice were considered negative if no tumour tissue was identified. Only doses that resulted in a mix of positive and negative mice were used to calculate the limiting dilution experiments.

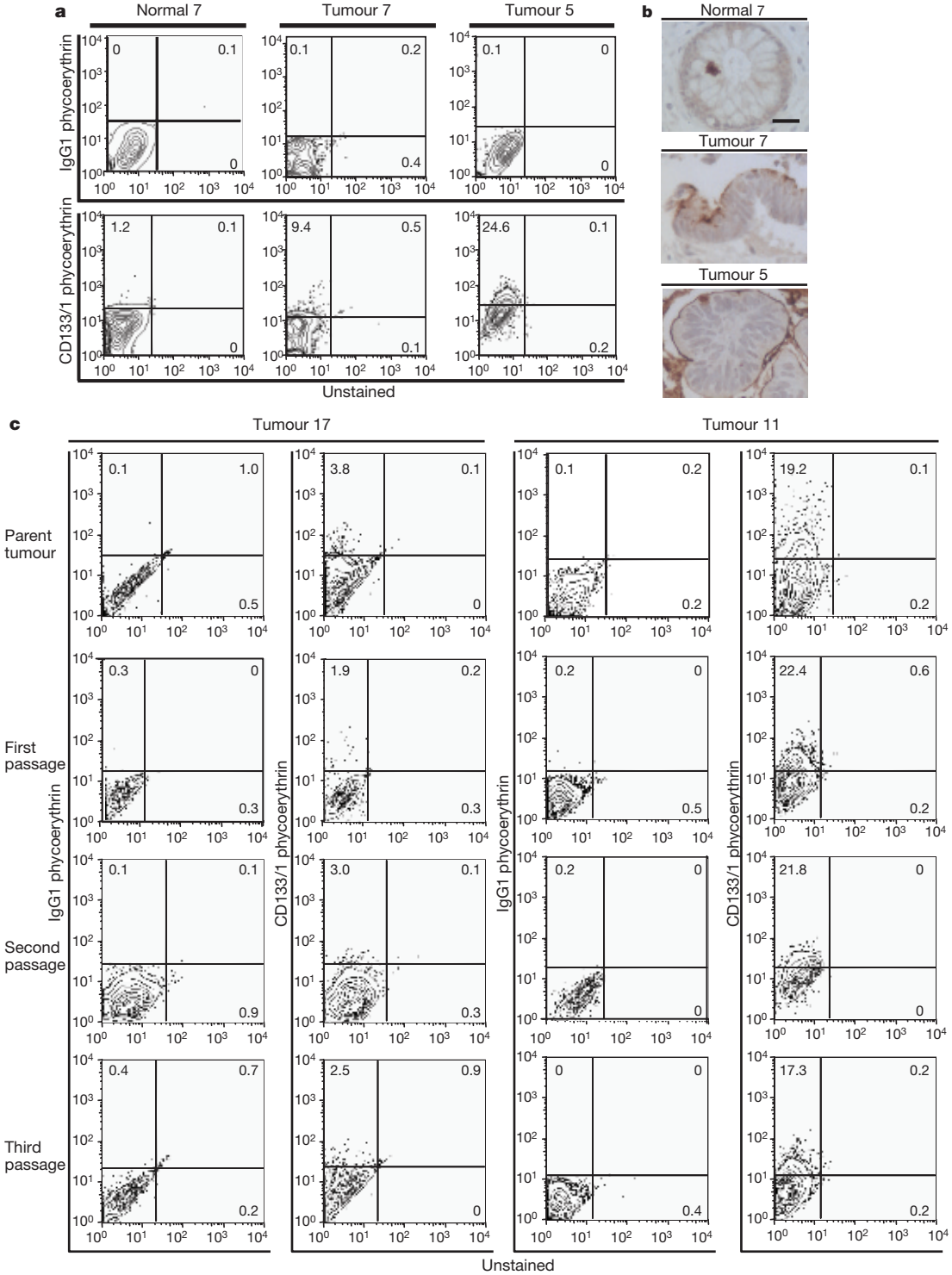

Figure 2 | Expression of CD133 in tumour and normal colonic tissue. a, Flow cytometric contour plots demonstrating the variable expression of CD133 between normal colon tissue (normal 7) and colon cancer tissue from the same patient (tumour 7) and a representative third tumour (tumour 5) showing higher CD133 expression. The upper and lower panels depict isotype controls and CD133 staining, respectively. **b**, Immunohistochemical staining for CD133: normal 7, and tumours 7 and 5 (all images are taken at the same magnification; scale bar represents 20 μm). **c**, Flow cytometric contour plots demonstrate preservation of CD133 expression through primary, secondary and tertiary passages as exemplified by tumours 17 and 11. Tumours from each passage were stained with CD133 phycoerythrin and an isotype-specific antibody (IgG1 phycoerythrin) and the proportion of $CD133^+$ cells is shown in each quadrant. CD133 expression varied between tumours 11 and 17. Each tumour maintained consistent levels of CD133 expression through three separate tumour passages. The first passage represented injection of bulk colon cancer cells but subsequent passages involved the isolation and injection of $CD133^+$ colon cancer cells.

Furthermore, as our understanding of normal colon stem and progenitor cell biology improves, it should be possible to gain insight into the cells that are the origin of colon cancer and the cellular context within which the well-characterized sequence of genetic events occurs[24,25].

The existence of tumorigenic and non-tumorigenic cells within colon cancers implies that not all the cells within a tumour are able to initiate and sustain neoplastic growth. This concept has important therapeutic implications, and may explain the observation that small numbers of disseminated cancer cells can be detected in the circulation of patients that never develop metastatic disease[18]. The identification of CC-ICs provides a powerful tool with which to develop a better understanding of tumour progression and the metastatic process, given that the CSC model predicts that the unit of selection in tumour progression would be the CSC itself. Moreover, because CC-ICs are the driving force sustaining tumour growth, developing adjuvant therapies directed at specifically eliminating the CC-IC fraction may prove to be a more effective strategy for reducing both local and distant recurrence[6,26]. The model described here will provide the means of further purifying and functionally characterizing the biological properties of the CC-IC fraction, with the goal of developing new therapeutic strategies directed specifically against CC-ICs.

