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# Recherche de nouveaux biomarqueurs tumoraux à l'aide de lignées cellulaires issues de cellules tumorales circulantes dans le cancer du côlon

Laure Cayrefourcq

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# THÈSE POUR OBTENIR LE GRADE DE DOCTEUR DE L'UNIVERSITÉ DE MONTPELLIER

En Biologie Santé

École doctorale Sciences Chimiques et Biologiques pour la Santé

Unité de recherche EA2415 - Aide à la Décision pour une Médecine Personnalisée

Recherche de nouveaux biomarqueurs tumoraux à l'aide de  
lignées cellulaires issues de cellules tumorales circulantes  
dans le cancer du côlon

Présentée par Laure CAYREFOURCQ

Le 11 Décembre 2020

Sous la direction de Catherine Alix-Panabières  
et Said Assou

Devant le jury composé de

Pr. Christian SIATKA Professeur Associé, Université de Nîmes

Dr. Françoise FARACE, Responsable de la plateforme Cellules circulantes rares, Institut Gustave Roussy, Villejuif

Pr. Louis BUSCAIL, Professeur des Universités et Patricien Hospitalier, CHU Toulouse

Pr. Jean-Michel FABRE, Professeur des Universités et Patricien Hospitalier, CHU Montpellier

Dr. Catherine ALIX-PANABIÈRES, Maître de Conférences des Universités et Patricien Hospitalier, CHU Montpellier

Dr. Said ASSOÛ, Ingénieur de recherche, Université de Montpellier

Président et Rapporteur

Rapporteur

Examineur

Examineur

Directrice de thèse

Co-directeur de thèse



UNIVERSITÉ  
DE MONTPELLIER





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## Liste des abréviations

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5FU	5-Fluorouracile
ACE	antigène carcino-embryonnaire
aFP	alpha fœto-protéine
ALDOB	aldolase B
CCR	cancer colorectal
CCRm	cancer colorectal métastatique
CDA	cytidine deaminase
CP	cancer de la prostate
CRPC	cancer de la prostate résistant à la castration
CT	chimiothérapie
CTC	cellule tumorale circulante
DEP	diélectrophorèse
DLA	produit de cytophérèse
EMP	plasticité épithélo-mésenchymateuse
EMT	transition épithélo-mésenchymateuse
EpCAM	molécule d'adhésion des cellules épithéliales
EPIDROP	EPIspot in a DROP
EPISPOT	Epithelial ImmunoSPOT
FDA	Food and Drug Administration
FGF2	fibroblast growth factor 2
FOLFIRI	traitement à base de 5FU + Irinotecan
FOLFOX	traitement à base de 5FU + Oxaliplatine
HR	hazard ratio
HT	hormonothérapie
LOH	Loss Of Heterozygosity
MBC	cancer du sein métastatique
MEC	matrice extracellulaire
MET	transition mésenchymateuse-épithéliale

MMP	métalloprotéases de matrice
MSI	instabilité des microsatellites
MSS	sans instabilité des microsatellites
PD-1	Programmed Death-1
PD-L1	Programmed Death-1 Ligand 1
SCLC	cancer du poumon à petites cellules
SG	Survie Globale
SSP	Survie Sans Progression
TGF $\beta$	Transforming Growth Factor
TIMP	inhibiteurs de métalloprotéase
VEGF	Vascular Endothelial Growth Factor
WGA	amplifications du génome entier

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## Résumé

La recherche sur les cellules tumorales circulantes (CTCs) est un large champ de recherche en oncologie, susceptible de fournir des informations importantes tant fondamentales que cliniques. Les CTCs représentent une population hétérogène des divers clones de cellules tumorales, provenant de la tumeur primaire et/ou des métastases, ayant la capacité de transiter dans la circulation sanguine. La détection des CTCs donne des informations importantes sur la prolifération tumorale et les propriétés moléculaires des lésions tumorales. Cette information participe à l'évaluation pronostique, la stratification des patients pour des thérapies ciblées, le suivi en temps réel de l'efficacité du traitement, l'identification de cibles thérapeutiques et de mécanismes de résistance. Néanmoins, la dissémination tumorale est le résultat d'un processus de sélection qui favorise la survie d'une infime proportion de cellules cancéreuses qui possèdent la capacité d'auto-renouvellement des cellules souches ainsi que des propriétés initiatrices de métastases, pour finalement coloniser des organes à distance de la tumeur primaire. La compréhension de la biologie de la cascade métastatique et le développement de nouvelles stratégies thérapeutiques nécessitent des modèles précliniques permettant l'étude de la fonctionnalité tumorale. Les lignées cellulaires cancéreuses obtenues à partir de CTCs représentent alors le modèle idéal pour comprendre la biologie cellulaire des cellules tumorales initiatrices de métastases et les mécanismes de la maladie métastatique, pour *in fine* développer de nouveaux traitements.

Le but de mon projet de thèse a été de contribuer à la compréhension des mécanismes du processus métastatique par l'analyse approfondie du matériel unique que représentent les lignées de CTCs et potentiellement d'identifier les biomarqueurs d'intérêts pour le traitement personnalisé du cancer colorectal.

L'obtention et la caractérisation de ces lignées CTCs coliques nous ont permis de comprendre certains des mécanismes intervenant dans la dissémination tumorale, mettant en avant une signature spécifique des CTCs, les différenciant totalement des tumeurs primaires et des métastases. Nous avons pu observer que le système de réparation de l'ADN, la plasticité cellulaire et différentes voies métaboliques (mitochondrie, xénobiotique) faisaient partie des processus clés de la cascade métastatique. L'analyse approfondie de ces mécanismes nous a permis d'identifier deux biomarqueurs potentiels : un marqueur de résistance au traitement (CDA) et un marqueur de métastases hépatiques (ALDOB). Par ailleurs, nous avons mis en évidence l'état de plasticité de ces CTCs qui possèdent, dès leur invasion et circulation au sein du compartiment sanguin, les effecteurs clés de la transition mésenchymateuse-épithéliale (MET) nécessaires à une éventuelle colonisation d'organes à distance.

**Mots clés :** Cellules tumorales circulantes, cancer du côlon, lignées cellulaires CTCs, cascade métastatique, biomarqueurs



## Abstract

Research on circulating tumor cells (CTCs) is a large field of research that can provide important fundamental and clinical information. CTCs represent a heterogeneous population of the diverse clones of tumor cells, originating from the primary tumor and / or metastases and having the capacity to pass through the bloodstream. CTC detection provides important information on tumor proliferation and the molecular properties of tumor lesions. This information helps for prognostic assessment, patient stratification to targeted therapies, real-time monitoring of treatment efficacy, and identification of therapeutic target and mechanism of resistance. Nevertheless, tumor dissemination is the result of a selection process which promotes the survival of a small proportion of cancer cells possessing stemness capacities as well as properties of metastasis to colonize distant organs. The understanding of the biology of the metastatic process and the development of new therapeutic strategies need reliable preclinical models to study tumor functionality. Cell lines obtained from CTCs represent then the ideal model to understand cell biology of metastatic initiating cells and disease mechanisms, to *in fine* develop new treatments.

The aim of my thesis project was to contribute to the understanding of the mechanisms involve in the metastatic process through an in-depth analysis of the unique material which are the CTC lines and to identify, potentially, biomarkers of interest for the personalized treatment of colorectal cancer.

Obtaining and characterizing these colon CTC lines has allowed us to understand some of the mechanisms involved in the metastatic cascade, highlighting a specific signature of the CTCs, totally differentiating them from primary tumors and metastases. We observed that the DNA repair system, cell plasticity and different metabolic pathways (mitochondria, xenobiotic) were among the key processes of the metastatic cascade and the in-depth analysis of these mechanisms allowed us to highlight two potential biomarkers, a marker of resistance to treatment (CDA) and a marker of hepatic metastases (ALDOB). In addition, we have demonstrated the state of plasticity of these cells which, from circulation, possess the important effectors of MET necessary to colonize distant organs.

**Keywords:** Circulating tumor cells, colon cancer, CTC lines, metastatic cascade, biomarkers

## Préambule

La recherche sur les cellules tumorales circulantes (CTCs) est un large champ d'investigation susceptible de fournir des informations importantes tant fondamentales que cliniques. Les CTCs représentent une population hétérogène des divers clones de cellules tumorales, provenant de la tumeur primaire et/ou des métastases, ayant la capacité de transiter dans la circulation sanguine. De par leur rareté dans le sang, environ une CTC parmi des millions de cellules hématopoïétiques, leur analyse approfondie reste technologiquement complexe nécessitant des méthodes d'enrichissement et de détection très sensibles et spécifiques.

De nombreuses études cliniques et méta-analyses ont démontré l'intérêt pronostique du dénombrement des CTCs, notamment dans les cancers du sein, de la prostate et du côlon. Actuellement, les recherches en cours visent à évaluer l'utilisation de ce facteur pronostique et à démontrer l'utilité clinique des CTCs en tant que '*biopsie liquide*' comme par exemple avec le STIC CTC METABREAST dans le cancer du sein métastatique.

Un autre enjeu majeur de la recherche sur les CTCs est la compréhension de la biologie du processus métastatique, afin de définir quels sont les mécanismes clés permettant à ces cellules tumorales d'aller former des métastases à distance. Cette connaissance nous permettrait (i) de découvrir de nouveaux biomarqueurs reflétant mieux l'hétérogénéité tumorale intra- et inter-patient(s), (ii) d'identifier les mécanismes de résistance qui se mettent en place, étape après étape, au cours de la sélection clonale, et (iii) de développer de nouveaux traitements ciblant particulièrement les sous-populations agressives qui sont à l'origine des rechutes chez les patients, le tout visant à s'inscrire dans une démarche de médecine de précision.

Le développement récent de modèles de culture de CTCs, *in vivo* et *in vitro*, a ouvert de nouvelles perspectives d'études des CTCs à propriété de cellules souches. Le nombre important de cellules obtenues dans ces modèles permet une analyse plus approfondie des divers mécanismes intervenant mais surtout permet l'analyse de la fonctionnalité de ces sous-populations de cellules tumorales bien particulières. Les premiers modèles ont déjà (i) montré la tumorigénicité de ces cellules reflétant leur propriété à initier des métastases ainsi que (ii) leur résistance/sensibilité face à différents médicaments afin de prédire la réponse à un traitement en clinique.



# Introduction





## 1. Le cancer colorectal

### 1.1 Généralités

Le cancer colorectal (CCR) est la 2<sup>ème</sup> cause de mortalité par cancer dans le monde après celui du poumon. Il reste donc encore aujourd'hui un problème de santé publique. Le CCR se développe à partir des cellules qui tapissent la paroi interne du côlon ou du rectum. Le plus souvent, ces tumeurs malignes proviennent d'une tumeur bénigne, appelée polype adénomateux, qui évolue lentement et finit par devenir cancéreuse. Cependant, tous les polypes n'évoluent pas en cancer du côlon (amelie.fr).

C'est un cancer fréquent aussi bien chez l'homme que chez la femme. On estime qu'environ 43 000 nouveaux cas sont diagnostiqués chaque année en France (43 336 en 2018). Il représente 11,2 % des nouveaux cas de cancers masculins et 11,3 % des nouveaux cas de cancers féminins. C'est le troisième cancer le plus fréquent chez l'homme et le second chez la femme. Actuellement, on estime que 200 000 personnes vivant dans notre pays sont ou ont été atteintes de cette forme de cancer (<https://www.roche.fr/fr/patients/info-patients-cancer/comprendre-cancer/cancer-colorectal-colon.html>). Il survient plutôt tardivement au cours de la vie : l'âge moyen au moment du diagnostic est de 71 ans chez les hommes et de 73 ans chez les femmes. Le CCR est rare avant 50 ans.

La mortalité par CCR est en baisse depuis les années 1990 alors que l'incidence est restée stable chez la femme, et qu'elle diminue chez l'homme (<https://www.santepubliquefrance.fr/maladies-et-traumatismes/cancers/cancer-du-colon-rectum>). L'allongement de l'espérance de vie a entraîné une augmentation du nombre de patients âgés atteints de CCR. La survie des personnes atteintes d'un CCR s'est améliorée ces dernières années, notamment par une amélioration du système de dépistage. Le CCR peut être découvert à un stade précoce grâce à un dépistage par recherche de sang occulte dans les selles. Il existe en France, un programme de dépistage organisé, proposé à toutes les personnes âgées de 50 à 74 ans (amelie.fr).

Plusieurs facteurs augmentent le risque d'avoir un CCR, notamment ceux ayant trait au mode de vie : consommation d'alcool, tabagisme, sédentarité, inactivité physique, surpoids et obésité, alimentation avec une consommation faible en fibres, excessive de viande rouge ou de viandes transformées. En 2015, on estime en France métropolitaine, qu'environ 21,0 % des cancers colorectaux (hors cancers de l'anus) chez les plus de 30 ans étaient attribuables à la consommation d'alcool [Circ 2018]. Cependant, les maladies inflammatoires chroniques de l'intestin, notamment la maladie de Crohn et la rectocolite hémorragique, sont aussi des facteurs de risques (<https://www.ameli.fr/herault/assure/sante/themes/cancer-colorectal/comprendre-cancer-colorectal>) ; surtout dans les cas où la maladie est étendue à tout le côlon et évolue depuis plus de 10

ans. Et il existe également, des formes familiales génétiques de cancers colorectaux avec ou sans polypose liées à des mutations génétiques (moins de 5 % des cas). Mais, le CCR dans sa forme familiale ou héréditaire est une maladie rare.

## 1.2 Classification anatomique

Les données épidémiologiques ont montré que l'évolution de l'incidence du CCR variait en fonction de la localisation tumorale. Ainsi, on note une augmentation de l'incidence du côlon droit et du côlon gauche plus rapide chez l'homme (+ 11,7 % et 10,3 % par période de 5 ans) que chez la femme (+ 5,9 % et 6,1 %) et une stabilité globale des cancers du sigmoïde et du rectum (Chauvenet, Cottet et al. 2011). Les hypothèses avancées suggèrent que les aliments et nutriments agissent de manière spécifique et variable suivant la localisation colique. Ainsi, les fibres qui sont dégradées dans le côlon entraînent une acidification du milieu et une inhibition des enzymes de transformation des acides biliaires primaires en secondaires. Cette action s'exerce principalement sur le côlon droit. L'action anti-oxydante des vitamines A, C et E agit principalement sur la muqueuse du côlon transverse et du côlon gauche. Le calcium, en se fixant aux sels biliaires, les transforme en savons insolubles avec une action pro-inflammatoire sur le côlon droit principalement. Ces éléments expliquent au moins en partie les variations géographiques d'incidence, le gradient nord-sud du CCR (<https://www.fmcgastro.org/texte-postu/postu-2018-paris/cancer-du-colon-classifications-moleculaires-et-anatomiques-necessaires-a-la-decision-therapeutique/>).

### 1.2.1 Impact pronostique de la localisation tumorale

Les tumeurs du côlon du côté droit ont une incidence plus faible, sont plus fréquentes chez les femmes, sont plus fréquemment porteuses de mutations BRAF, ont un stade de tumeur / ganglions / métastases plus élevé à la présentation et sont associées à un pronostic plus mauvais que les tumeurs colorectales du côté gauche (Burke, Gasdaska et al. 1989, Benedix, Kube et al. 2010). Une explication possible de ces différences est l'origine embryologique différente des parties proximale et distale du côlon et du rectum. Les deux parties sont réunies au niveau des deux tiers proximaux et distal du tiers du côlon transverse et présentent des apports sanguins, des innervations et des drainages lymphatiques différents (Lee, Malietzis et al. 2015).

Les résultats d'une méta-analyse de 13 essais randomisés et d'une étude prospective confirment le caractère prédictif et pronostique de la localisation tumorale (**Tableau 1**). Parmi les 6 652 patients avec un CCR métastatique (CCRm) traités en 1<sup>ère</sup> ligne, la Survie Globale (SG) et la Survie Sans Progression

(SSP) était plus faible pour les cancers du côlon droit (respectivement HR : 1,56 [1,43-1,70] et HR : 1,33 [1,2-1,48]) indépendamment du traitement reçu (Holch, Ricard et al. 2017).

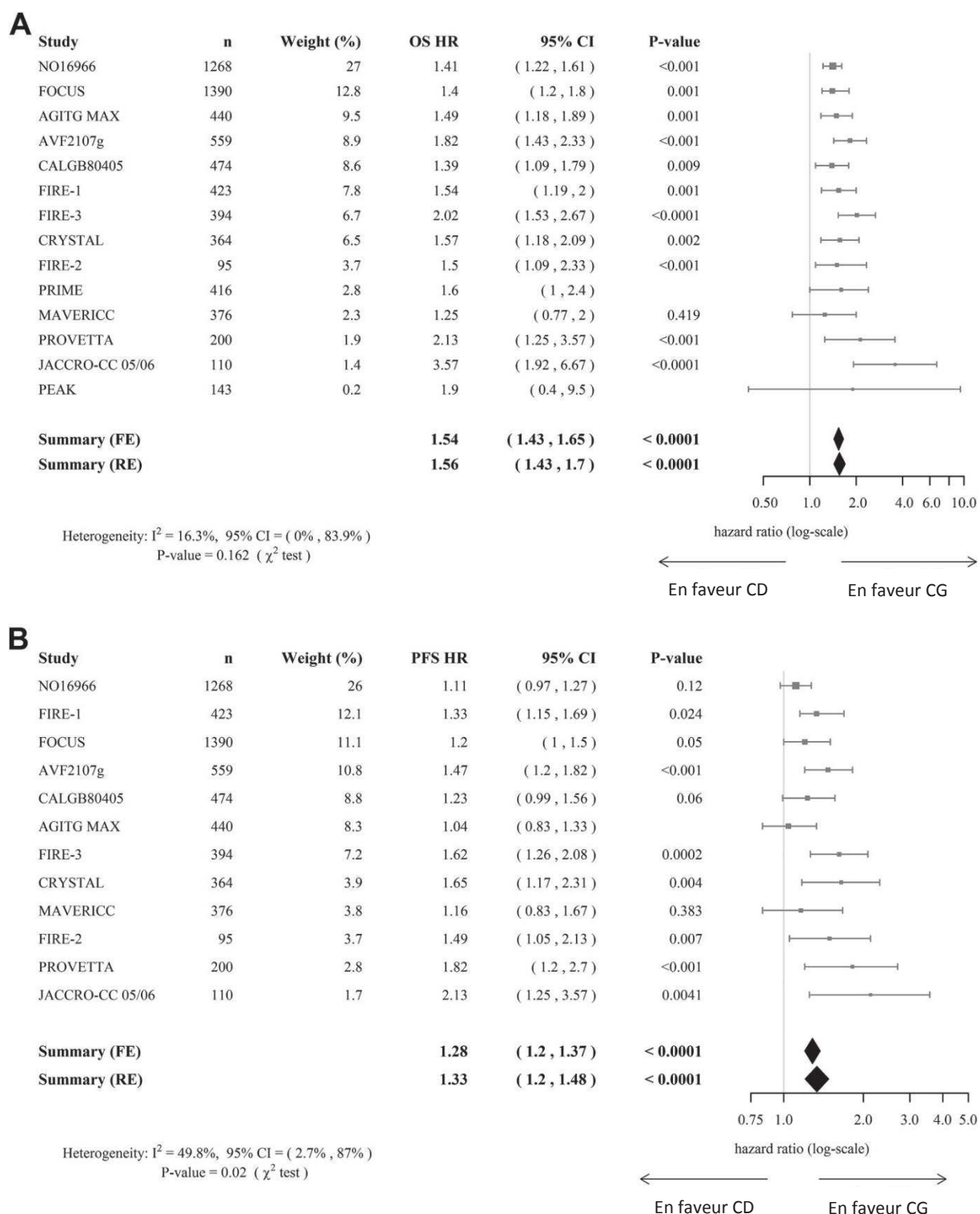


Tableau 1. Impact pronostique (A) et prédictif (B) de la localisation tumorale dans le cancer du côlon (côlon gauche vs côlon droit).

Légende : CD = cancer colorectal du côté droit; CG = cancer colorectal du côté gauche; FE = modèle à effets fixes; RE = modèle à effets aléatoires.



### 1.2.2 Impact prédictif de la localisation tumorale

L'effet prédictif d'efficacité de la chimiothérapie cytotoxique de la localisation tumorale n'a jamais été mis en évidence. À l'inverse des données récentes issues d'essais de phase III suggèrent un effet prédictif de la localisation tumorale (droite vs gauche) sur l'efficacité des biothérapies anti-EGFR (cetuximab et panitumumab) et anti-VEGF (bevacizumab).

Une méta-analyse de l'étude PRIME (NCT00364013) et CRYSTAL (NCT00154102) suggère que la localisation tumorale était prédictive du bénéfice de survie par ajout d'anticorps anti-EGFR à la chimiothérapie standard chez les patients atteints d'une tumeur de type RAS sauvage (survie globale, HR pour CG : 0,69 ; IC à 95% : 0,58 -0,83 ; P <0,0001 et HR pour CD : 0,96 ; IC à 95% : 0,68-1,35 ; P = 0,802) (**Tableau 2**). Une méta-analyse de l'étude FIRE-3 (NCT00433927), CALGB / SWOG 80405 (NCT00265850) et PEAK (NCT00819780) indique que les patients atteints de CG de type RAS sauvage avaient un bénéfice de survie significativement plus élevé avec le traitement anti-EGFR par rapport au traitement anti-VEGF lorsqu'il était ajouté à la chimiothérapie standard (HR 0,71 ; IC à 95% : 0,58-0,85 ; P = 0,0003) (Holch, Ricard et al. 2017). En revanche, chez les patients atteints de CD, le bénéfice du traitement standard était faible et le traitement à base de bevacizumab était numériquement associé à une survie plus longue (HR 1,3; IC à 95%: 0,97-1,74; P = 0,081).

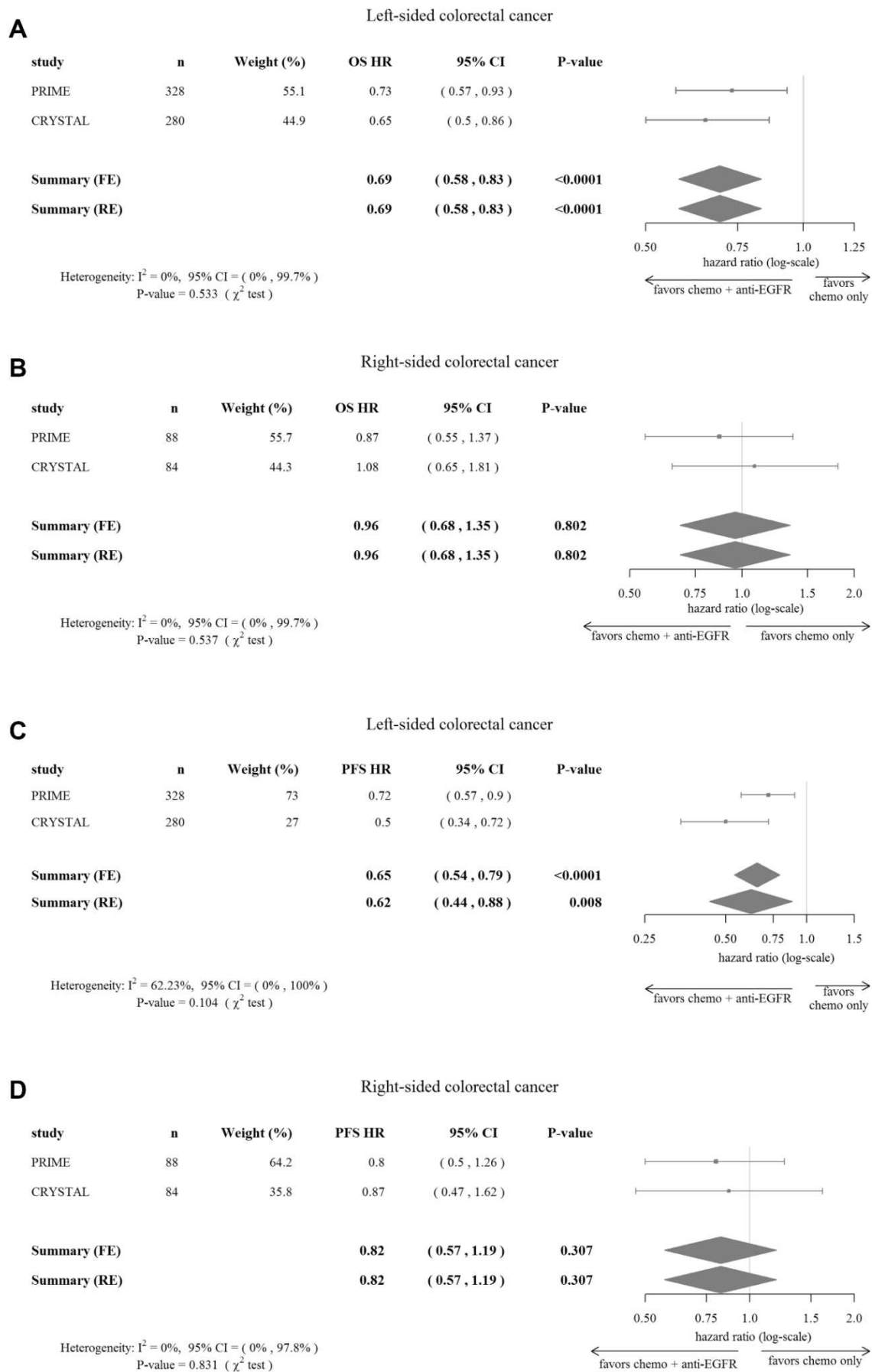


Tableau 2. Impact de la localisation tumorale sur l'efficacité du cetuximab (SSP et SG).

### 1.3 Classification moléculaire

Les altérations génétiques ou chromosomiques du CCR permettent de distinguer 2 grandes familles (Barault, Charon-Barra et al. 2008, Barault, Veyrie et al. 2008) :

- Les tumeurs avec instabilité génétique représentant 15 % des CCR. Il s'agit de tumeurs présentant une instabilité des microsatellites (MSI) c'est-à-dire porteuses de mutations sur les gènes du système MMR (MisMatch Repair) impliqués dans la réparation des erreurs de réplication de l'ADN (réparation du mésappariement de l'ADN). Le phénotype MSI confère un excellent pronostic si bien que très peu d'entre elles vont évoluer vers le stade métastatique (5 % de tumeur MSI seulement sur l'ensemble des CCRm).

Ces tumeurs se développent principalement dans le côlon droit et chez la femme. Elles sont associées à un profil biologique faisant intervenir des mutations diverses tout au long de la carcinogénèse : hyperméthylation du promoteur de MLH1 (formes sporadiques), mutations de BAX, de la  $\beta$ caténine, de TGF $\beta$  et de BRAF.

- Les tumeurs avec instabilité chromosomique (CIN+) qui représentent 85 % des CCR. Elles sont caractérisées par une perte d'un morceau de chromosome (Loss Of Heterozygosity - LOH) : il s'agit principalement d'un fragment du bras long du chromosome 5 (LOH 5q) mais aussi des chromosomes 2p, 17p, 18q. On y trouve les mutations et perte d'APC, la plupart des mutations RAS, de P53 et peu de mutations BRAF. Elles se développent préférentiellement dans le côlon gauche et chez l'homme.

Un consortium international d'experts a récemment permis de dégager, à partir de 6 classifications moléculaires et plus de 4 000 patients, 4 sous-types de cancers colorectaux. Il s'agit de la classification CMS en 4 groupes caractérisés par des facteurs moléculaires, biologiques et cliniques (Guinney, Dienstmann et al. 2015). Il faut noter que 21 % des CCR n'appartiennent à aucun de ces 4 groupes (**Table 3**).

CMS1 MSI Immune	CMS2 Canonical	CMS3 Metabolic	CMS4 Mesenchymal
14%	37%	13%	23%
MSI, CIMP high, hypermutation	SCNA high	Mixed MSI status, SCNA low, CIMP low	SCNA high
<i>BRAF</i> mutations		<i>KRAS</i> mutations	
Immune infiltration and activation	WNT and MYC activation	Metabolic deregulation	Stromal infiltration, TGF $\beta$ activation, angiogenesis
Worse survival after relapse			Worse relapse-free and overall survival

Tableau 3. Classification CMS.

- **Groupe CMS-1** (MSI, Immune) : Représente 14 % des CCR et caractérisés par des tumeurs avec instabilité des microsatellites (MSI high), par une infiltration immune marquée, souvent CIMP élevé (CIMP high = phénotype méthylateur) et par des tumeurs mutées BRAF. La localisation est préférentiellement à droite. Leur pronostic est bon en l'absence de métastase et mauvais en cas d'évolution métastatique (survie après rechute faible).
- **Groupe CMS-2** (Canonical) : Représente 37 % des CCR localisés plutôt à gauche sans instabilité des microsatellites (MSS), marqués par des mutations somatiques fréquentes (SCNA high) avec une surexpression de l'EGFR et activation de la voie WNT/MYC fréquentes. Leur pronostic est inter-médiaire.
- **Groupe CMS-3** (Metabolic) : Représente 13 % des CCR, de type épithélial, MSS dans 90 % des cas, et caractérisés par des mutations RAS fréquentes et peu de mutations somatiques. Elles sont réparties de manière assez homogène entre le côlon gauche et le côlon droit. Leur pronostic est intermédiaire.
- **Groupe CMS-4** (Mesenchymal) : Représente 23 % des CCR. Elles sont caractérisées par de nombreuses altérations somatiques (SCNA high), une activation fréquente du TGF $\beta$  et de l'angiogénèse, des tumeurs plus souvent MSI et se situent préférentiellement sur le côlon gauche. Leur pronostic est mauvais en situation métastatique.

Le nombre d'options de traitement est limité dans le CCR. Il existe donc un grand potentiel d'amélioration d'efficacité des traitements par l'utilisation guidée des thérapies ciblées selon la classification moléculaire.

## 1.4 Les biomarqueurs dans le cancer colorectal

### 1.4.1 Biomarqueurs sanguins

Les principaux marqueurs tumoraux sanguins utilisés en cancérologie digestive sont l'antigène carcino-embryonnaire (ACE), le CA19.9 et l'alpha foeto-protéine (aFP). Ce sont des substances protéiques endogènes dont la sécrétion peut être augmentée lors d'un processus pathologique et notamment lors de l'activation de certains gènes au cours de l'oncogenèse. L'intérêt de ces 3 marqueurs utilisés en routine clinique depuis des décennies fait encore l'objet de controverses quant à leur apport et parfois même leur utilité. Leur intérêt a évolué dans le temps car leur impact est fortement dépendant de l'évolution des prises en charges cliniques, thérapeutiques et techniques. Leur apport en termes d'aide à l'évaluation de l'efficacité des traitements doit être réservé aux tumeurs sans cible clairement mesurable (Jean-Marc, Léa et al. 2013).

### 1.4.2 Biomarqueurs moléculaires

Comme discuter en amont la classification moléculaire des CCR a pris de l'importance ces dernières années aux vues de l'apport important pour l'utilisation des thérapies ciblées.

#### 1.4.2.1 RAS

Les mutations les plus recherchées sont celles du gène RAS pour les traitements avec anti-EGFR. Environ 55 % des CCR présentent une mutation RAS. En conséquence, les thérapies anti-EGFR (panitumumab et cetuximab) sont restreintes aux tumeurs RAS sauvages (sans mutations des exons 2, 3 et 4 de KRAS et NRAS) (Douillard, Oliner et al. 2013). Cependant, l'absence de mutation RAS ne garantit pas une réponse aux anti-EGFR (30 à 40 % des patients restent non répondeurs).

#### 1.4.2.2 BRAF

La mutation BRAF V600E (5 % à 10 % des CCR) est un facteur pronostique péjoratif, surtout dans le CCR métastatique (Thiel and Ristimäki 2013). Son rôle prédictif de non réponse aux anti-EGFR n'est pas encore établi et sa recherche n'est donc pas à ce jour indispensable à leur prescription. Cependant, la connaissance de ce statut tumoral peut inciter à un traitement de chimiothérapie intensifiée dès la 1re ligne (Loupakis, Cremolini et al. 2014). Les patients peuvent être adressés vers des essais de thérapie ciblée anti-BRAF. Dans ce but, la détection de cette mutation est systématique depuis 2011.

#### 1.4.2.3 MSI

Les MSI sont présentes dans 15 % des CCR sporadiques et est une caractéristique constante des CCR développés dans le cadre d'un syndrome de Lynch. L'instabilité micro-satellitaire est utilisée dans le dépistage (pré-criblage tumoral) de cette forme héréditaire de CCR (Olschwang, Bonaiti-Pellié et al.

2006). Parmi les tumeurs avec instabilité microsatellitaire, la mise en évidence d'une mutation BRAF et/ou d'une méthylation du promoteur du gène MLH1 permet d'éliminer un syndrome de Lynch. L'instabilité micro-satellitaire est un facteur de bon pronostic des CCR de stade localisé (Ogino, Shima et al. 2012). Les cancers du côlon de stade II avec instabilité microsatellitaire n'ont pas d'indication à une chimiothérapie adjuvante par 5FU seul du fait d'un excellent pronostic lié à l'instabilité microsatellitaire et de l'effet délétère démontré du 5FU en monothérapie en adjuvant dans cette population (Sargent, Marsoni et al. 2010).

#### 1.4.2.4 HER2

Depuis l'essai de phase III ToGA (NCT01041404) démontrant l'efficacité du trastuzumab (anti-HER2) dans les cancers gastriques sur-exprimant HER2, l'association trastuzumab/chimiothérapie est devenue un standard des traitements de première ligne des cancers gastriques et de la jonction œsogastrique métastatiques (Gomez-Martín, Lopez-Rios et al. 2014). Seuls les patients dont la tumeur est positive pour HER2 sont éligibles à un traitement par trastuzumab (AMM 2010).

## 2. Processus métastatique

La formation de métastases est un événement déterminant dans la genèse d'un cancer et s'accompagne d'un pronostic clinique sombre. Le terme métastase, du grec *metastasis* « je change de place », est apparu au XVI<sup>e</sup> siècle pour désigner les tumeurs secondaires à une tumeur primitive. Ce phénomène devient un objet de recherche au XIX<sup>e</sup> siècle notamment avec la publication de J. Paget dans *Lancet* en 1889 de l'hypothèse de la graine et sol « seed and soil » (Paget 1889). Le processus métastatique est actuellement défini comme « une dissémination de cellules néoplasiques dans un site secondaire non contigu et distant, au sein duquel ces cellules prolifèrent pour former une masse extravasculaire de cellules incomplètement différenciées » (Bidard and Poupon 2012). La distance entre l'organe principal et les sites métastatiques implique un déplacement de cellules tumorales soit directement par voie hématogène, soit après invasion du tissu normal voisin, par voie lymphatique et par conséquent le franchissement de diverses barrières.

Malgré sa complexité et des modes d'actions qui restent encore discutés, il existe un consensus sur les étapes du processus métastatiques (**Figure 1**).

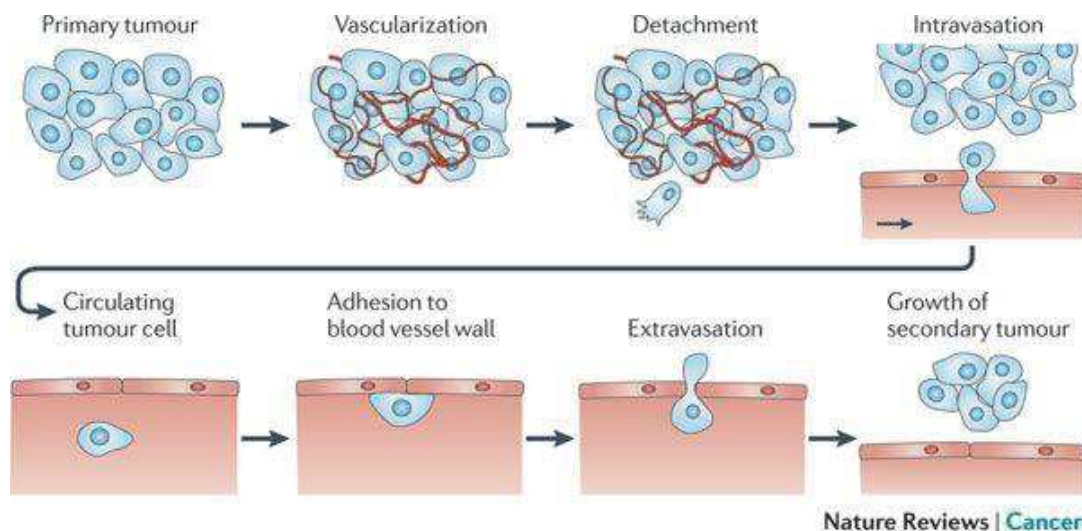


Figure 1. Étape du processus métastatique. (Wirtz, Konstantopoulos et al. 2011)

## 2.1 Invasion tumorale

L'invasion locale et la vascularisation de la tumeur représentent les premières étapes du processus. La tumeur primaire n'est pas composée exclusivement de cellules tumorales, en effet la tumeur s'appuie sur le microenvironnement dans lequel elle évolue et utilise toutes les ressources à sa portée. Ainsi les fibroblastes, cellules endothéliales et certaines cellules du système immunitaires, et aussi la matrice extracellulaire (MEC) participent à la progression tumorale. La croissance de la tumeur perturbe l'organisation des interactions intercellulaires présentes dans les tissus adultes normaux. La tumeur acquiert la capacité de contourner de manière chronique les signaux de normalisation du microenvironnement et à son tour, le microenvironnement évolue pour accueillir la tumeur en croissance (**Figure 2**) (Quail and Joyce 2013).

La dégradation de la MEC est assurée par des protéases, métalloprotéases (MMP) et leurs inhibiteurs (TIMP). Cette protéolyse permet une « activation » de la MEC, via la libération de nombreuses cytokines et facteurs de croissance (TGF $\beta$  [transforming growth -factor], VEGF [vascular endothelial growth factor], FGF2 [fibroblast growth factor 2] etc.), qui ont alors un rôle trophique et chimio-attractant pour les cellules tumorales mais aussi pour les cellules endothéliales et immunitaires. Ce stroma activé comporte donc une activité protéolytique élevée, une angiogenèse accrue, une quantité importante de facteurs de croissance, un afflux de cellules inflammatoires. Tous ces facteurs concourent au développement tumoral initial.

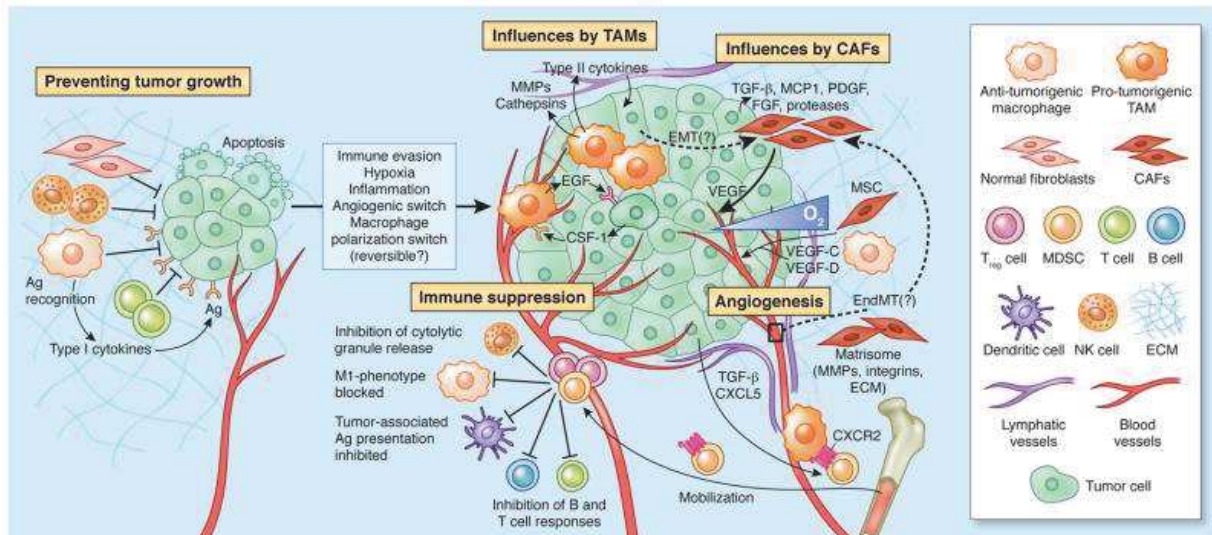


Figure 2. Rôle du stroma dans l'invasion et la néo-angiogénèse tumorale (Quail and Joyce 2013).

Lors de la croissance de la tumeur, certaines cellules tumorales vont perdre de leur adhésion intercellulaire et acquérir un phénotype migratoire. Cela correspond à l'étape de détachement de la tumeur. Ce processus apparait assez tôt dans le développement tumoral et peut survenir avant même la détection et le diagnostic de la tumeur primaire (**Figure 3**) (Talmadge 2007).

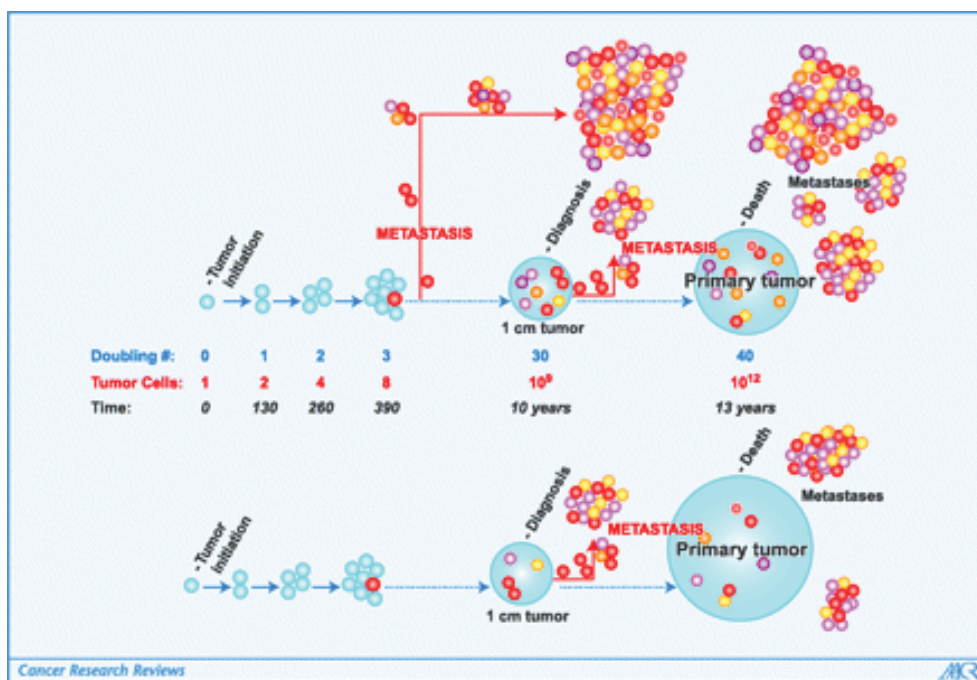


Figure 3. Dissémination métastatique au cours de la vie d'une tumeur (Talmadge 2007).



Pour les tumeurs épithéliales, le détachement des cellules de la tumeur n'est possible qu'après perte des liaisons adhérentes cellules-cellules et cellules-stroma. La communauté scientifique a donc émis l'hypothèse que lors du processus métastatique, les cancers d'origines épithéliales doivent acquérir des caractéristiques mésenchymateuses, semblables à celles décrites dans le développement embryonnaire. Ainsi les cellules se détachant de la tumeur modifieraient leur phénotype épithélial pour un phénotype plus mésenchymateux, c'est la transition épithélo-mésenchymateuse (EMT) (Nieto, Huang et al. 2016, Yang, Antin et al. 2020). Parmi les protéines épithéliales, E-cadhérine, est un acteur central de la polarité cellulaire et de l'organisation épithéliale. Cette cadhérine joue un rôle dans le maintien des jonctions adhérentes cellules-cellules, et sa perte est un des phénomènes les plus connu et décrit dans l'EMT. Il a été démontré que la dérégulation d'E-cadhérine corrélait avec la progression tumorale et un mauvais pronostic de survie (Moll, Mitze et al. 1993).

## 2.2 Intravasation et circulation des cellules tumorales

L'entrée des cellules dans la lumière d'un vaisseau sanguin, ou intravasation, marque ensuite le début d'une étape brève et difficile à observer. L'intravasation est un phénomène naturel se produisant lors du développement, qui met en jeu de multiples signaux et facteurs de croissance. L'intravasation des cellules tumorales est facilitée par les cellules stromales (Chiang, Cabrera et al. 2016). Comme pour l'invasion les cellules stromales libèrent de nombreuses cytokines et facteurs de croissance permettant la migration à travers la barrière endothéliale. Ce phénomène est aussi étroitement lié à l'EMT qui outre la perte de l'adhésion cellulaire induit également une augmentation de la capacité migratoire et une résistance à l'apoptose (Kalluri and Weinberg 2009).

Une fois la barrière des vaisseaux franchie les cellules se retrouvent dans la circulation sanguine et on les appelle les cellules tumorales circulantes (CTCs) (Pantel and Alix-Panabieres 2019). Cette circulation peut se faire sous la forme de cellules isolées et/ou d'amas cellulaires (clusters) (Aceto, Bardia et al. 2014). L'analyse des CTCs a connu ces 10 dernières années un intérêt croissant car elles sont un biomarqueur représentatif des fonctions biologiques du développement métastatique et leur analyse a le potentiel de révéler les mécanismes clés de la progression tumorale (Pantel and Alix-Panabieres 2019).

Dans la circulation sanguine, les CTCs font face à plusieurs obstacles naturels qui entravent le processus métastatique. Elles doivent résister à de nombreuses contraintes et attaques pour survivre et nécessitent des propriétés de (1) résistance mécanique aux pressions élevées, (2) résistance à l'apoptose induite par la perte d'un substrat d'adhésion (ce type d'apoptose est appelé anoïkis), (3) échappement au système immunitaire (Fares, Fares et al. 2020).

### 2.2.1 *Résistance à l'anoïkis*

Dans les tissus normaux, une adhésion appropriée aux protéines de la matrice extracellulaire est essentielle pour la survie des cellules. La perte de cette adhérence induit une mort cellulaire qui a été appelée « anoïkis ». L'anoïkis est un processus physiologiquement important pour l'homéostasie tissulaire et le développement car il empêche les cellules épithéliales détachées de coloniser d'autres tissus, inhibant ainsi la croissance des cellules dysplasiques ou l'attachement à une matrice inappropriée (Kim, Koo et al. 2012). La dérégulation de l'anoïkis, comme la résistance à cette mort cellulaire programmée, est un des mécanismes clé du processus métastatique. Si les cellules acquièrent des signaux oncogènes capables de surmonter cette machinerie, elles acquièrent la capacité de survivre en dehors de leur environnement normal en l'absence d'adhésion à la matrice extracellulaire. Les cellules tumorales qui ont acquis un tel phénotype peuvent survivre au détachement de leur site principal, voyager à travers les systèmes circulatoire et lymphatique pour disséminer vers des sites ectopiques (Simpson, Anyiwe et al. 2008). Différentes études ont montré que la voie de signalisation de mort cellulaire médiée par l'activation des caspases régule l'anoïkis. Ainsi, des défauts dans cette voie comme la surexpression de l'inhibiteur de la caspase-8 FLIP peut rendre les cellules résistantes à l'anoïkis. De même, la résistance à l'anoïkis peut être conférée par des barrages dans la voie de signalisation mitochondriale, tels que la surexpression de la famille de protéines anti-apoptotiques Bcl-2 (Simpson, Anyiwe et al. 2008).

