

Establishment and Characterization of a Cell Line from Human Circulating Colon Cancer Cells

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Abstract

Circulating tumor cells (CTC) in blood are promising new biomarkers potentially useful for prognostic prediction and monitoring of therapies in patients with solid tumors including colon cancer. Moreover, CTC research opens a new avenue for understanding the biology of metastasis in patients with cancer. However, an in-depth investigation of CTCs is hampered by the very low number of these cells, especially in the blood of patients with colorectal cancer. Thus, the establishment of cell cultures and permanent cell lines from CTCs has become the most challenging task over the past year. Here, we describe, for the first time, the establishment of cell cultures and a permanent cell line from CTCs of one patient with colon cancer. The cell line designated CTC-MCC-41 has been cultured for more than one year, and the cells

have been characterized at the genome, transcriptome, proteome, and secretome levels. This thorough analysis showed that CTC-MCC-41 cells resemble characteristics of the original tumor cells in the patient with colon cancer and display a stable phenotype characterized by an intermediate epithelial/mesenchymal phenotype, stem cell-like properties, and an osteomimetic signature, indicating a bone marrow origin. Functional studies showed that CTC-MCC-41 cells induced rapidly *in vitro* endothelial cell tube formation and *in vivo* tumors after xenografting in immunodeficient mice. The establishment of this first colon cancer CTC line allows now a wealth of functional studies on the biology of CTCs as well as *in vitro* and *in vivo* drug testing. *Cancer Res*; 75(5); 1–10. ©2015 AACR.

Introduction

The use of circulating tumor cells (CTC) as real-time liquid biopsy has received major attraction over the past years (1, 2). Blood samples can be obtained at the time of diagnosis and during the course of a systemic therapy. Several large scale clinical trials and meta-analyses have shown that the number of CTCs is an important indicator of the risk of progression or death in patients with metastatic solid tumors (3–7). In addition, the

molecular characterization of single CTCs has revealed important information on the genotype and phenotype of these tumor cells and demonstrated a striking heterogeneity of CTCs (8). Thus, the current challenge is now to identify the functional properties of the different CTC subsets. Functional assays are mandatory to discover the biology of CTCs with particular emphasis on the discovery of the metastasis-initiator cells. At present, such assays are limited by the very low concentration and yield of CTCs.

Besides the development of improved CTC capture methods with higher yields, it will be important to expand the number of CTCs in cell culture systems for functional analyses. Short-term cultures of CTCs have become possible by the EPISPOT assay 10 years ago (9); this assay detects specific proteins secreted during the *in vitro* culture of CTCs (10), and clinical studies in breast and colon cancer indicate that the detection of viable CTCs is associated with an unfavorable outcome (11, 12). First *ex vivo* expansion of CTCs has been described in breast cancer by Zhang and colleagues (13) and more recently by Yu and colleagues (14).

Another approach to expand the number of CTCs is xenotransplantation of patient-derived CTCs into immunodeficient mice. The first report by Baccelli and colleagues (15) showed that grown metastases after xenotransplantation of breast cancer CTCs into the bone of immunodeficient mice had an EpCAM^{low}, C-met^{high}, CD47^{high} CD44^{high} phenotype, which may be characteristic of metastasis-initiator cells. The second report was published on patients with small-cell lung cancer, demonstrating that CTCs from patients with either chemosensitive or chemorefractory tumors are tumorigenic in immune-compromised mice, and the resultant CTC-derived explants mirrored the donor patient's response to platinum and etoposide chemotherapy (16). However, these *in vivo* assays require at present very high CTC

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concentrations in the transplanted blood sample (e.g., > 1,000 cells per 7.5 mL in breast cancer), which were so far only achieved in a few index patients.

To our best knowledge, there is no report on the *in vitro* or *in vivo* expansion of CTCs from patients with colon cancer. Here, we report on the first establishment of CTC cultures from colon cancer–derived CTCs and their in-depth characterization including xenografts into immunodeficient mice.

Materials and Methods

Patient samples and blood collection

After informed consent was given, peripheral blood from patients with a nonresectable metastatic colorectal adenocarcinoma was collected before the start of first line of chemotherapy combining FOLFIRI and bevacizumab under the COLOSPOT study (NCT01596790-Patients inclusion period: 2011–2014). Blood was collected in CellSave tubes (Janssen; 10 mL) for CTC detection (Supplementary Materials and Methods) as well as in EDTA tubes (10 mL) for *ex vivo* CTC culture (Supplementary Materials and Methods).

Cell cultures

Culture conditions of primary endothelial cells, mammary cancer cell lines MCF7, MDA-MB-231, and BCM1, and colorectal cancer cell line HT-29 are detailed in the Supplementary Materials and Methods.

Immunocytochemical analyses

Single CTCs and CTC spheres were taken under a microscope with a STRIPPER micropipetter (ORIGIO) and put on a blue alcyan coated slide. After fixation and permeabilization with PFA 3.7% + Triton 0.2% in PBS solution, cells and spheres were labeled with a large panel of antibodies (Supplementary Table S1) and analyzed under a light microscope.

Flow cytometry experiments

The same panel of proteins (Supplementary Table S1) was tested by flow cytometry to characterize the CTC cell line. A fixation/permeabilization kit (Beckman Coulter) was used for the detection of the expression of intracellular proteins, whereas extracellular proteins were directly revealed by adding antibodies without fixation. Labeled CTCs were analyzed and detected by using the Cyan cytometer (Beckman Coulter), and the data were analyzed with the Kaluza software (Beckman Coulter).

Histopathologic and immunohistochemical analyses of the original tumor cells of the patient with colon cancer and the xenografts

Paraffin-embedded tumor tissues: (i) primary tumor biopsy of the patient with colon cancer, (ii) lymph node biopsy of the patient with colon cancer, and (iii) subcutaneous CTC-MCC-41 xenografts in SCID mice were cut in 3- μ m sections and analyzed for CK20 expression (Supplementary Materials and Methods).

EPISPOT assays

The fluoro-EPISPOT assay was performed as previously described (17). For the new proteins analyzed, i.e., VEGF, EGFR, and osteoprotegerin, corresponding protocols are detailed in Supplementary Materials and Methods.