METHODS

More detailed methods are in the Supplementary Information.

Tumour cell preparation. Colon cancer specimens were obtained from consenting patients, as approved by the Research Ethics Board at The University Health Network in Toronto. Tumour tissue was mechanically dissociated and incubated with Collagenase Type IV (Sigma) followed by magnetic bead separation to remove dead cells (Miltenyi Biotec).

Magnetic cell sorting and flow cytometry. Human colon cancer cells were magnetically labelled and separated by double passage using a CD133 Cell Isolation Kit (Miltenyi Biotec). Before separation, samples were assessed using a FACSCalibur flow cytometer (BD Biosciences), mouse IgG1s conjugated to phycoerythrin or fluorescein isothyiocyanate were used as isotype controls (BD Biosciences). CD133 expression was assessed using anti-CD133/1 phycoerythrin (Miltenyi-Biotec). To confirm the cells as human colon cancer, samples were tested using anti-epithelial specific antigen fluorescein isothyiocyanate (Biomeda) (Supplementary Fig. 2). At least 10,000 events were acquired for each sample and all cells positive for propidium iodide were gated out. After magnetic bead separation, samples were assessed by flow cytometry for purity.

Transplantation of human colon cancer cells into NOD/SCID mice. NOD/LtSz-scid/scid (NOD/SCID) mice were bred and maintained under defined conditions at the Ontario Cancer Institute under conditions approved by the Animal Care Committee of the Ontario Cancer Institute. Colon cancer cells were suspended in a 1:1 mixture of media and matrigel (BD Biosciences) and injected under the renal capsule of mice (8 weeks of age) that were sublethally irradiated (350 centiGray). Mice were anaesthetized while cells were injected under the renal capsule. All mice were killed when the tumour measured 1 cm, at the first sign of suffering, or between 15 and 21 weeks post-transplantation.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Contributions C.A.O'B. planned the project, carried out experimental work, analysed data and prepared the manuscript. A.P. provided pathology analysis. S.G. provided clinical information and human tissues. J.E.D. planned the project, analysed data, and prepared the manuscript.

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