L'étude des mécanismes moléculaires impliqués dans la survie des cellules cancéreuses pendant qu'elles quittent le microenvironnement adhérent de la tumeur vers le système circulatoire est importante pour comprendre le processus par lequel le cancer peut se propager à des organes distants, ainsi que pour développer de nouvelles thérapies pour inhiber la propagation de la maladie.

### 2.2.2 *Échappement au système immunitaire*

L'un des principaux obstacles auxquels les CTCs sont confrontés dans le sang est l'attaque du système immunitaire. Beaucoup d'études ont été réalisées pour comprendre les mécanismes impliqués dans la bataille entre les capacités du système immunitaire à lutter contre le cancer et les processus immunosuppresseurs qui favorisent la croissance tumorale. Plusieurs biomarqueurs sont ressortis de ce travail (**Figure 4**) ; par exemple, dans le CCR, un échappement au système immunitaire a pu être observé par la régulation à la hausse de CD47, un signal « don't eat me » qui empêche les macrophages et les cellules dendritiques d'attaquer les CTCs (Steinert, Scholch et al. 2014). Les biomarqueurs d'échappement immunitaire plus avancés cliniquement sont Programmed Death-1 (PD-1) et son ligand

(PD-L1). Il a été mis en évidence que l'expression de PD-L1 dans les tumeurs représentait un élément clé du cycle cancer-immunité en empêchant le système immunitaire de détruire les cellules cancéreuses. Le récepteur PD-1 est une protéine de surface exprimée sur les lymphocytes T activés et son ligand PD-L1 est exprimé à la surface des cellules présentatrices d'antigène. La formation du complexe PD-1 / PD-L1 induit un fort signal inhibiteur dans la cellule T, ce qui conduit à une réduction de la production de cytokines et à une suppression de la prolifération des lymphocytes T (Ohaegbulam, Assal et al. 2015) : le système immunitaire est induit en erreur par les cellules cancéreuses exprimant PD-L1 et ne les détruit pas. Cette compréhension a conduit au développement d'immunothérapies : « immune check-point inhibitors », avec des anticorps anti-PD-1 et anti-PD-L1. Des réponses cliniques remarquables ont été observées dans plusieurs tumeurs malignes différentes, notamment, mais sans s'y limiter, le mélanome, les cancers du poumon, du rein et de la vessie (Ohaegbulam, Assal et al. 2015).

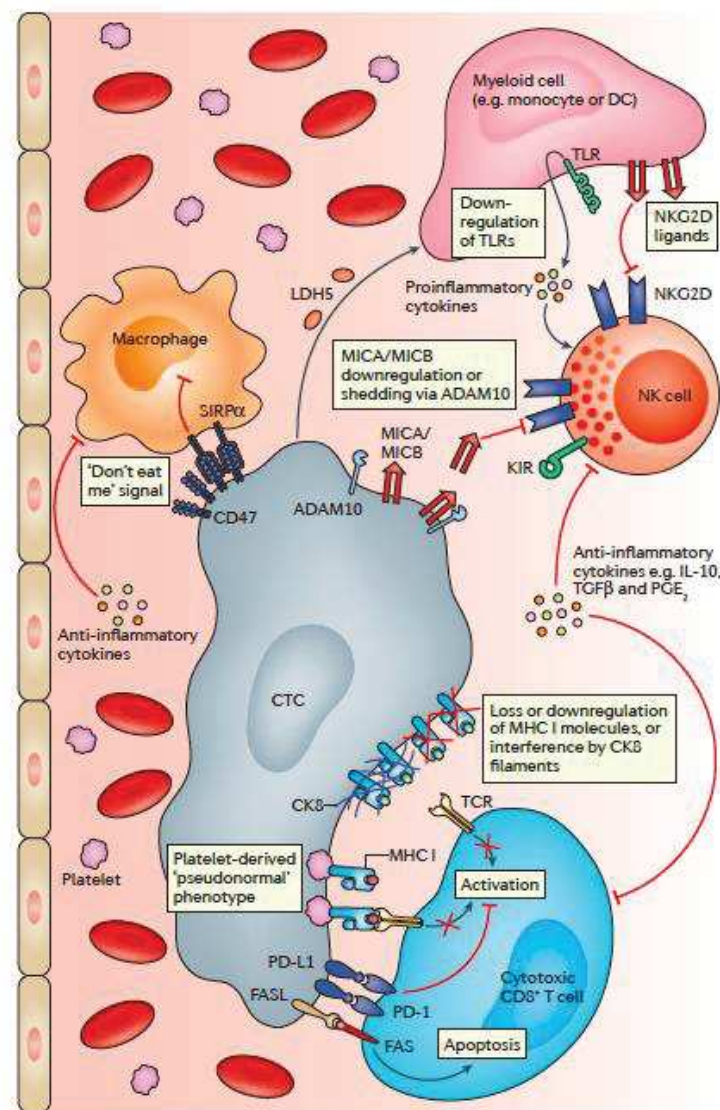


Figure 4. Mécanismes d'échappement au système immunitaire (Mohme, Riethdorf et al. 2017).

Il existe également d'autres mécanismes décrits, que les CTCs peuvent utiliser pour survivre dans le système circulatoire. Par exemple, ces cellules peuvent se coupler à des plaquettes activées. Plusieurs hypothèses proposent que les CTCs s'entourent de plaquettes, comme un revêtement de surface, qui servirait de bouclier contre les agressions immunitaires ou que ce revêtement de plaquettes peut servir à imiter le complexe majeur d'histocompatibilité des cellules hôtes et ainsi éviter la surveillance immunitaire (Lou, Sun et al. 2015). L'agrégation des CTCs avec des plaquettes, des fibroblastes stromaux et des leucocytes conduit à la formation de complexes et augmente la survie des CTCs dans la circulation sanguine en évitant l'anoïkis et en les protégeant des attaques des cellules immunitaires (Hong, Fang et al. 2016). De plus, la sécrétion par les plaquettes du VEGF, capable d'affecter la maturation des cellules dendritiques, joue un rôle clé dans le mécanisme de présentation de l'antigène (Abbott, Rönnbäck et al. 2006).

### 2.3 Plasticité épithelio-mésenchymateuse

L'EMT, que l'on a évoqué précédemment et qui est caractérisée par la régulation négative des protéines épithéliales et régulation positive des protéines mésenchymateuses, est un processus complexe qui soutient la capacité migratoire des cellules tumorales épithéliales et dont on pense qu'il joue un rôle crucial dans la promotion des métastases cancéreuses. L'EMT a conduit à une augmentation de la motilité cellulaire par réarrangement des jonctions de contact cellulaire et perte d'adhérence cellules-cellules (c.-à-d. E-cadhérine, N-cadhérine, claudines), mais aussi à un changement morphologique des cellules épithéliales par modification des protéines du cytosquelette (c.-à-d. cytokératine, vimentine, fibronectine, etc.) via de nombreuses voies de signalisations (**Figure 5**) (Nieto, Huang et al. 2016, Lu and Kang 2019).

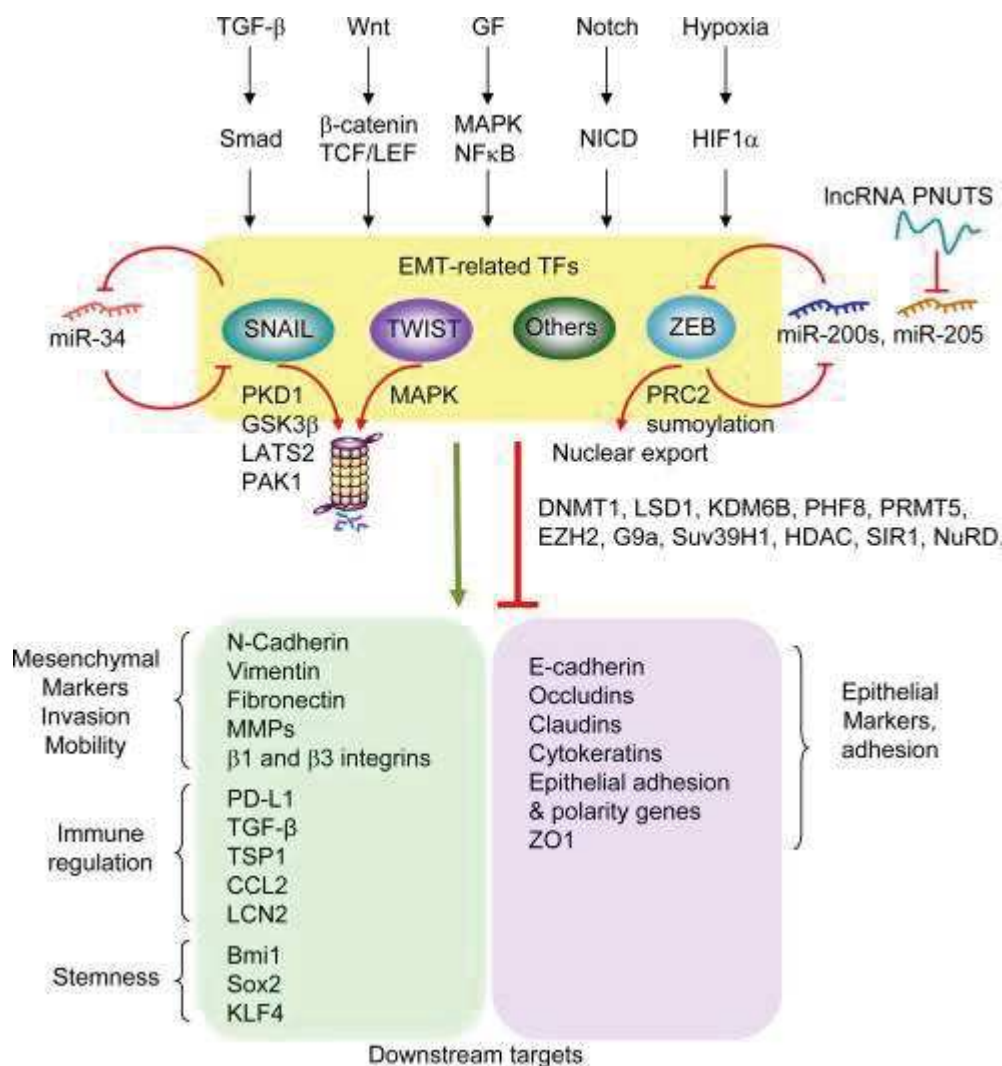


Figure 5. Mécanismes de régulation de l'EMT (Lu and Kang 2019).

Ce phénotype invasif permet aux cellules cancéreuses de traverser la membrane basale et les barrières endothéliales des vaisseaux sanguins pour atteindre la circulation sanguine. Cependant, on ne sait toujours pas quel degré d'EMT est nécessaire dans les cellules tumorales pour atteindre la circulation et son processus démontre une grande complexité et diversité selon le contexte cellulaire et tissulaire. Par exemple, il a été démontré que les facteurs de transcription EMT, SNAI1 et TWIST1, favorise la promotion de métastases dans le cancer du sein en utilisant des modèles murins génétiquement modifiés ou knock-out ainsi que des lignées cellulaires avec une expression génique modifiée (Yang, Mani et al. 2004, Tran, Luitel et al. 2014, Xu, Lee et al. 2017). Cependant ces facteurs se sont révélés inutiles pour génération de métastases dans un modèle de cancer du pancréas avec knock-out de SNAI1 ou TWIST1 (Zheng, Carstens et al. 2015).

Malgré la richesse des données expérimentales, le rôle exact de l'EMT chez les patients atteints de cancer reste toujours activement débattu. D'autre part, pour le développement efficace de métastases dans des organes distants, les cellules cancéreuses doivent inverser leur phénotype en ré-exprimant les protéines épithéliales et perdre leurs propriétés mésenchymateuses, c'est ce qu'on appelle la transition mésenchymateuse-épithéliale (MET). Bien que les processus EMT et MET aient un effet opposé dans la dissémination du cancer, ils utilisent les mêmes voies de signalisation et les facteurs de transcription décrits dans chacun jouent un rôle dans des processus cellulaires similaires, tels que le développement embryonnaire et la différenciation cellulaire (Lu and Kang 2019).

Au cours des 10 dernières années, le développement de technologies sensibles permettant la détection et la caractérisation moléculaire des CTCs ont contribué à apporter une nouvelle lumière sur l'importance de l'EMT pour la dissémination des cellules tumorales humaines (Alix-Panabieres, Mader et al. 2017, Jie, Zhang et al. 2017). L'accumulation de connaissance sur les processus de l'EMT et MET conduisent désormais à une nouvelle tendance, centrée sur la plasticité de la cellule tumorale : plasticité épithélio-mésenchymateuse (EMP) associée à des caractéristiques de cellules souche. Ce processus est aujourd'hui considéré comme un acteur central de la cascade métastatique, offrant aux cellules tumorales la capacité de s'adapter aux différents microenvironnements rencontrés pendant la propagation métastatique aux organes colonisés (c.-à-d. stroma adjacent, sang, organes colonisés) par manipulation de d'une multitude de mécanismes de contrôles (**Figure 6**).

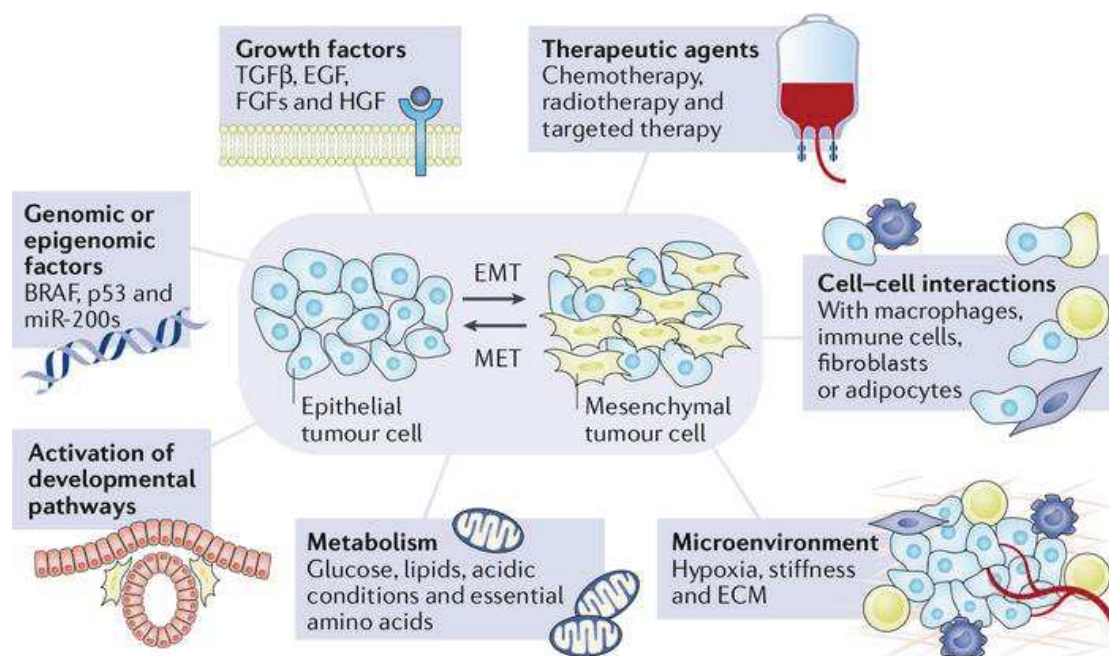


Figure 6. Représentation des divers mécanismes intervenant dans l'EMP (Williams, Gao et al. 2019).

## 2.4 Extravasation et colonisation d'organe distant

L'arrêt des CTCs se fait de manière soit passive, blocage dans un capillaire de petit taille (effet « filtre » du foie et des poumons par exemple), soit de manière active. Dans ce dernier cas, on peut supposer qu'il existe des mécanismes de reconnaissance spécifique et d'arrêt sur l'endothélium similaires à ceux qu'utilisent les leucocytes (Alvarez-Zarate, Matlung et al. 2015). Cette dernière reconnaissance fait intervenir des protéines d'adhésion membranaires sur les cellules tumorales et endothéliales, ces protéines jouent un rôle probablement important dans l'existence de sites métastatiques préférentiels pour chaque type de cancer. Cette spécificité tissulaire des localisations métastatiques est un phénomène très connu en clinique et que décrivait Paget grâce à une comparaison botanique, les « graines » (seeds) de tumeur se développant dans un « terreau » favorable (soil) (Paget 1889). L'existence de niches pré-métastatiques au niveau des organes cibles, au sein desquelles les CTCs s'arrêteraient préférentiellement a été rapportée (Peinado, Aleckovic et al. 2012). La libération d'exosomes par les CTCs serait un évènement crucial pour l'induction de la niche pré-métastatique. Hoshino *et coll.* ont montré que la composition des exosomes en intégrines pouvait prédire les organes cibles pour la formation de métastases et que les exosomes relargués par les tumeurs participent à la préparation de la niche pré-métastatique (Hoshino, Costa-Silva et al. 2015). Par exemple, l'intégrine  $\alpha\beta5$  indiquerait une cible hépatique, ou  $\alpha6\beta4$ , et  $\alpha6\beta1$  ciblerait les poumons (Costa-Silva, Aiello et al. 2015) ; c'est l'organotropisme. Rodrigues *et coll.* ont montré que le pré-conditionnement du microenvironnement cérébral avec des exosomes dérivés de cellules métastatiques cérébrales génère une niche métastatique qui favorise la colonisation. L'intégration par les cellules de l'organe cible, de ces exosomes, stimulent l'expression des gènes qui favorisent la migration et l'inflammation (Rodrigues, Hoshino et al. 2019). Cependant, une niche pré-métastatique favorable ne suffit pas à maintenir la prolifération des cellules cancéreuses. Par conséquent, les CTCs doivent avoir des fonctionnalités supplémentaires pour promouvoir leur croissance au niveau du microenvironnement de l'organe hôte, qui peut être très différent de celui du site primitif, comme des caractéristiques de cellules souches (Agnoletto, Corrà et al. 2019). Il peut également survenir une étape de dormance cellulaire, pendant laquelle les cellules tumorales disséminées persistent sans proliférer pendant un temps indéterminé, avant de reprendre - ou non - une nouvelle croissance aboutissant à l'apparition d'une métastase. Les mécanismes qui régulent l'entrée et la sortie de la phase de dormance restent obscurs, du fait de la difficulté de modéliser cette étape dans des modèles précliniques murins.

Les étapes citées ci-dessus font intervenir un nombre important d'effecteurs moléculaires différents, et, pour une cellule tumorale donnée, la probabilité de pouvoir franchir avec succès toutes ces étapes est extrêmement faible. D'anciennes expériences ont montré que le taux de formation de métastases à partir de cellules tumorales injectées dans la circulation serait de l'ordre de 1/1 000 000 [12], et suggéraient donc une inefficacité métastatique des cellules tumorales (Chambers, Groom et al. 2002, Massagué and Obenauf 2016).

### 3. Cellules tumorales circulantes (CTCs), en tant que *biopsie liquide*

Il y a quelques années, l'analyse des CTCs dans le sang de patients atteints de cancer a été définie par le terme de « *biopsie liquide* » (Pantel and Alix-Panabieres 2010)]. Des prélèvements sanguins peuvent être réalisés et analysés tant au moment du diagnostic que pendant la phase systémique de traitement. La détection des cellules tumorales en circulation donne des informations importantes sur la prolifération tumorale et les propriétés moléculaires des lésions tumorales. Cette information contribue à la détection précoce des lésions métastatiques et participe à la personnalisation des traitements des patients cancéreux tels que l'évaluation pronostique, la stratification des patients pour des thérapies ciblées, le suivi en temps réel de l'efficacité du traitement, l'identification de cible thérapeutiques et de mécanismes de résistance (**Figure 7**).

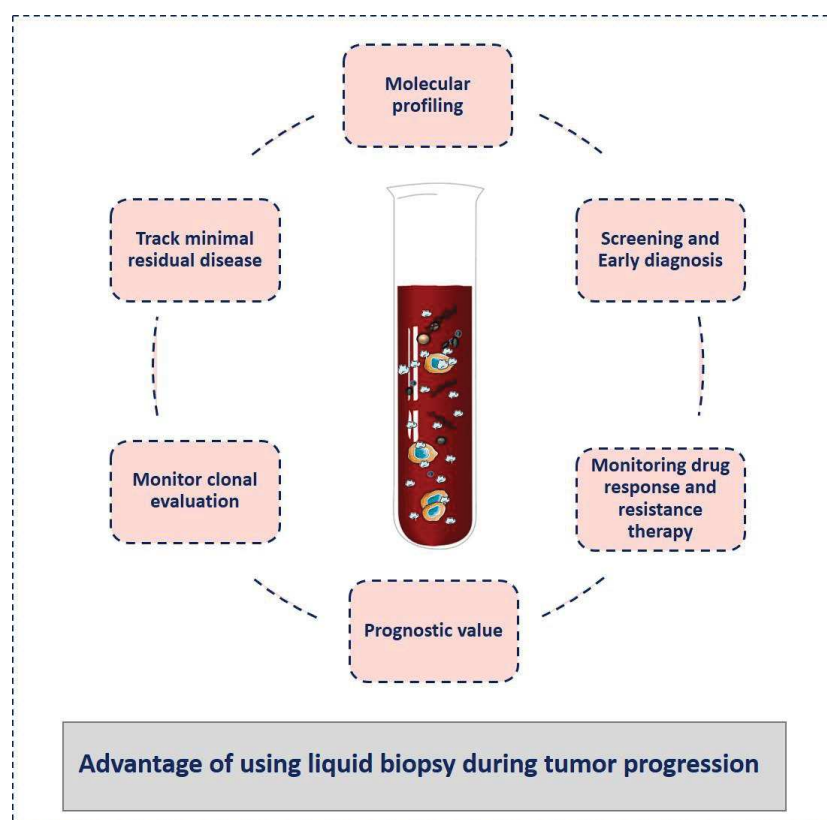


Figure 7. Applications possibles de l'analyse des CTCs en tant que biopsie liquide (Eslami, Cortés-Hernández et al. 2020).



L'analyse de la *biopsie liquide* offre de nouvelles perspectives pour l'étude de la biologie des métastases avec des implications importantes pour la prise en charge clinique des patients. De nombreuses études cliniques et méta-analyses, y compris des cohortes importantes de patients ont montré que le nombre de CTC est un indicateur important du risque de progression ou de décès chez les patients atteints d'un cancer solide métastatique (par exemple, sein, prostate, côlon, etc.) [2–6].

Malgré les progrès remarquables réalisés ces dernières années, à l'heure actuelle, l'analyse de la *biopsie liquide* est rarement mise en œuvre dans les tests de routine pour les patients. L'étude approfondie des CTCs reste techniquement difficile. Les CTCs sont présentes à de très faibles concentrations dans la circulation, on parle d'une cellule tumorale pour des millions de cellules sanguines. Leur identification et leur caractérisation nécessitent des méthodes analytiques extrêmement sensibles et spécifiques (Cayrefourcq and Alix-Panabières 2019).

De plus, jusqu'à présent, les résultats obtenus avec l'analyse de *biopsie liquide* n'ont pas encore conduit à des lignes directrices validées pour le traitement et la prise en charge des patients. Néanmoins, les avancées techniques et certaines études cliniques encourageantes ont démontré que la *biopsie liquide* représente une grande promesse pour la prise en charge du cancer dans un futur proche.

Due à la rareté des CTCs dans la circulation sanguine, leur étude reste techniquement difficile. Leur identification et la caractérisation nécessitent des méthodes analytiques extrêmement sensibles et spécifiques, qui sont généralement une combinaison de procédures d'enrichissement et de détection **(Figure 8)**.

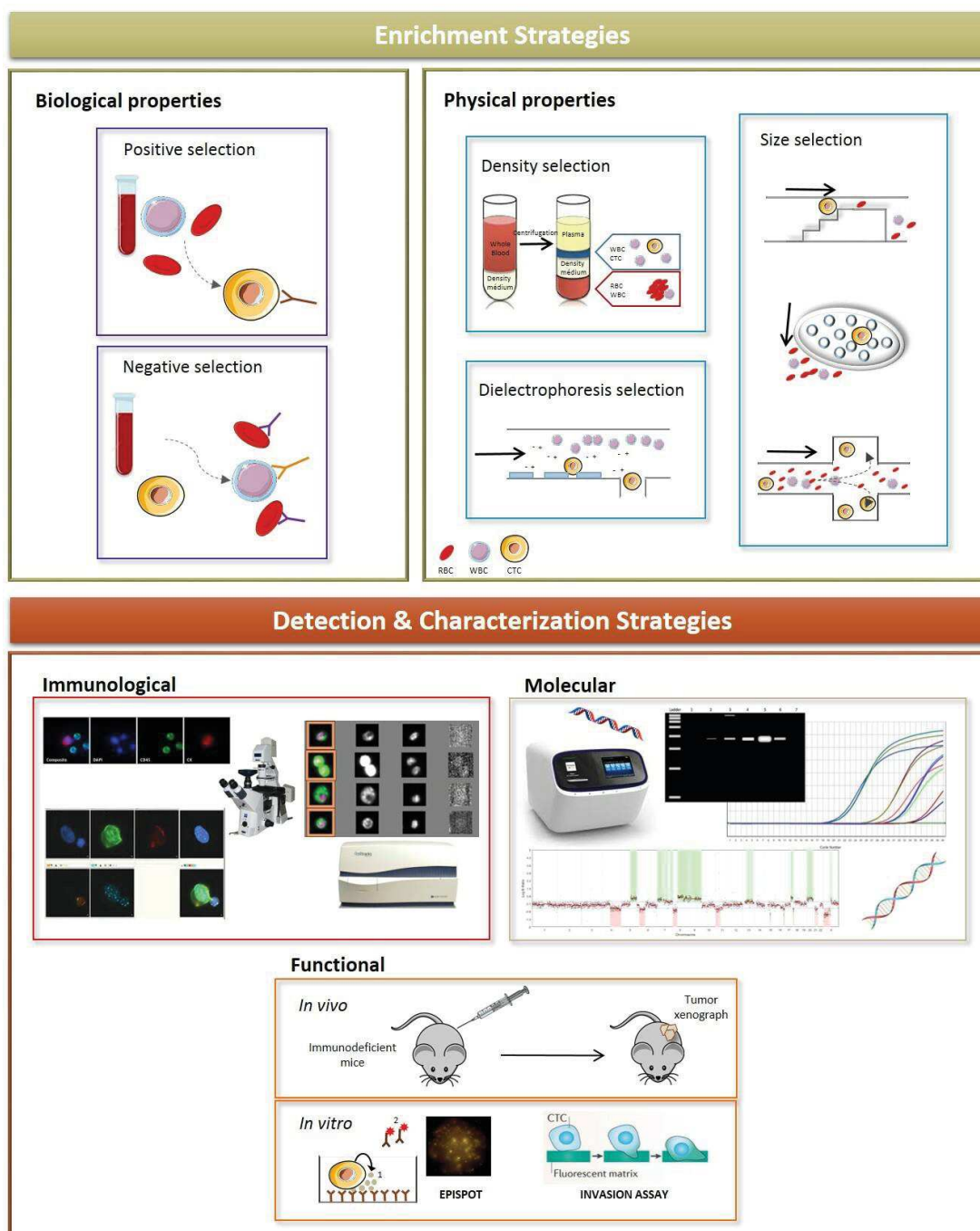


Figure 8. Stratégies pour l'enrichissement, la détection et la caractérisation des CTCs (Cayrefourcq and Alix-Panabières 2019).

### 3.1 Méthodes d'enrichissement

L'enrichissement en CTCs comprend un large panel de technologies basées sur des propriétés qui les différencient des cellules hématopoïétiques normales : (1) propriétés biologiques (par exemple, expression des protéines de surface et capacité d'invasion) et (2) propriétés physiques (par exemple, taille, densité, charges électriques et déformabilité).

### 3.1.1 Propriétés biologiques

Les propriétés biologiques sont principalement utilisées dans des procédures immunologiques avec des anticorps dirigés contre les antigènes associés aux cellules tumorales (sélection positive) ou contre l'antigène leucocytaire commun CD45 (sélection négative ou déplétion). L'enrichissement positif atteint généralement une pureté cellulaire élevée, qui dépend de la spécificité des anticorps. Les technologies d'enrichissement négatif échappent à certains des pièges de l'enrichissement positif ; par exemple, les CTCs ne sont pas marquées avec un anticorps difficile à éliminer, elles ne sont pas activées ou modifiées via une interaction anticorps-protéine et la sélection d'anticorps ne biaise pas la sous-population de CTCs capturées. Cependant, ces avantages se font au détriment de la pureté, car les stratégies d'enrichissement négatif ont généralement une pureté bien inférieure à celle de l'enrichissement positif mais un meilleur rendement (Lara, Tong et al. 2004, Yang, Lang et al. 2009, Baccelli, Schneeweiss et al. 2013) et nécessitent une étape de détection des CTCs appropriée.

#### 3.1.1.1 Sélection positive

Les systèmes immunomagnétiques sont principalement utilisés pour ce type de sélection. Ils ciblent un antigène d'intérêt avec un anticorps qui est couplé à une bille magnétique, et le complexe antigène-anticorps est isolé par exposition à un champ magnétique. La sélection positive est généralement effectuée avec des anticorps contre la molécule d'adhésion des cellules épithéliales (EpCAM). Parmi les technologies actuelles basées sur EpCAM, le système CellSearch® est la seule méthode d'enrichissement et de détection approuvée par la *Food and Drug Administration* (FDA – USA). Cette méthode a été utilisée et validée dans de nombreuses études analysant les CTCs, au cours des 10 dernières années, et est comparée à toute nouvelle méthode de détection des CTCs comme méthode gold standard. Cependant, certaines CTCs n'expriment pas ou peu EpCAM (expression tissu-dépendante + perte due à l'EMT). Leur capture est permise par l'utilisation de cocktails d'anticorps contre divers autres antigènes de surface des cellules épithéliales (par exemple EGFR, MUC1), ou contre un antigène spécifique de tissu (par exemple, PSA, HER2) et contre des antigènes mésenchymateux ou de cellules souches (par exemple, SNAIL, ALDH1) (Alix-Panabieres, Mader et al. 2017). De nombreuses plateformes commerciales de détection des CTCs par enrichissement magnétique positif sont disponibles : (i) basées sur EpCAM: MagSweeper (Powell, Talasaz et al. 2012) ou les technologies MACS de Miltenyi (Giordano, Gao et al. 2012); (ii) en utilisant différents cocktails d'anticorps : IsoFlux™ (Ramirez, Saenz et al. 2016), AdnaTest d'Adnagen (Bredemeier, Edimiris et al. 2016). Actuellement, l'accent est mis sur le développement de dispositifs microfluidiques (« puces ») comme Ephesia Chip (Autebert, Coudert et al. 2015) ou CTC-iChip (Karabacak, Spuhler et al. 2014).

Il est intéressant de noter qu'il existe également un dispositif *in vivo*, GILUPI CellCollector®, développé pour capturer les CTCs directement dans la veine des patients, à l'aide d'un cathéter médical spécialisé possédant des anticorps ciblant EpCAM pour piéger les CTCs. Il est indiqué que ce dispositif permet de cribler un grand volume sanguin pendant les 30 minutes de période de prélèvement, mais les avis divergent sur ce sujet (Dizdar, Fluegen et al. 2019). Plus récemment, une nouvelle technologie très intéressante a pu surmonter la faible sensibilité des dosages CTCs en analysant des volumes sanguins plus importants. C'est une plate-forme de cytométrie en flux photoacoustique *in vivo* qui utilise un laser à fréquence de pouls élevée et des transducteurs à ultrasons focalisés pour la détection transcutanée de CTCs dans le mélanome (CMCs) (Galanzha, Menyaev et al. 2019). Cette méthode, appelée « plateforme cytophone », a détecté des CMCs individuelles ou en clusters chez 27 / 28 patients atteints de mélanome testé dans l'étude.

La sélection positive des CTCs nécessite d'émettre une hypothèse sur la nature des CTCs dans un échantillon de sang individuel. Ce biais est évité par une sélection négative dans laquelle l'échantillon sanguin est dépourvu de cellules indésirables.

#### 3.1.1.2 Sélection négative

L'enrichissement négatif utilise une méthode indirecte pour isoler les CTCs : il cible et élimine les cellules sanguines non désirées, telles que les leucocytes, en utilisant des anticorps contre le CD45 (qui n'est pas exprimé sur les cellules de carcinomes ou autres tumeurs solides) et d'autres antigènes leucocytaires (par exemple, CD19, CD4), pour obtenir un échantillon enrichi en CTCs.

Le cocktail d'enrichissement RosetteSep™ CTC (STEMCELL Technologie) offre une méthode unique pour la déplétion des cellules indésirables en couplant un enrichissement basé sur l'immunoaffinité avec la centrifugation par gradient de densité. RosetteSep™ capture les cellules indésirables et forme un réseau avec les globules rouges grâce à des complexes d'anticorps tétramériques qui ciblent un vaste panel d'antigènes leucocytaires (Brungs, Minaei et al. 2020). La version immunomagnétique est également disponible avec le système EasySep™, qui contient des nanoparticules magnétiques et des complexes d'anticorps tétramères ciblant également un panel d'antigènes leucocytaires.

Parmi les technologies utilisant les propriétés biologiques des CTCs, listées ci-dessus dans les sections « Sélection positive » ou « Sélection négative », certaines d'entre elles sont « flexibles » et peuvent fonctionner soit comme des systèmes d'enrichissement positifs, soit comme des systèmes d'enrichissement négatifs en utilisant différents anticorps (par exemple, en remplacement de l'anti-EpCAM, spécifique des cellules épithéliales avec anti-CD45, spécifique des leucocytes). Par exemple, la

CTC-iChip peut s'utiliser avec des billes sensibilisées contre EpCAM ou CD45 et CD66b (Karabacak, Spuhler et al. 2014).

### 3.1.2 Propriétés physiques

Ces dernières années, de nombreuses techniques d'enrichissement indépendantes des marqueurs présents sur les cellules ont été développées pour l'isolement et la détection des CTCs. Les processus d'enrichissement sans marquage mais basés sur des propriétés physiques des cellules, telles que la densité, la taille, la déformabilité et les charges électriques, sont apparus pour éviter les biais induits par la variabilité de l'expression des biomarqueurs cellulaires associée à l'hétérogénéité tumorale.

#### 3.1.2.1 Sélection par densité

La centrifugation en gradient de densité est l'une des premières méthodes utilisées pour l'isolation de CTCs (Seal 1959). Cette méthode, Ficoll-Paque®, était initialement développée pour la séparation et l'isolation de cellules mononucléées. Ce processus pré-analytique non spécifique, permet l'obtention de CTCs parmi un grand nombre de leucocytes ; en effet, seuls les érythrocytes et les polynucléaires sont éliminés par une centrifugation sur gradient de Ficoll-Paque®. Pour améliorer cet enrichissement, une étape positive ou négative ultérieure est généralement requise et réalisée. Conçu pour l'isolation CTCs, OncoQuick (Greiner Bio-One) utilise un milieu de séparation liquide qui a été optimisé pour l'enrichissement spécifique des CTCs uniquement en fonction de leur densité de flottabilité dans des conditions appropriées et aucune étape supplémentaire n'est requise car même les leucocytes sont éliminés de la monocouche cellulaire entre le plasma et le Ficoll.

#### 3.1.2.2 Sélection basée sur la taille

Les technologies de microfiltration, basées sur le précédent selon lequel les CTCs présentent généralement une morphologie plus large que les leucocytes, telles que ScreenCell® (Desitter, Guerrouahen et al. 2011), ISET® de RareCell® (Farace, Massard et al. 2011), CellSieve™ (Adams, Zhu et al. 2014, Adams, Stefansson et al. 2015) ou Parsortix™ (Xu, Mao et al. 2015) impliquent une circulation de l'échantillon à travers les pores ou des canaux microfluidiques de taille calibrée pour piéger les cellules de grand taille (c'est-à-dire les CTCs) tandis que les cellules de plus petites tailles passent à travers et sont éluées puis éliminées.

Certains autres dispositifs microfluidiques basés sur la taille utilisent la focalisation inertielle pour séparer les CTCs du sang. La technologie Vortex repose sur la microfluidique inertielle et les vortex laminaires à échelle microscopique pour positionner les cellules le long des parois des canaux en amont des micro-vortex conçus pour piéger de manière stable les CTCs (Lemaire, Liu et al. 2018). De la même

manière, la technologie ClearCell FX utilise le « Dean Flow » et la focalisation hydrodynamique dans un canal courbe pour collecter les flux différentiels de CTCs et WBC (Hou, Warkiani et al. 2013). Cependant, cette méthode comprend une étape de lyse préalable des globules rouges avant l'isolation des CTCs.

De telles technologies ou approches ont l'avantage d'être peu complexes, parfois rapides, et nécessitent un équipement minimal. Cependant, certaines peuvent être sujettes au bouchage et la récupération des CTCs pour une analyse ultérieure est difficile.

### 3.1.2.3 Sélection par diélectrophorèse

Une nouvelle approche innovante pour la séparation cellulaire, la diélectrophorèse, exploite les empreintes électriques distinctes de chaque cellule. Cela prend en compte la composition (par exemple, membrane cellulaire, noyau, organites), la morphologie (par exemple, taille, forme) et le phénotype des cellules. ApoStream<sup>®</sup>, un système commercial d'enrichissement en CTC, est la première stratégie utilisant un dispositif microfluidique combinant un champ diélectrophorétique au flux de la puce pour la séparation des cellules (Balasubramanian, Kinders et al. 2017).

## 3.2 Méthodes de détection

Après enrichissement, la fraction CTCs contient encore un nombre important de leucocytes. Les CTCs doivent être spécifiquement identifiés par une méthode robuste et reproductible qui peut les distinguer des cellules sanguines normales.

### 3.2.1 Techniques immunologiques

Les CTCs peuvent être détectées en utilisant une combinaison d'anticorps membranaires et / ou intracytoplasmiques : anti-épithéliaux, anti-mésenchymateux, anti-tissu-spécifiques (Alix-Panabieres, Mader et al. 2017). Cependant, de nombreux tests 'CTC' utilisent la même étape d'identification que le système CellSearch<sup>®</sup> : les cellules sont colorées par fluorescence pour les cytokératines 8-18-19 (CK), l'antigène leucocytaire commun CD45 et un colorant nucléaire (DAPI). Grâce à l'analyse d'images multicolores avec un microscope à fluorescence, les CTCs sont définies comme des cellules CK<sup>+</sup> / CD45<sup>-</sup> / DAPI<sup>+</sup>. Bien que certains antigènes soient communs à divers types de cancer (par exemple, CK pour le cancer du sein, du côlon et de la prostate et d'autres tumeurs épithéliales), les antigènes spécifiques de tissu sont également appropriés (par exemple, PSA pour le tissu prostatique ou la mammaglobine pour le tissu mammaire).

La microscopie numérique automatisée à très haute vitesse utilisant la technologie de balayage à fibres optiques (FAST) a été développée pour détecter les CTCs marqués par des anticorps avec des

conjugués fluorescents (Krivacic, Ladanyi et al. 2004, Somlo, Lau et al. 2011). D'autres types de microscopes à balayage automatisés ont été introduits pour la détection des CTCs, du système d'imagerie Ikoniscope® ou du système Ariol® (Ntouroupi, Ashraf et al. 2008, Paillet, Oulhen et al. 2016), et montrent des résultats prometteurs qui doivent encore être validés dans de grandes études cliniques. Certains chercheurs utilisent également la technologie de cytométrie en flux, qui offre une détection et un marquage à haut débit de plusieurs cibles en même temps (jusqu'à 9 ou 12 cibles) (Lustberg, Balasubramanian et al. 2014). De plus, certains cytomètres en flux permettent le tri cellulaire pour une analyse post-détection (Zhang, Ridgway et al. 2013). En effet, la détection immunologique offre l'avantage de permettre l'isolation de CTCs selon leur marquage pour une caractérisation moléculaire ultérieure. Si l'isolation manuelle par micromanipulation des CTCs est possible (Heitzer, Auer et al. 2013), elle est plutôt ardue et prend du temps. Un dispositif automatisé de sélection et tri de cellule unique a donc été développé. La technologie DEPArray™ basée sur une stratégie de diélectrophorèse en piégeant des cellules individuelles dans des cages diélectrophorétiques (Abonnenc, Manaresi et al. 2013), est conçue pour la récupération monocellulaire des CTCs. De multiples études cliniques ont utilisé le DEPArray™ pour détecter et isoler des CTCs uniques dans le but d'effectuer des analyses génétiques approfondies (ex : séquençage après amplification du génome entier – WGA) (Fabbri, Carloni et al. 2013, Mesquita, Rothwell et al. 2017, Paolillo, Mu et al. 2017). Cependant, l'utilisation de cette technologie pour la récupération des CTCs reste limitée aux échantillons avec un nombre relativement élevé de CTCs en raison d'une perte de cellules d'environ 40% pendant le chargement de l'échantillon (Peeters, De Laere et al. 2013).

### *3.2.2 Stratégies basées sur la détection d'acides nucléiques*

Les méthodes de détection basées sur la détection d'acides nucléiques sont les alternatives aux tests immunologiques les plus largement utilisées pour identifier les CTCs. Ces techniques identifient des séquences d'ADN ou d'ARNm spécifique de tumeur pour confirmer indirectement la présence de CTCs (Esmailsabzali, Beischlag et al. 2013). Cette détection implique la conception d'amorces spécifiques censées être associées à des gènes spécifiques de CTCs. Ces gènes codent pour des protéines spécifiques d'un tissu, d'un organe ou d'une tumeur ou, plus spécifiquement, contiennent des mutations, des translocations ou des profils de méthylation connus dans les cellules cancéreuses (Riethdorf, Wikman et al. 2008).

Les méthodes basées sur la détection d'acide nucléique ont une sensibilité élevée mais manquent de spécificité, en raison des potentiels faux positifs générés par des cellules non cancéreuses résiduelles, diminuant ainsi la précision globale. Selon des travaux antérieurs, toutes les cibles de détection ne sont pas spécifiques de la tumeur, car les mêmes cibles ont pu être trouvées dans les cellules sanguines

(Fehm, Hoffmann et al. 2009, Punnoose, Atwal et al. 2010). Pantel *et coll.* ont rapporté que CK-19, un marqueur majeur de la détection de CTC, est également faiblement présent dans les cellules immunitaires (Pantel, Brakenhoff et al. 2008). Les PCR multiplex, telle que le kit AdnaTest (AdnaGen AG), peuvent surmonter cette limitation grâce à la combinaison de plusieurs cibles (Fehm, Hoffmann et al. 2009, Chebouti, Kuhlmann et al. 2017). De plus, compte tenu de l'hétérogénéité génétique des CTCs, un test PCR multiplex peut fournir une meilleure sensibilité et des taux de spécificité améliorés.

### 3.2.3 Essais fonctionnels

Les tests fonctionnels exploitent des aspects de l'activité cellulaire pour la détection de CTCs « vivantes ». En effet, seules les cellules vivantes ont une activité cellulaire fonctionnelle. Or les détections par immunofluorescence ou basées sur la détection d'acide nucléique ne peuvent pas distinguer les cellules viables et actives des cellules mortes qui ne font que transiter par la circulation. Ainsi, ces méthodes fonctionnelles ont la particularité de cibler la détection de « metastasis-competent cells » soit « des cellules compétentes à initier des métastases ». Afin de détecter uniquement les CTCs viables, le test Epithelial ImmunoSPOT (EPISPOT) a été introduit pour la détection *in vitro* de CTCs (Soler, Cayrefourcq et al. 2017). Cette technologie évalue la présence de CTCs en fonction de la sécrétion, de l'excrétion ou de la libération de protéines spécifiques pendant 24 à 48 heures de culture (Cayrefourcq, Mazard et al. 2015). La technique EPISPOT a été appliquée à des échantillons de sang et de moelle osseuse et a été validée dans plusieurs cancers différents, par exemple, les cancers du sein et du côlon avec le test EPISPOT-CK19 ou de la prostate avec le test EPISPOT-PSA (Alix-Panabieres 2012, Deneve, Riethdorf et al. 2013, Kuske, Gorges et al. 2016, Markou, Lazaridou et al. 2018). Ce test est actuellement en train d'être développé dans un format microfluidique avec micro-gouttelettes (appelé EPIDROP pour un EPISpot in a DROP) qui permettra la visualisation de toutes les CTCs présentes de manière individuelle dans leur gouttelette, plus la visualisation des cellules fonctionnelles parmi la totalité.

Un autre test fonctionnel *in vitro*, le Vita-Assay™ (Vitatex) exploite l'adhésion préférentielle des cellules invasives à une matrice spécialisée pour enrichir les CTC viables du sang (Tulley, Zhao et al. 2016). Cette méthode a également été testée dans le cancer métastatique de la prostate (Friedlander, Ngo et al. 2014) et du sein (Lu, Fan et al. 2010).

Plus récemment, Tang et coll. ont décrit un test métabolique à haut débit pour la détection rapide de cellules tumorales rares métaboliquement actives dans l'épanchement pleural et le sang périphérique de patients atteints d'un cancer du poumon (Tang, Wang et al. 2017).



*In vivo*, des informations importantes peuvent être obtenues par transplantation de CTCs provenant de patients chez des souris immunodéficientes. Les tumeurs et métastases qui se développent après xéno greffe de CTCs enrichies ont des caractéristiques des cellules initiateuses de métastases (Bacelli, Schneeweiss et al. 2013, Faugeroux, Pailler et al. 2020) et reflètent les réactions de la tumeur d'origine du patient.

### 3.3 Caractérisation des CTCs

Les CTCs détiennent la clé pour comprendre la biologie des métastases et sont un bon biomarqueur pour mesurer de manière non invasive l'évolution des sous-clones tumoraux pendant le traitement et la progression de la maladie (**Figure 9**). En effet, à l'heure actuelle, les choix pour les thérapies ciblées sont faits à partir de l'analyse de biopsies de tumeurs primaires, or celles-ci ne reflètent pas toujours parfaitement l'ensemble des clones tumoraux, et ne peuvent pas représenter l'évolution de la maladie au cours du traitement. Par exemple, Babayan *et coll.* ont montré la présence de CTCs négatives pour le récepteur aux œstrogènes (ER) chez des patients ayant un cancer du sein métastatique avec tumeur primitive ER, après une certaine période de traitement, ce qui peut refléter un mécanisme d'évasion thérapeutique (Babayan, Hannemann et al. 2013).