In vitro angiogenesis: endothelial cell tube formation

A 24-well plate coated with 1.3 mL Matrigel (BD Biosciences) per well was solidified at 37°C for 30 minutes. Endothelial cells (10^5 cells/well) were seeded into the plate and cultured for 6 hours. For tube formation experiments, cells were cultured under different media conditions (in duplicates): (1) RPMI 1640, a basal medium (negative control), (2) an endothelial complete medium with additional endothelial growth factors and heparin (positive control), and (3) RPMI 1640 basal medium used for a 48-hour CTC culture (cell culture supernatant).

Single-cell RT-PCR analysis of CTCs

AmpliSpeed technology (Beckman Coulter) was used for complementary DNA (cDNA) obtention by the reverse transcription at the single cell level (Supplementary Materials and Methods).

Next-generation sequencing for copy-number variations

Two spheres and one single cell were transferred each to an individual PCR tube (0.2 mL), after which whole genome amplification was performed using the PicoPlex WGA Kit according to manufacturer's protocol (Rubicon Genomics). Next, library preparation was performed using the TruSeq DNA preparation Kit according to manufacturer's protocol. Next-generation sequencing was performed by a HiSeq 2500 (Illumina) using single reads for 100 cycles. Analyses and identification of copy-number alterations were performed using Control-FREEC and a custom script in MatLab.

Subcutaneous tumorigenicity assay

To investigate the tumorigenicity of the CTC cell line, five SCID mice (Charles River; background BALB/c, aged 12 weeks) were injected subcutaneously with 10^6 CTC-MCC-41 cells per 200 μ L medium into their right shoulder. The mice were constantly monitored on tumor growth, and the animals were sacrificed if the total tumor burden reached 10% of the total body weight. From the sacrificed mice, tumors were collected for their histological analysis.

Analyses of KRAS and BRAF mutations

We compared mutations in the *KRAS* gene (codons 12 and 13) and *BRAF* gene in the primary tumor and the lymph node metastasis of the patient with colon cancer as well as the CTC-MCC-41 cell line and the xenografts (Supplementary Materials and Methods).

Results

Ex vivo culture of isolated CTC from patients with metastatic colon cancer

Peripheral blood samples from 71 patients were analyzed by the CellSearch system. Fifty of 71 (70.4%) patients were positive for CTCs with a CTC count of ≥ 1 using the CellSearch System (mean, 19.9; median, 4; range, 1–516) and 38 of 71 (53.5%) patients with a CTC count of ≥ 3 (mean, 36.6; median, 9; range, 3–516), which is the prognostic cutoff for patients with metastatic CRC (Table 1; ref. 4). Only 18 patients (25.4%) had a number of CTCs exceeding 10 CTCs and 3 patients (4.2%) had more than 100 CTCs in their blood samples.

Table 1. CTC detection and *ex vivo* culture

Patient ID	CTC detection by the CellSearch system		<i>Ex vivo</i> CTC culture
	N = 21	N = 50	
1	0		
2		6	
3	0		
4		2	
5 ^a		516	Yes
6		46	
7		2	
8	0		
9		11	
10		16	
11		2	
12		34	
13		11	
14		5	
15		2	
16		7	
17	0		
18		3	
19		52	
20		26	
21		15	
22	0		
23		11	
24		5	
25		11	
26	0		
27		5	
28		1	
29	0		
30		7	
31	0		
32		43	
33	0		
34	0		
35		1	
36	0		
37 ^a		302	Yes ^a
38		7	
39		1	
40		15	
41		1	
42		15	
43	0		
44		4	
45		4	
46		10	
47		129	
48		12	
49	0		
50		4	
51	0		
52		1	
53		22	
54	0		
55		4	
56	0		
57		2	
58		1	
59		8	
60	0		
61	0		
62	0		
63		5	
64	0		
65		4	

(Continued on the following column)

Table 1. CTC detection and *ex vivo* culture

Patient ID	CTC detection by the CellSearch system		<i>Ex vivo</i> CTC culture
	N = 21	N = 50	
66		4	
67	0		
68		2	
69		6	
70		4	
71		3	

^aFrom the marked patients, we were able to expand CTCs in cell culture.

Long-term CTC cultures could be established from two patients who had a CTC count of ≥ 300 . Concerning the first patient (no. 5), we could expand the CTCs in medium 1, cf. Materials and Methods) for more than 2 months, but after that period they gradually died off. Pathologists characterized the expanded cells as tumor cells, but we did not further analyze them. In contrast, from a second patient (no. 37), we were able to establish a permanent cell line. Indeed, viable CTCs were still observed after 4 days of cell culture in medium 1 under hypoxic conditions (cf. Materials and Methods) then, after 10 days, CTCs started to proliferate and formed spheres. Hypoxia is an important growth stimulus in particular for stem cells, and recent understanding of cancer stem cell biology and their similarities to somatic stem cells suggests that hypoxia acts to regulate the cancer stem cell phenotype (18). Another important culture condition issue was to switch them to medium 2 (cf. Materials and Methods) where CTC-sphere formation continued (Fig. 1A). Moreover, immunocytochemistry experiments have been performed with anti-EpCAM, anti-CK20, and anti-CD45 Abs, showing that these CTC spheres were of epithelial origin (Fig. 1B). Comparing cell culture growth in both media, we observed a significantly increased proliferation with a doubling time in 20 hours in the medium 2 as compared with a doubling time of 37 hours in the medium 1. Because of this rapid expansion, a change of the medium 2 was required every two days to obtain good viability of tumor cells. This procedure led to the establishment of the first permanent CTC colon cancer cell line that we decided to name "CTC-MCC-41," which is now growing in culture for >16 months with a maintained high doubling time of 20 hours with medium 2. The CTC-MCC-41 cell line can be frozen, banked, and thawed for successful regrowth.

Clinicopathologic characteristics of the patient and tumor giving rise to CTC-MCC-41

Standard histopathologic analysis of diagnostic biopsies performed on the primary tumor and one lymph node revealed a poorly differentiated adenocarcinoma with a lot of isolated single cells that lost their adhesive properties. The primary tumor was also characterized as *KRAS* wild-type (codons 12 and 13) and *BRAF* mutated (V600E mutation) according to the standard-of-care analyses.

