Establishment and characterization of a cell line from human circulating colon cancer cells

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Abstract

Circulating tumor cells (CTCs) 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 cancer patients. However, an in-depth investigation of CTCs is hampered by the very low number of these cells, especially in the blood of colorectal cancer patients. 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 colon cancer patient. 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 colon cancer patient 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.
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Introduction

The use of circulating tumor cells (CTCs) 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 ten 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 et al. (13) and more recently by Yu et al. (14).

Another approach to expand the number of CTCs is xenotransplantation of patient-derived CTCs into immunodeficient mice. The first report by Bacelli et al. showed that grown metastases after xenotransplantation of breast cancer CTCs into the bone of immunodeficient mice, had an EpCAM\textsuperscript{low}, C-met\textsuperscript{high}, CD47\textsuperscript{high} CD44\textsuperscript{high} phenotype.
which may be characteristic of metastasis-initiator cells (15). The second report was published on patients with small-cell lung cancer, demonstrating that CTCs from patients with either chemo-sensitive 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 concentrations in the transplanted blood sample (e.g., > 1,000 cells per 7.5mL 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 non-resectable 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, 10mL) for CTC detection (Supplementary Materials and Methods) as well as in EDTA tubes (10mL) for ex vivo CTC culture (Supplementary Materials and Methods).

Cell cultures

Culture conditions of primary endothelial cells (ECs), 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 permeabilisation with PFA 3,7% + Triton 0.2% in PBS solution, cells and spheres were labeled with a large panel of antibodies (Table S1) and analyzed under a light microscope.
Flow cytometry experiments

The same panel of proteins (Table S1) was tested by flow cytometry to characterize the CTC cell line. A fixation/permeabilisation 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).

Histopathological and immunohistochemical analyses of the original tumor cells of the colon cancer patient and the xenografts.

Paraffin-embedded tumor tissues: (1) primary tumor biopsy of the colon cancer patient, (2) lymph node biopsy of the colon cancer patient and (3) subcutaneous CTC-MCC-41 xenografts in SCID mice were cut in 3µm 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., vascular endothelial growth factor (VEGF), epithelial growth factor receptor (EGFR) and osteoprotegerin (OPG), corresponding protocols are detailed in the Supplementary Materials and Methods.

In vitro angiogenesis: Endothelial cell tube formation

A 24-well plate coated with 1.3mL Matrigel (BD Biosciences) per well was solidified at 37°C for 30 min. ECs endothelial cells (10^5 cells/well) were seeded into
the plate and cultured for 6hrs. 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), (3) RPMI 1640 basal medium used for a 48hrs-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 (RT) 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 (0.2mL) 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 perparation 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**

In order to investigate the tumorigenicity of the CTC cell line, five SCID mice (Charles River - background BALB/c, aged of 12 weeks) were injected subcutaneously with
10^6 CTC-MCC-41 cells per 200µL 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 (codon 12 and 13) and BRAF gene in the primary tumor and the lymph node metastasis of the colon cancer patient as well as the CTC-MCC-41 cell line and the xenografts (Supplementary Materials and Methods).
Results

*Ex vivo* culture of isolated CTC from metastatic colon cancer patients.

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 cut-off for patients with metastatic CRC (4) (Table 1). 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.

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 analyse 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 suggest 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 hrs in the Medium 2 as compared to a doubling time of 37 hrs in the Medium 1. Due to 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 hrs with Medium 2. The CTC-MCC-41 cell line can be frozen, banked and thawed for successful re-growth.

**Clinico-pathological 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 (codon 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
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months after the diagnosis due to a disease progression at the peritoneal (carcinosis) and pulmonary (lymphangitis) sites.

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 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
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(Vimentin, Snail, Twist), stem cells (ALDH-1, 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-micoglobulin) 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. While 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 OPG, a protein characteristic for the osteomimetism 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 non-hematopoietic 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 intra-cytoplasmic proteins were screened and the main results are shown in Fig. 3B.
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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. 4 A and B show that viable tumor cells were able to release CK19 and OPG as well as to secrete FGF2 and VEGF. Compared to the positive controls, CTC-MCC-41 cells released similar amounts of
CK19, secreted lower amounts of FGF2 and higher amounts of VEGF and OPG. 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 ECs were cultured for 6 hrs 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 ECs were then cultured with the basal medium used for a 48 hrs-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).