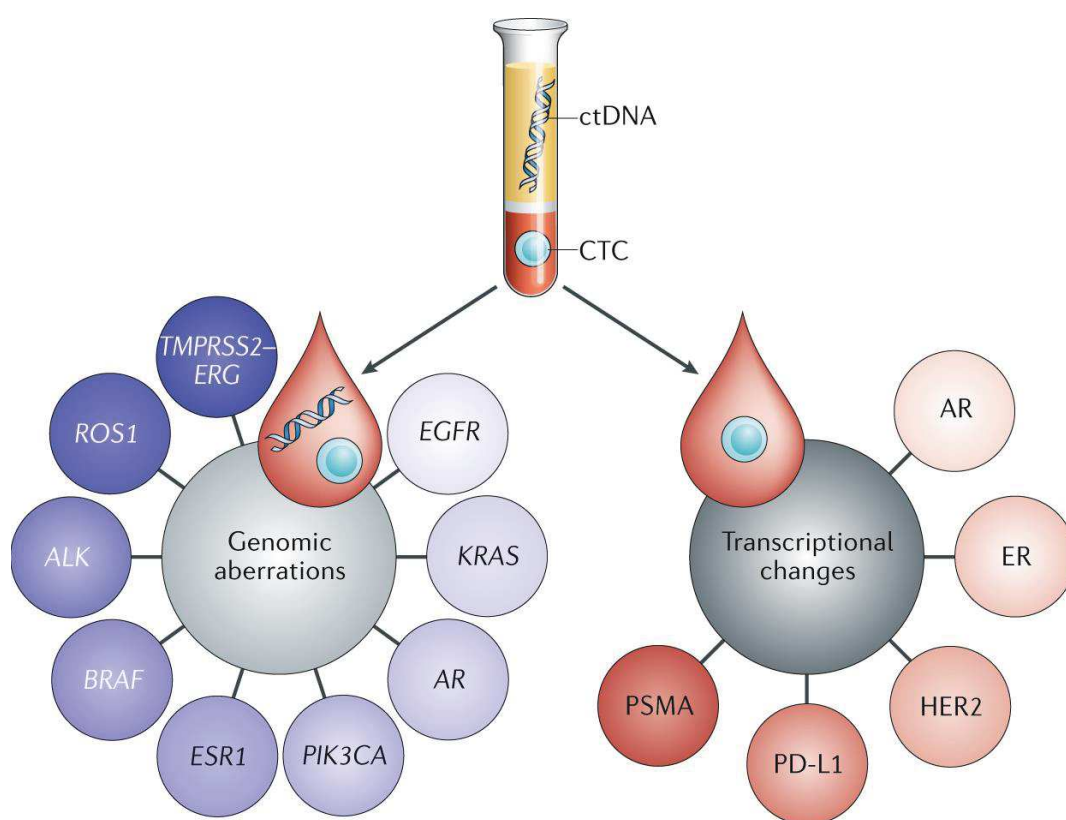


Figure 9. Représentation de cibles thérapeutiques et de mécanismes de résistance détectables sur l'ADN tumoral circulant et lors de la caractérisation des CTCs (Pantel and Alix-Panabieres 2019).

Les améliorations technologiques des dernières années permettent aujourd'hui l'analyse des CTCs à la cellule unique tant par immunofluorescence que CGH array ou séquençage nouvelle génération (NGS) de l'ADN et de l'ARN mais aussi l'hybridation *in situ* par fluorescence (FISH).

La caractérisation de la présence ou non de certaines protéines sur des CTCs est préférentiellement effectuée par immuno-marquage avec des anticorps dirigés contre la protéine d'intérêt. Un marquage multiple est possible mais généralement limité à quelques protéines d'intérêt reflétant la biologie des cellules tumorales (ex : marqueur de cellules mésenchymateuses, d'apoptose, etc...) et / ou d'intérêt pour les traitements (ex : PD-L1, Her2, etc...) (Pantel and Alix-Panabieres 2019). Il a été démontré que chez des patientes atteintes d'un cancer du sein, le statut Her2 des CTCs montre des divergences avec le statut de la tumeur primaire (Riethdorf, Muller et al. 2010, Jaeger, Neugebauer et al. 2017). Ainsi les patientes dont la tumeur primitive apparaît négative pour HER2 pourraient bénéficier d'un traitement par thérapie ciblée si elle a des CTCs Her2+. Plus récemment, les régulateurs de point de contrôle immunitaire tels PD-L1 sont devenus de nouvelles cibles thérapeutiques passionnantes. L'évaluation de leur présence chez les CTCs pourraient être analysés pour stratifier les patients pour qui ces thérapies seraient bénéfiques ou en suivie d'efficacité chez les patients traités par de telles thérapies (Mazel, Jacot et al. 2015, Topalian, Drake et al. 2015). Récemment, Jacot *et coll.* ont démontré que les CTCs PD-L1<sup>(+)</sup> corrélaient avec la survie des patientes dans le cancer du sein métastatique contrairement aux au statut PD-L1 des tumeurs primaires (Jacot, Mazel et al. 2020). Cela suggère que le « statut biologique » des CTCs pourrait être exploité pour la stratification des patients et guider les stratégies de traitement anti-PD-1 / PD-L1 en onco-immunothérapie.

La détection et la caractérisation immunologiques des CTCs offrent l'avantage de permettre l'isolation des différentes sous population de CTCs pour une caractérisation moléculaire ultérieure. Si l'isolation manuelle par micromanipulation est possible (Heitzer, Auer et al. 2013), elle est assez longue et difficile. Un nouveau dispositif automatisé de sélection et tri de cellule unique a été développé. La technologie DEPArray<sup>™</sup> basée sur une stratégie de diélectrophorèse (DEP) piégeant des cellules individuelles dans des cages DEP (Abonnenc, Manaresi et al. 2013), est conçue pour la récupération monocellulaire de CTCs. Plusieurs études cliniques ont utilisé le DEPArray<sup>™</sup> pour détecter et récupérer des CTCs uniques et ont démontré la faisabilité d'analyses génétiques ultérieures (Fabbri, Carloni et al. 2013, Mesquita, Rothwell et al. 2017, Paolillo, Mu et al. 2017).

Parmi les analyses moléculaires réalisables sur cellule unique pour identifier les caractéristiques génomiques et transcriptomiques des CTCs, la plupart des études se sont concentrées sur les analyses génomiques après amplifications du génome entier (WGA) pour augmenter la quantité d'ADN. Cette méthode permet par la suite l'analyse de mutations spécifiques et de variations du nombre de copies

(CNV) utilisant des technologies de séquençage conventionnelles et de nouvelle génération (Heitzer, Auer et al. 2013, Sho, Court et al. 2017, Paoletti, Cani et al. 2018). À titre d'exemple, les CTCs possédant une mutation du gène KRAS peuvent échapper aux thérapies anti-EGFR et leur détection précoce pourrait aider à guider la thérapie chez les patients.

Une autre approche pour la caractérisation des CTCs est l'analyse par FISH de cellules préalablement identifiées par immunocytochimie (Obermayr, Bednarz-Knoll et al. 2017, Podolak, Eilers et al. 2017). Une telle approche immuno-FISH peut être combinée avec la détection automatisée des CTCs et pourrait être plus facile à mettre en œuvre dans les futurs diagnostics cliniques. Plus récemment, la technologie de sonde Padlock, qui permet une analyse *in situ* de l'AR-V7 dans les CTCs, a montré que 71% (22 sur 31) des patients CRPC avaient une expression détectable d'AR-V7 allant d'une expression faible à élevée (El-Heliebi, Hille et al. 2018). Les patients avec des CTCs AR-V7<sup>+</sup> bénéficient plus d'un traitement par chimiothérapie à base de taxane par rapport aux nouvelles thérapies hormonales, indiquant l'efficacité de ce biomarqueur pour la sélection du traitement (Antonarakis, Lu et al. 2015, Scher, Lu et al. 2016).

Enfin, ces dernières années, de nombreuses équipes ont tenté d'obtenir des lignées de CTCs en cultivant des CTCs *ex vivo* (*in vitro* et chez la souris immunodéficiente). En effet, les lignées 'CTC' pourraient être utilisées pour identifier les protéines et les voies de signalisation impliquées dans la dissémination des cellules cancéreuses, ainsi que pour tester de nouveaux médicaments permettant de cibler les CTCs initiatrices de métastases. L'établissement de cultures *in vitro* et de lignées permanentes à partir de CTCs est une tâche difficile et nous y reviendrons dans ce manuscrit.

### 3.4 Intérêt clinique

Malgré de nombreuses études de validation clinique, les CTCs n'ont pas encore été incluses dans les lignes directrices cliniques (par exemple, les lignes directrices de l'ASCO à <http://www.asco.org/practice-guidelines/quality-guidelines/guidelines>). Bien que l'analyse des CTCs puissent contribuer à la détection précoce des lésions métastatiques et participer au traitement personnalisé des patients tels que l'évaluation pronostique, la stratification des patients pour des thérapies ciblées, le suivi en temps réel de l'efficacité du traitement, l'identification de cibles thérapeutiques et de mécanisme de résistance (**Figure 10**) (Alix-Panabières and Pantel 2016), l'utilité clinique des CTCs reste à être abordée dans les études interventionnelles (c'est-à-dire sa capacité à décider d'adopter ou de rejeter une action thérapeutique).

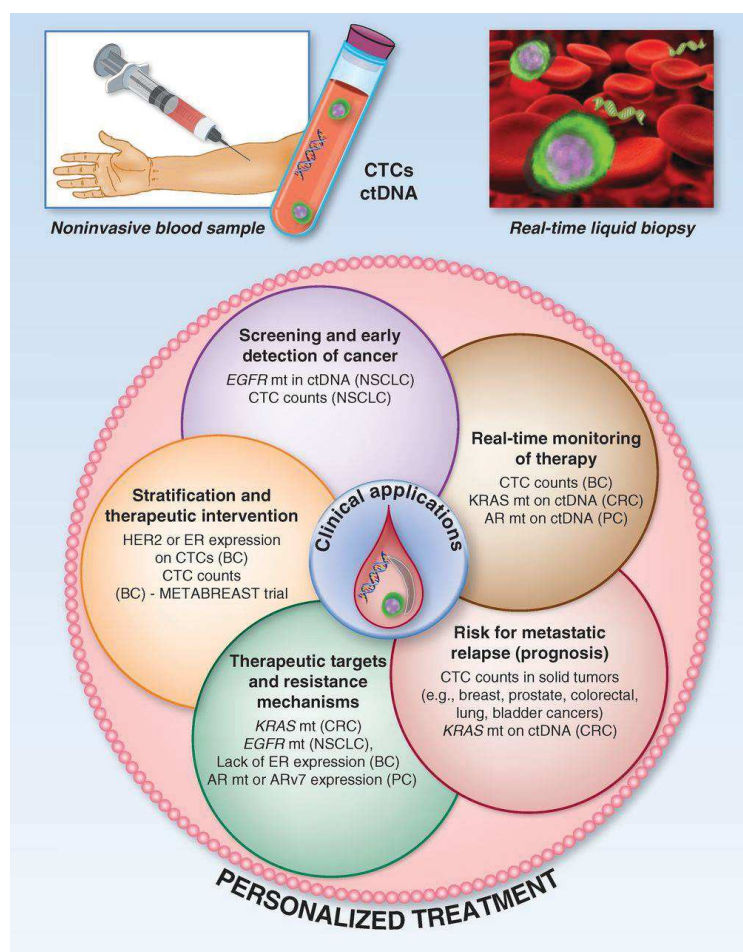


Figure 10. Représentation de l'intérêt clinique des CTCs pour la médecine personnalisée (Alix-Panabières and Pantel 2016).

Les études les plus avancées concernant la validité clinique des CTCs, ont été effectuées dans le cancer du sein métastatique (MBC). En effet, le dénombrement séquentiel de CTCs a été démontré dans une grande étude pronostique multicentrique comme étant supérieur aux marqueurs conventionnels des protéines sériques (CA-15-3, CEA) pour la détection précoce de l'échec thérapeutique dans le MBC (Bidard, Peeters et al. 2014). Cependant, dans l'essai interventionnel SWOG 0500 (NCT00382018), bien que la signification pronostique des CTCs ait été confirmée, le changement de traitement pour une thérapie cytotoxique alternative selon l'évolution du nombre de CTCs, n'a pas montré d'efficacité pour prolonger la survie globale des patients MBC (Smerage, Barlow et al. 2014). Cependant le biais de ce type d'études interventionnelles, basées sur les modifications de traitement selon des biomarqueurs, est le fait que le résultat dépend de l'efficacité thérapeutique. Cette stratégie ne peut fonctionner que s'il existe une thérapie efficace pour la cohorte identifiée par le test.

L'approche la plus prometteuse actuellement est la stratification de patients vers la chimiothérapie (CT) ou l'hormonothérapie (HT) basée sur le dénombrement des CTCs grâce à l'essai clinique

interventionnel STIC CTC METABREAST (NCT0 1710605). Les premiers résultats présentés lors de l'AACR de 2019 ont révélé que chez les patients du bras CTC avec HT planifiée modifiée en CT en raison du nombre élevé de CTCs, la survie sans progression (SSP) était significativement plus longue que dans le bras standard (choix de traitement clinique), et avec une tendance non significative vers une survie globale (SG) plus longue. À l'inverse, chez les patients du bras CTC avec CT planifié modifié en HT en raison d'un faible nombre de CTCs, la SSP n'était pas statistiquement différente par rapport au bras standard (Bidard, Jacot et al. 2019). Il s'agit d'une étude clé car elle démontre pour la première fois l'utilité clinique des CTC détectés par le système CellSearch® dans le cancer du sein métastatique. Actuellement, des études similaires sont en cours pour le cancer de la prostate (CP). Par exemple, l'essai TACTIK (NCT03101046) recrute des patients pour démontrer l'utilité clinique du dénombrement de CTCs pour la détection de l'apparition d'une résistance au docétaxel, dans le CP métastatique résistant à la castration. Dans la même dynamique, l'essai CTC STOP (NCT03327662) compare le dénombrement des CTCs et le taux de PSA pour déterminer la réponse au traitement dans le CP métastatique résistant à la castration.

Dans le CCR, le nombre d'étude portant sur l'intérêt clinique des CTCs est relativement faible comparé à d'autres cancers majeurs comme, le sein, la prostate ou le poumon. En 2008, Cohen *et coll.* ont démontré la valeur pronostique et prédictive indépendante des CTCs pour les patients débutant une chimiothérapie dans le cadre d'un CCRm (Cohen, Punt et al. 2008). Depuis cette première publication définissant un seuil de 3 CTCs, différentes méta-analyses ont confirmé que les niveaux de base du nombre de CTCs sont un facteur pronostique important pour la SSP et la SG chez les patients atteints de CCRm (Rahbari, Aigner et al. 2010, Groot Koerkamp, Rahbari et al. 2013, Huang, Gao et al. 2015).

Malgré les preuves solides d'une signification pronostique du nombre de CTCs, il n'a pas été fait de démonstration de l'intérêt du nombre de CTCs pour la stratégie thérapeutique, et ce biomarqueur est rarement utilisé dans la prise en charge des patients atteints de CCRm. Cependant, il existe actuellement une étude de phase II dans laquelle les patients recrutés ayant un nombre élevé de CTCs pourraient bénéficier d'un schéma chimiothérapeutique plus intense (Krebs, Renehan et al. 2015). Ces données préliminaires nécessiteront une validation dans des essais randomisés.

#### 4. Les lignées de cellules tumorales circulantes

Ces dernières années la révolution de l'analyse génomique à haut débit a ouvert une nouvelle ère dans la recherche translationnelle sur le cancer permettant l'analyse et la caractérisation d'évènement rare. Néanmoins, la dissémination tumorale est le résultat d'un processus de sélection qui favorise la survie d'une petite proportion de cellules cancéreuses possédant la capacité d'auto-renouvellement des cellules souches ainsi que des propriétés initiatrices de métastases, qui leur permet de coloniser des

organes distants. La compréhension du processus métastatique et le développement de nouvelles stratégies thérapeutiques nécessitent des modèles précliniques permettant l'étude de la fonctionnalité tumorale (**Figure 11**). Les modèles *ex vivo* représentent pour cela le modèle idéal et peuvent être utilisés pour comprendre la biologie cellulaire et les mécanismes de la maladie et également pour le développement de traitements.

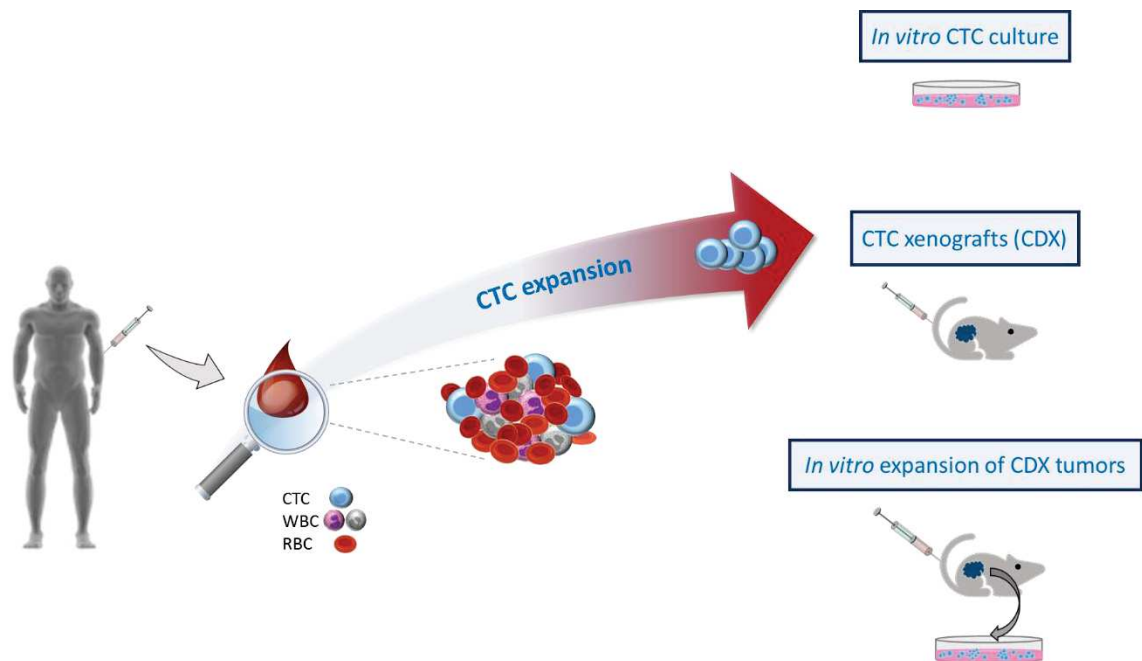


Figure 11. : Approches utilisées pour la culture de CTCs *ex vivo*. Adapté de (Cortés-Hernández, Eslami et al. 2020)

#### 4.1 Culture de CTCs *in vivo*

Les premières cultures de CTCs *ex vivo* ont été effectuées lors de xénogreffes, en effet en inoculant une fraction enrichie en cellules tumorales à partir de sang chez des souris immunodéficientes afin d'observer, si oui ou non, il y a une prolifération des cellules et la formation de tumeur, on génère une culture de CTCs *in vivo*. Dans un premier modèle de xénogreffe, Baccelli *et coll.* ont injecté des CTCs provenant de patients atteints d'un cancer du sein métastatique dans des souris immunodéficientes et ont obtenu un développement tumoral avec des métastases osseuses, pulmonaires et hépatiques (Baccelli, Schneeweiss et al. 2013). Ces métastases exprimaient uniformément EpCAM, CD44, CD47 et MET, des marqueurs connus chez les cellules souches et corrélant avec un mauvais pronostic clinique. À l'aide de ce modèle *in vivo*, ils ont démontré l'existence de cellules initiatrices de métastases dans des sous populations de CTCs chez des patients ayant un cancer du sein. Cependant, ces modèles n'ont pu être obtenus uniquement que chez des patients de stade avancé avec plus de 1000 CTCs (dénombrés avec le système CellSearch®).

Dans une autre étude, Rossi *et coll.* ont étudié le potentiel des CTC EpCAM<sup>(+)</sup> (Dénombrement et enrichissement basés sur le système CellSearch®) à développer des tumeurs chez la souris immunodéficiente avec des patients ayant un cancer métastatique du sein ou de la prostate (Rossi, Rugge et al. 2014). Aucune souris n'a développé de tumeur visible entre 6,5 et 12 mois après injection de CTCs, mais des CTCs humaines ont pu être détectées dans le sang périphérique, la moelle osseuse et la rate des souris, même chez celles ayant reçu une faible quantité de CTCs (n = 50).

Les patients atteints d'un cancer du poumon à petites cellules (SCLC) présentent des taux de CTCs les plus élevés parmi toutes les tumeurs solides (cut-off CellSearch à 50 CTCs vs 5 CTCs pour le cancer du sein ou de la prostate), ce qui constitue les meilleures conditions pour développer des modèles de culture *in vivo* (Hou et al. 2012). Hodgkinson *et coll.* ont démontré que les CTCs provenant de patients ayant un SCLC sont tumorigènes chez les souris immunodéficientes, et les xénogreffes dérivées de CTCs reflètent la réponse du patient à la chimiothérapie (Hodgkinson, Morrow et al. 2014). Ce modèle représente une opportunité pour les tests de sensibilité thérapeutique et pourrait permettre d'évaluer la réponse médicamenteuse du patient. Cependant, ces modèles sont longs à obtenir et ne sont permis qu'à partir d'échantillons de sang avec un nombre élevé de CTCs. Le même groupe a également généré un modèle de xénogreffe à partir d'un patient non-fumeur ayant un cancer métastatique du poumon mais non à petites cellules cette fois-ci (Morrow, Trapani et al. 2016). Dans ce cas, les CTCs ont été dénombrées avec le CellSearch® et enrichies à l'aide de la Plateforme ISET® (enrichissement basé uniquement sur la taille des CTCs). De cette façon, ils ont démontré que des xénogreffes peuvent être générées à partir de CTCs isolées d'échantillons de sang dans lesquels il n'y avait pas de cellules EpCAM<sup>(+)</sup>/CK<sup>(+)</sup> détectables.

Plus récemment, Drapkin *et coll.* ont développé en parallèle des xénogreffes d'échantillons de tissus et de CTCs à partir de mêmes patients atteints de SCLC (n=17) (Drapkin, George et al. 2018). Le séquençage du génome entier des modèles obtenus a démontré une similitude des caractéristiques génomiques dans les tumeurs originales et dans les modèles issus de tissus et de CTCs. De plus, l'analyse *in vivo* de la sensibilité aux médicaments a montré que le développement d'une résistance correspondait à une expression accrue de MYC. Enfin, les modèles de xénogreffes générés à partir de CTCs du même patient à différents stades de la maladie ont mimé les changements de sensibilité aux médicaments du patient au fil du temps.

Un groupe a également généré un modèle à partir du produit de cytophérèse (DLA) d'un patient ayant un cancer de la prostate résistant à la castration (CRPC). L'analyse des DLA a été initiée dans le cadre du programme européen FP7 'CTCTrap' qui visait à augmenter le rendement en CTCs pour effectuer la caractérisation moléculaire de la tumeur (Andree, Mentink et al. 2018). La xénogreffe s'est développée

encore une fois à partir d'un échantillon contenant un nombre très élevé de CTCs (~ 20 000 CTCs) et son analyse a mis en évidence l'acquisition de facteurs génétiques clés (c.-à-d. TP53, PTEN et RB1) qui régissent la trans-différenciation du CRPC en tumeur maligne CRPC-neuroendocrine (CRPC-NE) (Faugeroux, Pailler et al. 2020).

Les cultures de CTCs *in vivo* sont un bon système pour évaluer la tumorigénicité des CTCs, mais ils ont certaines limites. En effet, l'utilisation d'animaux immunodéficients, dans lesquels le système immunitaire n'est pas développé par définition, induit un biais car il ne parvient pas à imiter pleinement la physiologie des patients et ils ne peuvent pas être utilisés pour les études dans lesquelles il est important d'évaluer le système immunitaire. Les méthodes d'injection des CTCs posent également problème car elles sont invasives et l'acte en lui-même peut affecter la santé et la survie des animaux. Il est aussi nécessaire d'avoir des nombres élevés de CTCs pour établir des xénogreffes. De plus, les délais pour obtenir ces xénogreffes sont long, au minimum 5 semaines (mais cela peut être beaucoup plus long). Ainsi, cette méthode n'est pas adaptée pour le traitement personnalisé des patients en temps réel.

#### 4.2 Expansion *in vitro* de cellules tumorales de xénogreffes générées par des CTCs

La culture à court terme de cellules dérivées de xénogreffe peut être utilisée pour tester de nouvelles thérapies anticancéreuses, identifier de nouveaux biomarqueurs et étudier mécanismes de réaction aux traitements. De plus cette méthode représente une alternative plus rapide et moins coûteuse que le maintien d'animaux. Cette approche a été utilisée dans le SCLC et ces cellules ont conservé les caractéristiques génomiques des tumeurs des donneurs ainsi que leurs profils de sensibilité aux médicaments (Lallo, Schenk et al. 2017).

Le groupe ayant généré un modèle à partir du produit de DLA d'un patient CRPC a également dérivé une culture *ex vivo* permanente à partir de ce modèle. La lignée cellulaire dérivée *in vitro* obtenue récapitule fidèlement les caractéristiques génétiques et la tumorigénicité du modèle *in vivo* et mime la réponse du patient aux traitements standard de soins (Faugeroux, Pailler et al. 2020).

Dans l'ensemble, ces résultats indiquent que les cultures de cellules de xénogreffe conservent la plupart des caractéristiques des tumeurs. Cependant, au fil du temps, certaines différences pourraient survenir en raison du changement majeur d'environnement. Néanmoins, cette approche peut être utilisée pour réduire les coûts et gagner du temps lors d'étude d'efficacité de nouveaux traitements.

#### 4.3 Culture de CTCs *in vitro*

Le modèle *in vitro* offre un modèle plus simple et généralement moins coûteux et chronophage que les modèles *in vivo*. De plus, il permet un contrôle de l'environnement expérimental ainsi que



l'évaluation de différentes combinaisons de paramètres, ce qui n'est pas facilement réalisable dans les études *in vivo*. Au cours de la dernière décennie, différentes stratégies ont été utilisées pour l'expansion de CTCs sur des périodes plus ou moins longues. Mais la plupart des études se sont concentrées sur des cultures de CTCs à court terme, et seulement quelques laboratoires ont réussi à maintenir des cultures de CTCs à long terme.

En 2013, Zhang *et coll.*, ont été les premiers à décrire l'établissement de cultures primaires à partir de CTCs isolées chez des patientes ayant un cancer du sein métastatique. Les CTCs ont été isolées par cytométrie de flux selon leur phénotype CD45<sup>-</sup>, ALDH1<sup>+</sup> et EpCAM<sup>+/-</sup>. Ils ont pu obtenir 3 lignées de CTCs mammaires appelées CTC-1, CTC-2, CTC-3 établies à partir de 3 patientes différentes mais seule la population ALDH<sup>+</sup>/EpCAM<sup>-</sup> a pu être maintenue plus de 14 jours en culture. L'analyse de ces cellules leur ont permis d'identifier une signature de métastases cérébrales : HER2<sup>+</sup>/EGFR<sup>+</sup>/HPSE<sup>+</sup>/Notch1<sup>+</sup>. Lorsque ces cellules tumorales ont été injectées par voie intracardiaque ou dans la veine caudale de souris immunodéficientes, elles ont formé des tumeurs dans le cerveau et les poumons 6 semaines après injection, montrant l'agressivité de ces cellules. Malheureusement, les auteurs ne décrivent pas le temps maximum de culture de ces lignées et précisent seulement qu'elles ont été maintenues plus de 21 jours (Zhang, Ridgway et al. 2013).

Toujours dans le cancer du sein, Yu *et coll.* ont établi des lignées CTCs à plus long terme en utilisant des échantillons de sang de six patientes atteintes d'un cancer du sein positif aux récepteurs des œstrogènes (RE). Pour isoler les CTCs, la CTC-iChip<sup>®</sup> a été utilisée puis les cellules ont été cultivées en hypoxie dans des conditions non adhérentes pendant plus de 6 mois. Trois des cinq lignées CTCs testées étaient tumorigènes *in vivo*, donnant lieu à des tumeurs présentant des similitudes histologiques et immuno-histochimiques avec la tumeur primaire du patient. Une analyse du génome entier par séquençage a confirmé des mutations préexistantes dans la tumeur primaire et de nouvelles au niveau du gène PIK3CA ainsi que des mutations du gène ER, ESR1, et du récepteur du facteur de croissance, FGFR2. Ceci démontre l'importance de surveiller l'évolution mutationnelle de la tumeur tout au long de la maladie. De plus, ces lignées CTCs avec des profils mutationnels différents ont montré une réponse différente aux tests de viabilité aux drogues anti-cancéreuses (Yu, Bardia et al. 2014).

Très récemment, Koch *et coll.* ont décrit l'obtention d'une première lignée permanente dans le cancer du sein. Cette lignée est issue d'une patiente ayant un cancer du sein métastatique RE<sup>+</sup> résistante aux thérapies endocrines. Nommée CTC-ITB-01, cette lignée CTCs a conservé son phénotype RE<sup>+</sup> en culture, et le profil CNV est resté concordant avec celui des CTCs présentes chez la patiente au moment du prélèvement sanguin. Les données de séquençage de l'ARN indiquent que CTC-ITB-01 a une

signature d'expression principalement épithéliale, et cette lignée est tumorigène chez des souris immunodéficientes (Koch, Kuske et al. In press).

Dès 2014, des modèles de culture 3D pouvant mieux mimer le microenvironnement tumoral ont été proposés dans le cancer de la prostate et du poumon. Dans le cancer de la prostate, Gao *et coll.* ont généré sept lignées organoïdes dont une à partir de CTCs d'un patient présentant plus de 100 CTCs dans 8 ml de sang. L'analyse du séquençage de l'exome entier a montré que tous les modèles 3D récapitulaient la diversité moléculaire des sous-types de cancer de la prostate et se prêtaient à des tests pharmacologiques. Les xénogreffes des organoïdes *in vivo* ont abouti à des tumeurs possédant un profil histologique similaire à celui des tumeurs primaires (Gao, Vela et al. 2014). Dans le cancer du poumon, Zhang *et coll.* ont développé un nouveau modèle de capture et culture de CTCs *ex vivo* en utilisant un système de co-culture 3D qui recrée un micro-environnement propice à la croissance tumorale. Les CTCs sont enrichies et amplifiées pendant une courte période de 14 jours maximum puis libéré pour analyse. Ils sont parvenus à amplifier les CTCs de 14/19 patients atteints d'un cancer du poumon à un stade précoce. Le séquençage NGS de ces cultures a détecté plusieurs mutations, y compris TP53, trouvées à la fois dans des CTCs en culture et des tumeurs primaires de patients (Zhang, Shiratsuchi et al. 2014).

Depuis la description des premières lignées de CTCs de nombreuses équipes ont tenté d'obtenir de telles lignées dans différents cancers (ORL (Kulasinghe, Perry et al. 2016, Liao, Hsieh et al. 2019), Pancréas (Arnoletti, Fanaian et al. 2018, Rivera-Báez, Lohse et al. 2020)), en utilisant des échantillons concentrant plus de CTCs comme les DLAs (Franken, Driemel et al. 2019) et en créant de nouveaux dispositifs spécialisés dans la culture de CTCs (Khoo, Greci et al. 2018). Cependant, toutes les études publiées ne concernent que des cultures à court terme (ou cultures primaires) et, pour l'essentiel seulement, reproduisent les premiers résultats obtenus, à savoir les lignées CTCs sont représentatives de l'hétérogénéité tumorale de la tumeur primaire et elles reflètent la réaction tumorale des patients face aux traitements.

Dans le CCR, notre équipe a été la première à décrire l'établissement d'une lignée permanente de CTCs et la seule à avoir plusieurs lignées CTCs représentant longitudinalement la maladie colorectale. A notre connaissance ces lignées CTCs sont les seules existantes pour le CCR. Récemment, une nouvelle étude a mis en évidence l'obtention de lignées CTCs du tract gastro-intestinal (Brungs, Minaei et al. 2020). Deux lignées CTCs permanentes issues de patients atteints d'un cancer gastro-œsophagien, dénommées UWG01CTC et UWG02CTC, ont démontré une croissance tumorale rapide chez des souris immunodéficientes ainsi que des profils génotypiques et phénotypiques distincts qui sont cohérents avec les tumeurs d'origine. UWG02CTC présente un phénotype EpCAM<sup>+</sup>, cytokératine<sup>+</sup>, CD44<sup>+</sup>, tandis

que UWG01CTC, qui a été dérivé d'un patient atteint d'un cancer neuroendocrinien métastatique, présente un phénotype EpCAM-, cytokératine faible, avec une forte expression de marqueurs neuroendocriniens. En outre, les deux lignées CTCs présentent des différences distinctes de sensibilité aux médicaments et aux radiations qui correspondent aux voies d'expression génique différentielles associées au cancer. Cette preuve impliquant des CTCs négatives pour l'expression d'EpCAM dans la formation de métastases, ne représente cependant pas la majorité des cellules tumorales capables d'initier des métastases à distance (Eslami, Cortés-Hernández et al. 2020).

## 5. La technologie Affymetrix™

Nous utilisons au laboratoire la technologie Affymetrix™ pour effectuer les études transcriptomiques des CTCs (voir partie résultats). Les microarrays Affymetrix sont de plus en plus utilisées par les équipes de recherche car elles sont dotées d'une très bonne reproductibilité et rendent possible la comparaison des données inter laboratoires. Les microarrays Affymetrix™ HG-U133 Plus 2.0 sont dites « pangénomique » puisqu'elles permettent la mesure du niveau d'expression de ≈ 47 000 transcrits couvrant l'essentiel des 30 000 gènes humains (Le Brigand, Russell et al. 2006).

Pour le principe : La puce à ADN Affymetrix, est constituée d'oligonucléotides synthétisés directement sur un substrat solide par photolithographie. Dans ce procédé, une lumière dirigée sur des sites spécifiques de la puce active la réaction d'oligo-synthèse. Les sondes sont des oligonucléotides courts composés de 25 paires de bases synthétisées *in situ* par des dépôts de couches successives de quatre nucléotides (c'est-à-dire les empilements de bases A, T, C, G, dans l'ordre choisi). La technique débute par l'attache à la surface de la puce de liaisons synthétiques munies de groupes chimiques qui peuvent être enlevés sous l'effet de la lumière. On envoie ensuite de la lumière à certaines localisations définies via un masque photolithographique afin de déprotéger les liants. Le rajout de déoxynucléosides avec un groupe photolabile permet la liaison avec les groupes déprotégés. Un autre masque dont la configuration varie pour chaque couche déposée et qui assure ainsi une succession correcte des bases est ensuite mis en place et ainsi de suite jusqu'à la synthèse complète des oligonucléotides. L'emplacement des différentes sondes oligonucléotidiques sur la puce et l'enchaînement des bases qui les composent sont très précisément connus. L'ARN total est transformé en ADNc, avec incorporation du promoteur de la T7 ARN polymérase. Une ADN-polymérase transforme cet ADNc simple brin en ADNc double brin. Celui-ci est alors transcrit en ARN grâce à la T7 ARN polymérase en présence de ribonucléotides biotinylés puis hybrider sur la puce après fragmentation.

Les puces à ADN fournissent d'abondantes quantités de données. Après traitement des données grâce au logiciel Affymetrix (Transcriptome Analysis Console (TAC)), les résultats sont collectés sous la forme

d'un tableau avec autant de lignes que de sondes présentes sur la puce. Par conséquent, il est nécessaire d'en extraire une information accessible, intelligible et intuitive. Pour cela, on fera appel à des outils biostatistiques. L'étape de l'analyse est une étape appréhendée par les biologistes, car elle est considérée comme la plus décourageante.

L'intérêt de cette technique réside dans son efficacité et sa vitesse d'obtention des résultats ainsi que dans sa capacité à étudier la biologie d'un système dans sa globalité. L'établissement de signatures moléculaires, fondées sur les puces à ADN, offre de nombreux espoirs pour améliorer les outils diagnostiques dans le cas des CTCs et les thérapies ciblées. De plus en plus d'essais cliniques incluent des projets utilisant des puces à ADN pour identifier des profils d'expression spécifiques des pathologies étudiées, des signatures prédisant la sensibilité à un médicament, de nouvelles cibles thérapeutiques ou de nouveaux biomarqueurs (Riker, Enkemann et al. 2008, Huet, Tesson et al. 2018, Bruinius, Dykema et al. 2019, Lin, Li et al. 2019).



## Objectifs du travail de thèse

Le but de mon projet de thèse était de contribuer à la compréhension des mécanismes du processus tumoral par l'analyse approfondie du matériel unique que représente les lignées de CTCs et potentiellement de relever certains biomarqueurs d'intérêts pour le traitement personnalisé du CCR. Ce travail s'articule autour de trois axes de recherche :

- Dans **la première partie** de ma thèse, je résume nos efforts fournis pour obtenir et caractériser les 9 lignées CTCs coliques obtenues au cours de la progression du cancer et sous la pression des différents traitements jusqu'au décès du patient.

- Dans la **deuxième partie** de ma thèse, je rapporte les résultats obtenus lors d'une étude approfondie du transcriptome de ces 9 lignées de CTCs. La première étude traite de la comparaison de la première lignée, CTC-MCC-41, à la lignée commerciale non agressive HT-29 et à des lignées cellulaires cancéreuses coliques commerciales issues de tumeurs primaires et de métastases, pour mettre en évidence les caractéristiques spécifiques de ce matériel unique. L'analyse du transcriptome de toute les lignées CTC et la comparaison selon le moment d'obtention au cours de la maladie, effectuée dans la deuxième étude, montre l'évolution clonale due à la pression de sélection des traitements et la progression de la maladie. Enfin la troisième étude décrit l'analyse des transcrits impliqués dans les processus d'EMT et de MET dans les lignées CTC en comparaison avec la lignée colique primaire HT-29 et la lignée colique métastatique SW620.

- La **troisième et dernière partie** de ma thèse traite d'un travail effectué en collaboration avec l'équipe du Pr Manfred Jücker à Hambourg pour l'analyse de la sensibilité de la lignée CTC-MCC-41 aux inhibiteurs de d'AKT et mTOR. Ces voies de signalisation étant dérégulées chez les lignées CTCs.



# Résultats







## PARTIE 1 – Obtention et caractérisation des lignées CTCs

Précédemment, nous avons vu que l'obtention de lignées CTCs est devenu un challenge dans la recherche sur le cancer et plus particulièrement dans la compréhension de la biologie de la cascade métastatique car ces lignées cellulaires permettent d'étudier en profondeur ces cellules potentiellement initiatrices de métastases. Nous avons également compris qu'il était extrêmement difficile d'obtenir de telles lignées cellulaires cancéreuses et surtout d'être capable de trouver les conditions adéquates pour les maintenir sur du long terme.

Dans ce premier chapitre, je décris nos efforts mis en œuvre pour parvenir à l'obtention de nos 9 lignées de CTCs coliques ainsi que la première caractérisation que nous avons effectuée pour confirmer l'origine tumorale et appréhender les caractéristiques spécifiques confirmant ou non les hypothèses du processus métastatique.

Ainsi l'*article 1* décrit (i) les conditions de génération de la première lignée CTC colique obtenu chez un patient ayant un CCRm avant l'initiation de son traitement, dans le cadre d'un projet national : COLOSPOT (NCT01596790), et (ii) sa caractérisation *in vitro* et *in vivo* avec l'injection de cette lignée CTC chez des souris immunodéficientes. L'*article 2* détaille (i) l'obtention des 9 lignées CTC chez un même patient CRCm au cours de la progression et du traitement de son cancer et (ii) d'une caractérisation *in vitro* plus complète de ces 9 lignées CTCs.

L'obtention de ces lignées CTCs a été possible dans le cadre du projet COLOSPOT, dans lequel ont été inclus des patients ayant un CCRm non résécable, débutant une première ligne de traitement par FOLFIRI (5FU+ Irinotecan) + Avastin (anti-VEGF). L'étude principale du projet était l'énumération des CTCs pour l'évaluation précoce de progression de la maladie et les essais de culture *ex vivo* représentaient l'étude ancillaire. Parmi les 167 patients inclus dans l'étude (71 au moment de la publication de l'*article 1*), seuls les échantillons sanguins de 2 patients ont permis la culture primaire de CTCs. Ces 2 prélèvements correspondent aux prélèvements dénombant le plus grand nombre de CTC avec la technique CellSearch®, reflétant l'extrême difficulté d'isoler spécifiquement ces cellules initiatrices de métastases.

En nous basant sur des études sur les cellules souches colorectales, nous avons choisi de cultiver les cellules en hypoxie (condition favorable au développement tumoral et à la prolifération des cellules souches) dans un milieu pour cellules souches (*Milieu 1*: DMEM/F12 + insuline (20 µg/mL), complément N2 (1%) + L-Glutamine (2mM) + EGF (20 ng/mL) + FGF2 (10 ng/mL) + sérum (2%)). Dans ces conditions, la première lignée CTC n'a pas pu être conservée plus de 2 mois et seule une analyse

par les cytopathologistes a pu confirmer l'origine tumorale. Concernant la seconde lignée, nommée CTC-MCC-41, après démarrage de la prolifération cellulaire, dans les conditions 1, nous avons testé une deuxième condition de culture en normoxie dans un milieu spécifique pour la culture de cellules tumorales disséminées (*Milieu 2* : RPMI + L-Glutamine + ITS + EGF + FGF + 10% sérum). Dans ces conditions 2, les cellules ont montré une croissance plus rapide et ont pu être cultivées plusieurs mois consécutifs sans signe de mort cellulaire. Ceci confirme l'importance de choisir les bonnes conditions de cultures permettant la prolifération de ces cellules initiatrices de métastase.

Le projet COLOSPOT permettait également d'obtenir des échantillons sanguins de suivi, ainsi nous avons pu tester la culture de CTCs au long de la progression tumorale et de la prise de traitement. Nous avons donc obtenu une seconde lignée, nommée CTC-MCC-41.4, après échec de la deuxième ligne de traitement par FOLFOX (5FU + Oxaliplatine) + Avastin et 7 lignées supplémentaires, nommées CTC-MCC-41.5 [A-G], avant le décès du patient. Ces 8 lignées consécutives ont été obtenues directement dans les conditions de culture 2, signifiant que l'hypoxie n'est pas obligatoirement nécessaire pour stimuler la prolifération de ces cellules immédiatement après les avoir isolées du sang.

La comparaison des différentes lignées CTCs coliques avec la tumeur primaire correspondante a révélé une homogénéité des caractéristiques principales de ces cellules : elles possèdent principalement des propriétés de cellules épithéliales colique (EpCAM, CK20), et avec un profil mutationnel identique à celui de la tumeur primaire (KRAS non muté et BRAF muté V600E). L'analyse de certaines caractéristiques décrites chez les CTCs, comme la présence de marqueurs de cellules souches (CD133, ALDH...) ou de marqueurs de l'EMT (SNAIL, Vimentine, Fibronectine...), nous a permis de définir que ces lignées CTCs ont des caractéristiques de cellules souches et présentent un phénotype EMT intermédiaire par la présence de certains marqueurs (SNAIL, Fibronectine...) et absence d'autres (Vimentine, ZEB1, Twist...). Ceci confirme les dernières hypothèses indiquant que le processus métastatique nécessite plutôt une plasticité des cellules avec des facultés de cellules souches plutôt qu'une transition franche vers un profil mésenchymateux.

La néo-angiogenèse est également une étape clé du processus métastatique, nous avons donc testé la capacité de nos lignées CTCs à stimuler et mimer l'angiogenèse *in vitro* : la totalité des lignées CTCs sécrètent les facteurs nécessaires à la formation de tubulures par les cellules endothéliales. Avec les marqueurs de plasticité et de cellules souches, plus la capacité à stimuler l'angiogenèse, nos lignées possèdent les caractéristiques clés ('hallmarks') des cellules initiatrices de métastases. Pour l'instant, seule la première lignée, CTC-MCC-41, a été inoculée chez des souris immunodéficientes, ce qui a démontré la tumorigénicité de cette lignée cancéreuse avec la formation rapide de xénogreffes chez ces animaux.

Finalement, malgré un comportement similaire *in vitro* et une multitude de caractéristiques communes, il existe une hétérogénéité dans les degrés d'expression de différentes protéines, au sein des 9 lignées CTCs coliques, probable reflet de la sélection clonale au cours de l'évolution du cancer sous traitement.

Article 1

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

**Laure Cayrefourcq**, Thibault Mazard, Simon Joosse, Jérôme Solassol, Jeanne Ramos, Eric Assenat, Udo Schumacher, Valérie Costes, Thierry Maudelonde, Klaus Pantel, and Catherine Alix-Panabières

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dans *Cancer Research*

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

Laure Cayrefourcq<sup>1,2</sup>, Thibault Mazard<sup>3</sup>, Simon Joosse<sup>4</sup>, Jérôme Solassol<sup>5</sup>, Jeanne Ramos<sup>6</sup>, Eric Assenat<sup>3</sup>, Udo Schumacher<sup>7</sup>, Valérie Costes<sup>6</sup>, Thierry Maudelonde<sup>2,5</sup>, Klaus Pantel<sup>4</sup>, and Catherine Alix-Panabières<sup>1,2</sup>

### 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<sup>low</sup>, C-met<sup>high</sup>, CD47<sup>high</sup> CD44<sup>high</sup> 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

<sup>1</sup>Laboratory of Rare Human Circulating Cells (LCCRH), Department of Cellular and Tissular Biopathology of Tumors, University Medical Centre, Montpellier, France. <sup>2</sup>EA2415 - Help for Personalized Decision: Methodological Aspects, University Institute of Clinical Research, Montpellier University, Montpellier, France. <sup>3</sup>Department of Medical Oncology, University Medical Centre, Montpellier, France. <sup>4</sup>Department of Tumor Biology, University Medical Center Hamburg-Eppendorf, Hamburg, Germany. <sup>5</sup>Laboratory of Hormonal and Cell Biology, Department of Cellular and Tissular Biopathology of Tumors, University Medical Centre, Montpellier, France. <sup>6</sup>Laboratory of Pathology, Department of Cellular and Tissular Biopathology of Tumors, University Medical Centre, Montpellier, France. <sup>7</sup>Department of Anatomy and Experimental Morphology, University Medical Center Hamburg-Eppendorf, Hamburg, Germany.

**Note:** Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

L. Cayrefourcq and T. Mazard contributed equally to this article.

**Corresponding Author:** Catherine Alix-Panabières, Laboratory of Rare Human Circulating Cells (LCCRH), Saint-Eloi Hospital, University Medical Centre, 80, Avenue Augustin Fliche, Montpellier, France. Phone: 33-4-67-33-05-05; Fax: 33-4-67-33-01-13; E-mail: panabieres@yahoo.fr

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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 alcyon 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- $\mu$ 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  $\mu$ 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  $\geq 1$  using the CellSearch System (mean, 19.9; median, 4; range, 1–516) and 38 of 71 (53.5%) patients with a CTC count of  $\geq 3$  (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 = 71	N = 21	
1		0	
2			6
3		0	
4			2
5 <sup>a</sup>			516
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 <sup>a</sup>			302
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 = 71	N = 21	
66			4
67		0	
68			2
69			6
70			4
71			3

<sup>a</sup>From 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  $\geq 300$ . 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.