Clinically, the patient presented at diagnosis a widespread disease with numerous abdominal and mediastinal metastatic lymph nodes as well as metastatic lesions in the liver. No bone metastasis was detected during the cancer follow-up, but no sensitive examination, such as a bone scan, was performed due to the absence of any clinical signs. The patient did not respond to any of both administered treatments (FOLFIRI+bevacizumab followed by FOLFOX+bevacizumab) and died about 6 months after the diagnosis due to a disease progression at the peritoneal (carcinosis) and pulmonary (lymphangitis) sites.

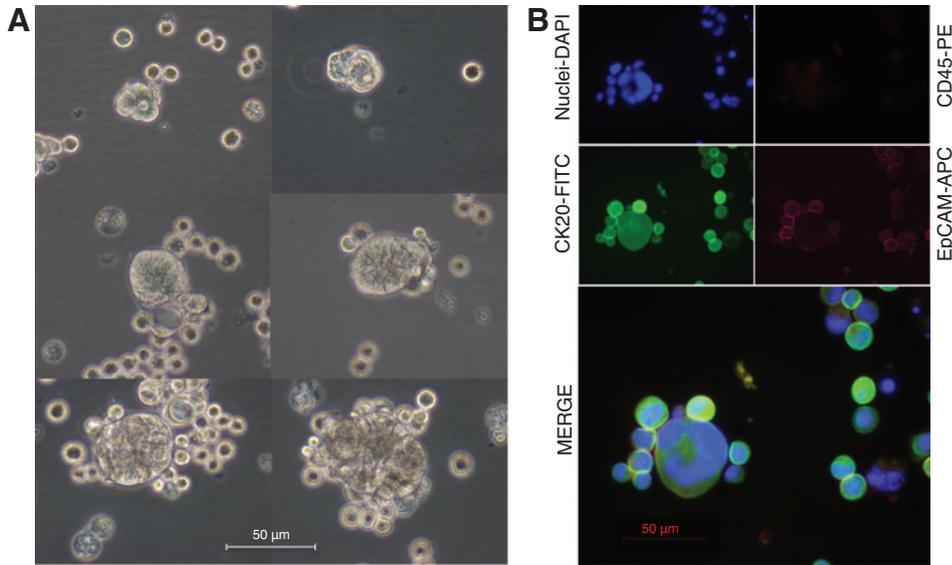


Figure 1. Sphere formation with *ex vivo* culture of colon CTCs. A, representative images of CTC spheres, with different sizes obtained at the same time in medium 2 (magnification, $\times 40$). B, immunocytochemical staining of the CTC spheres using anti-EpCAM-APC, anti-CK20-FITC, and anti-CD45-PE Abs.

Genome analysis of CTC-MCC-41 cells

Genomic analysis of the CTC-MCC-41 cell line using next-generation sequencing revealed a wide spectrum of chromosomal aberrations, as can be seen in Fig. 2. Typical colorectal cancer-related chromosomal gains and losses were found along the

whole genome in all investigated cells, such as gains of chromosome 7, 8q, and 20q, as well as loss of chromosome 8p.

Although the two CTC spheres and the one single CTC analyzed shared most of these aberrations, several distinctions could be made. Chromosome 2p is gained in both CTC sphere 1 and the