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

We compared KRAS and BRAF mutations in the primary tumor and the lymph node metastasis of the colon cancer patient 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 (codon 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 colon cancer patient 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, 6F) were homogeneously and more strongly stained.
Discussion

Subgroups of cancer cells can leave the primary tumour, 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 metastatic colon cancer patient 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 metastatic colon cancer patients 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 colon cancer patients 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
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days of cell culture and improved the index of proliferation of the tumor cells and their long-term survival. This important step has been 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 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 OPG which is involved in the osteomimicry. OPG was also found to be expressed on the BCM1 cancer cell line derived from disseminating tumor cell present in bone marrow of a breast cancer patient (22). Previous analyses of samples of primary tumor and matched bone metastases of breast cancer patients showed that only the metastatic cells express proteins of bone marrow origin such as OPG (23, 24). The adaptation of CTCs arriving in the bone marrow and the subsequent crosstalk 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 OPG when they colonize the bone (25). Therefore, expression of OPG 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 re-circulate in the peripheral blood. This result is consistent with the fact that DTCs are frequently found in the bone marrow of colon cancer patients (26), while 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 et al. 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 tumour-initiating capability (29). In addition, studies on colorectal cancer cell lines demonstrated that CD44+/CD24+ cells showed greater clonogenic ability in vitro and tumour 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 OPG 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 supernantant of CTC cell culture.

Most importantly, to test the tumorigenicity of the CTC-MCC-41 cell line, we inoculated sub-cutaneously 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 clinico-pathological 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 colon cancer patient. These tumor cells were most likely derived from the bone marrow based on the OPG 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 colon cancer patients 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 cancer patients. Therefore, optimization of enrichment and *ex vivo* cell culture conditions of CTCs remain a key step in order to allow a larger number of patients to benefit from this personalized approach.
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**Table 1. CTC detection and ex vivo culture.**

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*From the marked patients we were able to expand CTCs in cell culture.
**Figure legends**

**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 X40). B, immunocytochemical staining of the CTC-spheres using anti-EpCAM-APC, anti-CK20-FITC and anti-CD45-PE Abs.

**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 intra-chromosomal copy number alterations.

**Figure 3. A, Transcriptome of the CTC-MCC-41 cell line at the single cell level.** Thirteen different mRNA have been analyzed covering different characteristics of tumor cells: (1) epithelial (in green); (2) mesenchymal (in purple); (3) stem cell (in red); (4) angiogenesis (in blue); (5) proto-oncogene (in orange) and (6) osteomimetism (in brown). As controls, we used the β2-μglobulin (housekeeping gene) and the CD45 (leukocyte marker). In parallel to the CTC-MCC-41 cell line, 3 different cancer cell lines have been 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. Ct: Cycle threshold values are means +/- standard deviation of 3 experiments performed on CTC cultures on month 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 X40) experiments. All experiments were repeated 5 times and the figure shows representative results.

Figure 4. Functional characterization of the CTC-MCC-41 cell line by fluoro-EPISPOT assays. Secretion, release and shedding of CK19, FGF2, VEGF and 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 OPG (magnification X5). 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 to the respective positive controls.

Figure 5. A. Endothelial cell tube formation induced by the CTC-MCC-41 cell line. Representative photos of in vitro angiogenesis (X5 magnification): ECs (endothelial cells, 10⁵ cells/well) were seeded into the plate and endothelial cell tube formation exceeding the tube formation of positive controls was observed within 6h. The negative control is ECs cultured with RPMI 1640 only (on the left), the positive control is ECs cultured with a complete ECs medium with EC growth factors (in the middle) and our experiment is ECs cultured with RPMI 1640 medium used for a 48 hrs-CTC culture (CTC-MCC-41 cell culture supernatant; on the right). B. Xenografts. A representative photo 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*).

**Figure 6. Immunohistochemical analyses of CK20 expression in tumor tissues.**

Representative images of HE coloration and CK20 expression of the primary tumor (A, B), the lymph node metastasis (C, D) of the colon cancer patient as well as the xenografts (E, 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 are counterstained with hematoxylin (*blue*).
Figure 1.
Figure 2.
**Figure 3.**

### Table A

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- **Negative ≥ 30 Ct**
- **Positive < 30 Ct**

### Table B

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Figure 4.

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Graph B:

% spot recovery / 4,000 cells plated

Markers: CK19, FGF2, VEGF, OPG

Legend:
- Positive controls
- CTC-MCC line
Figure 5.

A

Basal medium

Complete medium

CTC line supernatant

B

Figure 5.
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| **Figure 6.**
Establishment and characterization of a cell line from human circulating colon cancer cells

Cancer Res  Published OnlineFirst January 15, 2015.

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