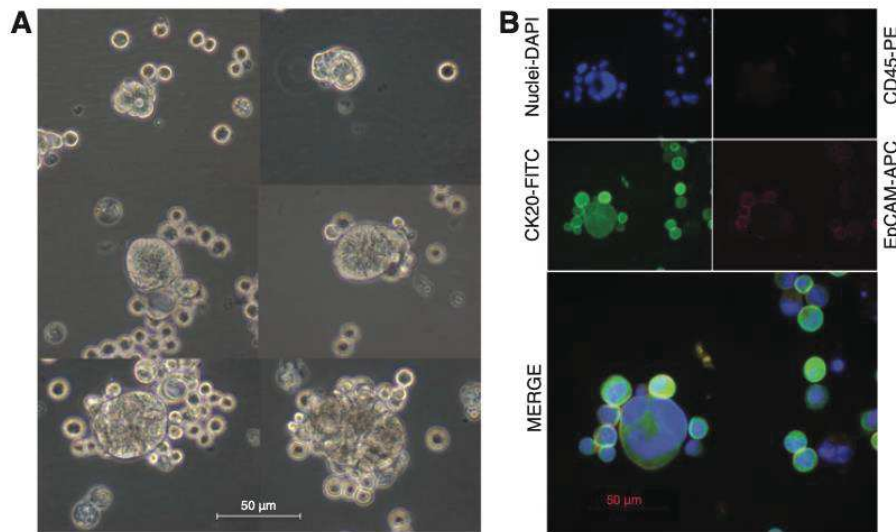
**Clinicopathologic characteristics of the patient and tumor giving rise to CTC-MCC-41**

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.



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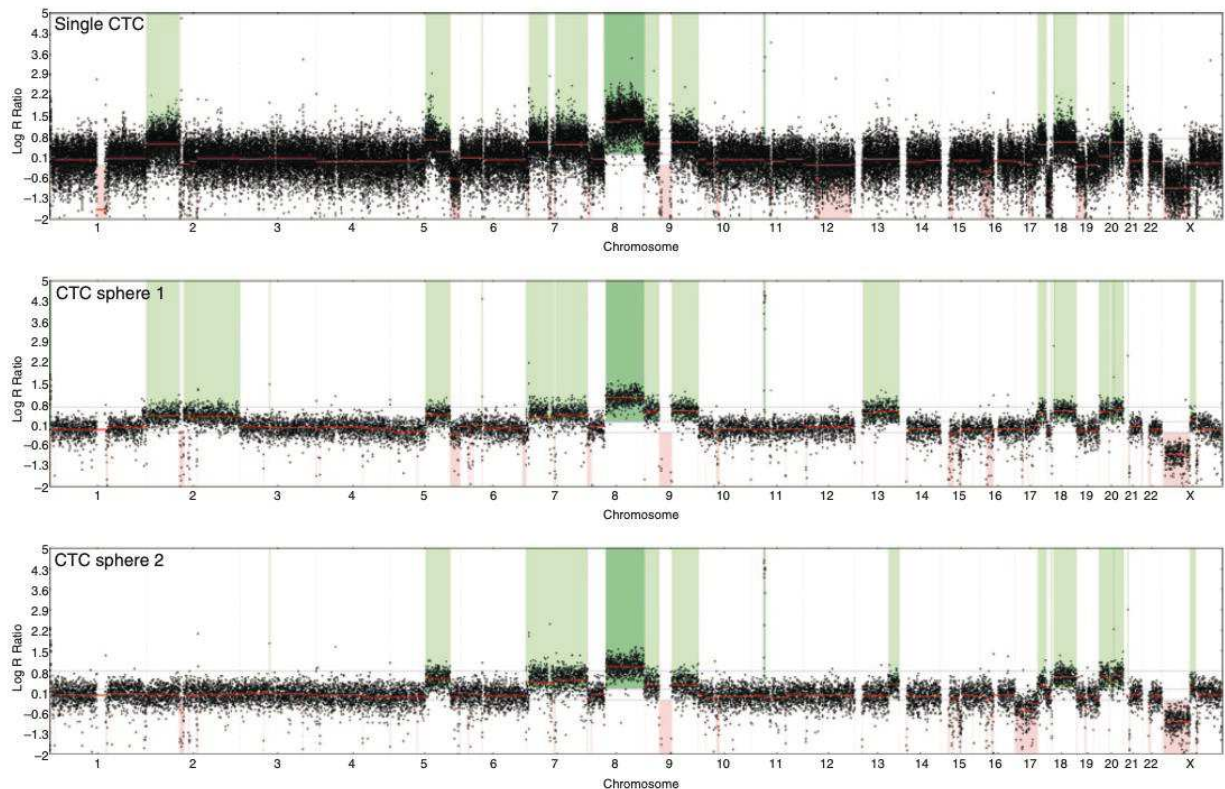
**Figure 1.** Sphere formation with *ex vivo* culture of colon CTCs. A, representative images of CTC spheres, with different sizes obtained at the same time in medium 2 (magnification,  $\times 40$ ). B, immunocytochemical staining of the CTC spheres using anti-EpCAM-APC, anti-CK20-FITC, and anti-CD45-PE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

**Genome analysis of CTC-MCC-41 cells**

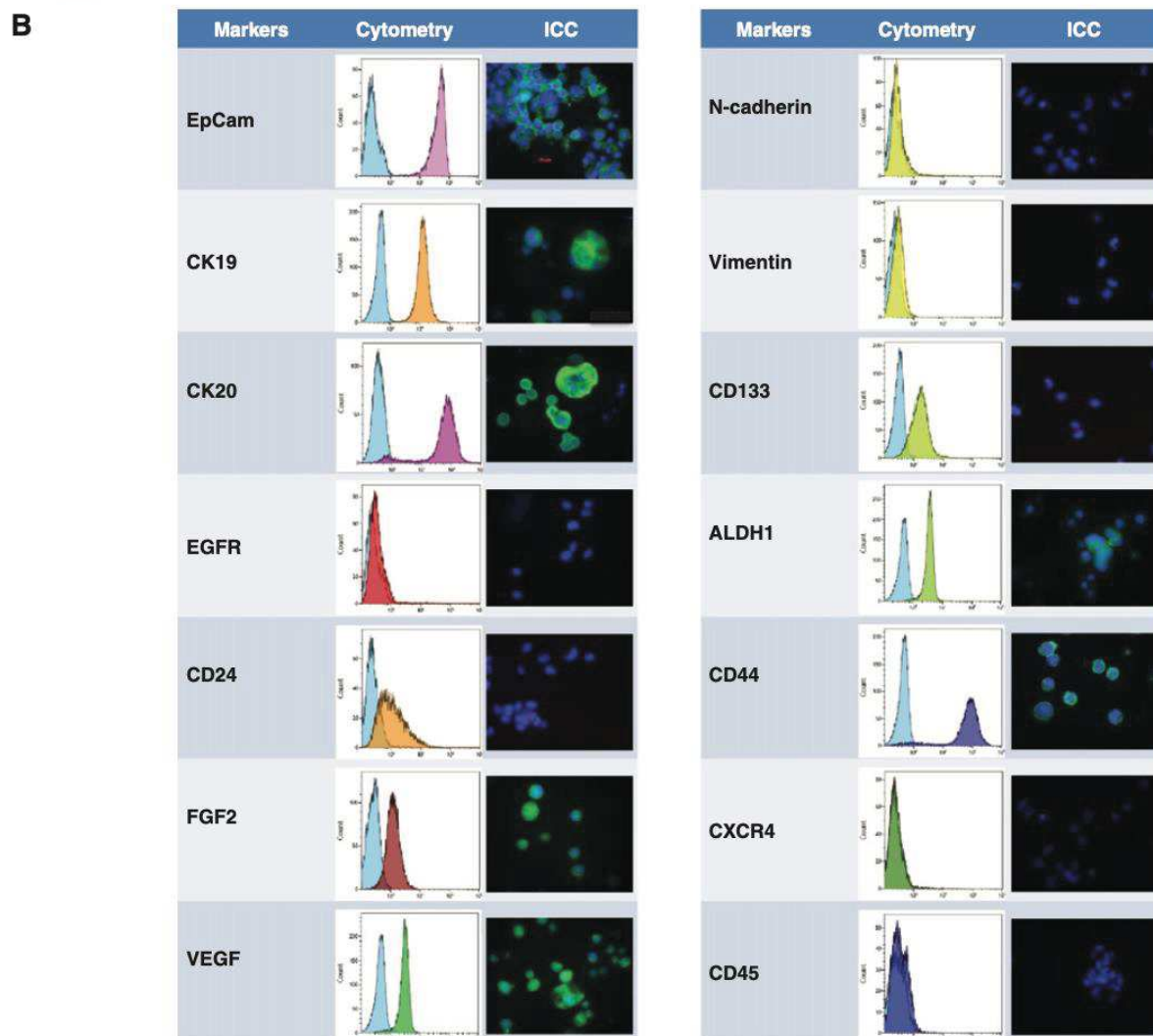
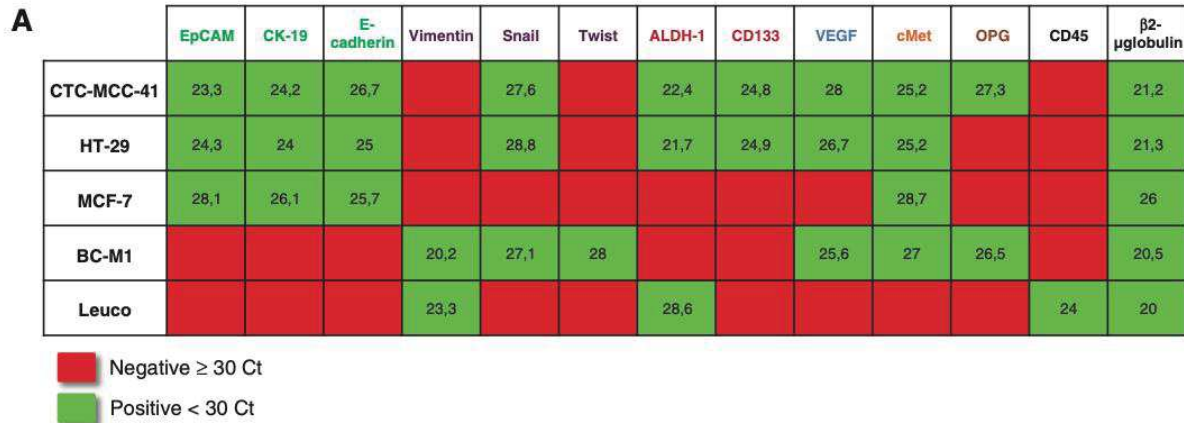
Genomic analysis of the CTC-MCC-41 cell line using next-generation sequencing revealed a wide spectrum of chromosomal aberrations, as can be seen in Fig. 2. Typical colorectal cancer-related chromosomal gains and losses were found along the

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.



**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) osteomimetism (brown). As controls, we used the  $\beta$ 2- $\mu$ 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 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 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<sup>high</sup>/CD24<sup>low</sup> 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).

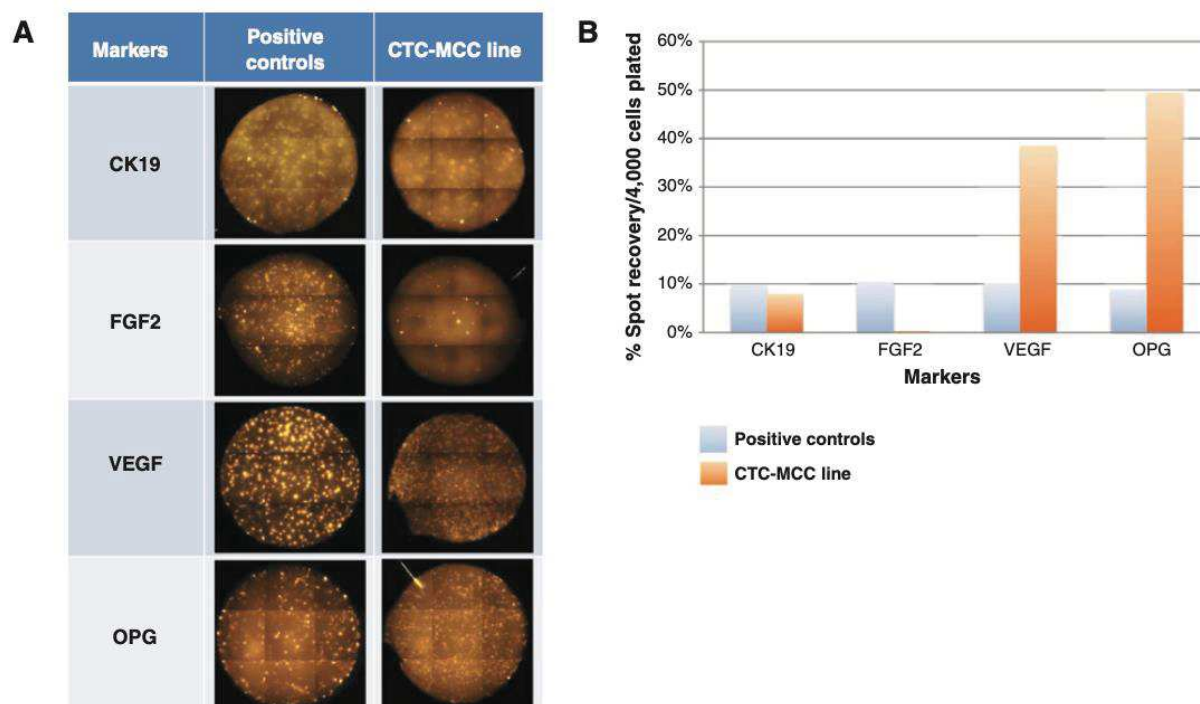
#### *In vitro* endothelial cell tube formation of CTC-MCC-41 cells

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**

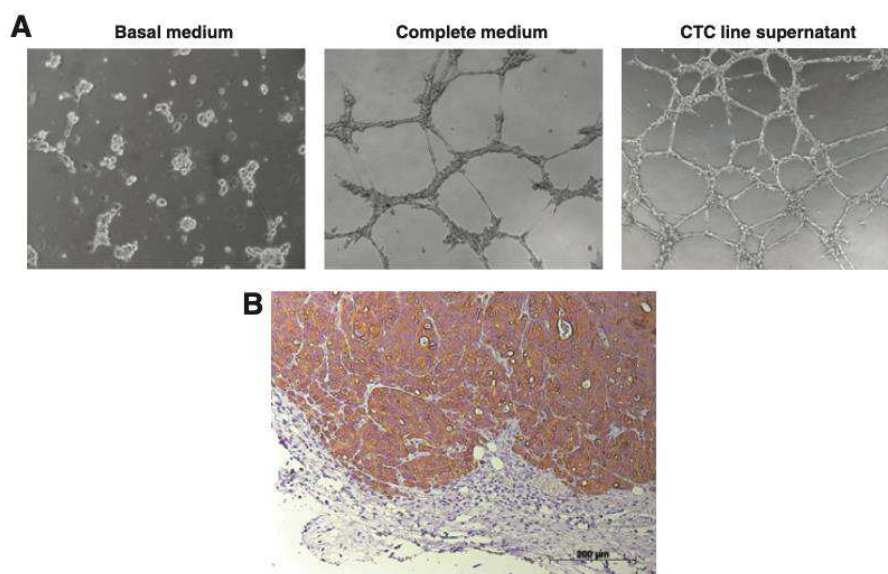
Subgroups of cancer cells can leave the primary tumor, travel as CTCs to a distant site in the body where they begin to colonize a new distant tissue, and form metastases (20). A subset of these CTCs might have a cancer stem cell phenotype, as recently indicated in xenograft models in breast and lung 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<sup>-</sup> remaining cells in nonadherent culture conditions. Even if 70.4% (CTC count  $\geq 1$ ) and 53.5% (CTC count  $\geq 3$ ) 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  $\geq 300$ . 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

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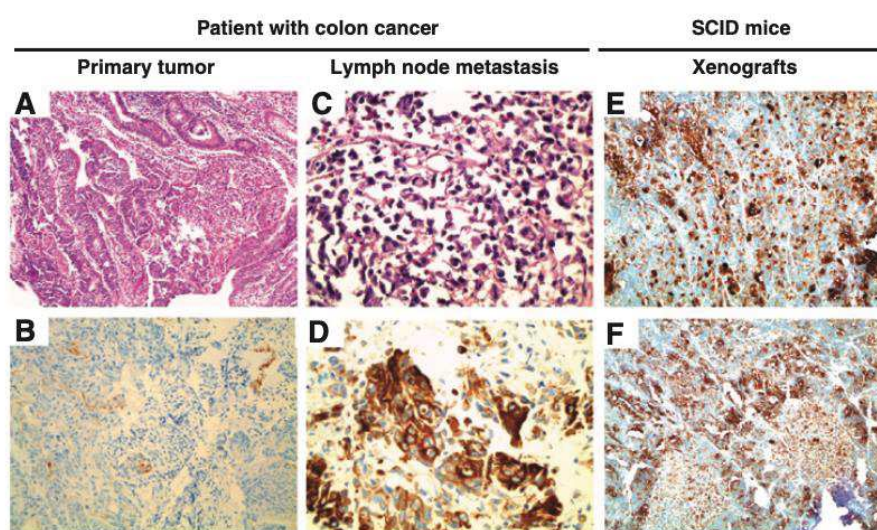
**Figure 5.** A, endothelial cell tube formation induced by the CTC-MCC-41 cell line. Representative photographs of *in vitro* angiogenesis (magnification,  $\times 5$ ). Endothelial cells ( $10^5$  cells/well) were seeded into the plate and endothelial cell tube formation exceeding the tube formation 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<sup>(+)</sup>, CK19<sup>(+)</sup>, E-Cadherin<sup>(+)</sup>, Vimentin<sup>(-)</sup>). However, it was interesting to observe that several cancer stem cell markers were also expressed: ALDH1<sup>(+)</sup>, CD133<sup>(+)</sup> as well as Snail<sup>(+)</sup>, 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<sup>+</sup>/CD24<sup>+</sup> 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

**Conception and design:** L. Cayrefourcq, T. Mazard, K. Pantel, C. Alix-Panabières

**Development of methodology:** L. Cayrefourcq, S. Joosse, V. Costes, K. Pantel, C. Alix-Panabières

**Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.):** L. Cayrefourcq, T. Mazard, S. Joosse, E. Assenat, U. Schumacher, V. Costes, K. Pantel

**Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis):** L. Cayrefourcq, T. Mazard, S. Joosse, U. Schumacher, V. Costes, K. Pantel

**Writing, review, and/or revision of the manuscript:** L. Cayrefourcq, T. Mazard, S. Joosse, K. Pantel, C. Alix-Panabières

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**Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases):** T. Mazard, S. Joosse, J. Solassol, J. Ramos, C. Alix-Panabieres

**Study supervision:** C. Alix-Panabieres

**Other (provided facilities):** T. Maudelonde

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Article 2

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Autologous cell lines from circulating colon cancer cells captured from sequential liquid biopsies as model to study therapy driven tumor changes

Alexandra Soler\*, **Laure Cayrefourcq\***, Thibault Mazard, Anna Babayan, Pierre-Jean Lamy, Said Assou, Eric Assenat, Klaus Pantel & Catherine Alix-Panabières.

\*Les auteurs ont contribué à part égale à ce travail.

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# SCIENTIFIC REPORTS



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## Autologous cell lines from circulating colon cancer cells captured from sequential liquid biopsies as model to study therapy-driven tumor changes

Alexandra Soler<sup>1</sup>, Laure Cayrefourcq<sup>1</sup>, Thibault Mazard<sup>2</sup>, Anna Babayan<sup>3</sup>, Pierre-Jean Lamy<sup>4</sup>, Said Assou<sup>5</sup>, Eric Assenat<sup>6</sup>, Klaus Pantel<sup>3</sup> & Catherine Alix-Panabières<sup>1</sup>

Circulating tumor cells (CTCs) are important clinical indicators for prognosis and treatment efficacy. However, CTC investigation is hampered by their low number, making the establishment of permanent CTC lines very challenging. We derived and characterized nine CTC lines using blood samples from a patient with metastatic colorectal cancer collected before and after chemotherapy and targeted therapy, and during cancer progression. These cell lines displayed an intermediate epithelial/mesenchymal phenotype, stem-cell like characteristics, angiogenesis potential, an osteomimetic signature and the capacity to escape from the immune system. Moreover, they showed changes in mRNA and protein expression (e.g., *DEFA6*, *ABCB1* and *GAL*), whereas analysis of chromosomal copy number aberrations revealed no significant variation over time. These data indicate that although CTC lines derived from sequential blood samples during therapy have common traits, treatment-resistant CTC clones with distinct phenotypic characteristics are selected over time.

Over the past years, liquid biopsy of circulating tumor cells (CTC) has received major attention as a strategy to obtain real time diagnostic and prognostic information<sup>1,2</sup>. Several large-scale clinical trials and meta-analyses have shown that CTC number is an important indicator of the risk of cancer progression or death in patients with metastatic solid tumors<sup>3-7</sup>. However, CTC investigation is hampered by their very low number, especially in blood of patients with colorectal cancer. Therefore, the establishment of *in vitro* cultures and permanent lines from CTCs has become the most challenging task over the past years. Indeed, CTC lines could be used to identify proteins and pathways involved in cancer cell stemness and dissemination, and also to test new drugs to inhibit metastasis-competent CTCs. *Ex vivo* CTC cultures have been established for breast<sup>8,9</sup>, prostate<sup>10</sup>, lung<sup>11</sup>, and head and neck cancer<sup>12</sup>. We previously described the first permanent cell line (CTC-MCC-41) from circulating colon cancer cells<sup>13</sup>. However, its establishment was very difficult (blood samples of 168 patients were tested). This could be partly explained by the much lower CTC number in the peripheral blood of such patients than in patients with breast or prostate cancer, making very difficult their enrichment and culture. In addition to its capacity to expand *in vitro* for more than 4 years, the CTC-MCC-41 cell line shows specific stem-cell like characteristics and shares some features of the original primary tumor and lymph node metastasis<sup>13</sup>.

We then established another eight CTC lines from blood samples collected from the same patient at different time points during his follow-up. This unique biological material represents a chance to study clonal selection and resistance mechanisms during tumor progression and treatment. Here, we report the establishment of these

<sup>1</sup>Laboratory of Rare Human Circulating Cells, University Medical Centre of Montpellier, EA2415, Montpellier, France.

<sup>2</sup>Department of Medical Oncology, Cancer Institute of Montpellier, Montpellier, France. <sup>3</sup>Department of Tumor Biology, University Medical Centre Hamburg-Eppendorf, Hamburg, Germany. <sup>4</sup>Imagenome, Labosud, Montpellier, France. <sup>5</sup>IRMB, Montpellier University, INSERM U1183, University Medical Centre of Montpellier, Montpellier, France. <sup>6</sup>Department of Medical Oncology, University Medical Centre of Montpellier, Montpellier, France. Alexandra Soler and Laure Cayrefourcq contributed equally. Correspondence and requests for materials should be addressed to C.A.-P. (email: [c-panabieres@chu-montpellier.fr](mailto:c-panabieres@chu-montpellier.fr))

new CTC lines from the same patient with metastatic colon cancer, and their characterization (genome, transcriptome, proteome, and functional analyses). Comparison of all nine autologous CTC lines (the previously described CTC-MCC-41 line and the eight new lines) highlighted their common features and main differences acquired over time.

## Results

**Establishment of colon CTC lines from a patient with metastatic colon cancer.** The national COLOSPOT project included 168 patients with metastatic colon cancer (NCT01596790). Before the first-line treatment, CTC number was evaluated in 7.5 mL of peripheral blood using the CellSearch system, and then another 10 mL of peripheral blood was used for CTC enrichment and culture. CTC number was  $\geq 1$  in 57.5% of patients and  $\geq 3$  in 39.4% (mean = 9; median = 1; range: 0–302). Only one colon CTC line (CTC-MCC-41) could be established<sup>13</sup> from the patient with the highest CTC number (302 CTCs/7.5 mL of blood) and with 38 CTC clusters ( $< 2$  to 5 CTCs/microemboli) (patient 044) (Fig. 1A).

During the follow-up, other blood samples were collected from this patient and long-term CTC cultures could be established at the time of the second and third relapse (Fig. 1B). At the time of the second relapse, the new CTC line CTC-MCC-41.4 was derived using a blood sample that contained 3,278 CTCs/7.5 mL and 962 CTC clusters ( $< 2$  to 13 CTCs/microemboli). At the time of the third relapse, seven new CTC-MCC-41.5 lines were established from a blood sample with 286 CTCs/7.5 mL (but only 3 clusters of 2 CTCs/microemboli): CTC-MCC-41.5A, CTC-MCC-41.5B, CTC-MCC-41.5C, CTC-MCC-41.5D, CTC-MCC-41.5E, CTC-MCC-41.5F, and CTC-MCC-41.5G. Analysis of the correlation between the CTC number in patient 044's blood samples and the successful establishment of CTC lines (Fig. 1C) suggests that, in colon cancer, around 300 CTCs are required to select metastasis-competent CTCs that can self-renew and grow *in vitro*. In addition, based on the CellSearch cell images, CTCs were present as single cells and clusters in the first samples, and as single cells in the last sample at the time of the third relapse (Fig. 1C). Moreover, in the first samples, giant and small CTCs could be detected simultaneously, whereas only very small (4–6  $\mu\text{m}$ ) and small (8  $\mu\text{m}$ ) CTCs were present in the last sample before the patient's death.

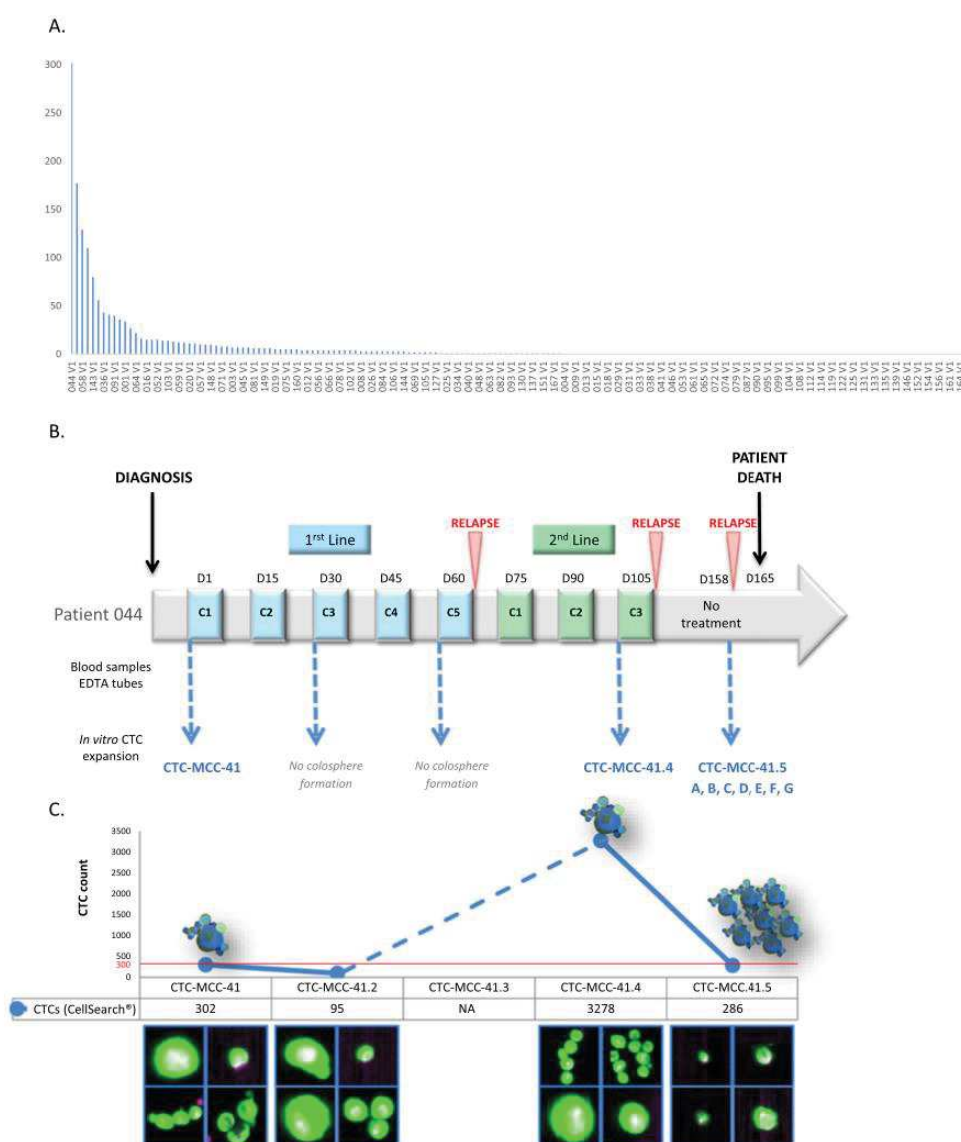
***In vitro* culture and morphological description of the nine colon CTC lines.** The CTC-MCC-41.4 line was obtained by pooling several CTCs that started to proliferate at the same time in all wells of the culture plate. This new CTC line showed isolated tumor cells with a mean diameter of 15  $\mu\text{m}$  (50%) and colospheres with a size that could reach 100  $\mu\text{m}$  (50%) (Fig. 2), as observed in the CTC-MCC-41 line.

In the CTC-MCC-41.5 lines, CTCs started to proliferate in different wells at different time points. Therefore, they were kept separated, giving rise to the different lines (CTC-MCC-41.5A, B, C, D, E, F and G). The first three CTC-MCC-41.5 lines (CTC-MCC-41.5D, CTC-MCC-41.5F and CTC-MCC-41.5G) appeared after 18 days of culture. They showed different morphologies and behaved differently. CTC-MCC-41.5D cells grew as isolated cells (50%) and also as big colospheres (up to 100  $\mu\text{m}$ ). The CTC-MCC-41.5F and CTC-MCC-41.5G lines were composed essentially of isolated cells (70%) and small colospheres (up to 50  $\mu\text{m}$ ) for the remaining 30%. After 24 days of culture, three additional CTC lines appeared: the CTC-MCC-41.5A line, characterized by the presence of isolated tumor cells and colospheres (50%–50%); the CTC-MCC-41.5B line, with 70% of small isolated cells and few small colospheres (30%); and the CTC-MCC-41.5C line, made mostly of large colospheres (80%, up to 200  $\mu\text{m}$ ) and few isolated cells (20%). Finally, after 34 days of culture, the last CTC line (CTC-MCC-41.5E) displayed isolated cells (50%) and big colospheres (50%) (Fig. 2).

**Characterization of the CTC lines.** *Genomic analysis.* To prove the tumor nature of the nine CTC-MCC-lines, copy number alterations (CNA) were assessed genome-wide in two single cells and one colosphere for each CTC line by next-generation sequencing (NGS). This analysis revealed a wide spectrum of chromosomal aberrations (Fig. 3). Typical colorectal cancer-related chromosomal gains and losses were found throughout the genome in all investigated samples, particularly gains in chromosomes 7, 8 and 20 and losses in chromosome 22. However, no specific CNA signature could be detected in the first CTC line (CTC-MCC-41), and in those established during disease progression (CTC-MCC-41.4 and -41.5A–G). Based on the phylogenetic analysis results using the copy number median ratios, samples (single cells and colospheres) could be clustered in six subgroups (Fig. 4). The largest group (on the left side on Fig. 4) contained all the CTC-MCC-41.5G samples, the colosphere sample and one single-cell sample from the CTC-MCC-41.5A, 5B and 5F lines, and the CTC-MCC-41.5D colosphere sample. These results demonstrate clearly the high genomic variability within of each colon CTC line, with the exception of the CTC-MCC-41 and -41.5E lines that clustered in two individual groups. Moreover, the absence of cell line-specific CNA signature suggests that CTC changes over time were not driven by additional CNAs and/or did not cause any new CNA.

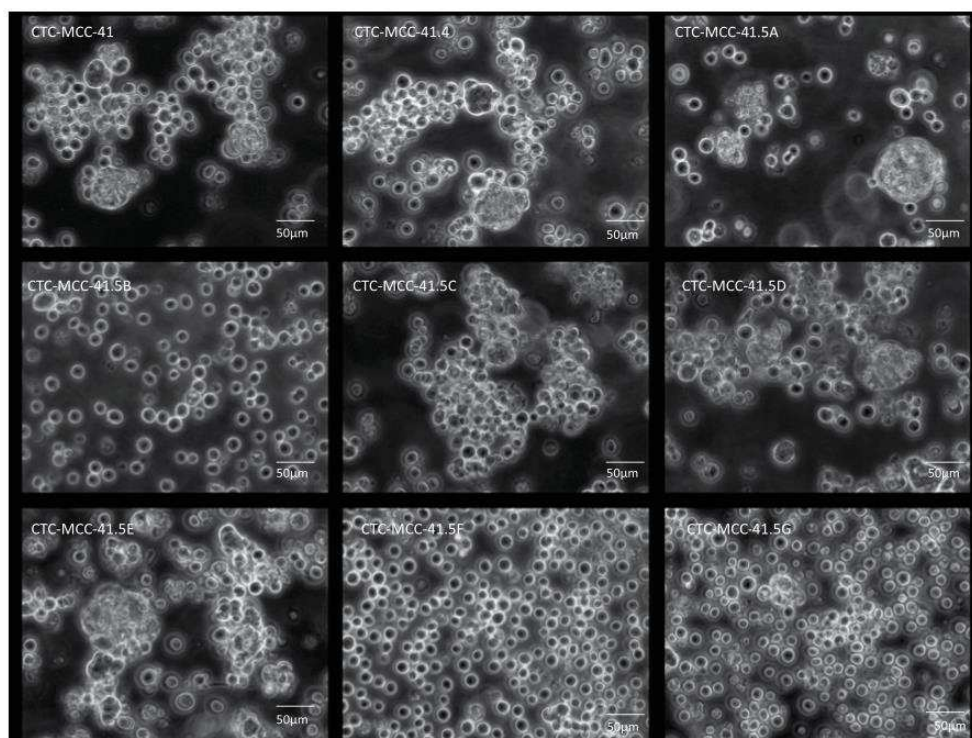
Concerning specific mutations, all samples carried the *BRAF*<sup>V600E</sup> mutation with a stable allele frequency of 50%, in agreement with a heterozygote mutation (Supplementary Table S1). *KRAS* was wild type (wt) in all CTC lines. This mutation profile (*BRAF*<sup>mut</sup>/*KRAS*<sup>wt</sup>) mirrors the one detected in the primary tumor, lymph node metastasis, and also tumor xenografts from immunodeficient mice after injection of CTC-MCC-41 cells<sup>13</sup>.

*Transcriptomic characterization.* To characterize these CTC lines, the transcriptome profiles of the eight new colon CTC lines were compared with those of the first line (CTC-MCC-41) and of two commercial colon cancer cell lines (HT-29 and SW-620; derived from a primary tumor and from a lymph node metastasis, respectively). The panel of messenger RNAs (mRNAs) to be analyzed (Supplementary Table S2) was chosen based on literature data and the transcriptomic profile of the CTC-MCC-41 line<sup>14</sup>, and included genes encoding molecules involved in many tumor cell features (e.g., epithelial, mesenchymal, stemness, energetic metabolism and immune system escape).



**Figure 1.** Blood samples collection for the establishment of CTC-derived colospheres and timeline of CTC line derivation from sequential blood samples of patient 044. (A) CTC number (assessed with the CellSearch system) in the blood sample of the 168 patients with metastatic colon cancer at V1 (before any treatment); (B) Nine colon CTC lines were established from blood samples of patient 044 at different time points: before treatment initiation (CTC-MCC-41 line), at the time of the second relapse at the end of second-line therapy (CTC-MCC-41.4 line), and at the time of the third relapse before the patient death (CTC-MCC-41.5 A-G lines); D, day; C, treatment cycle; (C) Correlation between CTC number detected with the CellSearch system and *in vitro* colosphere formation (in blue) for patient 044; CellSearch cell images representative of CTC morphology are shown for the blood samples from which CTC lines could be derived (cells in green). The red line shows the cut-off of approximately 300 cells per 7.5 mL of blood required for *in vitro* CTC expansion.

The transcriptome data could be stratified in four different groups according to the expression of each mRNA in the CTC lines (Supplementary Table S3). The first group included genes with an identical expression profile (present/absent) in all the cell lines analyzed (colon CTC, HT-29 and SW-620). All cell lines expressed epithelial cell adhesion molecule (*EPCAM*), cytokeratin 19 (*CK19*), E-cadherin (*ECAD*), *CMET*, carcinoembryonic antigen-related cell adhesion molecule 1 (*CEACAM1*), vascular endothelial growth factor (*VEGF*), snail family transcription repressor 1 (*SNAIL*) and aldehyde dehydrogenase 1 (*ALDH1*), but not *CD45*. This confirmed the epithelial origin of all these cell lines. A second group included transcripts that were differentially expressed in the colon CTC lines and in HT-29 and SW620 cells: (i) interleukin 33 (*IL33*), cyclin D2 (*CCND2*), semaphorin 6A (*SEMA6A*) and osteoprotegerin (*OPG*) that were expressed only in the colon CTC lines; and (ii) SWI/SNF related,



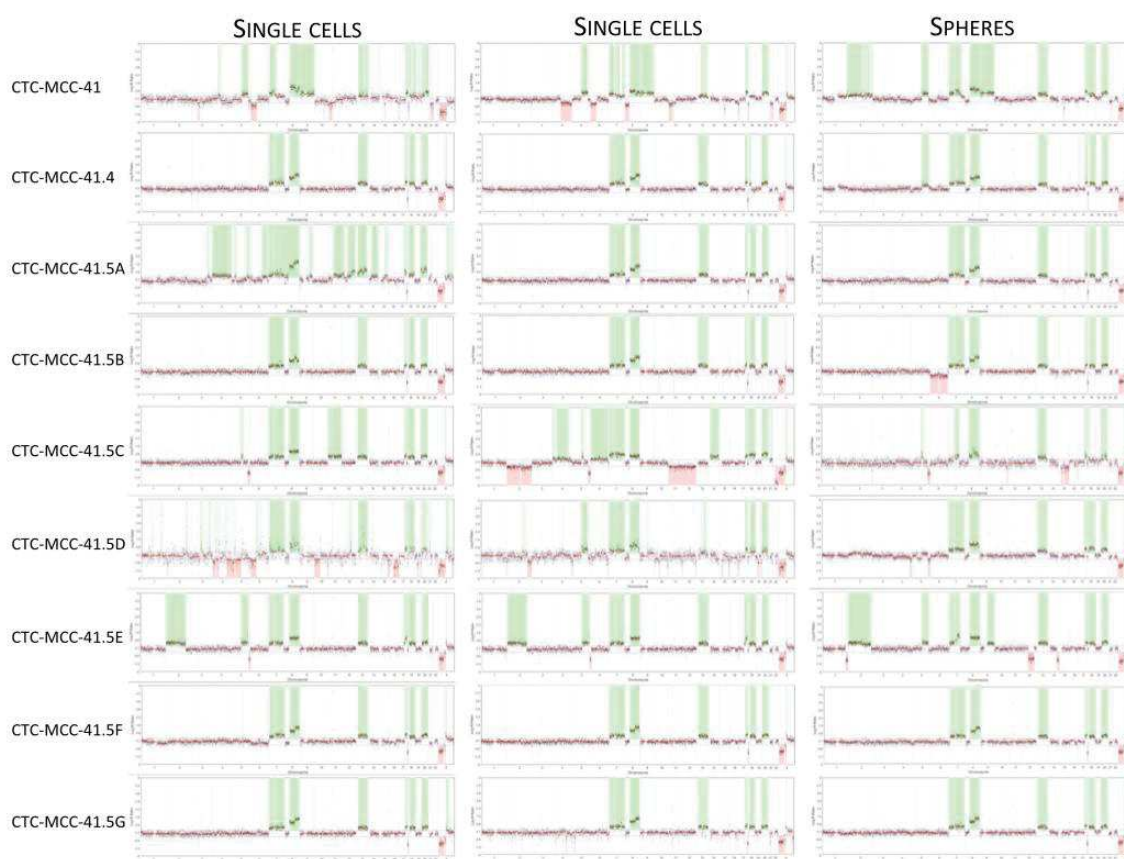
**Figure 2.** *In vitro* culture of the colon CTC lines. Representative images of each CTC line (Zeiss Axio Observer D1).

matrix associated, actin dependent regulator of chromatin, subfamily A, member 1 (*SMARCA1*) and dickkopf WNT signaling pathway inhibitor 1 (*DKK1*) that were expressed only in HT-29 and SW-620 cells. The third group included genes that (i) were expressed in the colon CTC lines and in SW620 cells: B-cell lymphoma 11A (*BCL11A*), tumor necrosis factor receptor superfamily 1B (*TNFRSF1B*), bone morphogenetic protein 7 (*BMP7*) and fibronectin 1 (*FN1*); and (ii) were not expressed in the CTC lines and SW620 cells: adam metalloproteinase with thrombospondin type 1 motif 6 (*ADAMTS6*), Gata binding protein 2 (*GATA2*), gap junction protein beta 6 (*GJB6*), transforming growth factor beta 2 (*TGFB2*), prostaglandin endoperoxidase synthase 2 (*PTGS2*), bone marrow stromal cell antigen 2 (*BST2*), Wnt family member 11 (*WNT11*) and *TWIST*. The last group included genes that were downregulated in all colon CTC lines and in HT-29 cells, but expressed in SW620 cells: SRY-related HMG-box gene 2 (*SOX2*) and vimentin (*VIM*). Hierarchical clustering analysis based on the mRNA expression profile clearly segregated the CTC lines from the HT-29 and SW-620 cell lines (Fig. 5). Moreover, the colon CTC lines could be clustered in several sub-groups based on their different expression profiles.

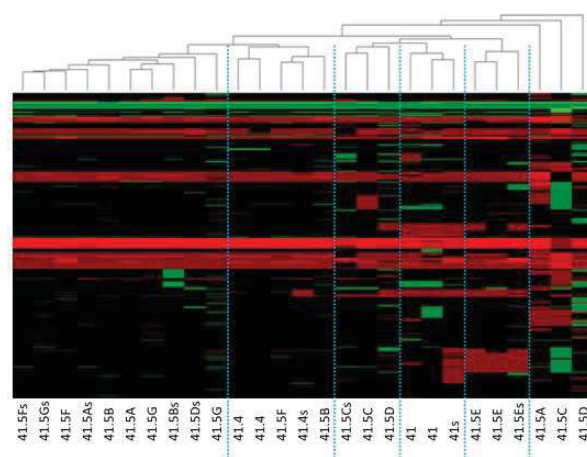
**Proteomic characterization.** The expression profile of a large panel of proteins to cover different properties of colorectal tumor cells (e.g., epithelial-mesenchymal plasticity, stem-cell properties, immunomodulation capacity and angiogenesis) was assessed in the nine colon CTC lines by flow cytometry (Fig. 6A). As already reported for the CTC-MCC-41 line<sup>13</sup>, the new CTC lines also strongly expressed epithelial markers, such as EpCAM and cytokeratins (CK8, 18, 19 & 20), but none of the tested mesenchymal markers (N-cadherin and vimentin). In addition, the expression of CD133 and CD44v6 and the CD24<sup>(low)</sup>/CD44<sup>(high)</sup> ratio indicated stem cell-like characteristics<sup>15</sup>. All CTC lines weakly expressed VEGF and PanCD66 (A, B, C & E), also known as CEACAM (1, 2, 3 and 5). The absence of CD45 expression confirmed that these cells were not leucocytes. Moreover, the eight new colon CTC lines did not express endothelial proteins (CD31 and CD105), hematopoietic progenitor markers (CD34) and C-X-C chemokine receptor type 4 (CXCR4), a receptor expressed on breast tumor cells and involved in the chemoattraction to specific organs<sup>16</sup> (Supplementary Table S4). All the data obtained by flow cytometry were confirmed by concomitant immunofluorescence analysis (Fig. 6B with examples of co-expression of specific proteins).

**Functional characterization.** Different functional assays were then performed to assess the biology of these tumor cells: EPISPOT assay, *in vitro* endothelial cell tube formation assay, and ALDH1 activity assay (Fig. 7).

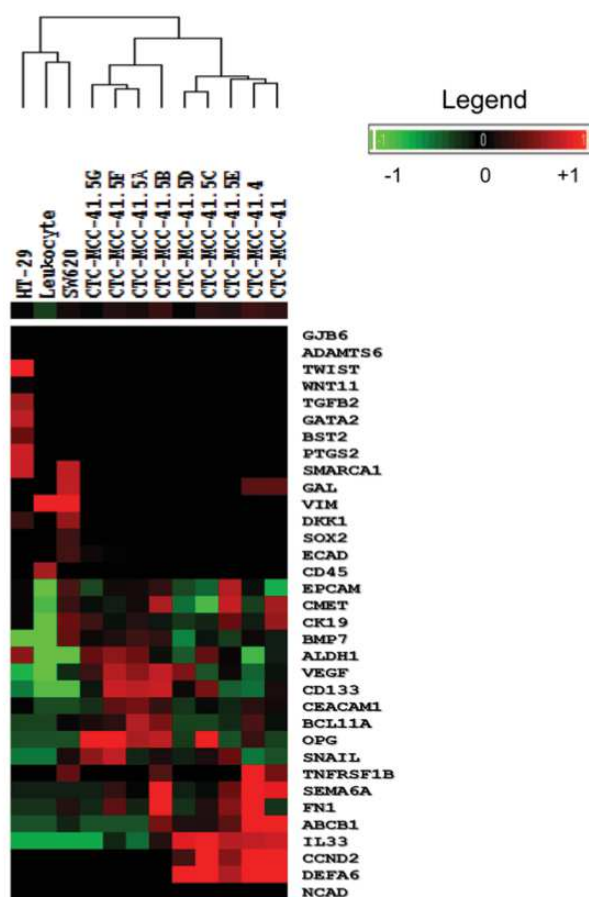
The fluoro-EPISPOT assay assesses the ability of viable tumor cells to secrete, shed or release specific proteins. The fluoro-EPISPOT assay results indicated that CTC-MCC-41, CTC-MCC-41.4 and all the CTC-MCC-41.5 lines could release CK19 (5% of cells), and secrete VEGF (95% of cells) (Fig. 7A, upper and lower panels). Moreover, about 80% of CTC-MCC-41.4, CTC-MCC-41.5A, CTC-MCC-41.5B, CTC-MCC-41.5C, CTC-MCC-41.5F and



**Figure 3.** Copy number alterations in the nine CTC cell lines determined by next-generation sequencing. Chromosomal aberrations in the whole genome (x-axis) were investigated in two single-cell and one colosphere sample for each colon CTC line. Copy number gains are shaded green, and copy number losses are shaded red. Red horizontal lines represent the estimated copy number value (y-axis) and mark the break points between intrachromosomal copy-number alterations.



**Figure 4.** Hierarchical clustering of the nine colon CTC lines. Whole genome analysis results, from chromosome 1 to X (bottom to top), of two single-cell and one colosphere (s) sample for each colon CTC line allowed their subdivision in different clusters. Red represents gains, and green represents losses of genomic material.



**Figure 5.** Hierarchical clustering of the nine colon CTC lines relative to the HT-29 and SW620 colorectal cancer cell lines and leukocytes. The expression of 34 mRNAs (Supplementary Table S2) was analyzed in the nine colon CTC lines, HT-29 cells (from a primary colorectal tumor), and SW620 cells (from a lymph node metastasis of colon cancer). These transcripts covered different properties of tumor cells: epithelial (*ECAD*, *CK19*, *EPCAM*), mesenchymal (*NCAD*, *VIM*, *FN1*), stemness (*CD133*, *ALDH1*), angiogenesis (*VEGF*), proto-oncogene (*MET*), osteomimicry (*OPG*, *BMP7*), EMT (*SNAIL*, *TWIST*, *WNT*, *DKK1*), immune system (*IL33*, *DEFA6*, *CEACAM1*), apoptosis (*CCND2*, *BCL11A*, *TNFRSF1B*), DNA repair (*GAL*, *SMARCA1*), cell growth (*BST2*, *SEMA6A*), cell interactions (*ADAMTS6*, *GJB6*, *GATA2*, *TGFB2*) and energy metabolism (*ABCB1*, *PTGS2*). *B2M* (housekeeping gene) and *CD45* (leukocyte marker) were used as reference genes. Leukocyte cDNA was used as negative control. Results were normalized to the expression level of the reference *B2M* gene. Cell lines were clustered based on their expression profile.