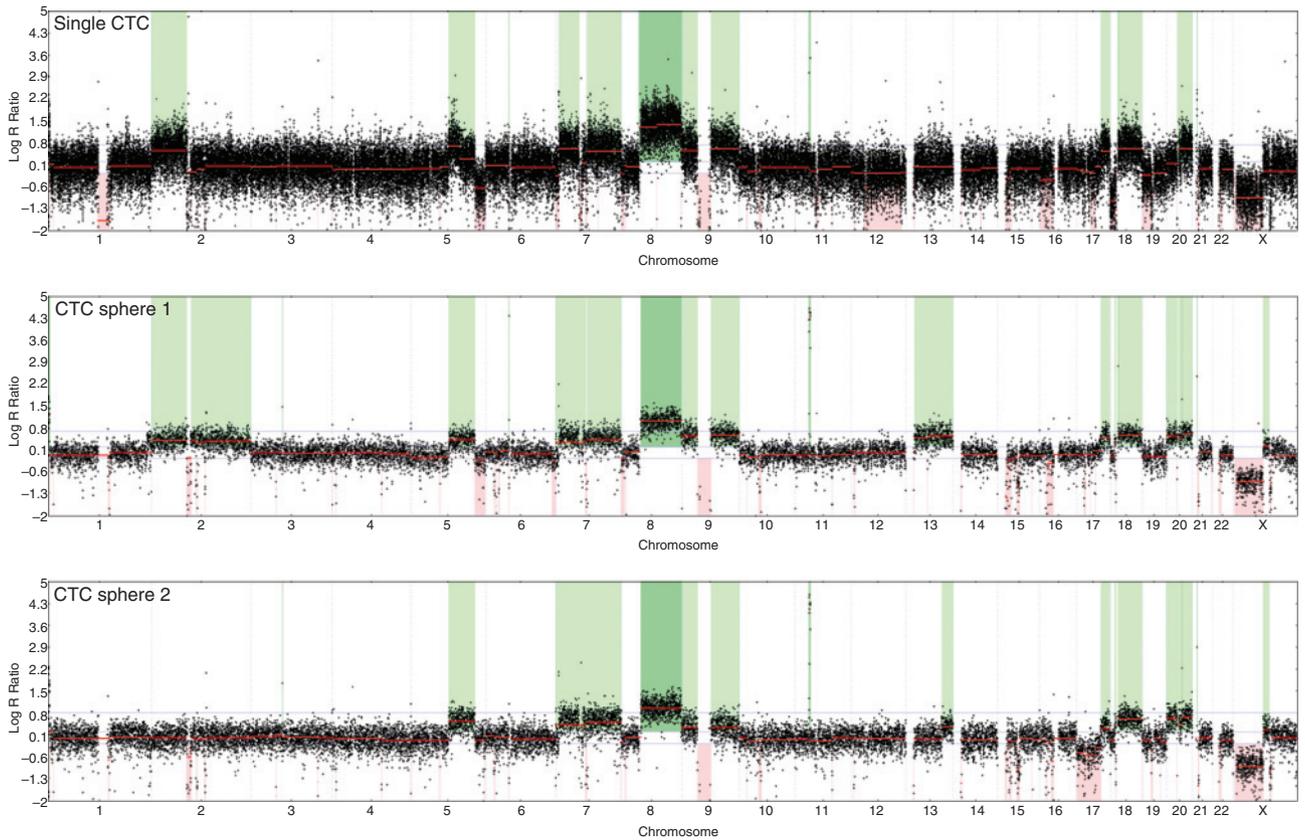


Figure 2. Copy-number variations in CTC-MCC-41 cells determined by next-generation sequencing. Chromosomal aberrations along the whole genome (x-axis) of a single CTC and two CTC spheres were investigated. Copy-number gains are shaded green, and copy-number losses are shaded red. Red horizontal lines represent the estimated copy-number level (y-axis) and mark the break points between the intrachromosomal copy-number alterations.

A

	EpCAM	CK-19	E-cadherin	Vimentin	Snail	Twist	ALDH-1	CD133	VEGF	cMet	OPG	CD45	β 2-globulin
CTC-MCC-41	23,3	24,2	26,7		27,6		22,4	24,8	28	25,2	27,3		21,2
HT-29	24,3	24	25		28,8		21,7	24,9	26,7	25,2			21,3
MCF-7	28,1	26,1	25,7							28,7			26
BC-M1				20,2	27,1	28			25,6	27	26,5		20,5
Leuco				23,3			28,6					24	20

Negative \geq 30 Ct
 Positive $<$ 30 Ct

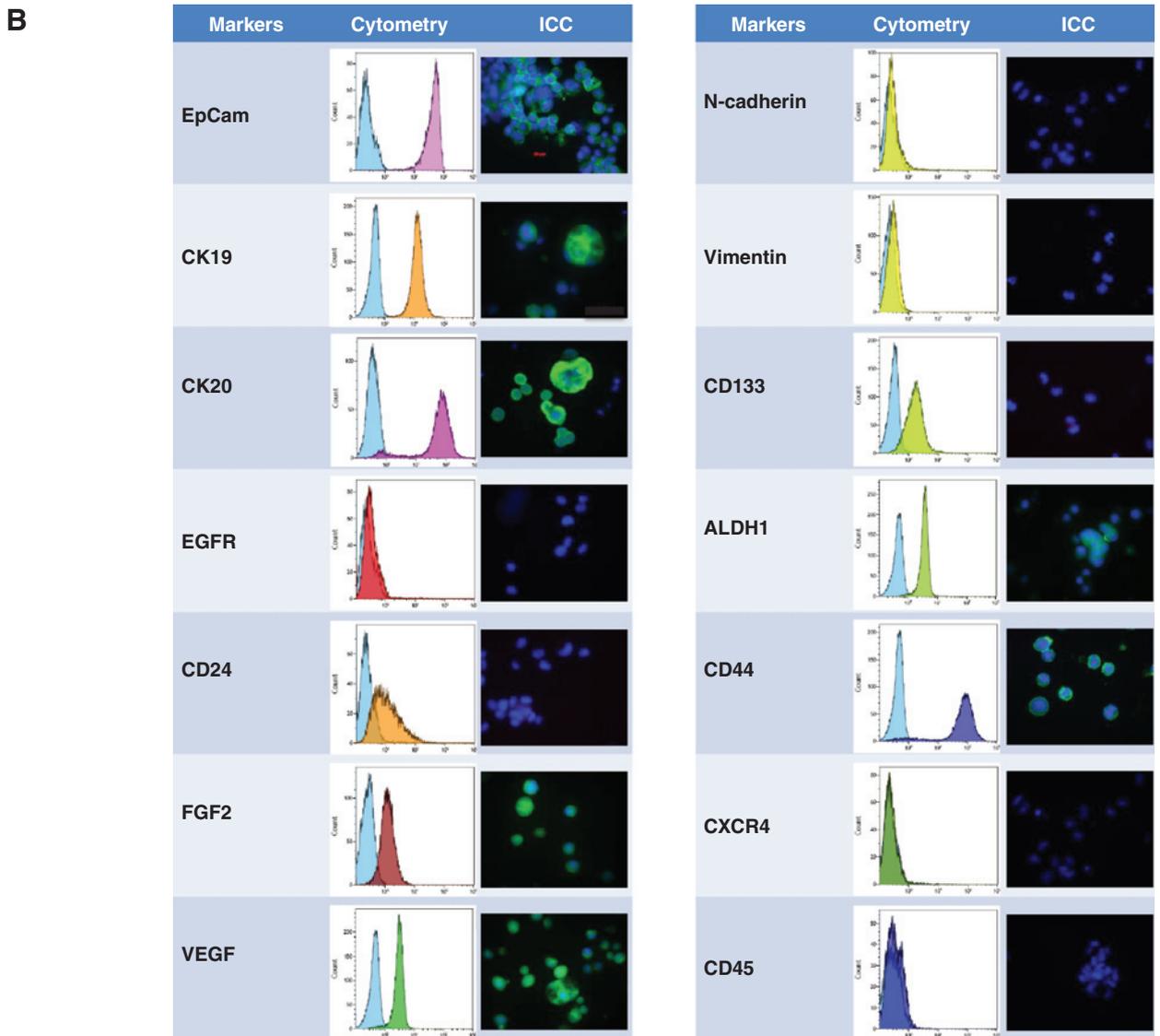


Figure 3. A, transcriptome of the CTC-MCC-41 cell line at the single-cell level. Thirteen different mRNAs were analyzed, covering different characteristics of tumor cells: (i) epithelial (green); (ii) mesenchymal (purple); (iii) stem cell (red); (iv) angiogenesis (blue); (v) proto-oncogene (orange), and (vi) osteomimeticism (brown). As controls, we used the β 2-globulin (housekeeping gene) and the CD45 (leukocyte marker). (Continued on the following page.)

single CTC, but chromosome 2q is only gained in CTC sphere 1, whereas no aberrations of chromosome 2 could be detected in CTC sphere 2. Similarly, chromosome 13q seems to be partly gained in CTC sphere 2, completely gained in CTC sphere 1, but not aberrated in the single CTC. Finally, the loss of chromosome 17p in CTC sphere 2 was not found in either CTC sphere 1 or the single CTC.