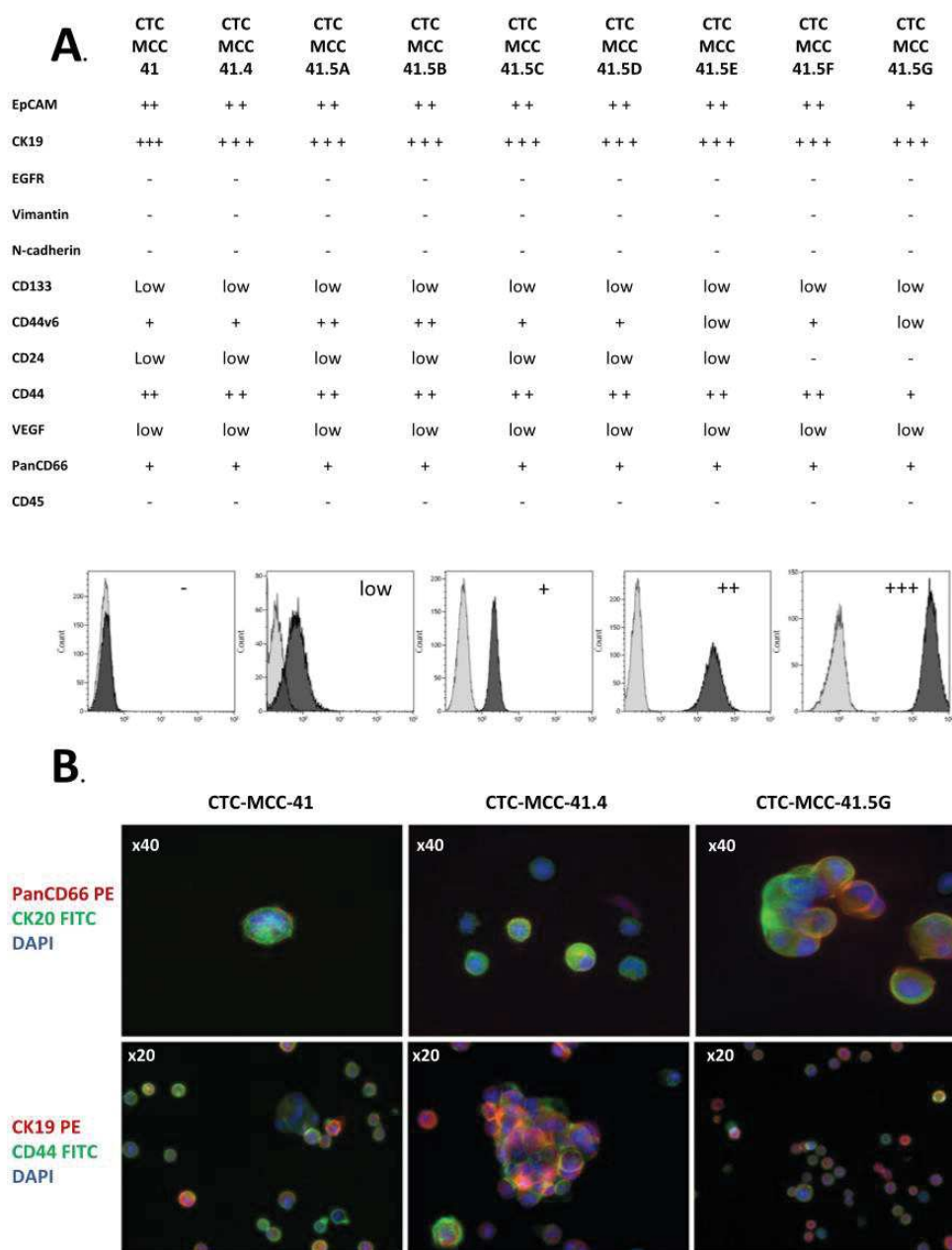
CTC-MCC-41.5G cells could actively shed OPG, whereas only 20% of CTC-MCC-41.5D and CTC-MCC-41.E cells showed OPG immunospots (Fig. 7A, middle panels).

As the colon CTC lines expressed and secreted VEGF, *in vitro* endothelial cell tube formation was also tested (Fig. 7B). Endothelial cell tubes were observed only when endothelial cells were cultured in endothelial cell medium for 6 hours (positive control), but not in the presence of basal medium (RPMI 1640 alone; negative control). Supernatants of all colon CTC lines also could induce endothelial cell tube formation, with a higher number of tube-like structures than the positive control for the CTC-MCC-41, CTC-MCC-41.4, CTC-MCC-41.5A, CTC-MCC-41.5B and CTC-MCC-41.5F cell supernatants (Fig. 7B).

Elevated ALDH activity is an important biomarker of stem and progenitor cells in epithelial cancers<sup>17</sup>. The ALDH activity assay (Fig. 7C) highlighted the presence of a subpopulation of ALDH-positive cells in all CTC lines, but for CTC-MCC-41.5E cells only 4.30% were ALDH-positive. CTC-MCC-41.5F and CTC-MCC-41.5G cells displayed the highest percentages of ALDH-positive cells (53.41% and 61.85%, respectively).

### Discussion

Subsets of cancer cells can disseminate in the body to a distant site where they colonize the new organ and form metastases<sup>18</sup>. Some of these CTCs have a cancer stem-cell phenotype, as indicated by different studies in breast, lung, prostate and colon cancer<sup>10,11,13,19–21</sup>.

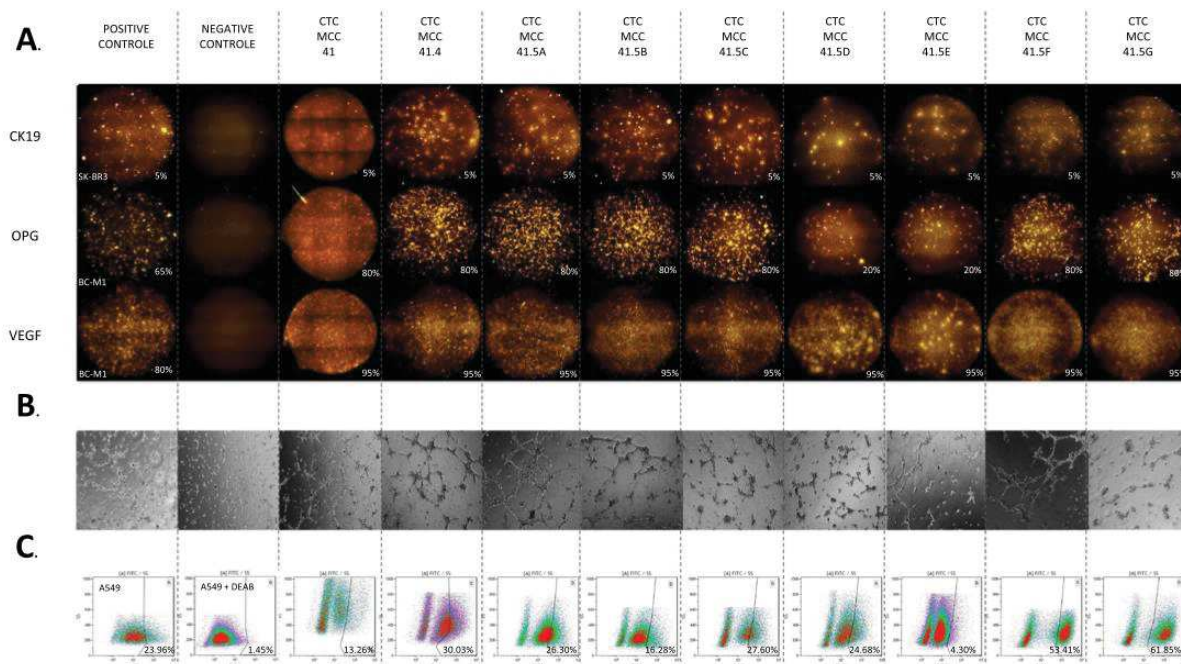


**Figure 6.** Protein expression analysis in the colon CTC lines. (A). Flow cytometry experiments. The expression of a set of proteins was analyzed using different fluorescent antibodies to characterize the phenotype of each CTC line: epithelial (EpCAM, CK19, EGFR), mesenchymal (vimentin, N-cadherin), stemness (CD133, CD44v6, CD44/CD24 ratio), angiogenic (VEGF), immune system escape (PanCD66) and hematopoietic (CD45). The histograms at the bottom show the expression levels (from negative to +++) used to describe the results. (B). Immunofluorescence experiments. Representative images of CTC-MCC-41, CTC-MCC-41.4 and CTC-MCC-41.5G single cells and colospheres showing co-expression of PanCD66 (CEACAM) and CK20, or CD44 and CK19. Nuclei were stained with DAPI (blue). PE, phycoerythrin; FITC, fluorescein isothiocyanate.

In 2015, our group published the first experimental proof that a stable colon CTC line (CTC-MCC-41) could be established from CTCs isolated from the blood of a patient with metastatic colon cancer<sup>13</sup>. The present study provides the characterization of the other eight CTC lines derived from CTCs isolated from the same patient at different times during his follow-up before/after treatment and disease progression. This unique material will allow assessing the clonal selection of aggressive metastasis-competent cancer cells during cancer progression.

Our previous<sup>13</sup> and present data on the establishment of colon CTC lines suggest that a CTC count of approximately 300 (using the CellSearch system) in blood samples from patients with colon cancer is a prerequisite





**Figure 7.** Functional analysis of the colon CTC lines. **(A)** Representative images of the EPISPOT results. Secretion, release and shedding of CK19 (upper), OPG (middle) and VEGF (lower panels) by viable CTCs were assessed using the fluoro-EPISPOT assay (C.T.L. Elispot reader). The breast cancer cell lines SKBR3 and BC-M1 were used as positive controls for CK19, and for OPG and VEGF, respectively. The negative control well was without cells (magnification X5). Each fluorescent immunospot corresponds to the protein fingerprint of one secreting cell. **(B)** Endothelial cell tube formation assay. Endothelial cell tube formation was observed after 6h (representative images; magnification X5) of cultures with endothelial cell medium (positive control), basal medium (RPMI 1640 alone; negative control), and supernatant from the nine CTC lines cultured in basal medium for 48h. **(C)** ALDH activity assay by flow cytometry. Gates for positive signal were designed for each CTC line based on the negative control performed with the DEAB reagent. The lung cancer cell line A549 was used as positive control. The percentage of ALDH-positive cells is indicated for each condition.

for successful CTC expansion in culture. However, the number of CTCs is lower in the blood of patients with colorectal cancer that with other cancer types. In patients with colon cancer, viable CTCs can be captured in mesenteric blood samples, indicating that many of them are trapped in the liver<sup>22</sup>. Furthermore, this specific patient had the BRAF mutation, which is relatively rare (about 10%) and highly aggressive in CRC<sup>23</sup>. Indeed, only 8 out of 95 patients were positive for this rare colorectal mutation. Interestingly, no correlation between the BRAF mutation and a high number of CTCs has been observed in the COLOSPOT study. All these data clearly show how challenging it is to establish a CTC line from patients with colon cancer even with distant metastases, and may explain why no CTC culture or cell line has been reported in the literature, with the exception by our group.

To verify that these cell lines are of cancer origin, we performed genotyping analyses. CNA analysis by NGS demonstrated that these CTC lines were cancer cell lines with cancer-specific genomic aberrations. This analysis highlighted CNA differences between samples (colosphere and single cells) from the same CTC line and also between lines, indicating that these lines have a polyclonal origin. However, we did not observe any CNA associated with disease progression. Indeed, the first CTC line (obtained at diagnosis) and the other lines (obtained at different time points after treatment) were all highly heterogeneous at the inter- and intra-line level. This suggests that therapy did not cause any significant change in the cancer CNA signature. However, we cannot exclude therapy-driven selection of resistance-conferring mutations, other than BRAF mutations that were detected with a stable frequency in all CTC lines.

Visual monitoring of the cultures showed again a clear intra- and inter-line heterogeneity: colospheres as well as small and big single cancer cells. This suggests the absence of a preferred morphology for these tumor cells, consistent with the observation that both small and large CTCs are linked to unfavorable prognosis<sup>24</sup>. Recent studies have emphasized that CTC clusters may have an increased malignant capacity<sup>25</sup>, and microemboli were observed in the blood samples of this patient with metastatic colon cancer during his follow-up.

Epithelial-to-mesenchymal transition (EMT) is a complex process that supports the migratory capacity of epithelial tumor cells. EMT also plays a crucial role in promoting cancer metastasis by various mechanisms, including CTC release from the primary tumor and distant metastases<sup>26</sup>. All nine CTC lines presented a stable epithelial phenotype, as indicated by the expression of epithelial markers, such as EPCAM and cytokeratins (8, 18, 19 & 20). Concerning markers of mesenchymal transition, all nine CTC lines expressed FN1 and SNAIL, but not vimentin or N-cadherin (mRNA and protein). Moreover, in all CTC cell lines, ECAD was weakly expressed

and *ADAMTS6*, *GATA2*, *GJB6*, *TGFB2*, *PTGS2* and *BST2*, genes encoding cell-cell junctions, focal adhesion or cytoskeletal proteins, were clearly downregulated<sup>14</sup>. This is in accordance with CTC loss of adherent cell properties to leave the tumor and reach the bloodstream. These data indicate that these colon CTC lines are tumor cells with an intermediate EMT phenotype. The acquisition of mesenchymal features by CTCs could reveal some plasticity required for dissemination to distant organs<sup>26</sup>. In addition, the expression of *TNFRSF1B* and *BCL11A*, linked to apoptosis suppression<sup>27</sup>, could indicate another CTC hallmark called «anoikis resistance», a mechanism to avoid cell death when tumor cells are not attached, but swim in the bloodstream<sup>18</sup>.

Stemness features, such as expression of CD133, CD44v6 (a colorectal stemness marker)<sup>15</sup>, *BMP7*, *GAL* and *SEMA6A*, and the CD44<sup>high</sup>/CD24<sup>low</sup> ratio<sup>28</sup>, were detected in all nine CTC lines. Moreover, they all showed ALDH activity, a well-known stem cell marker<sup>17</sup>. The combination of EMT phenotype and stem-cell characteristics should favor their dissemination and ability to form distant metastases<sup>29</sup>.

In addition, the expression of *OPG*, *BMP7* and *TNFRSF1B* (involved in osteogenic activity) suggests that these CTCs have the ability to lodge in the bone marrow. However, this patient developed liver metastases, without any evidence of bone metastases. Therefore, we hypothesize that these CTCs could have been released from the bone marrow where they were preserved in special niches as dormant tumor cells with the ability to mimic osteogenic cells.

Concerning the immune system escape and survival, the PD-1/PD-L1 axis has been extensively studied in the last years after the introduction of cancer immunotherapy. Recently, we demonstrated that CTCs in breast cancer can express PD-L1 as a camouflage against T-lymphocytes<sup>30</sup>. However, PD-L1 expression was not detected in any of the nine CTC lines from this patient with colon cancer. Indeed, PD-L1 expression can vary, and it is still debated whether this protein is expressed more at early<sup>31</sup> or late, metastatic stages<sup>32,33</sup>. Conversely, *CEACAM1*, *DEFA6* and *IL33*, three genes encoding proteins also involved in immune escape, were expressed by all nine CTC lines. *CEACAM1* upregulation at advanced stages of colorectal cancer is associated with invasiveness, metastasis, tumor progression and escape from the immune system<sup>34</sup>. Upregulation of *DEFA6*<sup>14</sup> and *IL33*<sup>35</sup> also is involved in immune mechanisms. Therefore, these three factors could be attractive targets for immunotherapy.

Another important feature of aggressive cancer cells is the capacity to induce neo-angiogenesis that is required to import nutrients and oxygen in the growing tumor. All the new CTC lines expressed and secreted VEGF, a key inducer of angiogenesis, as already shown for the CTC-MCC-41 line<sup>13</sup>. Moreover, a functional test based on *in vitro* tube formation using endothelial cells showed that CTC-MCC-41, 41.4, 41.5B, 41.5E and 41.5F cell supernatants induced a denser network of endothelial tubes than those of CTC-MCC-41.5C, D and G cells. These data highlight again the heterogeneity of the nine CTC lines. As the fluoro-EPISPOT assay showed that VEGF secretion was comparable in all nine CTC lines, other factors must be involved in CTC-induced angiogenesis. For instance, semaphorin 6A, a member of the semaphorin family involved in cell motility and proliferation, also contributes to vascular development and particularly to angiogenesis by modulating VEGFR2 signaling in endothelial cells<sup>36</sup>. The stronger *SEMA6A* expression in the CTC-MCC-41, 41.4, 41.5B, 41.5E and 41.5F lines could explain the heterogeneous results of the functional assay. Moreover, Loria *et al.* showed that forced *SEMA6A* overexpression induces anchorage-independent growth and significantly increases invasiveness. Interestingly, *SEMA6A* is overexpressed in *BRAF*<sup>V600E</sup> melanoma cells<sup>36</sup>. This *BRAF* mutation was detected (i) in the primary tumor, (ii) in all the CTC lines, (iii) the lymph node metastasis, and also (iv) in tumor xenografts obtained by injection of CTC-MCC-41 cells<sup>13</sup>. Thus, *SEMA6A* might represent a new potential therapeutic target in *BRAF*<sup>V600E</sup> cancers.

In conclusion, we established nine permanent colon CTC lines from the same patient with metastatic colon cancer at different time points during disease progression. We then thoroughly characterized these CTC lines, highlighting their heterogeneity, which suggests that they are derived from different clones, and also their common features. Indeed, they all show (i) an intermediate epithelial/mesenchymal phenotype, (ii) stem-cell like characteristics, (iii) the potential to induce angiogenesis, (iv) an osteomimetic signature, and (v) the capacity to escape from the immune system. These features suggest that they could initiate metastases at distant sites. Moreover, in these CTC lines, we observed changes in RNA and protein expression, while the analysis of chromosomal CNA revealed no significant pattern of selected genomic alterations. For instance, the stem-cell like characteristics progressively increased in the CTC lines isolated at later stages of cancer progression. These data indicate that CTCs cultured from sequential liquid biopsies during therapy have common traits, but that treatment-resistant clones with distinct phenotypic characteristics are selected.

## Methods

**Patients and blood collection.** After signature of the informed consent, peripheral blood samples from patients with non-resectable metastatic colorectal adenocarcinoma were collected before the start and during first-line chemotherapy (FOLFIRI and bevacizumab) in the framework of the COLOSPOT study (NCT01596790; 168 patients included between 2011 and 2015). The study was carried out in accordance with the World Medical Association Declaration of Helsinki and the guidelines for experimentation with humans by the Bioethical committee “Sud Méditerranée III”. The experimental protocol was approved (Approval reference No. 2011-A01130-41) by the Bioethical committee “Sud Méditerranée III”. All included subjects provided written informed consent for participation in this study and the publication of results. The Blood (10 mL) was collected in CellSave tubes (SILICON BIOSYSTEMS, Menarini, Bologna, Italy) for CTC enumeration and in EDTA tubes (10 mL) for *in vitro* CTC culture.

**Clinical characteristics and history of patient 044.** Patient 044 was diagnosed at an advanced stage of colon cancer (lymph nodes and liver metastases, but no evidence of bone metastasis). He showed good tolerance to the first-line treatment (FOLFIRI + bevacizumab), but, after 5 cycles, had to stop it because of cancer progression (RECIST criteria). A second-line treatment was immediately initiated (FOLFOX + bevacizumab), but again, the treatment was stopped after three cycles due to clinical progression (peritoneal carcinomatosis), and no other

treatment was planned. The patient was hospitalized for abdominal pain and occlusive syndrome, and a new relapse was diagnosed. He died 6 months after the diagnosis of cancer, 2 months after the last treatment, and 1 week after the last blood sample collection. Additional clinical data about this special patient are available in the Supplementary Table S5.

**CTC enrichment and *in vitro* culture.** The blood collected in EDTA tubes was immediately used for CTC culture. First, viable CTCs were enriched through depletion of hematopoietic CD45<sup>(+)</sup> cells using the RosetteSep Human Circulating Epithelial Tumor Cell Enrichment Cocktail (STEMCELL TECHNOLOGIES, Vancouver, Canada), according to the manufacturer's protocol. Then, CD45<sup>(-)</sup> CTCs were incubated at 37 °C in 24-well non-adherent plates with CTC culture medium (RPMI1640 with EGF and FGF-2, Insulin-Transferrin-Selenium supplement, L-Glutamine), as previously described<sup>13</sup>. Colospheres and tumor cells were observed during the first weeks of culture and were then transferred to new 24-well plates for further growth and to T25 flasks for cell expansion. In these conditions, CTCs could be quickly expanded and after a few months, billions of tumor cells were obtained.

**Cell culture.** The colorectal cell lines HT-29 (ATCC HTB-38) and SW620 (ATCC CCL-227) were used as control for several tests, as well as the lung cancer cell line A549 (ATCC CRM-CCL-185), the mammary cancer cell lines SKBR3 (ATCC HTB-30) and BC-M1 (an established breast cancer cell line from a single disseminated tumor cell in the bone marrow of a patient with non-metastatic breast cancer patient)<sup>37</sup>. HT-29 and SKBR3 cells were maintained in Dulbecco's Modified Eagle's medium (DMEM), 10% fetal bovine serum (FBS), and 1% penicillin/streptomycin. A549 cells were maintained in DMEM supplemented with 10 mM HEPES, 10% FBS and 1% penicillin/streptomycin. SW620 cells were maintained in RPMI 1640 with L-glutamine and 10% FBS, and the colon CTC lines and BC-M1 cells in CTC culture medium. Endothelial cells were cultured in M199 (M4530, SIGMA ALDRICH, St Louis, USA) containing 0.4 ng/ml endothelial growth factors ( $\beta$ ECGF; E1388, SIGMA ALDRICH), 20  $\mu$ g/ml heparin (H4784, SIGMA ALDRICH, Saint-Louis, USA), 1% penicillin/streptomycin and 20% FBS (endothelial cell medium).

**Next-generation sequencing for copy-number alterations.** One colosphere and two single cells were transferred to individual PCR tubes (0.2 mL) for whole genome amplification using the PicoPlex WGA Kit, according to the manufacturer's protocol (RUBICON GENOMICS, Ann Arbor, USA). Next, libraries were prepared using the TruSeq DNA Sample Prep Kit according to the manufacturer's protocol. NGS was performed on a HiSeq. 2500 apparatus (ILLUMINA, San Diego, USA) using single reads for 100 cycles. Sequence analyses and CNA identification were performed using Control-FREEC and a custom-made script in MatLab.

**CTC mutational status.** DNA was extracted from the nine colon CTC lines using the QIAamp DNA Mini Kit (QIAGEN GmbH, Hilden, Germany), according to the manufacturer's instructions. DNA yield and purity were assessed with a Qubit spectrophotometer (THERMO FISHER SCIENTIFIC, Wilmington, DE). DNA was stored at -20 °C in 10 mmol/L Tris and 0.5 mmol/L EDTA (pH 7.6).

Genomic DNA samples were genotyped using MassArray iPLEX Pro (AGENA BIOSCIENCE), a Matrix Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry assay. A specific colorectal cancer panel (Imagenome Labosud, France) was customized for the detection of mutations in six oncogenes (Supplementary Table S6), selected on the basis of their role in colorectal cancer diagnosis, treatment and follow-up. The Assay Design Suite software (ADS, AGENA BIOSCIENCE) was used to design four sets of PCRs and iPLEX primers. Briefly, 2  $\mu$ L (10 ng) of genomic DNA was amplified in 4 wells by multiplex PCR with specific primers for wild type (WT) and mutant DNA (mtDNA), followed by shrimp-alkaline-phosphatase (SAP) treatment to remove the surplus nucleotides. Then, a primer single base extension reaction (iPLEX Pro) was performed with mass-modified ddNTP terminators. The extension products were dispensed on a SpectroCHIP Array and detected by MassARRAY mass spectrometry. Data were analyzed with the MassARRAY Typer Analyzer software<sup>38</sup>.

**RT-qPCR analysis of colon CTC lines.** Messenger RNA (mRNA) was extracted from each CTC line using the RNeasy Mini Kit (74106, QIAGEN, Hilden, Germany), and complementary DNA (cDNA) was obtained by reverse transcription (RT) using the SuperScript III First-Strand Synthesis Super Mix kit (18080, INVITROGEN, Carlsbad, USA), according to the manufacturer's instructions. Semi-quantitative PCR that targeted a wide range of sequences (Supplementary Table S2) was used to assess the expression of a large panel of genes, including all the transcripts previously validated as up- or down-regulated after a microarray assay in the CTC-MCC-41 line<sup>14</sup>. In all experiments,  $\beta$ 2-microglobulin (*B2M*) was used as reference housekeeping gene. One  $\mu$ L of each RT product was added to 9  $\mu$ L of mix containing the two primers (6  $\mu$ M each) and SYBR Green (600882, Brilliant III Ultra-Fast SYBR Green Master Mix, AGILENT TECHNOLOGIES, Santa Clara, USA). PCR reactions were performed on a QuantStudio 5 real-time PCR instrument (THERMO FISHER, Waltham, USA) as follows: 95 °C for 3 min, followed by 40 cycles of 95 °C for 5 s, 60 °C for 10 s, and then 1 cycle at 95 °C for 1 min, 55 °C for 5 s, 95 °C for 30 s, 37 °C for 30 s. Results were analyzed using the QuantStudio Design and Analysis Software. Hierarchical clustering was computed with the Cluster and Treeview software packages (PMID: 9843981).

**Flow cytometry and immunofluorescence experiments.** A panel of antibodies (Supplementary Table S7) was used to characterize the nine colon CTC lines. The IntraPrep Permeabilization Reagent kit (A07803, Beckman Coulter, Brea, USA) was employed to study the expression of intracellular proteins, whereas conjugated antibodies were directly used for membrane proteins.

Labeled tumor cells were analyzed using a Cyan cytometer (BECKMAN COULTER) and data interpreted with the Kaluza software (BECKMAN COULTER). In parallel, labeled tumor cells were also loaded on glass slides, using a cytospin, and observed under a fluorescent microscope (Axio Observer D1, ZEISS, Oberkochen, Germany).

**Fluoro-EPISPOT assays.** The fluoro-EPISPOT assays for CK19, VEGF and OPG were performed as previously described<sup>13,39,40</sup>.

**Endothelial cell tube formation assay.** 24-well plates were coated with 250 µL/well Matrigel (BD BIOSCIENCES, San Jose, USA) and left at 37 °C for 30 min. Endothelial cells (10<sup>5</sup> cells/well) were then seeded and cultured for 6 h with different media: RPMI 1640 alone (basal medium; negative control), endothelial cell medium with 20% FBS (positive control), and supernatant from the CTC lines cultured with basal medium for 48 h.

**ALDH1 activity assay.** ALDH1 activity was measured using the ALDEFLUOR kit (01700 – STEMCELL TECHNOLOGIES) following the manufacturer's instructions. Briefly, 5 µL of ALDEFLUOR Reagent was added to the cell suspension and half of the cell mixture was immediately transferred to another tube with the ALDEFLUOR DEAB Reagent (an ALDH activity inhibitor) to serve as internal negative control to quantify ALDH activity in each cell line. A549 cells were used as positive control, as recommended by the manufacturer.

**Statement of Significance.** Only metastasis-competent CTCs lead to cancer relapse, but their specific signature is not known. The nine sequential autologous CTC lines we derived from a patient with metastatic colorectal cancer will help to study CTC changes during cancer progression, a prerequisite to discover new therapeutic targets and to propose personalized treatments.

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### Author Contributions


C.A.-P. and L.C. conceived the concepts of the research. E.A. and T.M. included the colon cancer patients in the COLOSPOT study. A.S. and L.C. performed the experiments for the characterization of the 9 CTC lines unless for the genomic characterization that has been done by A.B., K.P. and P.-J.L. A.S., L.C., A.B., P.-J.L., S.A., K.P. and C.A.-P. analyzed the data. All authors wrote and revised the manuscript.

### Additional Information

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## PARTIE 2 – Analyse du transcriptome des lignées CTC coliques et biomarqueurs

Afin d'appréhender les degrés de l'hétérogénéité clonale de nos 9 lignées CTCs, nous avons effectué une étude transcriptomique à l'aide de la plateforme Affymetrix en utilisant les puces 'Human U133 Plus'. Une étude approfondie du transcriptome de ces lignées CTCs nous a permis (i) d'avoir une vision globale des événements moléculaires impliqués dans la progression du cancer du côlon et (ii) de fournir potentiellement des biomarqueurs qui pourraient aider à développer de nouvelles thérapies ciblant spécifiquement les CTCs responsables de la génération de métastases et de rechute chez les patients atteints d'un cancer du colorectal.

Le premier article de ce chapitre (*article 3*) fait la comparaison du transcriptome de la première lignée CTC-MCC-41 avec celui d'une lignée cellulaire cancéreuse primaire du côlon HT-29 (considérée comme 'non agressive'). Cette étude a mis en évidence dans les cellules CTC-MCC-41, une surexpression de gènes impliqués dans des fonctions importantes pour le développement et la survie des cellules tumorales, telles que le métabolisme cellulaire, la croissance et mort cellulaire, des propriétés de cellules souches et de réparation de l'ADN. Cette étude a également permis de mettre en évidence une utilisation importante du métabolisme mitochondrial par les CTCs.

De manière très intéressante, la comparaison de ces données avec des données publiques de différents types de lignées cellulaires coliques (obtenues à partir de tumeurs primaires et de métastases coliques), a pu montrer que la lignée CTC se départageait complétement de ces autres lignées cellulaires cancéreuses coliques. On s'attendait à ce que cette lignée CTC-MCC-41 ait plus de caractéristiques communes avec des lignées cellulaires issues de métastases, car par définition les cellules formant les métastases ont été initialement des CTCs, or ce n'est pas le cas. On retrouve même plus de similitudes entre les lignées cellulaires issues de tumeurs primaires et de métastases que dans la comparaison de chaque type avec les lignées CTCs, démontrant que ces dernières ont un profil transcriptomique qui leur est propre et unique. Cela pourrait s'expliquer par la ré-acquisition par les lignées cellulaires de métastases d'un profil épithélial avec la présence de molécules de jonction et d'adhésion, comme cela était le cas au sein de la tumeur primaire, contrairement aux CTCs qui sont encore dans un état transitoire et de survie dans le compartiment sanguin qui leur est très drastique et sans attache possible.

La comparaison du transcriptome des lignées CTCs au cours de la maladie est rapportée dans l'*article 4*. Cette étude nous a permis d'apprécier les modifications transcriptomiques acquises au cours de la progression du cancer et les caractéristiques moléculaires spécifiques des derniers clones de CTCs représentatifs de la maladie juste avant le décès du patient. Une première analyse révèle un grand

nombre de transcrits différentiellement surexprimés dans les lignées CTC obtenues après traitement, suggérant l'acquisition d'un certain nombre de propriétés pour s'adapter et résister aux traitements. Les mécanismes de réparation de l'ADN restent sur-régulés dans cette comparaison alors que les gènes liés au métabolisme énergétique et respiratoire de la mitochondrie ressortent encore plus dérégulés après traitement, impliquant une potentielle altération des fonctions mitochondriales sous traitement. Parmi les lignées cellulaires obtenues après traitement, on a pu observer la dérégulation de voies métaboliques impliquées dans l'EMP et le métabolisme énergétique mais surtout dans le métabolisme des xénobiotiques qui est le mécanisme de détoxification des cellules en molécules chimiques (par ex, les traitements).

Outre une meilleure compréhension des voies de signalisation impliquées dans le processus métastatique, cette étude nous a permis de mettre en évidence la présence d'une surexpression de la protéine CDA dans les lignées obtenues après traitement et particulièrement dans la CTC-MCC-41.4, obtenues au moment de la dernière cure de traitement du patient. CDA est une enzyme intervenant dans le maintien de la quantité de pyrimidine au sein de la cellule, et le 5FU (qui a été donné au patient dans les 2 lignes de chimiothérapie) est un traitement par analogue de la pyrimidine. Ceci suggère une adaptation des cellules tumorales pour éliminer spécifiquement ce médicament. De plus, cette protéine est sécrétée par les cellules, elle pourrait donc représenter un '*candidat biomarqueur*' plasmatique pour surveiller le développement d'une résistance au 5-FU. L'analyse des derniers clones sélectionnés nous a également permis de distinguer 2 sous-groupes parmi les dernières CTC-MCC-41.5 : [ABFG] et [CDE]. Parmi les gènes dérégulés dans ces 2 sous-groupes, nous avons trouvé ALDOB, une enzyme du métabolisme du fructose exprimé dans le foie, qui a été décrite comme acquise par les cellules tumorales coliques générant des métastases hépatiques. Ainsi, parmi les dernières lignées de CTCs, nous avons le sous-groupe [CDE] dont les cellules proviennent de la tumeur primaire et le groupe [ABFG] qui sont des clones originaires de métastases hépatiques.

Finalement dans le dernier article de ce chapitre (*article 5*), nous avons utilisé les données générées par les puces Affymetrix pour observer plus spécifiquement le comportement des gènes décrits dans les mécanismes de transition EMT et MET. Cette étude a montré la dérégulation à la hausse de plusieurs gènes impliqués dans le processus reverse de la MET, alors que les effecteurs connus de l'EMT comme ZEB1 ou SIX1 ne montrent pas de dérégulation, même dans des conditions où l'on induit cette transition. De plus, la sur-régulation de certaines cibles des effecteurs EMT décrits révèle la complexité de ces mécanismes, suggérant des processus en plusieurs étapes. Cependant, l'augmentation significative de l'expression de gènes intervenant dans la MET nous a démontré que les cellules tumorales initiatrices de métastase acquièrent un phénotype d'invasion et de colonisation dès leur entrée dans le système circulatoire.





Article 3

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Molecular Portrait of Metastasis-Competent Circulating Tumor Cells in Colon Cancer Reveals the Crucial Role of Genes Regulating Energy Metabolism and DNA Repair.

Catherine Alix-Panabières, **Laure Cayrefourcq**, Thibault Mazard, Thierry Maudelonde, Eric Assenat and Said Assou.

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# Molecular Portrait of Metastasis-Competent Circulating Tumor Cells in Colon Cancer Reveals the Crucial Role of Genes Regulating Energy Metabolism and DNA Repair

Catherine Alix-Panabières,<sup>1,2\*</sup> Laure Cayrefourcq,<sup>1,2</sup> Thibault Mazard,<sup>3,4</sup> Thierry Maudelonde,<sup>5,2</sup> Eric Assenat,<sup>6</sup> and Said Assou<sup>7,8\*</sup>

**BACKGROUND:** Unraveling the molecular mechanisms that regulate the biology of metastasis-competent circulating tumor cells (CTCs) is urgently needed to understand metastasis formation and tumor relapse. Our group previously established the first cell line (CTC-MCC-41) derived from metastasis-competent CTCs of a patient with colon cancer.

**METHODS:** In this study, we analyzed the transcriptome of CTC-MCC-41 cells using Human Genome U133 Plus 2.0 microarrays with the aim of unraveling the molecular basis of their special features (stem cell properties and ability to initiate and support metastasis formation).

**RESULTS:** Comparison of the transcriptome data of metastasis-competent CTC-MCC-41 cells and of HT-29 cells (derived from a primary colon cancer) highlights the differential expression of genes that regulate energy metabolism [peroxisome proliferator-activated receptor  $\gamma$  coactivator 1A (*PPARGC1A*), peroxisome proliferator-activated receptor  $\gamma$  coactivator 1B (*PPARGC1B*), fatty acid binding protein 1 (*FABP1*), aldehyde dehydrogenase 3 family member A1 (*ALDH3A1*)], DNA repair [BRCA1 interacting protein C-terminal helicase 1 (*BRIP1*), Fanconi anemia complementation group B (*FANCB*), Fanconi anemia complementation group M (*FANCM*)], and stemness [glutaminase 2 (*GLS2*), cystathionine- $\beta$ -synthase (*CBS*), and cystathionine  $\gamma$ -lyase (*CTH*)]. The differential expression of 20 genes was validated by quantitative reverse transcription PCR.

**CONCLUSIONS:** This study gives a comprehensive outlook on the molecular events involved in colon cancer progression and provides potential CTC biomarkers that may help develop new therapies to specifically target CTCs with stem cell properties that cause metastases and tumor relapse in patients with colon cancer.

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Circulating tumor cells (CTCs)<sup>9</sup> are invasive tumor cells that disseminate from the primary tumor in the blood and might represent a fundamental prerequisite for metastasis formation (1). CTC analysis as a “real-time liquid biopsy” (1) in epithelial tumors (i.e., breast, prostate, lung, and colon cancer) for personalized medicine has received tremendous attention in recent years (2) and has important clinical implications (3). However, the low CTC concentration in blood samples, especially from patients with early stage cancer, is a crucial limiting factor for the identification of “metastasis-initiator cells.” Moreover, information on CTC functional properties is quite limited because their low number in the peripheral blood makes difficult the generation and maintenance of in vitro cultures of CTCs from patients with tumors (4, 5). Recently, we derived the first cell line, named CTC-MCC-41, from CTCs isolated from the blood of a patient with colon cancer (6). CTC-MCC-41 cells have a stable intermediate epithelial-mesenchymal phenotype and stem cell–like properties, and these cells can produce

<sup>1</sup> Laboratory of Rare Human Circulating Cells, Department of Cellular and Tissue Biopathology of Tumors, University Medical Centre, Montpellier, France; <sup>2</sup> EA2415 – Help for Personalized Decision: Methodological Aspects, University Institute of Clinical Research (IURC), University of Montpellier, Montpellier, France; <sup>3</sup> Department of Medical Oncology, Institut du Cancer à Montpellier (ICM), France; <sup>4</sup> Institut du Cancer à Montpellier (ICM), Montpellier, France; <sup>5</sup> Laboratory of Hormonal and Cell Biology, University Medical Centre, Montpellier, France; <sup>6</sup> Department of Medical Oncology, University Medical Centre, Montpellier, France; <sup>7</sup> University of Montpellier, UFR de Médecine, Montpellier, France; <sup>8</sup> INSERM U1183; Institute for Regenerative Medicine and Biotherapy, CHU Montpellier, Hôpital Saint-Eloi, Montpellier, France.

\* Address correspondence to C.A.-P. at Institut Universitaire de Recherche Clinique (IURC), 641, Ave. du Doyen Gaston Giraud, 34093 Montpellier Cedex 5, France. Fax +33-4-11-75-99-33; e-mail c-panabieres@chu-montpellier.fr. S.A. at Institute for Research in Re-

generative Medicine and Biotherapy, Hôpital Saint-Eloi, 80 Ave. Augustin Fliche, 34295 Montpellier Cedex 5, France. Fax +33-4-67-33-01-13; e-mail said.assou@inserm.fr. Received July 11, 2016; accepted October 24, 2016.

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<sup>9</sup> Nonstandard abbreviations: CTCs, circulating tumor cells; MIAME, minimal information about microarray experiment; GEO, gene expression Omnibus; AGCC, Affymetrix GeneChip Command Console software; SAM, significance analysis of microarray; FDR, false discovery rate; FC, fold change; PCA, principal component analysis; IPA, ingenuity pathway analysis; TAC, transcriptome analysis console; RT, reverse transcription; qPCR, quantitative PCR; DEs, differentially expressed transcripts; hESCs, human embryonic stem cells; FA, fanconi anemia; PSCs, pluripotent stem cells; GLS2, glutaminase 2; CBS, cystathionine- $\beta$ -synthase; CTH, cystathionine- $\gamma$ -lyase.

## Transcriptome of Metastasis-Competent CTCs in Colon Cancer

tumors when xenografted in immunodeficient mice (6). CTC lines can be used to identify proteins and pathways involved in cancer dissemination and stemness and also to test new drugs to inhibit metastasis-competent CTCs. For these purposes, it is now important to determine the molecular bases underlying the functional differences between CTC lines, such CTC-MCC-41 cells, and cancer cell lines derived from the primary tumor.

In this study, we used DNA microarray technology to compare the transcriptome of metastasis-competent CTC-MCC-41 cells and of HT-29 cells, a cell line established from a primary colon cancer. This comparison allowed us to identify a specific molecular signature of CTCs that gives crucial information on their stem cell properties and their ability to initiate and support the formation of distant metastases. These findings may provide a starting point for investigating specific molecular mechanisms and key pathways involved in colon cancer progression. Moreover, such data may supply insights for the discovery of new biomarkers to identify the most aggressive CTC subpopulations and for the development of new drugs to inhibit metastasis-initiator CTCs.

### Materials and Methods

#### CTC ISOLATION AND ESTABLISHMENT OF THE CTC-MCC-41 CELL LINE

We previously described the generation of the colon CTC line named CTC-MCC-41 (6) that now has been growing in culture for 3 years with a constant doubling time (see the Data Supplement that accompanies the online version of this article at <http://www.clinchem.org/content/vol63/issue3>).

#### CLINICOPATHOLOGIC CHARACTERISTICS OF THE PATIENT AND TUMOR GIVING RISE TO CTC-MCC-41

Standard histopathologic analysis of diagnostic biopsies and clinical data of the patient have been detailed in the online Supplemental Data.

#### HT-29 CELL LINE

The HT-29 cell line (ATCC® HTB-38™) is a human colorectal adenocarcinoma cell line that was established in 1964 by J. Fogh from the primary tumor of a 44-year-old Caucasian female (7) (see online Supplemental Fig. S1).

#### RNA ISOLATION

The RNeasy Mini Kit (ref.74106; Qiagen) was used to extract total RNA from each cell sample, according to the manufacturers' recommended protocol (see online Supplemental Data). CTC-MCC-41 cells were prepared at passages P6, P8, and P10 and HT-29 cells at passages P130, P132, and P134.

#### COMPLEMENTARY RNA PREPARATION AND MICROARRAY HYBRIDATION

Total RNA (200 ng) was used to prepare cRNA using the Affymetrix 3' IVT express protocol (ref.901229). The complete cDNA was amplified by in vitro transcription (see online Supplemental Data). Amplified RNA (aRNA) was quantified with a NanoDrop ND-1000 spectrophotometer. After fragmentation, 12  $\mu$ g of labeled antisense aRNA was hybridized to HG-U133 plus 2.0 GeneChip arrays (Affymetrix®). In total, 6 chips (3 chips for CTC-MCC-41 cell samples and 3 chips for HT-29 cell samples) were used for the microarray hybridization experiments. Microarray data were obtained and analyzed according to the minimal information about microarray experiment (MIAME) recommendations (8).

#### ACCESSION NUMBER

All our data are accessible at the gene expression Omnibus (GEO) repository (<https://www.ncbi.nlm.nih.gov/geo>) with the provisional accession series number GSE82198. The 14 publicly available Affymetrix data on other colon cancer cell lines are accessible through the accession numbers GSM224696, GSM224697, GSM224698, GSM274711, GSM274712, GSM1624316, GSM1624317, GSM274713, GSM274714, GSM800783, GSM274717, GSM274718, GSM274773, and GSM274774 (samples).

#### DATA PROCESSING AND BIOINFORMATICS ANALYSIS

After image processing with the Affymetrix GeneChip® Command Console® Software (AGCC), the CEL files were analyzed using the Affymetrix Expression Console™ software v1.3.1 and normalized with the MAS5.0 algorithm by scaling each array to a target value (TGT) of 100 using the global scaling method to obtain an intensity value signal for each probe set. First, microarray data were filtered based on the detection call (absent/present). Then, transcripts with significant differential expression profiles were identified using the 2-class Significance Analysis of Microarray (SAM) algorithm (<http://www.stat.stanford.edu/~tibs/SAM/>) with the Wilcoxon test and sample label permutations ( $n=300$ ). Only transcripts with a significant false discovery rate (FDR)  $<5\%$  and fold change (FC)  $\geq 2$  were retained. Hierarchical clustering analyses based on the expression levels of the differentially expressed genes were performed with the Cluster and TreeView software packages (9). Principal component analysis (PCA) was performed using GenomicScape (<https://www.genomicscape.com>). The Ingenuity Pathway Analysis (IPA) software (<http://www.ingenuity.com>) and Pathway Studio 9.0 (Ariadne Genomics) were used for the functional assessment of genes that were differentially expressed in CTC-MCC-41 and HT-29 cells (see online Supplemental Data). In addition, we used Affymetrix Tran-

scriptome Analysis Console (TAC) software to construct a volcano plot and chromosome view.

#### REVERSE TRANSCRIPTION-QUANTITATIVE PCR ANALYSIS

Reverse transcription (RT) and quantitative PCR (qPCR) were performed as recommended by the manufacturer (Invitrogen) (see online Supplemental Data). The primer sequences are shown in the online Supplemental Table S1.

#### STATISTICAL ANALYSIS

Each experiment was performed as least 3 times and data are shown as the mean  $\pm$  SE. The differences were evaluated using the Student *t*-test. Statistical analyses were performed with GraphPad InStat (Mann–Whitney *U*-test; GraphPad). A value of  $P \leq 0.05$  was considered to be statistically significant.

### Results

#### PHENOTYPIC AND MOLECULAR CHARACTERIZATION OF CTC-MCC-41 CELLS (A METASTASIS-COMPETENT COLON CTC CELL LINE) AND HT-29 CELLS

Despite the different sources (circulating CTCs vs primary tumor) and culture methods (suspension vs adherent culture), the analysis of specific epithelial markers by immunocytochemistry revealed that both CTC-MCC-41 and HT-29 cells expressed epithelial markers, such as EpCam and cytokeratin 20 (Fig. 1A). This suggests that these 2 colon cell lines have a similar phenotype. Then, the transcriptome profile of CTC-MCC-41 and HT29 cells was determined by using high-density oligonucleotide microarray analysis. The 3-dimensional distribution profile obtained by PCA of the variance for each gene showed that (a) samples from the same category (CTC-MCC-41 or HT29 cells) grouped together very tightly, corroborating the robustness of the microarray analysis, and that (b) CTC-MCC-41 cell samples were separated from HT-29 samples, demonstrating a significant difference in the molecular profile of these colon cancer cell lines (Fig. 1B). A volcano plot of the combined expression data of all transcripts grouped by fold change difference and *P* values (Fig. 1C) confirmed the results of the PCA analysis. The chromosomal distribution of transcripts expressed differently in CTC-MCC-41 than in HT-29 cells are shown in a chromosome summary graph (see online Supplemental Fig. S2).

#### IDENTIFICATION OF SETS OF TRANSCRIPTS THAT ARE DIFFERENTIALLY EXPRESSED IN CTC-MCC-41 AND HT-29 CELLS

To gain insight into the molecular basis of the invasive properties of CTCs with stem cell properties, the transcriptome profiles of CTC-MCC-41 and HT-29 cells were then compared. A first selection based on the

detection call retained 30 167 transcripts. Then, the SAM software with a 2-fold change cutoff and FDR  $< 5\%$  identified 7553 differentially expressed transcripts (DETs) that significantly distinguished CTC-MCC-41 from H29 cell samples. Specifically, 3670 transcripts (probe sets) were upregulated in CTC-MCC-41 cells and 3883 transcripts (probe sets) were upregulated in HT-29 cells (see online Supplemental Tables S2 and S3). Hierarchical clustering analysis based on the DETs clearly segregated CTC-MCC-41 from HT-29 cell samples (Fig. 2A). DETs were then functionally categorized using the IPA software. A large portion of DETs were involved in (a) cell morphology, cellular function, cellular assembly, and organization (score=30) (Fig. 2B-a); (b) cell cycle, cellular movement, and cancer (score = 26) (Fig. 2B-b); and (c) metabolism, cell-to-cell signaling, and interaction (score = 26) (Fig. 2B-c). As expected, various blood cell- and cell movement-related adhesion molecules were found among the top regulator networks.

#### MOLECULAR DIVERGENCE BETWEEN CTC-MCC-41 AND HT-29 CELLS

The SAM analysis revealed that CTC-MCC-41 and HT-29 cells had specific molecular signatures. The “colon CTC signature” included 2324 genes (see online Supplemental Table S4) that were significantly upregulated in CTC-MCC-41 cells, among which 29% showed the highest FC ( $5 \leq FC \leq 1900$ ). The “HT-29 signature” included 2294 genes (see online Supplemental Table S5) that were upregulated in HT-29 cells, among which 32% had the highest FC ( $5 \leq FC \leq 1102$ ) (Fig. 3A). The “colon CTC signature” included the defensin  $\alpha$  genes [defensin alpha 5 (*DEFA5*) and defensin alpha 6 (*DEFA6*)],<sup>10</sup> trefoil factors [trefoil factor 3 (*TFF3*) and

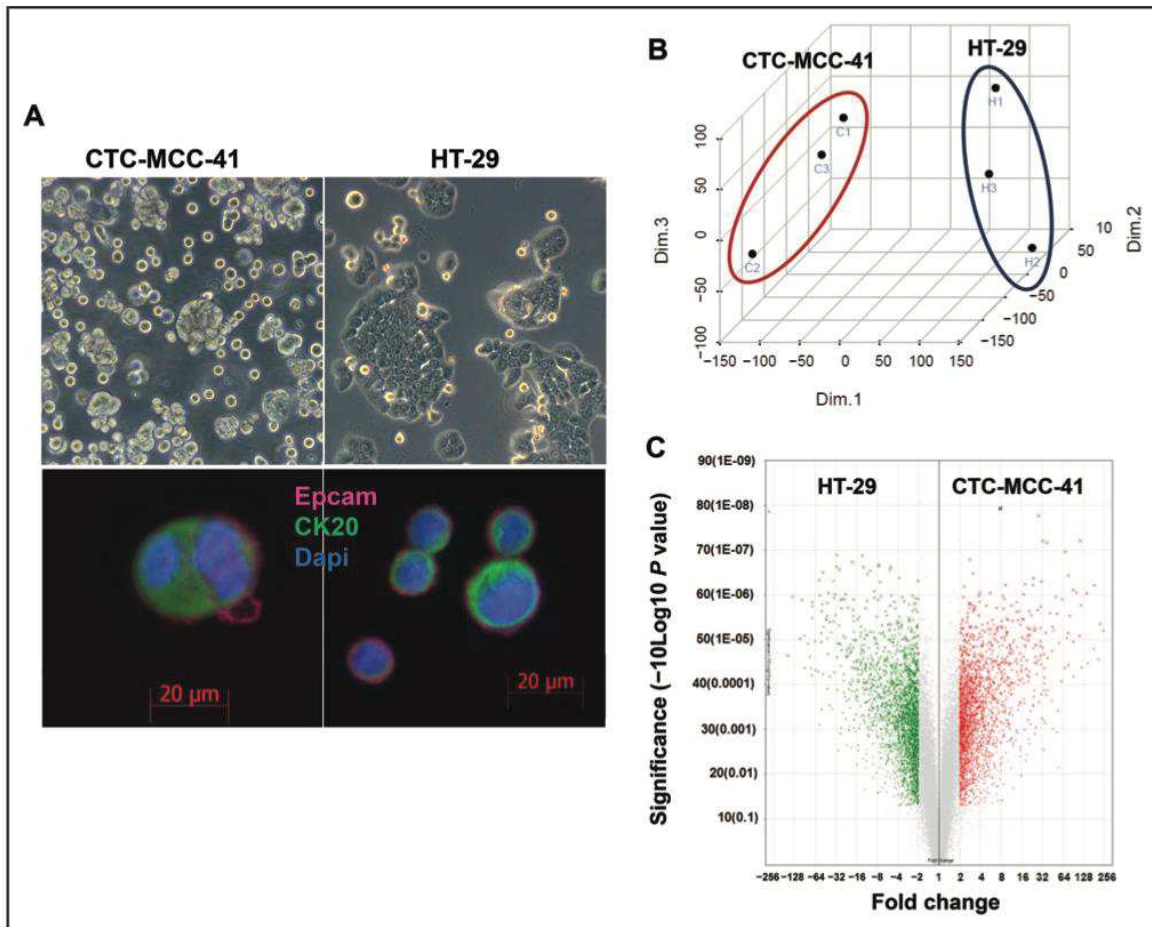
<sup>10</sup> Human genes: *DEFA5*, defensin alpha 5; *DEFA6*, defensin alpha 6; *TFF3*, trefoil factor 3; *TFF1*, trefoil factor 1; *KRAS*, *KRAS* proto-oncogene, GTPase; *IL-33*, interleukin-33; *IL-37*, interleukin-37; *IL12A*, interleukin 12A; *CXCL3*, C-X-C motif chemokine ligand 3; *CXCL16*, C-X-C motif chemokine ligand 16; *CCL24*, C-X-C motif chemokine ligand 24; *BMP5*, bone morphogenetic protein 5; *BMP7*, bone morphogenetic protein 7; *BMP8B*, bone morphogenetic protein 8B; *PPARGC1A*, peroxisome proliferator-activated receptor  $\gamma$  coactivator 1A; *PPARGC1B*, peroxisome proliferator-activated receptor  $\gamma$  coactivator 1B; *KLK1*, kallikrein 1; *KLK7*, kallikrein 7; *KLK8*, kallikrein 8; *KLK15*, kallikrein 15; *TNFRSF1B*, tumor necrosis factor receptor superfamily 1B; *TNFRSF10B*, tumor necrosis factor receptor superfamily 10B; *TNFRSF10A*, tumor necrosis factor receptor superfamily 10A; *BCL2L14* (*BCL-G*), BCL2 like 14; *BCL2L15* (*BFK*), BCL2 like 15; *BCL2L11*, BCL2 like 11; *BIK*, BCL2 interacting killer; *DAPK2*, death-associated protein kinase 2; *FAS*, fas cell surface death receptor; *BAX*, BCL2-associated X protein; *TP53*, tumor protein 53; *TP53INP1*, *TP53* inducible nuclear protein 1; *RRM2B*, ribonucleotide reductase regulatory TP53 inducible subunit M2B; *CCND2*, cyclin D2; *CCND1*, cyclin D1; *MDM2*, MDM2 proto-oncogene; *PLAGL1*, pleomorphic adenoma gene-like 1; *DRAM1*, DNA-damage regulated autophagy modulator 1; *GREM1*, gremlin 1; *INHBB*, inhibin beta B subunit; *PDGFC*, platelet derived growth factor C; *SMARCA1*, SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily A, member 1; *SMARCA2*, SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily A, member 2; *TIMP*, tissue inhibitor of metalloproteinases; *TIMP2*, metalloproteinase inhibitor 2; *ADAMTS6*, ADAM metalloproteinase with thrombospondin type 1 motif 6; *ADAM28*, ADAM metalloproteinase domain 28; *ADAM22*, ADAM metalloproteinase domain 22;

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trefoil factor 1 (*TFPI*), an oncogene [KRAS proto-oncogene, GTPase (*KRAS*)], interleukins [interleukin-33 (*IL-33*), interleukin-37 (*IL-37*), and interleukin 12A (*IL12A*)], chemokines [C-X-C motif chemokine ligand 3 (*CXCL3*), C-X-C motif chemokine ligand 16 (*CXCL16*), and C-X-C motif chemokine ligand 24 (*CCL24*)], bone morphogenetic proteins [bone morphogenetic protein 5 (*BMP5*), bone morphogenetic protein 7 (*BMP7*), and bone morphogenetic protein 8B (*BMP8B*)], peroxisome proliferator-activated receptor  $\gamma$  coactivators [peroxisome proliferator-activated receptor  $\gamma$  coactivator 1A (*PPARGC1A*) and peroxisome proliferator-activated receptor  $\gamma$  coactivator 1B (*PPARGC1B*)], and kallikrein-related peptidases [kallikrein 1 (*KLK1*), kallikrein 7 (*KLK7*), kallikrein 8 (*KLK8*), and kallikrein 15 (*KLK15*)]. Several genes encoding proteins involved in apoptosis were also significantly overexpressed in the “colon CTC signature,” such as members of the tumor necrosis factor receptor superfamily [tumor necrosis factor receptor superfamily 1B (*TNFRSF1B*), tumor necrosis factor receptor superfamily 10B (*TNFRSF10B*), and tumor necrosis factor receptor superfamily 10A

(*TNFRSF10A*)], apoptosis facilitators including BCL2 like 14 [*BCL2L14* (*BCL-G*)], BCL2 like 15 [*BCL2L15* (*BFK*)] and the BH3-only member *BIM* [BCL2 like 11 (*BCL2L11*)], apoptosis-inducers [BCL2 interacting killer (*BIK*)], death-associated protein kinase 2 (*DAPK2*), fas cell surface death receptor (*FAS*), and BCL2-associated X protein (*BAX*). Genes connected to p53 signaling, such as the genes encoding tumor protein 53 (*TP53*) inducible nuclear proteins [*TP53* inducible nuclear protein 1 (*TP53INP1*) and ribonucleotide reductase regulatory TP53 inducible subunit *M2B* (*RRM2B*)], cyclins [cyclin D2 (*CCND2*) and cyclin D1 (*CCND1*)], MDM2 proto-oncogene (*MDM2*), pleomorphic adenoma gene-like 1 (*PLAGL1*), and DNA-damage regulated autophagy modulator 1 (*DRAM1*) were also up-regulated in CTC-MCC-41 cells. Moreover, gremlin 1 (*GREM1*) and inhibin beta B subunit (*INHBB*) that encode ligands of the TGF- $\beta$  superfamily were among the most highly expressed transcripts in CTC-MCC-41 cells. Conversely, the “HT-29 signature” was enriched in genes associated with guidance and cytoskeleton signaling, such as platelet derived growth factor C (*PDGFC*), chromatin rearrangement genes [SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily A, member 1 (*SMARCA1*) and SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily A, member 2 (*SMARCA2*)], tissue inhibitor of metalloproteinases (TIMP) metalloproteinase inhibitor 2 (*TIMP2*), many ADAM metalloproteinases [ADAM metalloproteinase with thrombospondin type 1 motif 6 (*ADAMTS6*), ADAM metalloproteinase domain 28 (*ADAM28*), ADAM metalloproteinase domain 22 (*ADAM22*), ADAM metalloproteinase domain 19 (*ADAM19*), ADAM metalloproteinase domain 17 (*ADAM17*), and ADAM metalloproteinase domain 8 (*ADAM8*)], plexins [plexin D1 (*PLXND1*), plexin A2 (*PLXNA2*), and plexin A1 (*PLXNA1*)], integrins [integrin subunit alpha 1 (*ITGA1*), integrin subunit alpha 3 (*ITGA3*), integrin subunit alpha M (*ITGAM*), integrin subunit alpha V (*ITGAV*), integrin subunit beta 1 (*ITGB1*), integrin subunit beta 5 (*ITGB5*), and integrin subunit beta 8 (*ITGB8*)], and myosins [myosin light chain kinase (*MYLK*), myosin light chain 6 (*MYL6*), and myosin light chain 10 (*MYH10*)]. Interleukins [interleukin 8 (*IL8*), interleukin 6 signal transducer (*IL6ST*), interleukin 7 (*IL7*), interleukin 15 (*IL15*), and interleukin 1 receptor type 1 (*IL1RI*)] and chemokines [C-X-C motif chemokine ligand 2 (*CXCL2*), C-X-C motif chemokine ligand 6 (*CXCL6*), C-X-C motif chemokine ligand 10 (*CXCL10*), C-X-C motif chemokine ligand 11 (*CXCL11*), C-C motif chemokine ligand 5 (*CCL5*), and C-C motif chemokine ligand 28 (*CCL28*)] also were overexpressed in HT-29 cells. Finally, the Gene Set Enrichment Analysis showed that several tumorigenesis-related genes were enriched in the “colon CTC signature,” such as carcinoembryonic antigen-related cell adhesion molecule 5 (*CEACAM5*) and carcinoembry-

*ADAM19*, ADAM metalloproteinase domain 19; *ADAM17*, ADAM metalloproteinase domain 17; *ADAM8*, ADAM metalloproteinase domain 8; *PLXND1*, plexin D1; *PLXNA2*, plexin A2; *PLXNA1*, plexin A1; *ITGA1*, integrin subunit alpha 1; *ITGA3*, integrin subunit alpha 3; *ITGAM*, integrin subunit alpha M; *ITGAV*, integrin subunit alpha V; *ITGB1*, integrin subunit beta 1; *ITGB5*, integrin subunit beta 5; *ITGB8*, integrin subunit beta 8; *MYLK*, myosin light chain kinase; *MYL6*, myosin light chain 6; *MYH10*, myosin light chain 10; *IL8*, interleukin 8; *IL6ST*, interleukin 6 signal transducer; *IL7*, interleukin 7; *IL15*, interleukin 15; *IL1RI*, interleukin 1 receptor type 1; *CXCL2*, C-X-C motif chemokine ligand 2; *CXCL6*, C-X-C motif chemokine ligand 6; *CXCL10*, C-X-C motif chemokine ligand 10; *CXCL11*, C-X-C motif chemokine ligand 11; *CCL5*, C-C motif chemokine ligand 5; *CCL28*, C-C motif chemokine ligand 28; *CEACAM5*, carcinoembryonic antigen-related cell adhesion molecule 5; *CEACAM6*, carcinoembryonic antigen-related cell adhesion molecule 6; *PLA2G2A*, phospholipase A2 group 2A; *ALDH3A1*, aldehyde dehydrogenase 3 family member A1; *PTGS1*, prostaglandin-endoperoxide synthase 1; *EMILIN2*, elastin microfibril interfacer 2; *PLOD2*, procollagen-lysine 2-oxoglutarate 5-dioxygenase 2; *COL18A1*, collagen type 18 alpha 1; *BCL11A*, B-cell CLL/lymphoma 11A; *IL33*, interleukin 33; *ABC11*, ATP binding cassette subfamily B member 11; *FNT1*, fibronectin 1; *SEMA6A*, semaphorin 6A; *GAL*, galanin; *PTGS2*, prostaglandin-endoperoxide synthase 2; *WNT11*, Wnt family member 11; *TGFB2*, transforming growth factor beta 2; *DKK1*, dickkopf WNT signaling pathway inhibitor 1; *BST2*, bone marrow stromal cell antigen 2; *GATA2*, GATA binding protein 2; *GJB6*, gap junction protein beta 6; *ADAMTS6*, ADAM metalloproteinase with thrombospondin type 1 motif 6; *NRF1*, nuclear respiratory factor 1; *FABP1*, fatty acid binding protein 1; *FABP5*, fatty acid binding protein 5; *ALDH3A2*, aldehyde dehydrogenase 3 family member A2; *ALDH1B1*, aldehyde dehydrogenase 1 family member B1; *ALDH2*, aldehyde dehydrogenase 2; *HSD17B1*, hydroxysteroid 17-beta dehydrogenase 1; *CYP2B6*, cytochrome P450 family 2 subfamily B member 6; *AKR1C4*, aldo-keto reductase family 1 member C4; *GSTM1*, -2, -3, and -4, glutathione S-transferase mu 1-4; *AOX1*, aldehyde oxidase 1; *XDH*, xanthine dehydrogenase; *MAOA*, monoamine oxidase A; *MAOB*, monoamine oxidase B; *PON2*, paraoxonase 2; *PON3*, paraoxonase 3; *VDAC1*, voltage dependent anion channel 1; *ABC*, ATP-binding cassette; *IGFBP3*, insulin-like growth factor binding protein 3; *GLS2*, glutaminase 2; *CBS*, cystathionine-beta-synthase; *CTH*, cystathionine gamma-lyase; *CD200*, CD200 molecule; *SALL4*, spalt like transcription factor 4; *ID1*, inhibitor of DNA binding 1; *APTX*, aprataxin; *APLF*, aprataxin and PNKP like factor; *XRCC4*, X-ray repair cross complementing 4; *BRIP1*, BRCA1 interacting protein C-terminal helicase 1; *FANCB*, Fanconi anemia complementation group B; *FANCM*, Fanconi anemia complementation group M; *XPC*, XPC complex subunit, DNA damage recognition and repair factor; *DDDB2*, damage specific DNA binding protein 2; *MLH3*, mutL homolog 3; *POLM*, DNA polymerase mu; *RAD51*, (RAD51 recombinase; *ERCC1*, ERCC excision repair 1; *TP53BP1*, tumor protein p53 binding protein 1.



**Fig. 1. Characterization of CTC-MCC-41 and HT-29 cells.**

(A), Representative images of single CTC-MCC-41 cells and spheres and HT-29 cells obtained using a bright-field microscope (magnification,  $\times 40$ ) (upper panels). Immunocytochemical staining of CTC-MCC-41 and HT-29 cells with DAPI, anti-EpCAM-APC, anti-CK20-FITC, and anti-CD45-PE antibodies (lower panels). (B), PCA 3-dimensional (Dim.) scatter plots representing the gene expression patterns of the different samples (CTC-MCC-41 and HT-29 cells). Each dot represents a sample and the symbol its origin: C, CTC-MCC-41 cells; H, HT-29 cells. Samples can be divided in 2 distinct groups based on their gene expression profiles. (C), Volcano plots showing the distribution of gene expression fold changes and  $P$  values. A total of 54 675 transcripts were tested using the TAC software. Transcripts upregulated in CTC-MCC-41 are indicated in red, and transcripts upregulated in HT-29 are indicated in green.

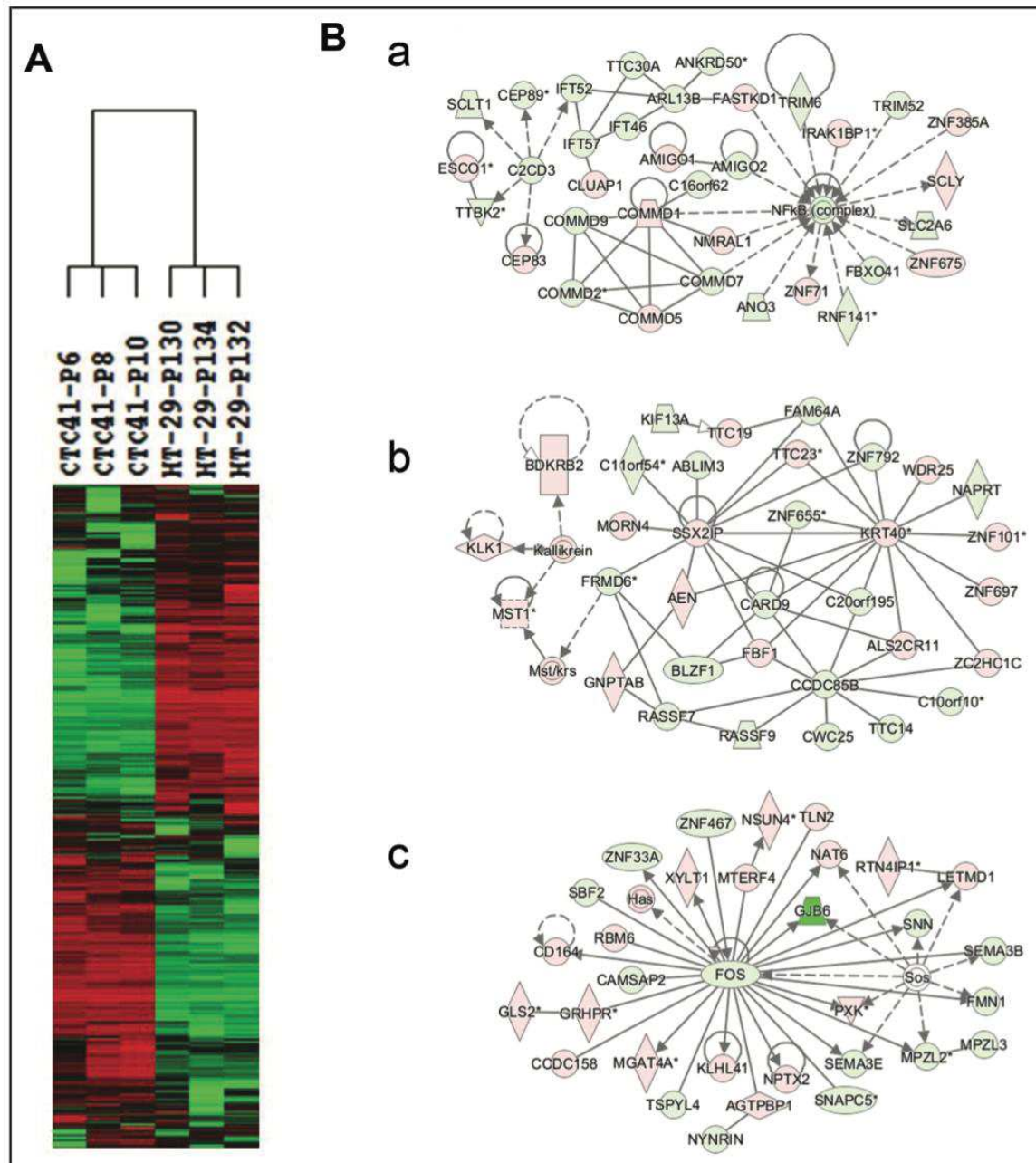
onic antigen-related cell adhesion molecule 6 (*CEACAM6*), phospholipase A2 group 2A (*PLA2G2A*), and aldehyde dehydrogenase 3 family member A1 (*ALDH3A1*). Conversely, genes related to matrix metalloproteinases, such as prostaglandin-endoperoxide synthase 1 (*PTGS1*), elastin microfibril interfacer 2 (*EMILIN2*), procollagen-lysine 2-oxoglutarate 5-dioxygenase 2 (*PLOD2*), and collagen type 18 alpha 1 (*COL18A1*) were overrepresented in the “HT-29 signature” (Fig. 3, B and C).

**MICROARRAY DATA VALIDATION BY RT-qPCR**

Among all the DETs, 20 genes were selected for validation by RT-qPCR. These genes were chosen based either

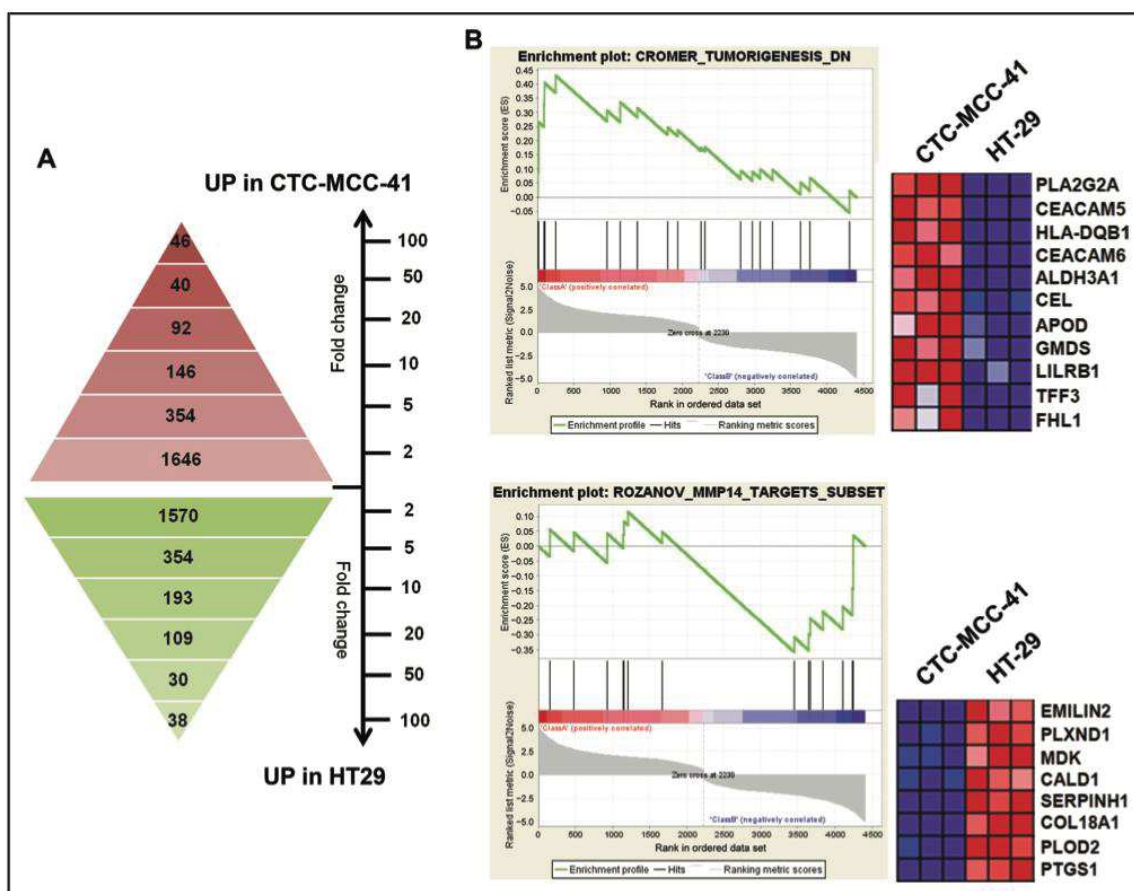
on their high FC or on their potential functions and involvement in a relevant biological pathway. Analysis of the qPCR data (Fig. 4) confirmed that *DEFA6*, B-cell CLL/lymphoma 11A (*BCL11A*), interleukin 33 (*IL33*), *BMP7*, ATP binding cassette subfamily B member 1 (*ABCB1*), *CCND2*, *TNFRSF1B*, fibronectin 1 (*FN1*), semaphorin 6A (*SEMA6A*), and galanin (*GAL*) were up-regulated and that *PDGFC*, prostaglandin-endoperoxide synthase 2 (*PTGS2*), Wnt family member 11 (*WNT11*), transforming growth factor beta 2 (*TGFB2*), dickkopf WNT signaling pathway inhibitor 1 (*DKKI1*), bone marrow stromal cell antigen 2 (*BST2*), GATA binding protein 2 (*GATA2*), gap junction protein beta 6 (*GJB6*),

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**Fig. 2.** CTC-MCC-41 and HT-29 gene signatures.

(A), Heat map. The molecular signatures of CTC-MCC-41 and HT-29 cells were visualized by hierarchical clustering based on the DETs. Genes are arranged in rows and the 6 cell samples are arranged in columns. All 3 CTC-MCC-41 cell samples clustered in 1 branch and all HT-29 cell samples in another branch. In each cell group, the tree represents the relationship among samples and the branch length reflects the degree of similarity between samples according to their gene expression profile. Red, upregulated genes; green, downregulated genes. (B), Functional network analyses based on the DETs. The most significant networks included genes related to: (a) cell morphology, cell function, cell assembly, and organization; (b) cell cycle, cell movement, and cancer; and (c) metabolism, cell-to-cell signaling, and interaction. Upregulated genes are in pink and downregulated genes are in green. Continuous lines between nodes indicate direct molecular interactions between connected genes and dotted lines indicate indirect functional interactions between genes.



**Fig. 3.** Differentially expressed genes in CTC-MCC-41 cells and HT-29 cells.

(A), Number of upregulated genes in CTC-MCC-41 cells and in HT-29 cells. SAM analysis identified a signature of 2324 genes that are upregulated in CTC-MCC-41 cells and 2294 genes that are upregulated in HT-29 cells. Of these, 46 and 38 genes showed a fold change  $\geq 100$  in CTC-MCC-41 cells and HT-29 cells, respectively. (B), Gene Set Enrichment Analysis (GSEA) was performed using the microarray data to compare the gene expression profiles of CTC-MCC-41 and HT-29 cells. Upper panels present GSEA results showing a significant enrichment of the tumorigenesis category among genes upregulated in CTC-MCC-41 cells. Lower panels present GSEA results showing that the *MMP14* signaling pathway is downregulated in CTC-MCC-41 cells compared with HT-29 cells. The heatmaps on the right show the gene expression level (red, high; blue, low) of each gene in the indicated cell sample. (C), Box-and-whisker plots comparing the expression level of DETs in CTC-MCC-41 and HT-29 cell samples. The signal intensity for each gene is shown on the y axis as arbitrary units determined by the AGCC software (Affymetrix).

Continued on page 707

*SMARCA1*, and ADAM metallopeptidase with thrombospondin type 1 motif 6 (*ADAMTS6*) were downregulated in CTC-MCC-41 cells compared with HT-29 cells. This agreement between microarray and qPCR data indicates that our microarray analysis was reliable.

**MITOCHONDRIAL GENE EXPRESSION PROFILE AND METABOLIC PATHWAYS IN CTC-MCC-41 CELLS**

Microarray data analysis also highlighted the upregulation of genes encoding proteins related to the “mitochon-

drial part” (GO: 0044429) and/or “mitochondrion” (GO: 0005739) GO categories in CTC-MCC-41 cells compared with HT-29 cells (Fig. 5A). The Pathway Studio analysis showed that the “colon CTC signature” was enriched in transcripts associated with localization in the “mitochondria” (Fig. 5B). The “colon CTC signature” also included genes known to promote mitochondrion biogenesis, particularly nuclear respiratory factor 1 (*NRF1*) and peroxisome proliferator-activated receptor  $\gamma$  coactivators (*PPARGC1A* and *PPARGC1B*). Several



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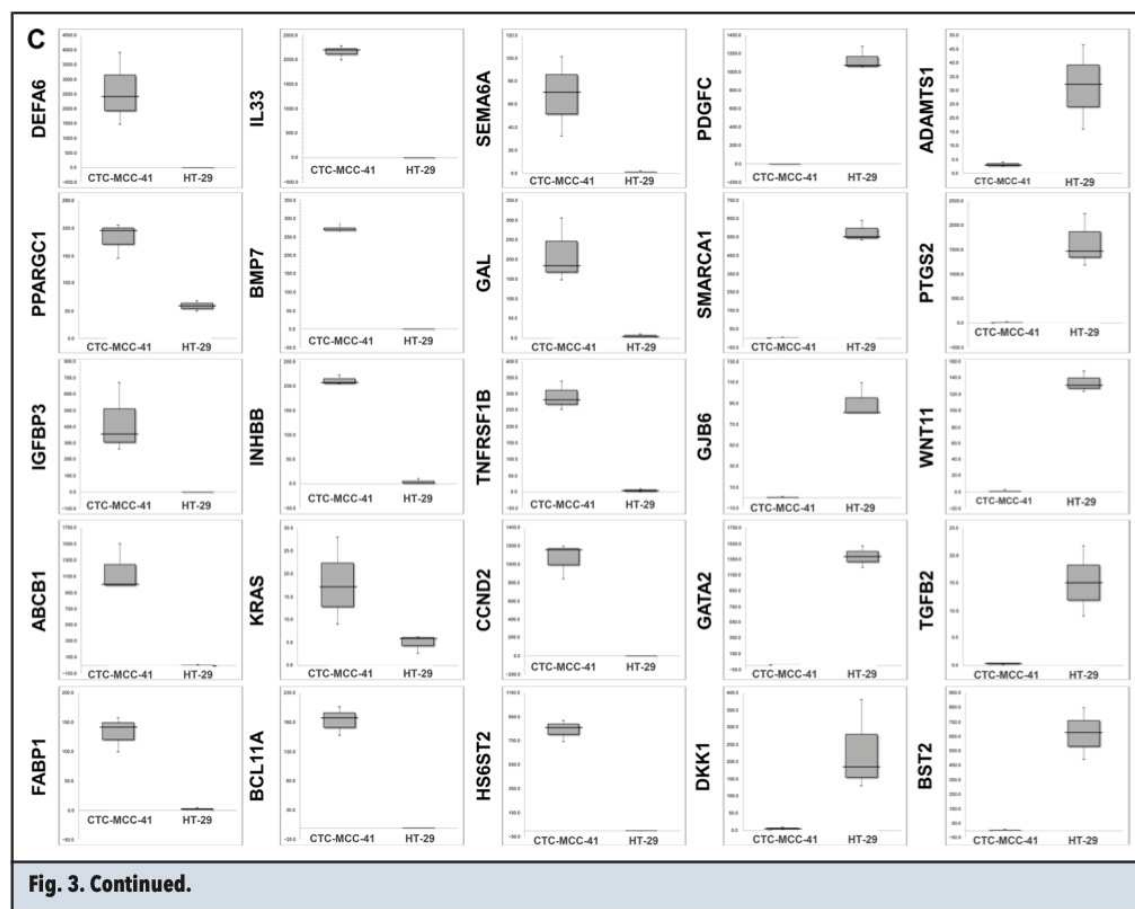


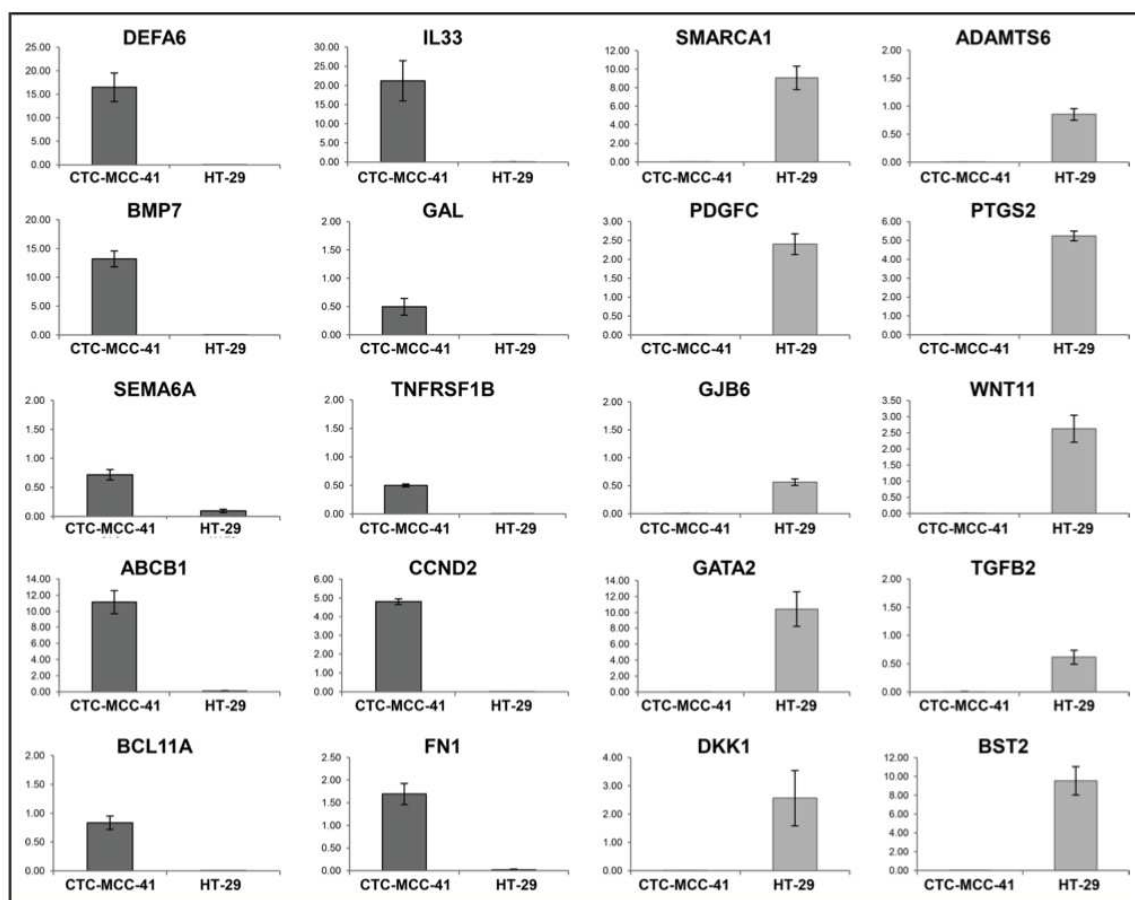
Fig. 3. Continued.

metabolic genes were also upregulated in the “colon CTC signature,” suggesting that different metabolic pathways are operating in metastasis-competent colon CTCs (Fig. 5C). Specifically, genes involved in fatty acid metabolism [fatty acid binding protein 1 (*FABP1*), fatty acid binding protein 5 (*FABP5*), *ALDH3A1*, aldehyde dehydrogenase 3 family member A2 (*ALDH3A2*), aldehyde dehydrogenase 1 family member B1 (*ALDH1B1*), and aldehyde dehydrogenase 2 (*ALDH2*)], estrogen biosynthesis [hydroxysteroid 17-beta dehydrogenase 1 (*HSD17B1*), cytochrome P450 family 2 subfamily B member 6 (*CYP2B6*), and aldo-keto reductase family 1 member C4 (*AKR1C4*)], PXR/RXR activation [glutathione S-transferase mu 1–4 (*GSTM1*, -2, -3, and -4)], energy production [aldehyde oxidase 1 (*AOX1*), xanthine dehydrogenase (*XDH*), monoamine oxidase A (*MAOA*), monoamine oxidase B (*MAOB*), paraoxonase 2 (*PON2*), paraoxonase 3 (*PON3*), and voltage dependent anion channel 1 (*VDAC1*)], and various ATP-binding cassette (ABC) transporters (*ABCB1*, *ABCA12*, *ABCA5*, *ABCA3*, *ABCC2*, *ABCC5*, and *ABCC6*) were up-regulated in CTC-MCC-41 cells. Finally, the gene encoding insulin-like growth factor binding protein 3 (*IGFBP3*)

was strongly overexpressed in CTC-MCC-41 cells. This protein regulates apoptotic pathways by modulating the expression of BCL-2 family members in mitochondria (10–12).

EXPRESSION OF STEMNESS-RELATED GENES IN CTC-MCC-41 CELLS

Analysis of the expression of stemness-related genes in CTC-MCC-41 and HT-29 cells, based on a previously published data set from human embryonic stem cells (hESCs) in which a consensus hESC stemness gene list (n=1076 genes) was defined (13), showed that CTC-MCC-41 cells shared 130 stemness-related genes with hESCs (see online Supplemental Table S6) and that HT-29 shared 109 genes. The key stemness genes glutaminase 2 (*GLS2*), cystathionine-beta-synthase (*CBS*), and cystathionine gamma-lyase (*CTH*) were enriched in CTC-MCC-41, whereas CD200 molecule (*CD200*), spalt like transcription factor 4 (*SALL4*), and inhibitor of DNA binding 1 (*ID1*) were over-represented in HT-29. Analysis of the functional relationship between the 130 stemness-related genes using IPA and Pathway Studio



**Fig. 4. Validation by RT-qPCR of the microarray data.**

A set (n=20) of upregulated and downregulated genes in CTC-MCC-41 cells were analyzed by RT-qPCR to validate the microarray data. All the RT-qPCR results were normalized to the expression level of  $\beta$ 2-microglobulin in each sample. The relative abundance for each gene is shown on the y axis in arbitrary units. Results are presented as the mean  $\pm$  SE. \*P value <0.05.

showed that 21 of them functionally interacted with each other, forming a tightly connected network, which we called “CTC core stemness circuitry” (Fig. 6A).

#### DNA REPAIR GENES IN CTC-MCC-41 CELLS

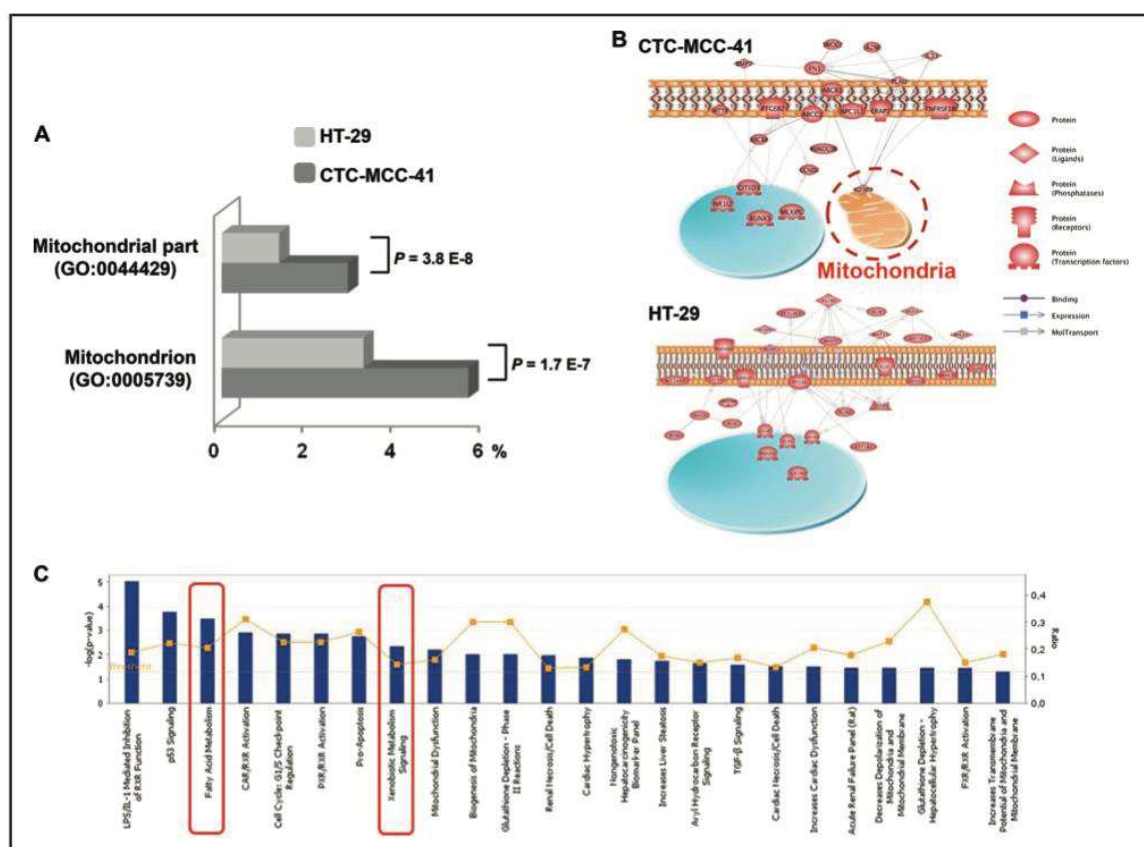
DNA repair pathways are crucial for the survival of cancer cells. The obtained microarray data were also used to evaluate the expression of a comprehensive list of 112 DNA repair genes (14) in CTC-MCC-41 and HT-29 cells (see online Supplemental Table S7). Among these genes, 9 [aprataxin (*APTX*), aprataxin and PNKP like factor (*APLF*), X-ray repair cross complementing 4 (*XRCCA*), BRCA1 interacting protein C-terminal helixase 1 (*BRIP1*), Fanconi anemia complementation group B (*FANCB*), Fanconi anemia complementation group M (*FANCM*), damage specific DNA binding protein 2 (*DDB2*), XPC complex subunit, DNA damage recogni-

tion and repair factor (*XPC*), and mutL homolog 3 (*MLH3*)] were over-expressed in CTC-MCC-41 cells and only 4 [DNA polymerase mu (*POLM*), RAD51 recombinase (*RAD51*), ERCC excision repair 1 (*ERCC1*), and tumor protein p53 binding protein 1 (*TP53BP1*)] in HT-29 cells. Analysis of the functional relationship between these DNA repair genes using the STRING software showed that they all displayed a documented functional interaction and that the Fanconi anemia (FA) pathway, which includes *BRIP1*, *FANCB*, and *FANCM*, was specifically upregulated in CTC-MCC-41 cells (Fig. 6B).