Transcriptome of single CTC-MCC-41 cells

To characterize the CTC-MCC-41 cell line at the gene expression level at different time points of cell culture, we performed single-cell transcriptome analyses exploring 13 different mRNAs. This panel has been selected to cover different known properties of tumor cells: epithelial (EpCAM, CK19, and E-Cadherin), mesenchymal (Vimentin, Snail, Twist), stem cells (ALDH-1 and CD133), and angiogenesis (VEGF) characteristics as well as the expression of the tyrosine kinase receptor c-Met. In addition, we included the detection of CD45 mRNA to exclude cells of hematopoietic origin and a housekeeping gene (beta-microglobulin) as the positive control of the experiment.

All results obtained with CTC-MCC-41 cells at 1, 6, and 13 months of culture were identical and are shown in Fig. 3A. Like MCF-7 breast cancer cells and HT-29 colon cancer cells used for comparison, CTC-MCC-41 cells expressed the epithelial markers analyzed. Although expression of the mesenchymal marker vimentin and Twist 1 was not detected, CTC-MCC-41 cells expressed the EMT inducers Snail, suggesting that CTC-MCC-41 cells have an intermediate E/M phenotype. In addition, CTC-MCC-41 cells are also able to express VEGF, a key player to induce angiogenesis, and c-Met, a proto-oncogene specific of tumor cells. Interestingly, CTC-MCC-41 cells expressed osteoprotegerin, a protein characteristic for the osteomimeticism in the bone marrow. This protein is also expressed by the BCM1 cancer cell line of bone origin used as a positive control. Finally, no CD45 expression was detected confirming the nonhematopoietic origin of CTC-MCC-41 cells.

Proteome analyses of the CTC-MCC-41 cells

To define a specific phenotype of the CTC-MCC-41 cell line, we performed flow cytometry and immunocytochemistry experiments at different times of cell cultures (at 1, 3, and 6 months). A large panel of membrane and intracytoplasmic proteins were screened and the main results are shown in Fig. 3B.

This CTC-MCC-41 cells expressed strongly epithelial markers like EpCAM, cytokeratins (CK19 and CK20), whereas no EGFR was observed. As CK20 protein expression in colon cancer has been reported for many years (19), we decided to add this protein marker to our immunocytochemical analyses. With regard to mesenchymal markers, the cell line showed no expression of vimentin and N-cadherin. In addition, stem cell markers were expressed as represented by the presence of FGF2 as well as

CD133, ALDH1, CD44^{high}/CD24^{low} in these tumor cells. Interestingly, CTC-MCC-41 cells expressed VEGF but not the chemokine receptor CXCR4. Finally, these cells were all negative for the expression of Her2-neu (EGFR2), CD105 (endoglin), CD146 (MUC18), CD309 (VEGFR2), and CD31 (Platelet endothelial cell adhesion molecule, PECAM-1; data not shown).

As expected from the RNA analysis, CTC-MCC-41 cells did not express the leukocyte marker CD45, and these cells were also negative for the early hematopoietic and vascular-associated tissue marker CD34, indicating that CTC-MCC-41 cells do not represent circulating hematopoietic progenitor cells or endothelial cells.

Secretome analysis of viable CTC-MCC-41 cells

To assess the cell capacity of the colon CTC line CTC-MCC-41 to secrete specific proteins, CTC spheres and single CTCs were analyzed by performing functional fluoro-EPISPOT assays. Results described in Fig. 4A and B show that viable tumor cells were able to release CK19 and osteoprotegerin as well as to secrete FGF2 and VEGF. Compared with the positive controls, CTC-MCC-41 cells released similar amounts of CK19, secreted lower amounts of FGF2, and higher amounts of VEGF and osteoprotegerin. Moreover, these tumor cells were unable to release EGFR (data not shown).

Interestingly, we found no differences in the secretion profile between CTC spheres and single CTCs (data not shown).

In vitro endothelial cell tube formation of CTC-MCC-41 cells

As the CTC-MCC-41 cell line expressed and secreted VEGF, it was interesting to perform *in vitro* endothelial cell tube formation. When endothelial cells were cultured for 6 hours with the basal medium, no tubes were observed, whereas when these cells were cultured with the complete medium containing the required growth factors (positive control), *in vitro* endothelial cell tube formation could be observed (Fig. 5A). When endothelial cells were then cultured with the basal medium used for a 48-hour CTC culture (CTC line culture supernatant), the formation of tube-like structures was observed and exceeded even the tube formation induced by the positive control medium. These findings showed the capacity of the CTC line to release factors that are able to induce *in vitro* angiogenesis (Fig. 5A).

Growth of CTC-MCC-41 cells as xenografts in immunodeficient mice

To evaluate the potential of the CTC-MCC-41 cells to grow *in vivo* and to generate colon tumors, these cells were injected subcutaneously into immunodeficient mice. After 40 and 42 days, respectively, two mice had to be sacrificed as the tumors had grown beyond 10% of their body weight (2.19 g and 2.04 g, respectively). The staining of the xenografts extracted from the mice with an anti-human keratin Ab showed that these xenografts contained human tumor cells with the typical morphology of an adenocarcinoma (Fig. 5B).

(Continued.) In parallel to the CTC-MCC-41 cell line, three different cancer cell lines were analyzed as positive controls: HT-29, colon cancer cells established from primary cancer; MCF-7, breast cancer cells established from pleural effusion; BCM1, breast cancer cells established from disseminated tumor cells in bone marrow. Leukocytes were used as negative control. Cycle threshold (Ct) values are mean \pm SD of three experiments performed on CTC cultures on months 1, 6, and 12 after initiation of culture. B, phenotypic characterization of the CTC-MCC-41 cell line by flow cytometry and immunocytochemistry (ICC; magnification, $\times 40$) experiments. All experiments were repeated five times, and the figure shows representative results. OPG, osteoprotegerin.

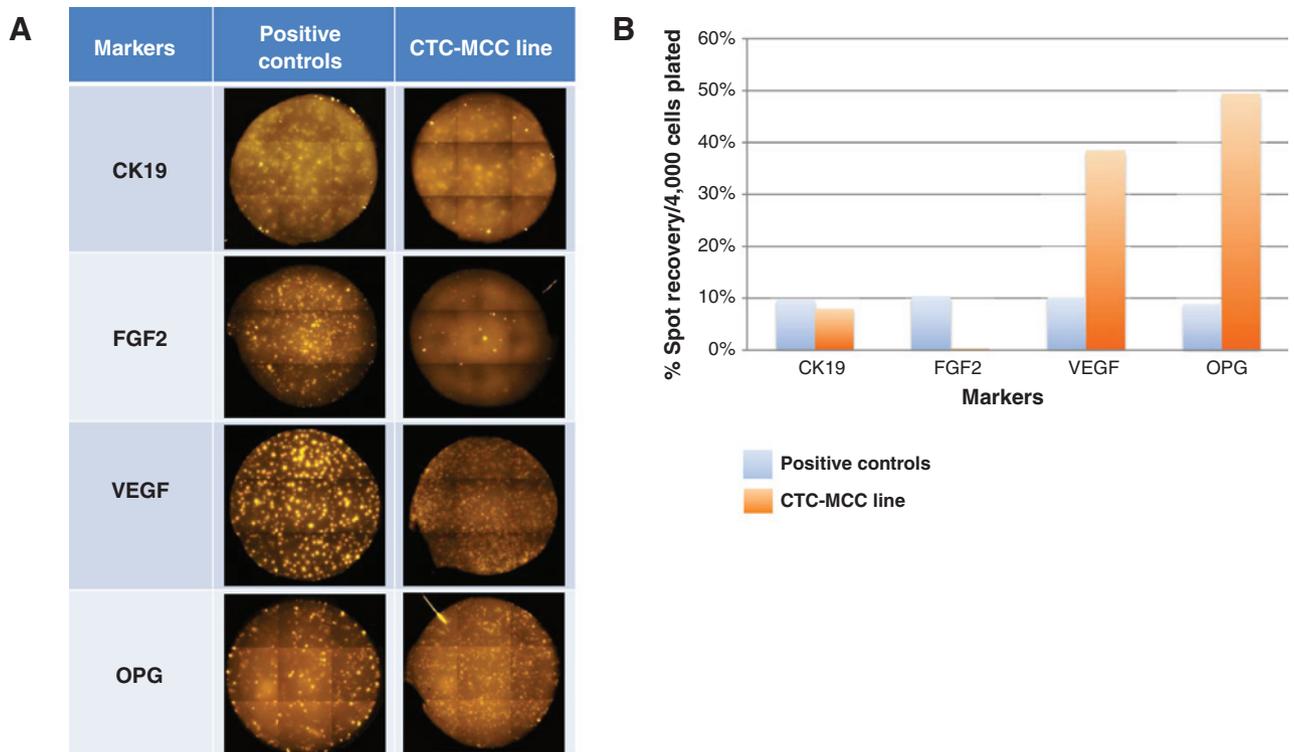


Figure 4. Functional characterization of the CTC-MCC-41 cell line by fluoro-EPISPOT assays. Secretion, release, and shedding of CK19, FGF2, VEGF, and osteoprotegerin (OPG) proteins by viable CTC-MCC-41 cells were investigated using the EPISPOT assay. The cancer cell lines used as positive controls were MCF-7 for CK19 and VEGF, NBTII for FGF2, and BCM1 for osteoprotegerin (magnification, $\times 5$). A, images of the EPISPOT membranes on which 4,000 cells were plated. B, quantitative analyses of the secreted markers in CTC-MCC-41 line compared with the respective positive controls.

Comparison of the CTC-MCC-41 cell line with tumor tissues of the patients with colon cancer and xenografts

We compared *KRAS* and *BRAF* mutations in the primary tumor and the lymph node metastasis of the patient with colon cancer as well as the CTC-MCC-41 cell line and the xenografts. We found the same mutational status in all of these tissues, i.e., *KRAS* wild-type status (codons 12 and 13) and *BRAF* mutation (V600E mutation, 1799T>A).

In addition, we performed comparative analyses of CK20 expression in the primary tumor and the lymph node metastasis of the patient with colon cancer as well as the CTC-MCC-41 cell line and the xenografts. CK20 expression was detected in all of these tissues (Fig. 6). The CK20 staining in the original primary tumor was focal (Fig. 6B) while the tumor cells of the lymph node biopsy (Fig. 6D) as well as the CTC-MCC-41 cells (Fig. 3B) and the xenografts of both mice (Fig. 6E and F) were homogeneously and more strongly stained.

Discussion

Subgroups of cancer cells can leave the primary tumor, travel as CTCs to a distant site in the body where they begin to colonize a new distant tissue, and form metastases (20). A subset of these CTCs might have a cancer stem cell phenotype, as recently indicated in xenograft models in breast and lung cancer (13–16).

This article provides, for the first time, the experimental proof that CTCs isolated from the blood of a patient with metastatic

colon cancer could give rise to an established stable colon CTC line. The present characterization at the genome, transcriptome, proteome, and secretome levels of this colon CTC line named "CTC-MCC-41" revealed a specific make-up with interesting potentials. Besides its capacity to expand *ex vivo* for more than 16 months, it showed (i) epithelial properties with stem cell-like characteristics, (ii) an intermediate epithelial/mesenchymal phenotype, (iii) an osteomimetic signature, (iv) *in vitro* induction of endothelial cell tube formation, and (v) *in vivo* tumor formation after xenografting into immunodeficient mice.