#### INTEGRATING AFFYMETRIX GENECHIP DATA SETS OBTAINED FROM OTHER COLON CANCER CELL LINES

To compare our data to those from other colon cancer cell lines, we used publicly available Affymetrix data on colon

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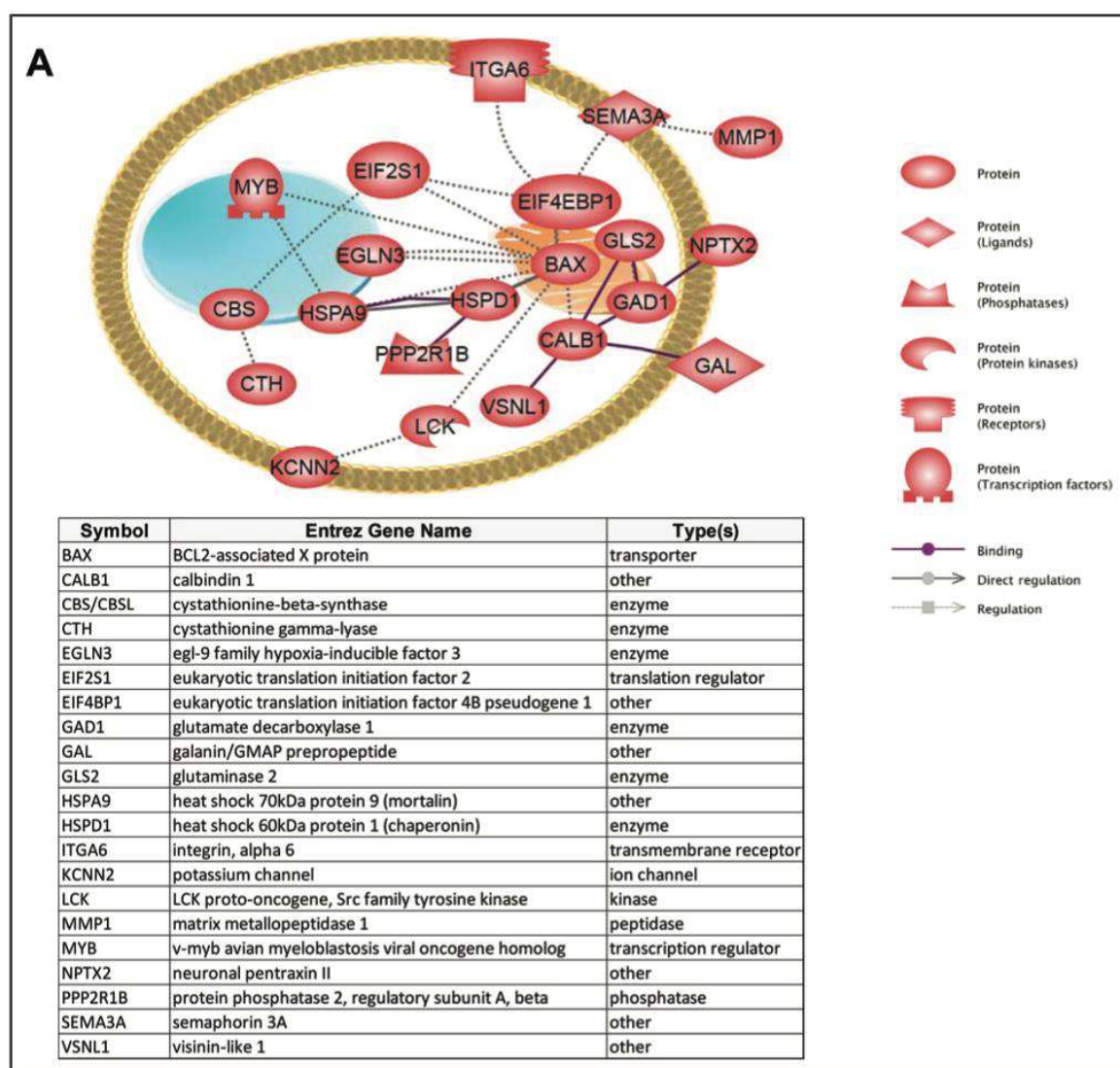


**Fig. 5.** Expression of mitochondrial-related genes in CTC-MCC-41 cells and HT-29 cells.

(A), Histograms show the percentage of genes annotated as “mitochondrial part” and “mitochondrion” (GO categories) among the genes overexpressed in CTC-MCC-41 (dark grey) and in HT-29 (light grey) cells. (B), Pathway studio analysis of differentially expressed genes in CTC-MCC-41 (CTC) and HT-29 cells. Only genes included in the “colon CTC signature” are connected to mitochondria. Each node represents either a gene entity or a control mechanism of the interaction. (C), Top canonical pathways that were significantly upregulated in CTC-MCC-41 cells, identified by Ingenuity® Pathway Analysis (IPA). The “fatty acid metabolism” and “xenobiotic signaling” pathways were significantly associated with genes overexpressed in CTC-MCC-41 cells. The ratio represents the number of analyzed genes in a given pathway divided by the total number of genes that form that pathway. The threshold represents the *P* value.

cell lines obtained from colon primary tumors (CaCo2, HCT116, and RKO) and colon cell lines obtained from metastatic distant sites: ascites and lymph node, respectively (COLO205 and SW620). A hierarchical clustering analysis using Affymetrix TAC software mapped the samples into 2 main major clusters. As observed in the dendrogram (Fig. 6C), a first branch indicates a clear segregation between the CTC-MCC-41 and the other colon cancer cell lines (all the samples of the CTC-MCC-41 group self-cluster into 1 branch). The HT-29 samples self-cluster into another branch and situated in the vicinity of the primary and metastatic colon cancer cell lines. These results highlight that CTC-MCC-41 line displays a very specific transcription program. We further investigated differences and similarities in the gene expression patterns between CTC-MCC-41

lines and other colon cancer cell lines in comparison to HT-29 samples. A SAM, with a 2-fold change cutoff and FDR < 5%, showed that 7380 transcripts were upregulated in the CaCo2 and HCT116 lines compared to HT-29 samples and 8924 transcripts in the COLO205 and SW620 lines. These lists of transcripts were then intersected with the CTC-MCC-41 signature (see online Supplemental Table S4) to determine their overlap (Fig. 6D). Interestingly, among the 1624 transcripts (see online Supplemental Table S8) exclusively upregulated in CTC-MCC-41 samples, key genes related to energy metabolism such as (*PPARGC1A*, *PPARGC1B*, *FABP1*, *ALDH3A*, *ALDH2*, *AOX1*, and *ABCB1*), DNA repair (*BRIP1*, *FANCM*, *XRCC4*, *DDB2*, and *XPC*), and stemness genes including *GLS2*, *ZNF93*, and *B3GNT7* were observed.



**Fig. 6. Core stemness circuitry and DNA repair genes.**

(A), Functional networks for stemness-related genes, identified using Pathway Studio, in CTC-MCC-41 cells. Interactions between nodes are denoted by colored arrows or lines. The 21 stemness related-genes included in the "colon CTC signature" are listed in the table. (B), DNA repair genes in CTC-MCC-41 cells and HT-29 cells. This figure was produced using the STRING software to generate automatically the interaction graph of DNA repair genes. Colored circles represent genes, and lines indicate interactions among genes. (C), Hierarchical clustering. Each horizontal line represents a transcript and each column represents a single sample. A tree represents relationship among samples whose branch lengths reflect the degree of similarity between the samples according to transcript expression profile. Transcripts up- and down-regulated were colored in red and blue, respectively. (D), Intersection with transcriptomes of colon cancer cell lines. Venn diagram representing the number of genes in each comparison and the overlaps between the 3 main comparison groups.

*Continued on page 711*

### Discussion

The analysis of the transcriptome profile of tumor cells is an important step for understanding cancer biology. In the present study, we compared the gene expression pro-

files of CTC-MCC-41 cells [derived from metastasis-competent CTCs of a colon cancer patient (6) and of HT-29 cells (from a primary colorectal cancer)]. We clearly identified genes and signaling pathways that are involved in cancer progression and that were differen-

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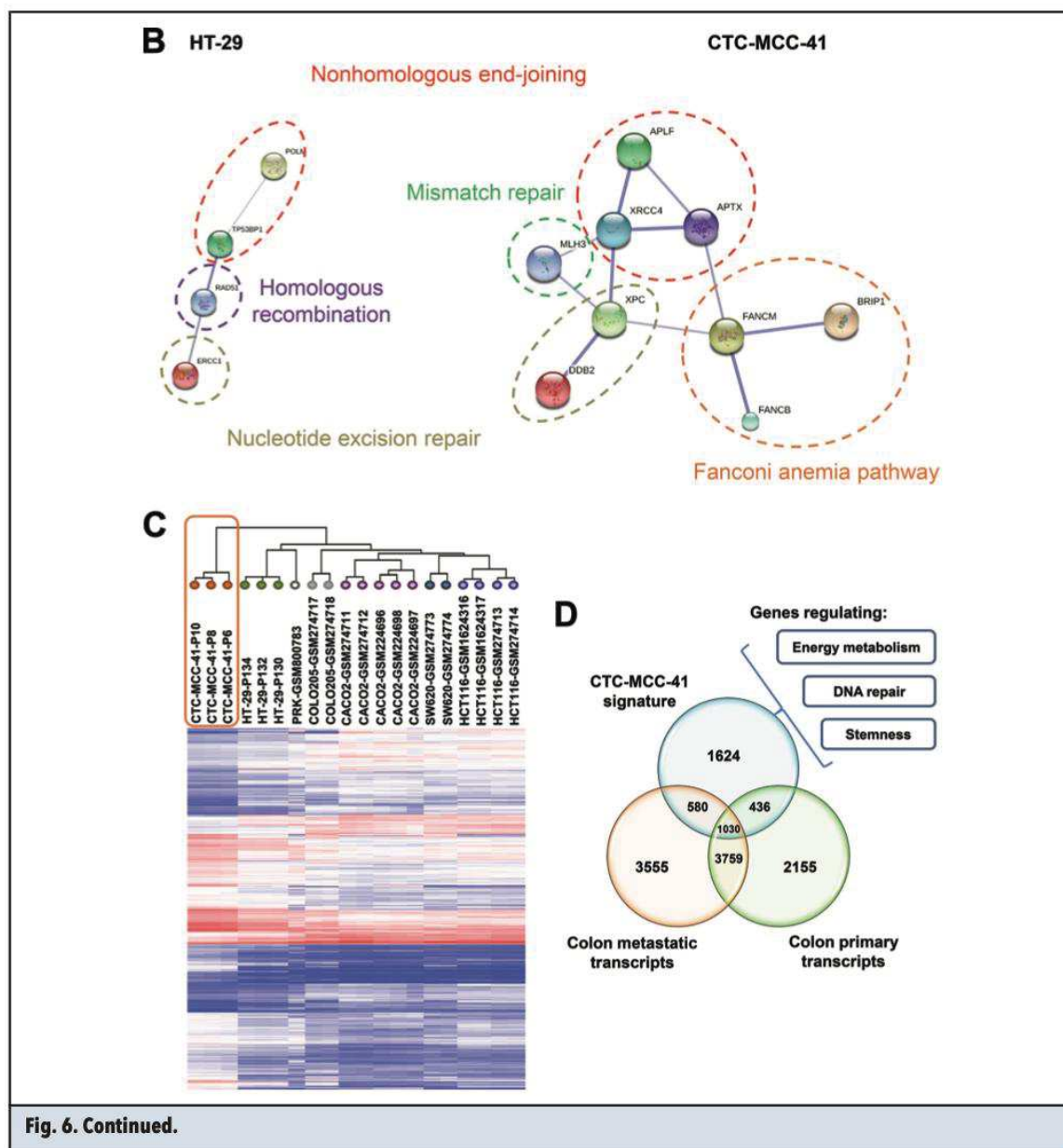


Fig. 6. Continued.

tially expressed in CTC-MCC-41 and HT-29 cells. The genes upregulated in CTC-MCC-41 cells are involved in functions that are important for tumor cell survival, such as cell metabolism, cell growth and death, signal transduction, and DNA repair.

Tumor cells adapt the activity of metabolic pathways in response to modifications in the microenvironment (15) and need to coordinate energy generation to promote cell proliferation (16). Our data show that the expression levels of *PPARGC1A* (or *PGC1α*) and *PPARGC1B* are much higher in CTC-MCC-41 than in

HT-29 cells and that fatty acids metabolism is the most representative pathway in these colon CTCs. *PPARGC1A* is a major regulator of several key metabolic pathways and coordinates the regulation of genes promoting the conversion of glucose to fatty acids to support tumor growth (17). It also plays a pivotal role in inducing the expression of oxidative phosphorylation genes in various tissues, including colon cancer (18, 19). Increased fatty acid synthesis is important in cancer (20). *PPARGC1A* is a key modulator of the tumor suppressor p53 that promotes cell survival in response to metabolic

stress (21) and can modulate *PPARGC1* activity (22). Remarkably, many genes connected to p53 signaling were upregulated in CTC-MCC-41 cells, suggesting a potential response to stress caused by their presence in the bloodstream as well as a quick adaptation to the new microenvironment and a p53 role in the regulation of their metabolism.

The “CTC molecular signature” also included many genes that are annotated as “mitochondrial,” demonstrating a strong link between CTCs and genes involved in energy production, cell proliferation, and apoptosis. Genes coding for BCL-2 family proteins are essential components of the intrinsic (mitochondrial) apoptotic pathway. The expression profile of apoptosis-related genes in CTC-MCC-41 cells suggests that the balance between anti- (*BCL2A1*) and pro-apoptotic factors (*BIK*, *BAX*, *BCL2L11*, *BCL2L14*, and *BCL2L15*) could be critical in CTCs. The antiapoptotic *BCL2* gene is upregulated in many tumors, including colon and breast cancers (23–25). Moreover, the BIK protein heterodimerizes with BCL2 to antagonize its antiapoptotic function (26). Additionally, the expression level of *TFF1*, a protein involved in cell protection against several forms of apoptosis (27), was much higher in CTC-MCC-41 than in HT-29 cells. Thus, understanding apoptosis regulation in CTCs with stem cell properties should be critical for the success of therapies targeting these cells.

Moreover, some ABC transporter genes, such as *ABCB1*, *ABCA12*, *ABCA5*, *ABCA3*, *ABCC2*, *ABCC5*, and *ABCC6*, were strongly upregulated in CTC-MCC-41 compared with HT-29 cells. Several studies suggest that ABC transporters modulate cancer initiation and progression (28, 29). For instance, *ABCA5* expression induction might correlate with the differentiation status of human colon cancers and contribute to tumor development (30) and *ABCC2* transports a wide range of unconjugated organic anions (31). Our data are in agreement with previous studies reporting the upregulation of ABC transporters in colorectal cancer (28, 32).

In CTC-MCC-41 and HT-29 cells, multiple DNA repair pathways are active to repair genome damages and prevent genomic alterations. Our microarray analysis clearly shows that the *BRIP1*, *FANCB*, and *FANCM* genes, which are part of the FA pathway, are specifically upregulated in CTC-MCC-41 cells, suggesting that metastases-competent colon CTCs require the FA pathway for survival in vivo in the bloodstream and efficient growth in distant organs. *BRIP1* alterations have been associated with the development of ovarian and breast cancers (33). However, the real function of FA pathway proteins in colon CTCs is not known yet. Similarly, many studies suggest that the FA pathway is especially important for pluripotent stem cells (PSCs) (34–37). Both PSCs and cancer cells can self-renew and proliferate, suggesting that the pathways controlling these bio-

logical processes might be shared between PSCs and cancer cells with metastasis-initiator properties. In this study, we used the stemness gene set previously identified by Assou et al. (13) in hESCs to analyze the “colon CTC signature.” We found that stemness-related genes, including several genes that encode enzymes (*GLS2*, *CBS/CBSL*, *CTH*, and *GAD1*), are preferentially upregulated in CTC-MCC-41 compared with HT-29 cells. Glutaminase 2 (*GLS2*) is a mitochondrial enzyme that converts glutamine into glutamate and is a p53 target gene that influences energy metabolism (38–40). Cystathionine- $\beta$ -synthase (*CBS*) and cystathionine- $\gamma$ -lyase (*CTH*) are the main enzymes that catalyze the production of hydrogen sulfide, which plays essential roles in many pathological processes (41). It was reported that colon cancer cells selectively over-express *CBS* to maintain the cell bioenergetics and to support tumor growth (42). Moreover, the WNT pathway regulates the expression of *CTH*, which plays important roles in colon cancer (43). Additional studies on the physiological function of these enzymes in CTC-MCC-41 cells will help better understand their role in colon cancer.

Another lesson from our transcriptomic analysis is that the CTC-MCC-41 lines display a very specific transcription program. Hierarchical clustering demonstrated that CTC-MCC-41 expression profiles were markedly different from those of HT-29 samples and other colon cancer cell lines (CaCo2, HCT116 and RKO, COLO205, and SW620). In addition, the Venn diagram revealed that the differences observed between CTC-MCC-41 and HT-29 do not just reflect of differences between 2 different cell lines, but were exclusive to the CTC-MCC-41 metastasis competent cells. Additional studies of other yet-to-be-characterized metastasis competent cell lines will be necessary to determine whether these differences are unique to metastasis competent cell lines in general.

In conclusion, by focusing on the identified pathways and biomarkers, new drugs to target metastasis-competent CTC with stem cell properties could be developed to inhibit metastatic growth and relapses in patients with cancer. Many of the genes identified in this study may provide innovative biomarkers for CTC detection, isolation, and characterization. The new challenge is to correlate gene functions to specific events in colon CTCs. This may ultimately help in developing new personalized treatments for patients with colon cancer.

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Transcriptome of Metastasis-Competent CTCs in Colon Cancer

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Article 4

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Selective treatment pressure in colon cancer sculpts the molecular portrait of resistant circulating tumor cell clones

**Laure Cayrefourcq**, Frédéric Thomas, Thibault Mazard, Eric Assenat, Said Assou\*, Catherine Alix-Panabières\*

\*Les auteurs ont contribué à part égale à ce travail.

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## LETTER TO THE EDITOR

## Open Access

# Selective treatment pressure in colon cancer drives the molecular profile of resistant circulating tumor cell clones



Laure Cayrefourcq<sup>1,2</sup>, Frédéric Thomas<sup>2</sup>, Thibault Mazard<sup>3</sup>, Eric Assenat<sup>4</sup>, Said Assou<sup>5\*</sup> and Catherine Alix-Panabières<sup>1,2\*</sup>

## Abstract

The characterization of circulating tumor cells (CTCs) holds promises for precision medicine because these cells are an important clinical indicator of treatment efficacy. We established the first and still only nine permanent colon CTC lines from peripheral blood samples of a patient with metastatic colon cancer collected at different time points during treatment and cancer progression. The study objectives were (i) to compare the gene expression profiles of these CTC lines, and (ii) to determine the main features acquired during treatment. The number of upregulated genes was higher in the CTC lines obtained after treatment, indicating that they acquired properties to escape treatment pressure. Among these upregulated genes, some are involved in the mTOR and PI3K/AKT signaling pathways. Moreover, cytidine deaminase expression was significantly increased in the CTC lines obtained after failure of the first- and second-line 5-fluorouracile-based treatments, suggesting that these CTCs can eliminate this specific drug and resist to therapy. Several enzymes involved in xenobiotic metabolism also were upregulated after treatment, suggesting the activation of detoxification mechanisms in response to chemotherapy. Finally, the significant higher expression of aldolase B in four of the six CTC lines obtained after treatment withdrawal and cancer progression indicated that these clones originated from liver metastases. In conclusion, these CTC lines generated at different time points during treatment of metastatic colon cancer in a single patient are characterized by the deregulation of different genes that promote (i) drug resistance, (ii) xenobiotic and energy metabolism, and (iii) stem cell properties and plasticity.

**Keywords:** Circulating tumor cells, Gene expression, Clonal evolution, Colon cancer, CDA, ALDOB

## Main text

Liquid biopsy-based strategies may transform oncology in the near future [1]. Many studies analyzed circulating tumor cells (CTCs) as a real-time liquid biopsy in epithelial tumors (i.e. breast, prostate, and colon cancer) [2] and showed that their study contributes to the

prognostic evaluation, patient stratification, and real-time monitoring of treatment efficacy, as well as to the identification of therapeutic targets and tumor resistance mechanisms. However, the low CTC concentration in blood is a crucial limiting factor for the identification of CTCs that may initiate a new tumor at a distant site (i.e. “metastasis-initiator CTCs” or “metastasis-competent CTCs”). Moreover, the establishment and long-term maintenance of in vitro CTC cultures are a major challenge, achieved only by few groups [3, 4].

We established the first nine permanent CTC lines from a 57-year-old patient with metastatic colon cancer

\* Correspondence: said.assou@inserm.fr; c-panabieres@chu-montpellier.fr

<sup>2</sup>IRMB, University of Montpellier, INSERM, CHU Montpellier, Montpellier, France

<sup>1</sup>Laboratory of Rare Human Circulating Cells, University Medical Center of Montpellier, University of Montpellier, Montpellier, France

Full list of author information is available at the end of the article



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(MCC). At diagnosis, he had unresectable widespread MCC with abdominal and mediastinal lymph node invasion and liver metastases. The patient was first treated with the 5-fluorouracile (5-FU)-irinotecan (FOLFIRI) combination and bevacizumab (5 cycles). The first biological progression was observed after the fifth FOLFIRI cycle, and second-line treatment was initiated with 5-FU-oxaliplatin (FOLFOX) and bevacizumab, but was stopped after the third cycle because of disease progression (clinical and morphological). The patient died about 6 months after diagnosis due to cancer progression. We derived these CTC lines from blood samples collected before treatment initiation (CTC-MCC-41) [5], after the first- and second-line treatments (CTC-MCC-41.4), and 1 week before the patient's death (CTC-MCC-41.5 [A-G]) [6]. Their phenotypic and molecular characterization indicated that they present common traits and all display epithelial-to-mesenchymal plasticity and stem cell-like characteristics [6, 7].

Here, we obtained and compared the transcriptomic profiles of these CTC lines to identify expression changes linked to tumor progression and treatment pressure, with the aim of identifying biomarkers and mechanisms involved in colon cancer progression.

## Results and discussion

### CTC selection during cancer progression and treatment pressure

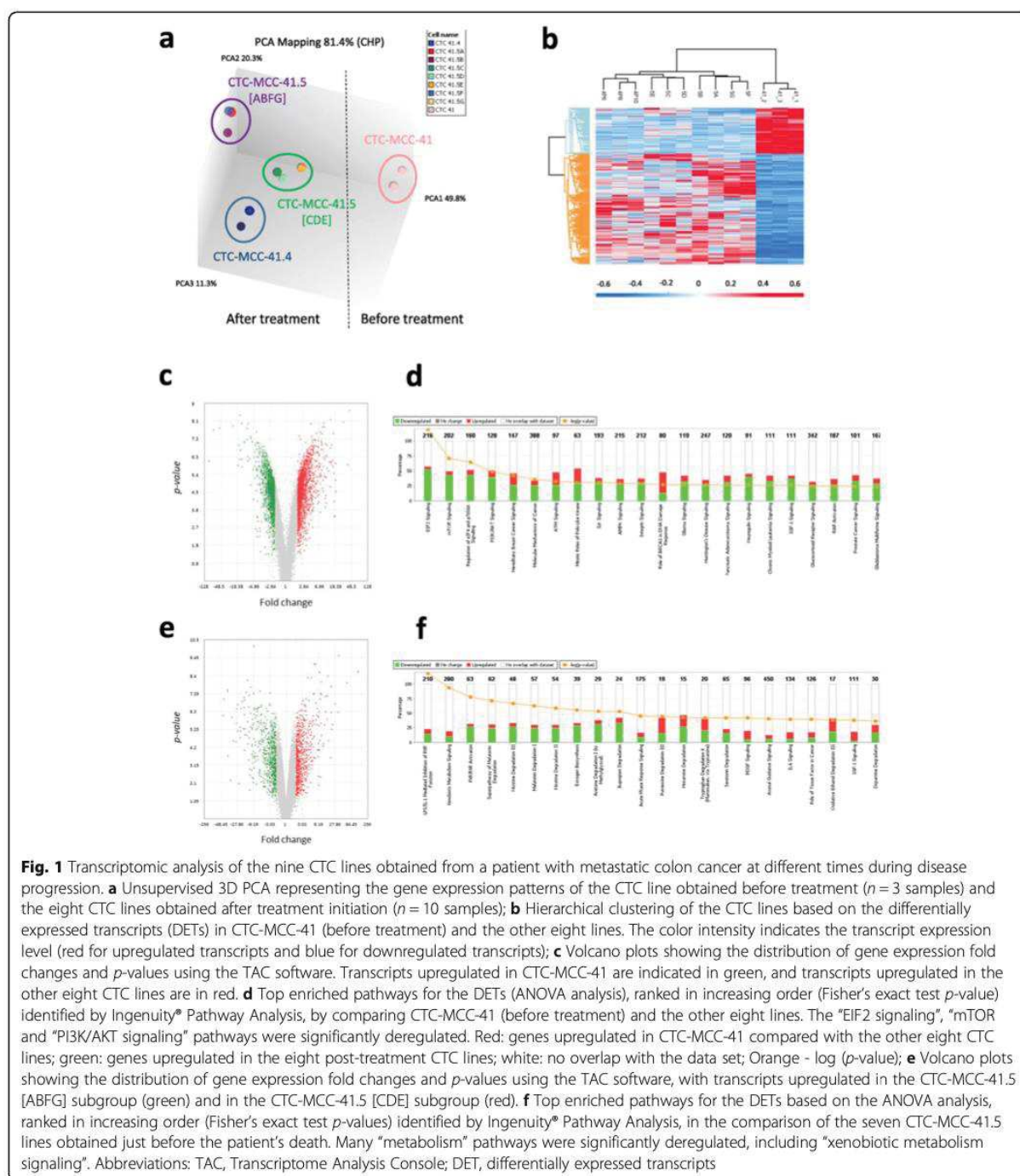
The analysis of the transcriptomic profiles of the nine CTC lines using Affymetrix HG-U133P microarray chips clearly separated them in four distinct groups: CTC-MCC-41, CTC-MCC-41.4, CTC-MCC-41.5 [ABFG], and CTC-MCC-41.5 [CDE] (Fig. 1a). We then compared the transcriptomic profiles of the pre-treatment CTC-MCC-41 line and of the other eight CTC lines (CTC-MCC-41.4 and CTC-MCC-41.5[A-G]) to understand the treatment impact on CTC clonal selection (Fig. 1b and Additional file 2: Tables S1–2). The significantly higher number of upregulated genes in the eight cell lines obtained after treatment initiation (Fig. 1c) suggests that they acquired properties to adapt and resist to treatment. For instance, genes involved in the mTOR and PI3K/AKT signaling cascades, which are implicated in cancer development by coordinating cell growth, survival and proliferation, and in resistance to chemotherapy, were upregulated in the post-treatment CTC lines (Fig. 1d). Colon cancer-specific mortality is higher in patients with tumors harboring mutated PIK3CA than wild type PIK3CA [8]. All our CTC lines harbored wild type PIK3CA and AKT [6], but these signaling pathways were deregulated. Some studies demonstrated that PI3K/mTOR pathway inhibitors could be used in primary and metastatic colorectal cancer [9]. We recently reported that CTC-MCC-41 cells also respond to mTOR and

AKT inhibitors, suggesting these therapies are effective even in the absence of mutations [10].

Conversely, we did not observe many differences between the CTC-MCC-41.4 line, obtained after the last treatment, and the seven CTC-MCC-41.5 [A-G] lines obtained before the patient's death (Additional file 2: Table S3). This suggests that, without drug pressure, the rapid cancer worsening was not linked anymore to a clonal evolution but is due to natural disease progression with the replication of the CTC clones already selected under treatment. Indeed, the seven last CTC lines seem to be already present in the pooled CTC-MCC-41.4 line and they have been selected in the *in vitro* culture from the last blood sample. However, we could clearly segregate the last seven CTC cell lines (CTC-MCC-41.5 [A-G]), in two groups, [ABFG] and [CDE] (Fig. 1a), with different gene expression profiles (Fig. 1e). Most of the significantly deregulated pathways were involved in metabolism signaling (Fig. 1f), including xenobiotic metabolism. This suggests that detoxification mechanisms were induced upon exposure to anti-cancer drugs, as indicated by the deregulation of the irinotecan/SN38 pathway specifically in the [CDE] group. Furthermore, lipid metabolism upregulation appeared to be more represented in the "CDE signature". Lipid metabolism is a key function on the basis of the enrichment of different signaling cascades leading to energy metabolism. Since Warburg's work, metabolic reprogramming is one of the main hallmarks of cancer cells and plays a critical role in the continued tumor growth and progression and is driven by a complex interplay between the tumor mutational landscape, epigenetic modifications, and microenvironmental influences [11]. This topic is actively studied and a high-throughput metabolic-based assay was developed for rapid detection of rare metabolically active disseminated tumor cells in pleural effusion of lung cancer [12].

### Cytidine deaminase as a drug resistance biomarker

Besides deregulation of the irinotecan/SN38 pathway in the [CDE] group, the gene encoding cytidine deaminase (CDA) was deregulated in the post-treatment CTC lines (Additional file 2: Table S2). This finding in a patient treated with 5-FU, a pyrimidine analogue, is interesting because CDA maintains the cellular pyrimidine pool by catalyzing the hydrolytic deamination of cytidine and deoxycytidine to uridine and deoxyuridine. Some studies showed that in patients treated with direct cytidine analogues, such as gemcitabine and cytosine arabinoside, CDA overexpression might be a marker of resistance [13]. Analysis of CDA expression in colorectal cancer using publicly available data [14] indicated that it was significantly downregulated in colon adenocarcinoma compared with normal colon (Fig. 2a). However, CDA was strongly upregulated in the CTC-MCC41.4 cell line

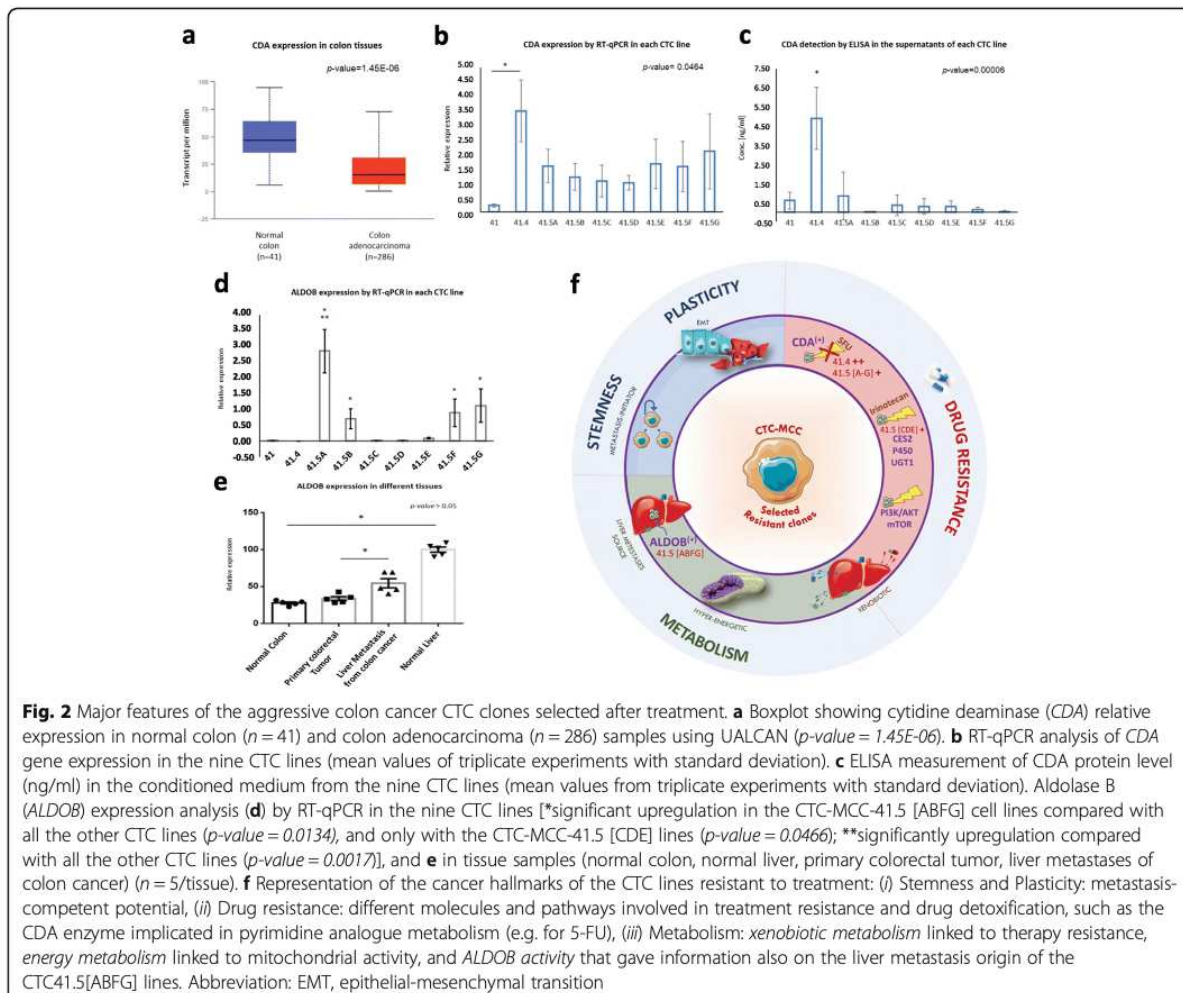


**Fig. 1** Transcriptomic analysis of the nine CTC lines obtained from a patient with metastatic colon cancer at different times during disease progression. **a** Unsupervised 3D PCA representing the gene expression patterns of the CTC line obtained before treatment ( $n = 3$  samples) and the eight CTC lines obtained after treatment initiation ( $n = 10$  samples); **b** Hierarchical clustering of the CTC lines based on the differentially expressed transcripts (DETs) in CTC-MCC-41 (before treatment) and the other eight lines. The color intensity indicates the transcript expression level (red for upregulated transcripts and blue for downregulated transcripts); **c** Volcano plots showing the distribution of gene expression fold changes and  $p$ -values using the TAC software. Transcripts upregulated in CTC-MCC-41 are indicated in green, and transcripts upregulated in the other eight CTC lines are in red. **d** Top enriched pathways for the DETs (ANOVA analysis), ranked in increasing order (Fisher's exact test  $p$ -value) identified by Ingenuity® Pathway Analysis, by comparing CTC-MCC-41 (before treatment) and the other eight lines. The "EIF2 signaling", "mTOR and "PI3K/AKT signaling" pathways were significantly deregulated. Red: genes upregulated in CTC-MCC-41 compared with the other eight CTC lines; green: genes upregulated in the eight post-treatment CTC lines; white: no overlap with the data set; Orange - log ( $p$ -value); **e** Volcano plots showing the distribution of gene expression fold changes and  $p$ -values using the TAC software, with transcripts upregulated in the CTC-MCC-41.5 [ABFG] subgroup (green) and in the CTC-MCC-41.5 [CDE] subgroup (red). **f** Top enriched pathways for the DETs based on the ANOVA analysis, ranked in increasing order (Fisher's exact test  $p$ -values) identified by Ingenuity® Pathway Analysis, in the comparison of the seven CTC-MCC-41.5 lines obtained just before the patient's death. Many "metabolism" pathways were significantly deregulated, including "xenobiotic metabolism signaling". Abbreviations: TAC, Transcriptome Analysis Console; DET, differentially expressed transcripts

obtained directly after failure of the first- and second-line 5-FU-based treatments (RT-qPCR analysis in Fig. 2b) and also, to a lower extent, in the CTC lines (CTC-MCC-41.5 A-G) obtained just before death, compared with the pre-treatment CTC-MCC-41 line.

As CDA is secreted in the extracellular compartment (<https://www.uniprot.org/uniprot/P32320>) and can be

detected in blood, we quantified CDA concentration by ELISA in conditioned medium from the nine CTC-MCC lines. As observed for the *CDA* gene, CDA protein level was highest in CTC-MCC-41.4 cells (Fig. 2c). These findings suggest that in this patient, CDA was directly produced and secreted by resistant and aggressive CTCs in response to 5-FU-based chemotherapy. Therefore,



CDA might represent a candidate plasmatic biomarker to monitor 5-FU efficacy and resistance development.

### Aldolase B as a marker to identify CTCs released only by liver metastases

Comparison of the signatures of the two CTC-MCC-41.5 sub-groups ([ABFG] and [CDE]) (Additional file 2: Tables S4–5) highlighted *ALDOB* upregulation in the [ABFG] group (Fig. 2d). This gene encodes the aldolase B enzyme (fructose-bisphosphate aldolase B or liver-type aldolase), one of three isoenzymes (A, B, and C) of the class I fructose 1,6-bisphosphate aldolase enzyme (EC 4.1.2.1) that plays a key role in glycolysis and gluconeogenesis. This enzyme is preferentially expressed in the liver and at lower extend in the kidney and the small intestine. *ALDOB* downregulation correlates with poor overall survival in liver and gastric cancer [15, 16], whereas *ALDOB* overexpression in colorectal cancer has been associated with poor overall survival and epithelial-

to-mesenchymal transition promotion [17]. Colorectal cancer is known to preferentially metastasize (~70% patients) to the liver [18], which is the main organ for glycogenesis and gluconeogenesis. In addition, this specific patient had liver metastases. Comparison of *ALDOB* expression in different tissues using publicly available data showed that: (i) *ALDOB* was strongly expressed in normal liver compared with normal colon, (ii) *ALDOB* expression was comparable in primary colon adenocarcinoma and normal colon samples, and (iii) *ALDOB* was upregulated in colon cancer liver metastases compared with primary tumors (Fig. 2e). Bu et al. showed that during liver colonization, colon cancer cells undergo metabolic reprogramming by upregulating *ALDOB* [19]. This enhances fructose metabolism and promotes the growth of colon cancer liver metastases. These data suggest that the [ABFG] lines were derived from CTCs released by liver metastases. It is thought that CTCs are released by the primary tumor and/or metastases; however, to our

knowledge, this is the first time that this could be demonstrated in a patient.

### Conclusions

From Nowell's work in 1976, it is acknowledged that cancer is an evolutionary process and that treatments exert selective pressures that drive the tumor cell evolution, favoring the appearance of resistant clones. The present results confirm that CTC line profiling is a relevant approach to study clonal selection during disease progression and to discover new CTC biomarkers for monitoring treatment response. The transcriptomic analysis of the CTC lines obtained after treatments (CTC-MCC-41.4 and CTC-MCC-41.5[A-G]) showed the progressive deregulation of genes involved in cancer aggressiveness hallmarks: (i) drug resistance, with the upregulation of molecules and pathways implicated in drug detoxification (e.g. CDA and the irinotecan/SN38 pathway), (ii) metabolism changes, including upregulation of genes implicated in the xenobiotic metabolism, linked to therapy resistance, and energy metabolism (mitochondrial activity to provide energy to hyper-metabolic cells), and the ALDOB gene linked to liver metabolism, and (iii) stemness and plasticity, highlighting their metastasis-competent potential (Fig. 2f). This work is, however, specific of this cancer patient and the biomarkers found and highlighted in these CTC lines need now to be validated on CTCs and plasma from independent patients with colorectal cancer.

### Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s12943-021-01326-6>.

**Additional file 1.** Supplementary materials and methods.

**Additional file 2: Table S1.** List of DETs upregulated in CTC-MCC-41 vs all the other CTC lines. **Table S2.** List of DETs downregulated in CTC-MCC-41 vs all the other CTC lines. **Table S3.** List of DETs upregulated in all CTC-MCC-41.5 cell lines vs the CTC-MCC-41.4 cell line. **Table S4.** List of DETs upregulated in the [ABFG] subgroup vs the [CDE] subgroup of the CTC-MCC-41.5 cell lines. **Table S5.** List of DETs downregulated in the [ABFG] subgroup vs the [CDE] subgroup of the CTC-MCC-41.5 cell lines. **Table S6.** Primer sequences.

**Additional file 3: Figure S1.** Box-plots showing the expression level of a set of differentially expressed transcripts (DETs) in the CTC-MCC-41, CTC-MCC-41.4, and CTC-MCC-41.5 [ABFG] and [CDE] cell lines. Abbreviations: CTC-BT, CTC line derived before treatment initiation; CTC-AT, CTC lines derived after treatment initiation. **Figure S2.** RT-qPCR validation of the microarray data. Abbreviations: CTC-BT, CTC line derived before treatment initiation; CTC-AT, CTC lines derived after treatment initiation.

### Abbreviations

5-FU: 5-fluorouracil; ALDOB: Aldolase B; CDA: Cytidine deaminase; CTC: Circulating tumor cell; EMT: Epithelial to mesenchymal transition

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### Authors' contributions

LC and SA analyzed and interpreted the data. LC, SA and CAP were major contributors in the conception of the study and in the writing of the manuscript. All authors read and approved the final manuscript.

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### Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request. The 20 publicly available Affymetrix datasets on normal colon ( $n = 5$  samples), primary tumor ( $n = 5$  samples), liver metastases ( $n = 5$  samples) and normal liver ( $n = 5$  samples) are accessible at the gene expression Omnibus (GEO) repository (<https://www.ncbi.nlm.nih.gov/proxy/ncbi/geo/>) through the accession numbers: GSM1198032, GSM1198034, GSM1198038, GSM1198047, GSM1198050, GSM1198030, GSM1198035, GSM1198037, GSM1198046, GSM1198048, GSM1198023, GSM1198031, GSM1198033, GSM1198042, GSM1198052, GSM557089, GSM557091, GSM557093, GSM557099, GSM557107.

### Ethics approval and consent to participate

The patient gave his consent to participate in the COLOSPOT study to investigate CTCs in metastatic colorectal cancer. The bioethics committee gave its approval (ID-RCB 2011-A01130-41).

### Consent for publication

The content of this manuscript has not been previously published and is not under consideration for publication elsewhere.

### Competing interests

CAP declare to have had an honorarium from Menarini. The other authors declare that they have no competing interests.

### Author details

<sup>1</sup>Laboratory of Rare Human Circulating Cells, University Medical Center of Montpellier, University of Montpellier, Montpellier, France. <sup>2</sup>CREEC (CREES), Unité Mixte de Recherches, IRD 224-CNRS 5290-University of Montpellier, Montpellier, France. <sup>3</sup>Department IRCM, Inserm, University of Montpellier, ICM, Montpellier, France. <sup>4</sup>Department of Medical Oncology, University Medical Center of Montpellier, University of Montpellier, Montpellier, France. <sup>5</sup>IRMB, University of Montpellier, INSERM, CHU Montpellier, Montpellier, France.

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Article 5

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Sequential circulating tumor cell lines from a colorectal cancer patient display increased epithelial-to-mesenchymal plasticity.

Pelin Balcik-Ercin\*, **Laure Cayrefourcq\***, Rama Soundararajan, Sendurai Mani, ..., Catherine Alix-Panabières.

\*Les auteurs ont contribué à part égale au travail.

Article soumis à *EMBO journal*, 2020 Oct



## **Sequential circulating tumor cell lines from a colorectal cancer patient display increased epithelial-to-mesenchymal plasticity**

**Pelin Balcik-Ercin<sup>1</sup>, Laure Cayrefourcq<sup>2</sup>, Rama Soundararajan, Sendurai Mani, Catherine Alix-Panabières<sup>2</sup>**

<sup>1</sup> Department of Molecular Biology and Genetics, University of Gebze Technical University, Kocaeli, Turkey

<sup>2</sup> Laboratory of Rare Human Circulating Cells, University Medical Center of Montpellier, University of Montpellier, Montpellier, France

### **Corresponding author:**

Catherine Alix-Panabières, Institut Universitaire de Recherche Clinique (IURC), 641, avenue du Doyen Gaston Giraud, 34093 Montpellier Cedex 5, France, Fax: 33-(0) 4-11-75-99-33 Email: [c-panabieres@chu-montpellier.fr](mailto:c-panabieres@chu-montpellier.fr)

**Running Title: Colon CTC lines display epithelial-to-mesenchymal plasticity**

**Keywords:** Circulating tumor cells, Epithelial-to-mesenchymal transition, Mesenchymal-to-epithelial transition, metastasis-competent cells

## **Abstract**

Metastasis is a complicated multistep process and remains partially understood. A subset of cancer cells is able to leave the primary tumor, intravasate, and circulate to reach eventually distant organs, these cells are called circulating tumor cells (CTCs). Multiple lines of evidence suggest that metastatic cancer cells have epithelial-to-mesenchymal plasticity (EMP). The epithelial-to-mesenchymal transition (EMT) and its reverse process, the mesenchymal-to-epithelial transition (MET), are crucial steps in embryonic development, wound healing, and also in metastasis. CTCs represent a unique source to understand biology of the metastatic cascade, such as the molecular signature of the EMP of CTCs. Our group previously established and characterized nine serial autologous CTC lines from a patient with metastatic colon cancer. In this article, EMT and MET markers were evaluated to understand the EMP profile in unique CTC cell lines. We reported that the oncogenes MYC and EZRIN were expressed by all CTC lines, whereas one of their common regulators, SIX1, an EMT inducer, was not. Besides, the MET activator transcription factor GRHL2 and its putative targets are highly expressed in the CTC lines, revealing the plasticity of these cell lines in favour of an increased MET state.

## Introduction

Cancer is the second leading cause of death in the world and metastasis is a major cause of cancer-related death (Steeg 2016, Bray, Ferlay et al. 2018). Despite of significant progress in the study, diagnosis, and treatment of cancer, molecular variation of the dissemination process still poorly understood (Fares, Fares et al. 2020). Metastasis is a dynamic complex process in which a subset of cancer cells dissociate from the primary tumor, gain migratory potential, enter the blood circulation, reach and colonize specific distant organs where they may find favorable conditions to grow and form a second tumor (Micalizzi, Maheswaran et al. 2017). This subset of cells are called circulating tumor cells (CTCs). CTCs are rare events that that could originate from primary or metastatic tumors (Alix-Panabières and Pantel 2013). CTCs reflect the primary tumor mutations and metastatic recurrence of cancer patients. These cells are also a unique and crucial source of information regarding the metastasis process between primary-to-secondary tumor formation (Alix-Panabières and Pantel 2014, Agnoletto, Corrà et al. 2019, Li, Dong et al. 2020). Although various biological and molecular tumor markers and mechanisms of the metastasis cascade have been revealed, our understanding of this process is still limited. To intravasate and rejoin the bloodstream, tumor cells should lose epithelial polarity and gain migratory capacity to pass through the basement membrane, this process is the epithelial-to-mesenchymal transition (EMT) (Pantel and Alix-Panabieres 2017). On the other hand, for successful development of metastasis in distant organs, cancer cells have to revert their phenotype by re-express epithelial proteins and lose mesenchymal properties, this is called mesenchymal-to-epithelial transition (MET). Although the EMT and MET processes have an adverse effect in cancer dissemination, EMT and MET specific transcription factors play a role in similar cellular processes, such as embryonic development and cellular differentiation. (Lu and Kang 2019). During the embryonic development, cells have an epithelial-mesenchymal plasticity (EMP) and this process contains dynamic flux of EMT/MET markers (Williams, Gao et al. 2019).

EMT inducer transcription factors are ZEB1/2, SNAIL1/2, TWIST1/2, FOXC2 and Sine oculis homeobox 1 (SIX1). Certain cytokines such as TGF $\beta$  and TNF $\alpha$  induce these EMT markers in various tumors (Jung, Fattet et al. 2015, Stemmler, Eccles et al. 2019). Of particular interest is the SIX1 protein, a member of the homeobox SIX transcription factor family, which is an evolutionarily conserved transcription factor necessary for embryonic development and organ formation (Wu, Huang et al. 2014). SIX1 co-activator is EYA2, and the direct interaction between specific domains of these two proteins gives rise to a transcriptional unit providing

DNA binding activity. The SIX1 and EYA2 complex regulates multiple hallmarks of cancer, including proliferation, invasion capacity and resistance to apoptosis (Blevins, Towers et al. 2015). SIX1 was also shown to activate transcription of important oncogenic genes, such as potent oncogenic and stem cell regulators c-MYC and EZRIN, that have a vital role in tumor invasion and signal transduction in many cancers (Weng, Åhlén et al. 2005, Yu, Davicioni et al. 2006, Deng, Tannehill-Gregg et al. 2007, Xie, Xu et al. 2009, Chen, Liu et al. 2018). SIX1 overexpression has been shown to promote the proliferation, invasion, and migration capacity and also correlated with poor prognosis in colorectal cancer (Behbakht, Qamar et al. 2007, Tan, Zhang et al. 2011, Ono, Imoto et al. 2012, Armat, Ramezani et al. 2016). . Regarding MET inducer transcription factors are GRHL2, ELF3, ELF5 and OVOL1/2 (Jolly, Somarelli et al. 2019). Besides, MET process, can be induced by some transcription factors which suppress EMT activator pathways.

Recent studies showed that EMT/MET characters could be observed *in silico*, *in vitro* and *in vivo* experiments, in multiple subtypes of cancer cell lines, and primary tumors. The CTCs are an important example of EMP phenotype (Armstrong, Marengo et al. 2011, Hamilton, Hochmair et al. 2016, Lowes and Allan 2018, Jolly, Somarelli et al. 2019). Xenograft of CTC in animal models represent an advance to understand the mechanism of cancer progression, but the limitation of these models is that they cannot fully recapitulate the complexity of a human cancer progression (Gómez-Cuadrado, Tracey et al. 2017). In parallel *in vitro* culture of CTCs was developed to understand the metastatic cascade. These models allow control of the experimental environment as well as the evaluation of different combinations of settings, which is not easily achievable in *in vivo* studies. Five years ago, our group established the first colon CTC line from a patient with metastatic colorectal cancer before treatment initiation and 8 other CTC lines during disease progression and after different therapies of the same patient (Cayrefourcq, Mazard et al. 2015, Soler, Cayrefourcq et al. 2018). The 9 CTC lines present an intermediate phenotype with epithelial and mesenchymal markers with varying degrees of EMP. This observation of partial EMT has been already observed in CTCs by different groups (Yu, Bardia et al. 2013, Pantel and Alix-Panabieres 2017, Lim, Yeo et al. 2019, Koch, Kuske et al. 2020). However, it is quite difficult to analyze in-depth this plasticity in rare single CTCs.