We applied a negative selection for CTC enrichment on blood samples from 71 patients with metastatic colon cancer and cultured all of the CD45⁻ remaining cells in nonadherent culture conditions. Even if 70.4% (CTC count ≥ 1) and 53.5% (CTC count ≥ 3) of the patients were positive for CTCs with the CellSearch system, only two times CTCs could be expanded *ex vivo*, and, interestingly, the required condition was to have a CTC count of ≥ 300 . These results showed how challenging it is to establish a colon CTC line from patients with colon cancer even with overt distant metastases and may explain why so far no CTC cultures or cell lines have been reported in the literature. In our study, only one cell line could be expanded for more than one year and these cells are still alive and grow well in culture. For this specific colon CTC-MCC-41 cell line, we switched from medium 1 to medium 2 after a few days of cell culture and improved the index of proliferation of the tumor cells and their long-term survival. This important step has been

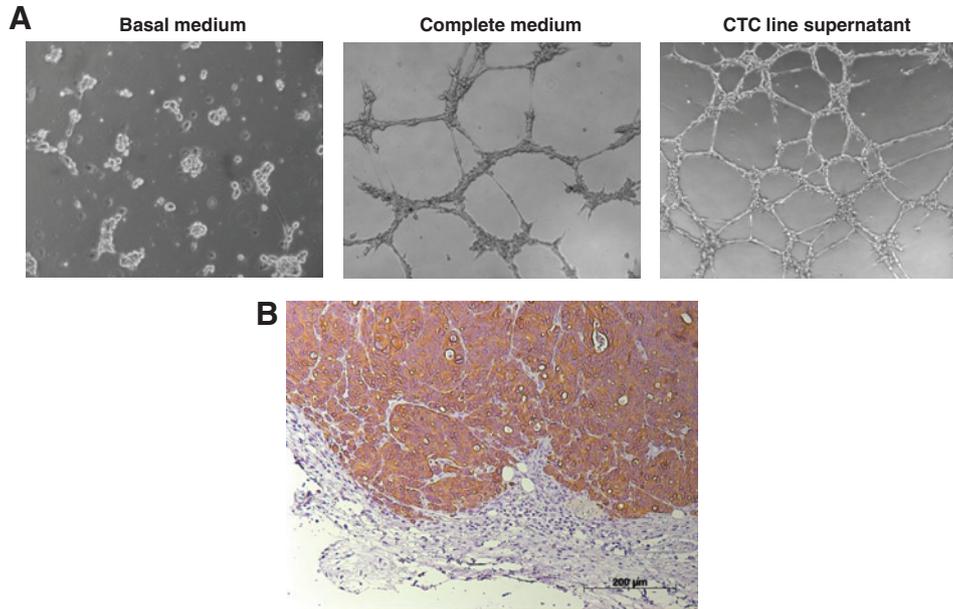


Figure 5.

A, endothelial cell tube formation induced by the CTC-MCC-41 cell line. Representative photographs of *in vitro* angiogenesis (magnification, $\times 5$). Endothelial cells (10^5 cells/well) were seeded into the plate and endothelial cell tube formation exceeding the tube formation of positive controls was observed within 6 hours. Endothelial cells cultured with RPMI 1640 only (left) were used as the negative control; endothelial cells cultured with a complete endothelial cell medium with endothelial cell growth factors (middle) were used as the positive control, and our experiment was endothelial cell cultured with RPMI 1640 medium used for a 48-hour CTC culture (CTC-MCC-41 cell culture supernatant; right). B, xenografts. A representative photograph of the tumor tissues taken from the sacrificed mice. On the paraffin block cut presented here, the keratin staining (brown) shows a clear reactivity with the cancer cells of human origin, whereas mouse stromal cells are unstained and located at the bottom. Nuclei are counterstained with hematoxylin (blue).

omitted for the first CTC line, which may explain its loss after 2 months.

To confirm the establishment of this cancer cell line, we performed genotyping analyses. Copy-number variation analysis of cultured cells using next-generation sequencing technology demonstrated that these CTC-MCC-41 cells were cancer cells with colon cancer-specific genomic changes. Interestingly, the two CTC spheres and the single CTC that were investigated showed

several subtle differences among the copy-number changes, indicating that the colon CTC-MCC-41 cell line has a polyclonal origin. Moreover, we can hypothesize that the changes observed in the stem cell-like spheres might be more relevant to the outgrowth of the CTCs.

To characterize the colon CTC-MCC-41 cell line at the gene expression level, we performed single-cell transcriptome analyses exploring 13 different mRNAs and covering different known

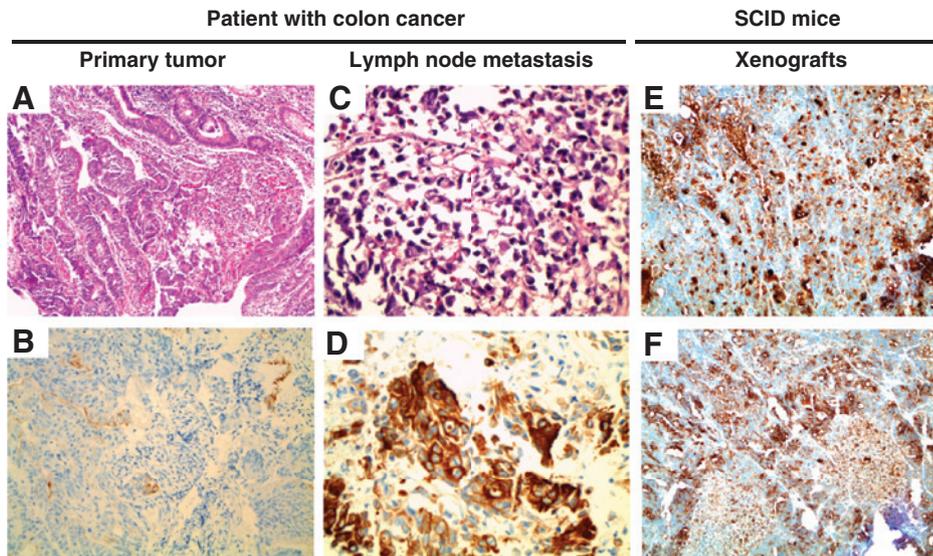


Figure 6.

Immunohistochemical analyses of CK20 expression in tumor tissues. Representative images of hematoxylin and eosin coloration and CK20 expression of the primary tumor (A and B), the lymph node metastasis (C and D) of the patient with colon cancer, as well as the xenografts (E and F). Each tissue reveals the presence of characteristic differentiated adenocarcinoma with cells of low nuclei-cytoplasmic ratio and irregular nuclei shape, as well as vacuoles. All tumor tissues show a positive staining for human CK20 in the cytoplasm (brown). Nuclei were counterstained with hematoxylin (blue).

properties of tumor cells: epithelial, mesenchymal, stem cells, and angiogenesis characteristics as well as the expression of the proto-oncogene *c-Met*. We defined this cell line as epithelial (EpCAM⁽⁺⁾, CK19⁽⁺⁾, E-Cadherin⁽⁺⁾, Vimentin⁽⁻⁾). However, it was interesting to observe that several cancer stem cell markers were also expressed: ALDH1⁽⁺⁾, CD133⁽⁺⁾ as well as Snail⁽⁺⁾, an important EMT inducer. Thus, as we have a mixture of epithelial and mesenchymal traits, we can suggest that these colon CTC-MCC-41 cells show an intermediate phenotype between epithelial and mesenchymal. This intermediate phenotype appears to have an increased potential to form metastases, as recently suggested by Weinberg and colleagues (21). In addition, the CTC-MCC-41 cell line was also able to express VEGF, a key inducer of angiogenesis and *c-Met*, a proto-oncogene specific of tumor cells.

Most interestingly, the CTC-MCC-41 cell line expressed osteoprotegerin which is involved in the osteomimicry. Osteoprotegerin was also found to be expressed on the BCM1 cancer cell line derived from disseminating tumor cell present in bone marrow of a patient with breast cancer (22). Previous analyses of samples of primary tumor and matched bone metastases of patients with breast cancer showed that only the metastatic cells express proteins of bone marrow origin such as osteoprotegerin (23, 24). The adaptation of CTCs arriving in the bone marrow and the subsequent cross-talk between these CTCs and the host tissue induces the expression of site-specific proteins. Thus, there is evidence from other groups that CTCs acquire expression of osteoprotegerin when they colonize the bone (25). Therefore, expression of osteoprotegerin by CTC-MCC-41 cells is of utmost importance, because it indicates that these colon CTCs have been already located in the bone marrow as DTC before to recirculate in the peripheral blood. This result is consistent with the fact that DTCs are frequently found in the bone marrow of patients with colon cancer (26), whereas overt bone metastases are rare in these patients and were also not detected in the patient from whom the CTC-MCC-41 cell line was established.

These results obtained at the transcriptome level were then confirmed and implemented with additional experiments at the protein level using flow cytometry, immunocytochemistry, and functional assays like the EPISPOT and the *in vitro* angiogenesis assays. We could show that the CTC-MCC-41 cell line was also expressing strongly CK20, consistent with the colon origin of these tumor cells, FGF2 as a well know stem cell growth factor important for the *ex vivo* growth of metastatic cells in breast cancer (27), and CD44 and CD133 as additional stem cell markers. Indeed, Du and colleagues indicate that CD44 as a potential marker for CSCs in colorectal cancer (28) and cells with high expression of CD44 along with CD133 in HCT116 showed tumor-initiating capability (29). In addition, studies on colorectal cancer cell lines demonstrated that CD44⁺/CD24⁺ cells showed greater clonogenic ability *in vitro* and tumor initiation *in vivo* (30). However, the source of cancer stem cells in colorectal cancer is still controversial (31).

To assess the viable tumor cells and their potential, we optimized new functional EPISPOT assays to show that they were able to release CK19 and osteoprotegerin as well as to secrete FGF2 and VEGF. These results clearly confirmed their epithelial character and potential bone marrow origin plus their capacity to secrete actively *in vitro* a stem cell growth factor and an angiogenic inducer to initiate tumor cell growth and recruitment of endothelial cells. These very last results were demonstrated by the *in*

vitro tube formation after cell culture of primary endothelial cells with the supernatant of CTC cell culture.

Most importantly, to test the tumorigenicity of the CTC-MCC-41 cell line, we inoculated subcutaneously tumor cells into immunodeficiency mice. CTC-derived xenografts were established and human-specific keratin staining demonstrated their human origin. During the last year, the growth of CTCs in immunodeficient mice was still a big challenge but has been successfully performed by four different groups in breast and lung cancer, directly after CTC isolation from blood sample or after short-term *ex vivo* CTC expansion (13–16).

Finally, the analysis of clinicopathologic characteristics of the patient from whom we were able to isolate CTCs and establish a CTC cell line showed that the presence of CTCs was associated with rapid disease progression. Indeed, this patient had a very short survival due to a chemorefractory tumor exhibiting several factors correlated with poorer outcome: a poor differentiation (32) and a *BRAF* mutation, well known to be a pejorative marker of poor prognosis regardless of the administered treatment (33, 34). Importantly, the mutation status of the primary tumor was also confirmed in the lymph node metastasis, in the CTC-MCC-41 cell line, and in the xenografts. At the protein level, we compared expression of CK20 and observed a focal expression in the primary tumor, whereas the lymph node metastasis, the CTC-MCC-41 cell line, and the xenograft exhibited a strong homogeneous expression of CK20, suggesting a selection of CK20 on metastatic tumor cells.

In conclusion, we were able to establish a colon CTC line stable for at least 16 months, and this cell line shares important features of the tumor cells in the patient with colon cancer. These tumor cells were most likely derived from the bone marrow based on the osteoprotegerin expression, showed an intermediate epithelial/mesenchymal phenotype with stem cell-like characteristics (e.g., growth as microspheres and expression of cancer stem cell markers), could induce *in vitro* angiogenesis and tumors in immunodeficient mice. Thus, it is conceivable that we have established a cell line with important properties relevant for the development and progression of metastatic disease. Further studies on these cells could be an important step to better predict the fate of CTCs in patients with colon cancer and to develop new treatments to target these aggressive tumor cells. Nevertheless, a current drawback is the low success rate at which CTC lines can be generated from patients with cancer. Therefore, optimization of enrichment and *ex vivo* cell culture conditions of CTCs remain a key step to allow a larger number of patients to benefit from this personalized approach.

Disclosure of Potential Conflicts of Interest

T. Mazard received a commercial research grant from Roche. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

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