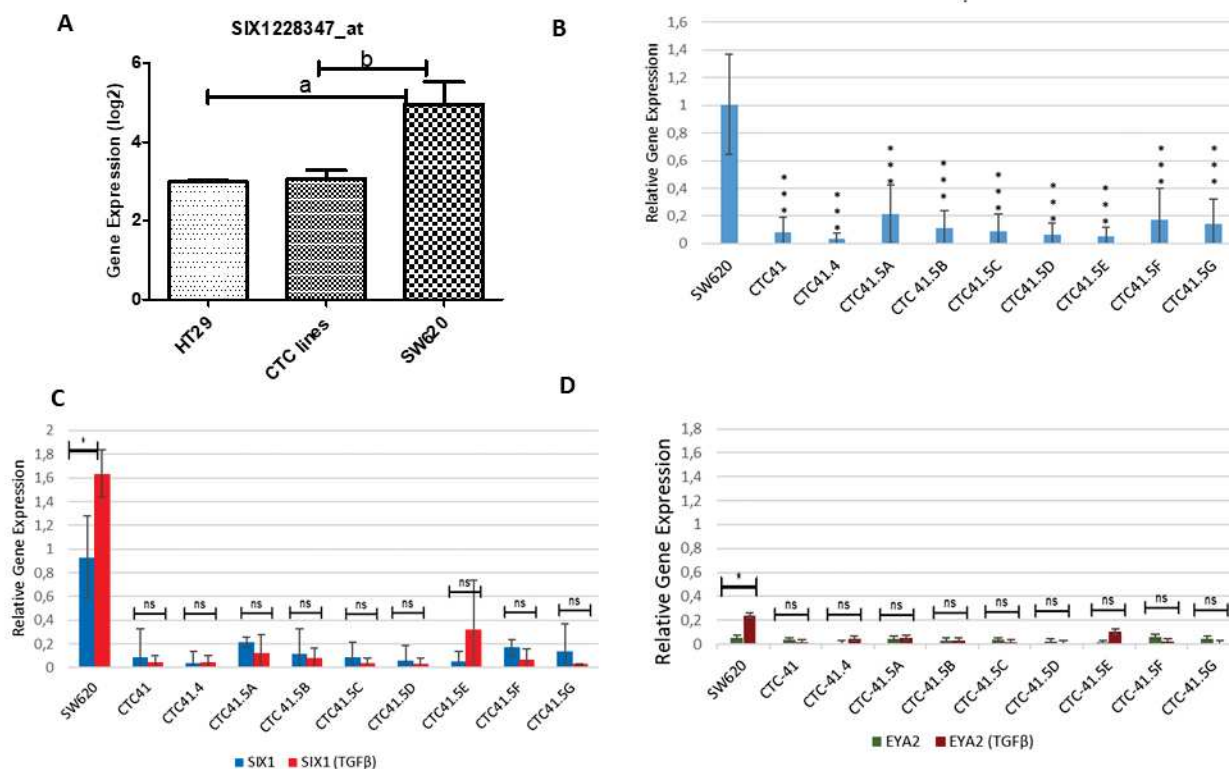
We propose thus to analyze the EMP in these 9 colon CTC lines with the advantage that (i) they are metastasis-competent CTCs, and that (ii) the amount of tumor cells is huge as they are growing very efficiently *in vitro*. In this article, we evaluated the transcriptomic profile of transcription factors involves in EMP, of the different CTC clones selected over time under the

therapy pressure (treatment-induced clonal selection process). Furthermore, we compared the colon CTC clones data to well-characterized primary (HT-29) and metastatic (SW620) colon cancer cell lines.

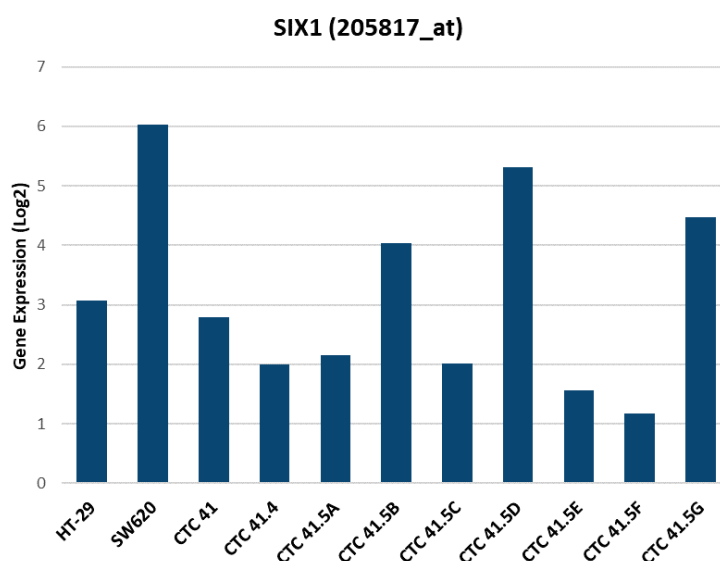
## Results

### **SIX1 and its co-activator EYA2 expression are suppressed in all colon CTC lines and TGF- $\beta$ induction do not alter their profiles.**

SIX1 was previously identified as an independent prognostic factor promoting metastasis in colorectal cancer (Kahlert, Lerbs et al. 2015, Xu, Zhang et al. 2017). In order to investigate the transcriptome profiles of SIX1 transcription factor expression in the 9 CTC lines, we compared it to those of the primary HT-29 and metastatic SW620 colon adenocarcinoma cell lines (**Fig EV1**). According to microarray transcriptomic analyses, *SIX1* expression was significantly down-regulated: 4.29 fold ( $\log_2$ ) in HT-29 cells and 3.92 fold ( $\log_2$ ) in CTCs compared to SW620 cells (**Fig 1A**). Next, *SIX1* microarray results were confirmed by RT-qPCR and we found that *SIX1* expression was significantly decreased in all 9 CTC lines compared to SW620 cells (**Fig 1B**). EMT inducer SIX1 transcription factor and its co-activator EYA2 are often co-overexpressed in tumors and activated by the TGF- $\beta$  signaling pathway (Micalizzi, Christensen et al. 2009, Blevins, Towers et al. 2015). To investigate this observation, cells were treated with TGF- $\beta$  and, then, *SIX1* and *EYA2* expression profiles were analyzed in these cells. CTC lines did not show any significant *SIX1* and *EYA2* modification due to the TGF- $\beta$  induction, only SIX1 and EYA2 expression profile in SW620 cells ( $P < 0.05$ ) induced significantly (**Fig 1C and 1D**). These results suggest that *SIX1* and *EYA2* are not expressed in CTC lines under normal or TGF- $\beta$  activation conditions.



**Figure 1. Expression pattern of *SIX1* and its co-activator *EYA2* under normal and TGF-β activation conditions in colorectal cell lines.** **A.** Microarray transcriptomic analysis of *SIX1* expression and comparison between colon CTC lines, colorectal primary and metastatic cancer cell lines. **B.** RT-qPCR analysis of *SIX1* expression in CTC lines compared to SW620. **C.** RT-qPCR analysis of *SIX1* expression with and without TGF-β activation. **D.** *EYA2* expression profile in TGF-β activated and unactivated cells. ‘a’ indicates P value:2.73E-09, ‘b’ indicates P value: 9.80E-11, \*, \*\*\* indicates P<0.05 and P<0.001, respectively.

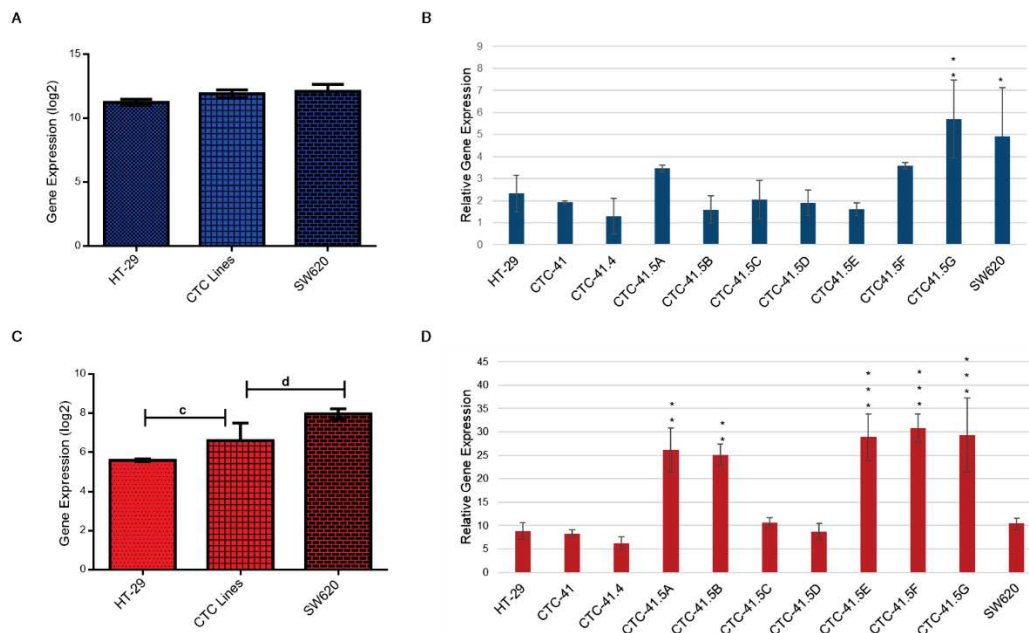


**Figure EV1. GeneChip assay of *SIX1* mRNA level in HT-29, SW620 and CTC cell clones.****SIX1 targets are expressed in all colon CTC lines**

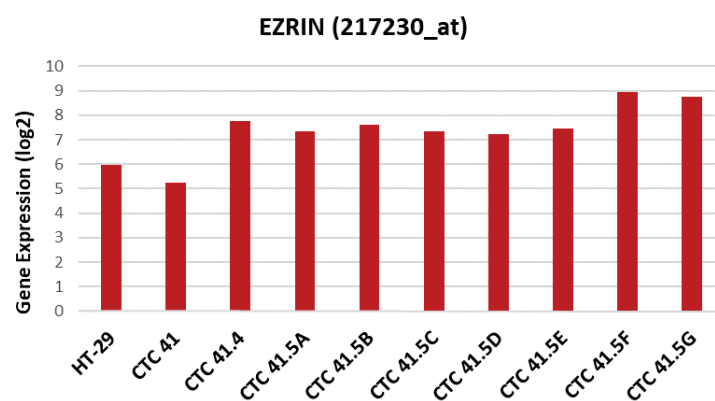
SIX1 plays a role by activating the expression of the pro-tumorigenic genes c-MYC and EZRIN in the tumorigenesis process (Yu, Davicioni et al. 2006). c-MYC regulates many important biological pathways that are involved in the cell cycle, cellular proliferation, survival, metabolic reprogramming growth, and stemness of tumor cells (Elbadawy, Usui et al. 2019). To explore this gene expression pattern, we first assessed the transcriptomic comparison on CTCs, HT-29 and SW620 cells. The expression of *MYC* was 1.8 fold ( $\log_2$ ) up-regulated in CTCs compared to HT-29 (**Fig 2A**). Next, we tested the identification of c-MYC expression profile in the CTC lines. Interestingly, *MYC* expression was variable among the 9 CTC lines. Indeed, although all CTC lines expressed *MYC*, the expression ratios in CTC41.5A, CTC41.5F, and CTC41.5G as well as in SW620 cells were higher than in the other CTC lines and HT-29 cells, especially CTC41.5G over-expressed *MYC* gene significantly (**Fig 2B**).

EZRIN, cytoskeleton regulator protein, has been shown to be a direct transcriptional target of SIX1 in rhabdomyosarcoma (Yu, Davicioni et al. 2006) and previous studies showed that SIX1 and EZRIN proteins are significant independent prognostic factor and also EZRIN expression is associated with metastasis in colorectal cancer analyses (Wang, Zhu et al. 2009, Lin and Chen 2013, Kahlert, Lerbs et al. 2015). EZRIN is well-documented to be associated with development, invasion and metastasis in a variety of human malignancies. A meta-analysis showed that an elevated EZRIN expression was associated with a worse prognosis in patients with different types of cancers (e.g., digestive cancers, head and neck squamous cell carcinoma, gynecologic cancer, osteosarcoma, hepatobiliary cancer and non-small cell lung cancer) (Li, Wei et al. 2015). The analysis of the expression of *EZRIN* gene in the CTC lines and HT-29 cells (**Fig EV2**) showed that the *EZRIN* expression was significantly 2.37 fold ( $\log_2$ ) over-expressed in all CTC lines combined compared to HT-29. The comparison of SW620 cells and the CTC lines showed that the CTC *EZRIN* profile was 2,23 fold lower than in the SW620 cells (**Fig 2C**). The in-depth analysis of CTC-MCC cells expression profile (qPCR) showed that CTC-41, 41.4, 41.5C and 41.5D cells have similar expression patterns compared to HT-29 cells whereas CTC-41.5A, 41.5B, 41.5E, 41.5F and 41.5G cells overexpressed *EZRIN* significantly (**Fig 2D**). All together, these data revealed that the oncogenic targets of *SIX1* transcription factor

showed an increased expression in some CTC clones, although surprisingly the expression of *SIX1* was not overexpressed in these colon cell lines compared to SW620 cell line.



**Figure 2. Increased *MYC* and *EZRIN* gene expression in CTC-MCC lines compared to HT-29 cells.** **A.** Comparison of *MYC* transcriptomic analyses between CTC lines and HT-29 cells. **B.** RT-qPCR analyses of *MYC* expression in each CTC line and HT-29 cells. **C.** Comparison of *EZRIN* transcriptomic analyses between CTC lines and HT-29 cells. **D.** *EZRIN* expression pattern in each CTC line and HT-29 cells. ‘c’ and ‘d’ indicate P values c:1.00E-04, d: 6.41E-07\*\*P value< 0.01, \*\*\*P value< 0.001.

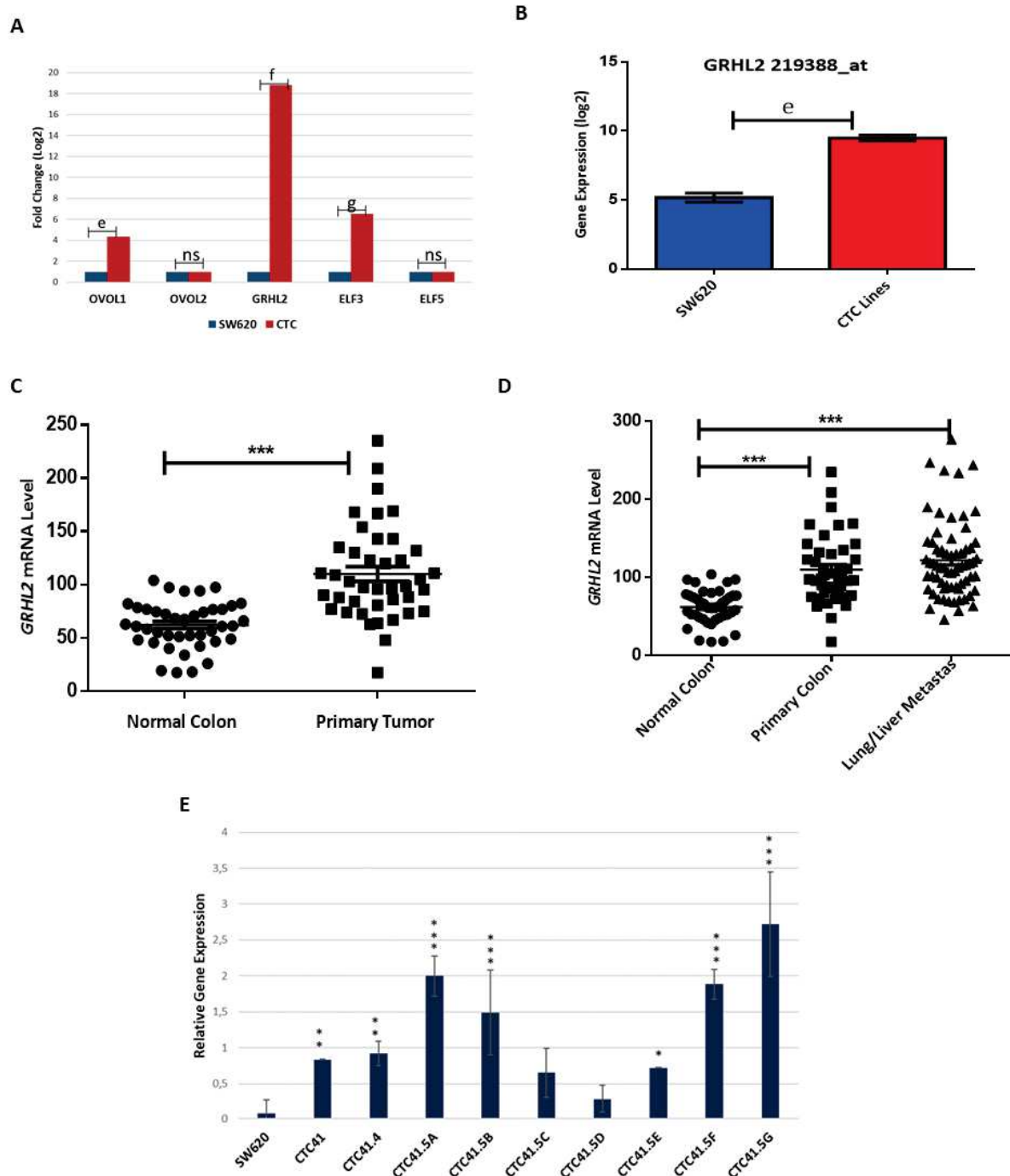


**Figure EV2. *EZRIN* gene expression profile by GeneChIP assay.**



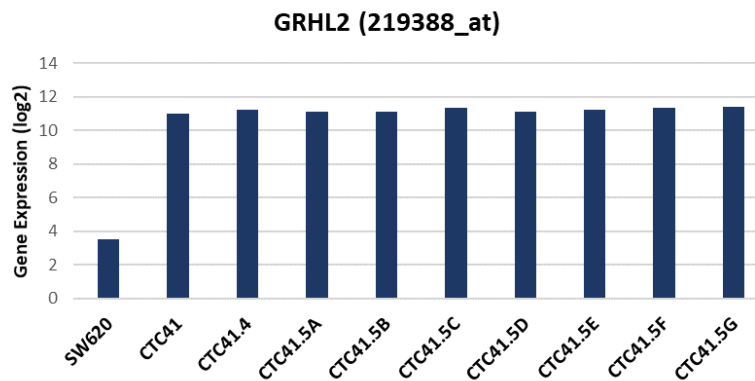
### **Analyses of MET marker expression pattern revealed that GRHL2 is upregulated in colon CTC lines.**

Given the heterozygous nature of the observed oncogenic and metastatic gene expression pattern in CTCs, we focused on the expression profiles of MET transcription factors GRHL2, ELF3/5 and OVOL1/2. Based on the data obtained with the microarrays in CTCs, the expression of *GRHL2*, *ELF3* and *OVOL1* were upregulated 17,8 fold (log<sub>2</sub>), 5.5 fold (log<sub>2</sub>) and 3.3-fold (log<sub>2</sub>), respectively, whereas a nearly equivalent pattern was detected for *OVOL22* and *ELF5* expression when compared to SW620 cells (**Fig 3A**). The highest significant difference observed in CTC lines for *GRHL2* compared to the metastatic colon cancer cell line was confirmed by RT-qPCR (**Fig 3B**). Our gene array data showed that all CTC-MCC lines had approximately a 4 fold (log<sub>2</sub>) increase of the *GRHL2* expression pattern compared to SW620 cells (**Fig EV3**). Consistent with our initial results, previous papers showed that *GRHL2* was suppressed by TGF- $\beta$  activation *in vitro* and *in vivo* analyses (Xiang, Fu et al. 2017, Chen, Kang et al. 2018). The upregulation of *GRHL2* in CTCs prompted us to study colorectal cancer *GRHL2* expression patterns in more details. We extended the analysis of *GRHL2* expression to datasets (GEO accession GSE41258) Israel cohort, consisting of primary colon adenocarcinomas, adenomas, metastasis and corresponding normal mucosae. The mRNA level of *GRHL2* expression significantly increased in colorectal cancer tumor tissues compared to the same patient's normal colorectal tissues (**Fig 3C**). Moreover, the significant increase of *GRHL2* mRNA levels was detected in primary and lung/liver metastatic CRC tissues compared to normal colorectal tissues (**Fig 3D**). Since we observed a higher expression of *GRHL2* in colorectal cancer tissues compared to normal tissues, we also determined the transcription profile of *GRHL2* in the CTC lines. We displayed that the 9 CTC lines have a higher expression profile of *GRHL2* but, more specifically, CTC-41, CTC-41.4, CTC-41.5A, CTC-41.5B, CTC-41.5E, CTC-41.5F, and CTC-41.5G are significantly up-regulated compared to SW620 cells (**Fig 3E**).



**Figure 3. Up-regulation of the MET marker transcription factor *GRHL2* in colorectal cancer CTC-MCC lines and its association with colorectal cancer.** **A.** The analysis of MET transcription factors expression profile and statistics analysis. **B.** The highest expression was detected in *GRHL2* MET marker. **C.** Statistics of the paired samples (n=27) *GRHL2* mRNA level in the normal colorectal epithelium and primary CRC. The mRNA levels were obtained from the Israeli cohort (GSE41258). **D.** The expression of *GRHL2* mRNA level in the same cohort as in C, and compared to the normal colorectal epithelium (n=41), primary CRC (n=41),

and lung & liver metastatic (n=66) CRC samples. E. *GRHL2* relative gene expression in SW620 and in each CTC line. ‘e’, ‘f’ and ‘g’ indicate P values, e: 2.71E-09, f: 1.80E-17, g: 1.54E-12. \* P value < 0.05, \*\*P value < 0.01, \*\*\* P value < 0.001.



**Figure EV3. GeneChip assay of *GRHL2* mRNA level in CTC-MCC lines and SW620 cells.**

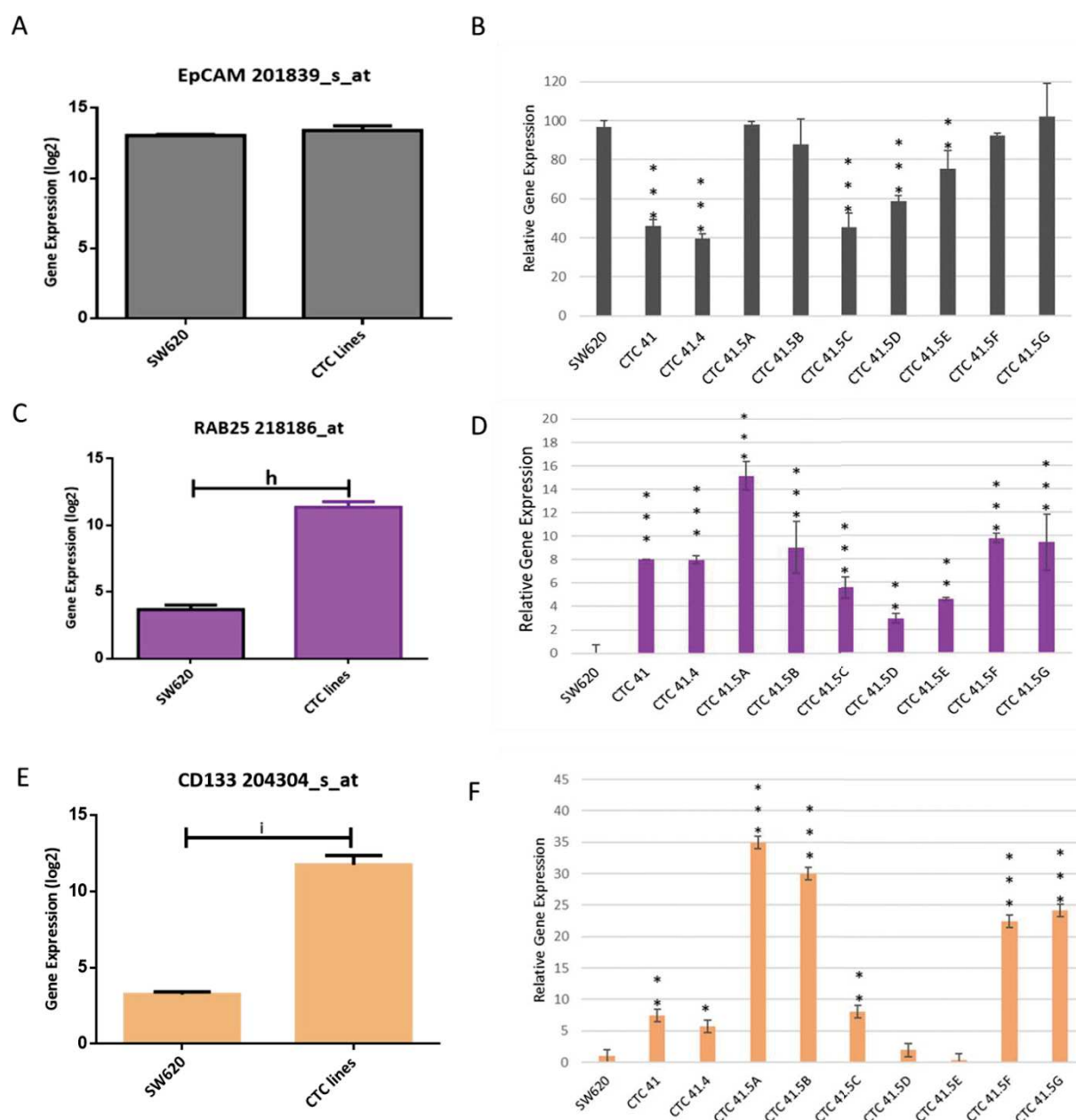
### **EpCAM and RAB25, the direct targets of *GRHL2*, and CD133, its indirect target are differentially regulated in the colon CTC lines**

A recent study showed that *GRHL2* is a key player for the EMP (Chung, Tan et al. 2019) and other studies pointed that *GRHL2* directly regulates the transcription of *EpCAM* and *RAB25*, which is correlated with cancer progression (Chung, Tan et al. 2016, Paltoglou, Das et al. 2017, Liang, Tso et al. 2018, Cho and Lee 2019). Previously, we demonstrated that all our colon CTC lines and the cancer cell lines HT-29 and SW620, highly express *EpCAM* (Soler, Cayrefourcq et al. 2018); this result has been confirmed by the microarray analyses. Considering the comparison of the CTC lines with metastatic colon cancer cells, no significant difference was observed when CTC lines data were pooled (**Fig 4A**): the high *EpCAM* expression profile was detected in both CTC lines and SW620 cells. However, when we analyzed the CTC lines individually, we observed that *EpCAM* expression was significantly lower for CTC-41, 41.4, 41.5C, 41.5D, and 41.5E cells when compared to SW620 (**Fig 4B**). In addition, we can observe that the *EpCAM* expression profile follows exactly the *GRHL2* profile in the CTC lines while, surprisingly, in SW620 cells we have a high expression of *EpCAM* with a low expression of *GRHL2* (**Fig 3E & 4B**).

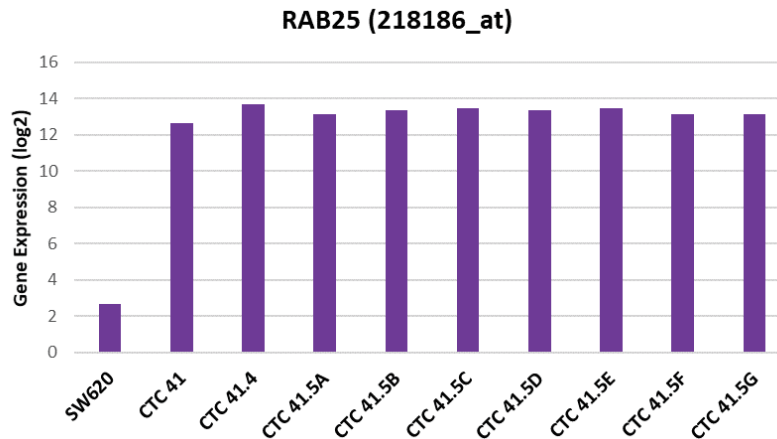
Another known direct target of *GRHL2* is *RAB25* and this gene was identified as a tumor-suppressor gene in colorectal cancer patients (Nam, Lee et al. 2010). Interestingly, *RAB25* has a role in anchorage-independent cell proliferation, resistance to apoptosis, stem cell capacity

and differentiation, which have a critical importance for CTCs (Cheng, Lahad et al. 2004, Zhang, Shang et al. 2012). To comprehensively characterize the significantly high increment in *RAB25* expression level in metastatic colon cancer, we analyzed its transcriptomic profile in all CTC lines (**Fig 4C, 4D** and **Fig EV4**). Considering the SW620 cell line, we did not observe *RAB25* expression, which is consistent with a previous study identifying that *RAB25* expression was down-regulated by hyper-methylation in SW620 cells (Letellier, Schmitz et al. 2017). Concerning the CTC lines, in our analyses, *RAB25* is expressed in all of them, implying an increased expression pattern compared to SW620 cells (**Fig 4D**).

Interestingly, previous papers reported that *GRHL2* directly suppresses miR-122 expression, which post-transcriptionally regulates *CD133* expression (Tanimizu, Kobayashi et al. 2014, Song, Kwon et al. 2015, Nishino, Takano et al. 2017). *CD133*, a cancer stem cell marker, is displayed as a significant prognostic factor for colorectal cancer patient survival (Glumac and LeBeau 2018). *CD133* was overexpressed in CTC lines compared to the SW620 cells (**Fig 4E**). Moreover, in the qPCR analyses, all CTC-MCC lines had a significantly high *CD133* expression, excepted for CTC-41.5D and 41.5E (**Fig 4F**). Even if, up to now, there is no data showing that *Grhl2* directly regulates *CD133*, we can observe that *CD133* and *GRHL2* expression patterns were similar in the different CTC lines.



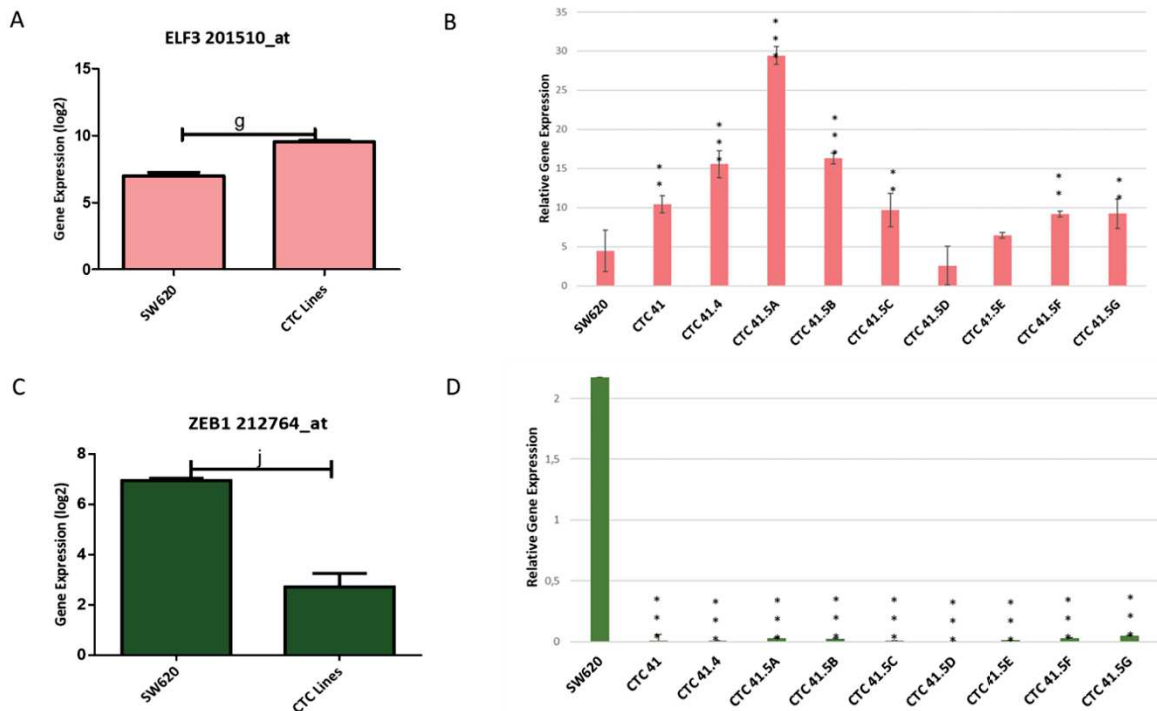
**Figure 4. GRHL2 direct and indirect targets EpCAM, RAB25, and CD133 are differentially expressed in each CTC-MCC line.** **A.** Transcriptomic analyses of *EpCAM* in CTC lines and SW620 cells. **B.** Relative gene expression of *EpCAM* in each CTC lines and SW620 cells. **C.** Transcriptomic analyses of *RAB25* in CTC lines and SW620 cells. **D.** *RAB25* gene expression profile in each CTC line and SW620 cells. **E.** Transcriptomic analyses of *CD133* in CTC lines and SW620 cells. **F.** *CD133* expression pattern in CTC lines and SW620 cells. ‘h’ and ‘i’ indicates P values, h: 1.57E-23, i: 1.34E-18. All the RT-qPCR results were normalized to the expression level of  $\beta$ 2-microglobulin in each sample. CTC expression statistically compared with SW620 values\* P value< 0.05,\*\*P value< 0.01,\*\*\* P value< 0.001.



**Fig EV4. *RAB25* gene expression profile by GeneChIP assay.**

**Inverse correlation between MET markers *GRHL2* and *ELF3* and their common target *ZEB1* in the CTC lines.**

The second highest MET marker is E74 Like ETS Transcription Factor 3, known as *ELF3*, and *ELF3* expression is also regulated by *GRHL2* (10.1038/srep19943). A previous study determined that *ELF3* binds and trans-activates a  $\beta$ -catenin promoter and is associated with poor prognosis in colorectal cancer (Wang, Chen et al. 2014). We determined the expression profile of *ELF3* at the transcriptomic level of each CTC lines, and showed that *ELF3* is highly expressed in all CTC lines, except in the CTC-41.5D, and significantly over-expressed compared to SW620, like it was observed for *GRHL2* (**Fig 5A and 5B**). Interestingly, *GRHL2* and *ELF3*, two important MET markers, share the same target *ZEB1*, the well-known inducer of EMT. We report here that *ZEB1* was significantly suppressed in our analyses (transcriptomic analyses and qPCR data) (**Fig 5C and 5D**).



**Figure 5. *ELF3* and *ZEB1* expression assays.** **A.** *ELF3* mRNA level was significantly up-regulated in all CTC-MCC lines compared to SW620 cells in GeneChip assay. **B.** RT-qPCR analysis of *ELF3* on CTC lines and SW620 cells. **C.** *ZEB1* expression pattern and statistical analyses in all CTC lines and SW620 cells. **D.** *ZEB1* expression analysis by RT-qPCR in CTC lines and SW620 cells. ‘g’ and ‘j’ indicates P values, g:1.54E-12, j: 1.20E-17. CTC lines expression statistically compared to SW620 values \* P value< 0.05,\*\*P value< 0.01,\*\*\* P value< 0.001.

## Discussion

EMT is an important cellular program for embryogenesis, wound healing and malignant progression. In the metastatic process, tumor cells have first to lose epithelial properties, so do EMT, to leave the tumor, migrate and intravase blood vessel to potentially invade a distant organ. Furthermore, tumor cells have to revert to epithelial characters, do MET, to settle in secondary distant tissue (Dongre and Weinberg 2019). Recent evidences indicated that cancer cells have hybrid mesenchymal and epithelial characters and that this EMT/MET plasticity also called EMP is clearly associated with a metastatic potential (Williams, Gao et al. 2019). CTCs as cancer cells which circulate through the bloodstream and source of metastasis precursor represent the ideal source to study the EMP process. It is of utmost importance to put our efforts to identify and characterize them to understand the biology of the metastatic cascade (Castro-Giner and Aceto 2020). Our group published the first experimental proof of long-term colon CTC lines in 2015 (Cayrefourcq, Mazard et al. 2015). Our previous work showed that the characterization of the 9 colon CTC lines, established from the same patient at different time-points during the disease progression and treatment, highlighted an intermediate EMT profile with a high plasticity (Soler, Cayrefourcq et al. 2018). In the current study, EMT and MET markers as well as associated genes were analyzed in the 9 CTC lines, and compared to the well-known primary and metastatic colon cancer cell lines, HT-29 and SW620, respectively.

As an EMT-marker, the expression profile of SIX1 has been performed in the serial CTC lines. This transcription factor is known to be important for the mesenchymal profile and drug resistance in various cancers (Jin, Cui et al. 2014, Lv, Cui et al. 2014, Kahlert, Lerbs et al. 2015). In our study, we observed that there was no SIX1 expression, by RT-qPCR validation of microarray data, in our colon CTC lines, even after TGF- $\beta$  activation, used as an EMT inducer. This result suggest strongly that these tumor cells did not used this specific driver during their EMT to leave the primary tumor. Similar to these data on SIX1, its co-activator EYA2 was not expressed neither in the 9 CTC lines, (i) either in normal or (ii) TGF- $\beta$  activation conditions. Furthermore, a previous paper identified an hyper-methylation of EYA2 in colorectal cancer samples (Zou, Harrington et al. 2007). Interestingly, in our CTC lines, SIX1 oncogenic target genes *MYC* and *EZRIN* were up-regulated without any SIX1 activation. On one side, *MYC* has an important role in cell proliferation, differentiation, survival, apoptosis, cancer stemness as well as in drug resistance in colorectal cancer stem cells (Elbadawy, Usui et al. 2019), and on the other side, *EZRIN* promotes invasion and metastasis of cancer cells (Ren, Hong et al. 2009). These results implice that even in absence of well-known and described



regulators, tumor cells still develop alternative processes to activate genes and to increase their plasticity and invasiveness.

EMT is a key recognized step for increasing the motility and the invasiveness of subsets of cancer cells in the primary tumor, however, for metastatic colonization in a distant organ, cancer cells need an epithelial phenotype to achieve this last crucial step (Brabletz, Kalluri et al. 2018, Aiello and Kang 2019). The reverse process of EMT, known as MET, is orchestrated by MET-transcription factors, such as *OVOL1/2*, *GRHL2*, *ELF3*, and *ELF5* (Jolly, Somarelli et al. 2019). In MET transcription factors analyses, we found that *GRHL2*, *ELF3*, and *OVOL1* were over-expressed in the CTC lines compared to the metastatic colorectal cancer cell line SW620; the highest expression was detected for the *GRHL2* transcription factor. Furthermore, we found that *GRHL2* was up-regulated in colorectal cancer as well as in liver and lung metastatic samples compared to normal colon tissue in our bioinformatics analyses.

Recent papers pointed out that a full mesenchymal phenotype is insufficient for metastasis while *GRHL2* may stabilize the hybrid epithelial/mesenchymal phenotype and support cell migration ability (Ye, Brabletz et al. 2017, Chung, Tan et al. 2019). It is known that *GRHL2* transcription factor regulates various cellular process in development, such as epithelial morphogenesis, barrier formation and wound healing (Walentin, Hinze et al. 2015). Then, we investigated *GRHL2* target genes that have different reported roles in cancer progression. We detected that *RAB25* expression was significantly increased in all CTC lines compared to SW620 cells. *RAB25* is involved in the regulation of epithelial morphogenesis through the control of *CLDN4* (Wang, Kumar et al. 2000) and its over-expression can be linked to metastasis capacity, anchorage-independent cell proliferation, prevention of apoptosis and anoikis, hallmarks that are crucial for CTC survival and metastatic competence (Cheng, Lahad et al. 2004, Amornphimoltham, Rechache et al. 2013). However, the role of *RAB25* still remains unclear. Indeed, in diverse cancer types (e.g., bladder, non-small cell lung, ovarian, prostate cancer and clear cell renal cell carcinoma) (Agarwal, Jurisica et al. 2009, Wang, Hu et al. 2017), elevated expression of *RAB25* was significantly associated with shorter OS; conversely, in colorectal cancer *RAB25* expression was decreased and this loss was associated with poorer survival (Goldenring and Nam 2011, Letellier, Schmitz et al. 2017). A recent study introduced a new perspective on the role of small GTPases in the dynamic EMP process, *RAB11* (an evolutionarily conserved and same family member with *RAB25*) has an important role in re-localization of epithelial proteins during EMP process and also promotes CTC clusters formation (Aiello, Maddipati et al. 2018).

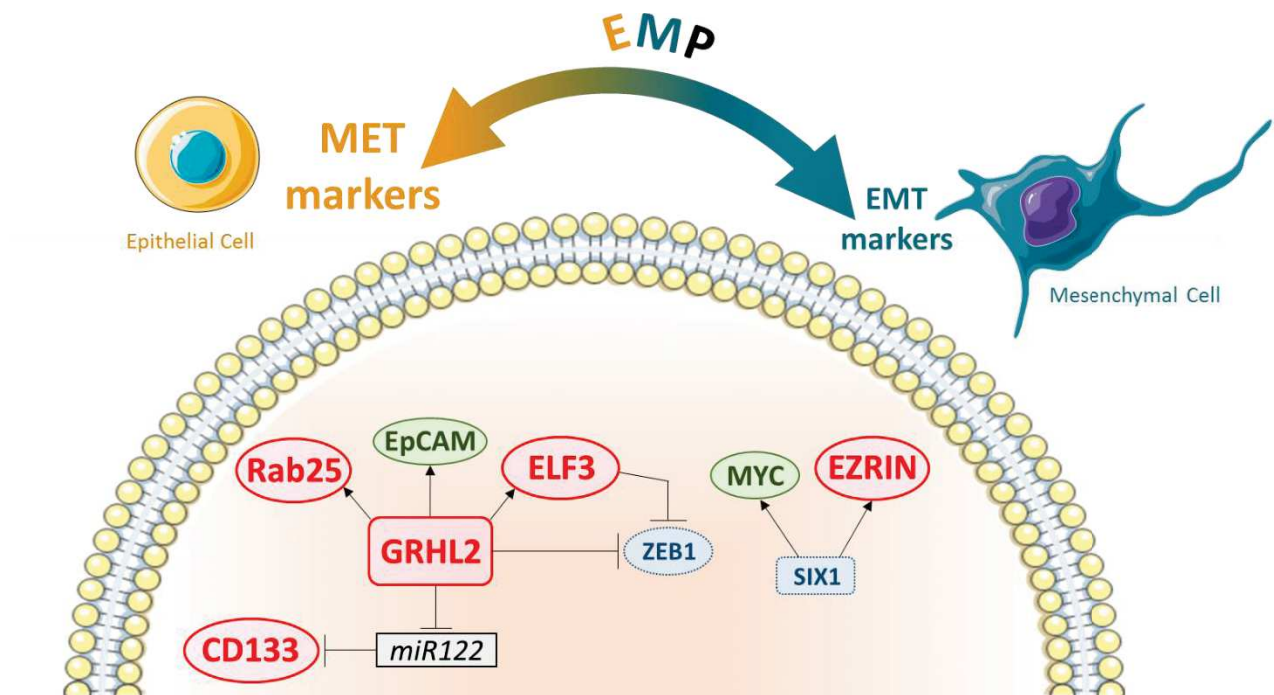
Interestingly, we observed that *CD133* is expressed in CTC lines and its expression profile was positively correlated with that of *GRHL2*. This connection may be explained by an indirect regulation through microRNAs such as miR-122 (Tanimizu, Kobayashi et al. 2014, Song, Kwon et al. 2015). *CD133* is a known cancer stem cell marker and *CD133*-expressing cells exhibited self-renewal potential and the ability to regenerate a histologically similar tumor mass following transplantation into immunodeficient mice (Glumac and LeBeau 2018). Moreover, *CD133* expression by cancer cells has been correlated with poor prognosis and reduced overall survival in a number of different cancers, including colorectal cancer (Horst, Kriegl et al. 2009, Chen, Song et al. 2013, Fang, Fan et al. 2017). We believe that *GRHL2*, an inducer of *MET*, activates *CD133* to prepare the stemness of the tumor cells to allow them to settle and grow in a new distant organ to form an overt metastases

Another *MET* transcription factor, *ELF3*, is overexpressed in our established CTC lines compared to the metastatic cell line SW620. This factor has been also identified as a *GRHL2* target. *ELF3* is described as overexpressed in colorectal cancer and promotes colorectal cancer progression by transactivation of  $\beta$ -catenin (Wang, Chen et al. 2014, Paltoglou, Das et al. 2017).

Finally, *ELF3* and *GRHL2* *MET* transcription factors are both negative regulators of *ZEB1*. In this study, we found that there was no *ZEB1* expression in all CTC lines, which is consistent with *ELF3* and *GRHL2* over-expression, but also with *SIX1* and *EYA2* results, implying that these cells are not in a full mesenchymal state.

Taken together, these results suggest that our colon CTC lines had acquired some mesenchymal markers to migrate and intravasate but they remain more in an epithelial state with a high degree of *MET* inducers to be able to colonize distant organs when required. Indeed, we reported EMT inducers that were down-regulated, whereas *MET* markers were observed to be up-regulated, even if some EMT inducer targets are still overexpressed (**Fig 6**).

In conclusion, this study improves our knowledge on the heterogeneity and the plasticity of these special subsets of CTC clones selected over cancer progression and treatment pressure. These findings should help to understand better the clonal evolution of CTCs over time in a metastatic colon cancer patient who was treated with different therapies and who relapsed sequentially until his death. Advances in the knowledge on the plasticity of these metastasis-initiator cells provide new ideas for the development of innovative personalized medicine in colon cancer.



**Figure 6. Schematic presentation of EMP markers expression network and their regulation in the serial CTC lines.** The significantly up-regulated genes are shown in red color (greater than 2 fold change in  $\log_2$ ), green color indicates up-regulated genes but without significant difference, blue-colored genes were significantly down-regulated, comparison of CTC lines to metastatic colorectal cancer cell line (SW620).

## Material and Methods

### Cell culture

The colorectal cell lines HT-29 (ATCC HTB-38) and SW620 (ATCC CCL-227) were used as control for analysis. HT-29 cells were maintained in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), SW620 cells, in RPMI 1640 with L-glutamine and 10% FCS, and the colon CTC lines were cultured in CTC culture medium which was previously described (Cayrefourcq, Mazard et al. 2015, Soler, Cayrefourcq et al. 2018).

### TGF- $\beta$ stimulation

For TGF- $\beta$  stimulation, cells were incubated with 10ng/ml of human TGF- $\beta$ 1 protein (Ref: PHG9214, Gibco by Life Technologies) in complete culture medium for 6 days in 24 well culture plate (Falcon). The culture medium was replaced every two days with fresh media containing TGF- $\beta$ 1 and cells were passaged just before reaching 70% confluence. All cell lines were maintained at 37°C in 5% CO<sub>2</sub>.

### RNA isolation and Microarray analysis

The total RNA from each sample was extracted using the RNeasy Mini Kit (74106-Qiagen), as recommended by the manufacturer. The RNA concentration of each sample was measured using the NanoDrop One (ThermoFisher) and a solution of 100ng/ $\mu$ l was prepared for Microarray analysis on Affymetrix platform. RNA quality was analyzed by Bioanalyzer 2100 (Agilent). Total RNA (200 ng) was used to prepare cRNA using the Affymetrix 3' IVT express protocol (ref.901229). The complete cRNA was amplified by in vitro transcription. Amplified RNA (aRNA) was quantified with a NanoDrop ND-1000 spectrophotometer. After fragmentation, 12  $\mu$ g of labeled antisense aRNA was hybridized to HGU133 plus 2.0 GeneChip arrays (Affymetrix®). Microarray data were obtained and analyzed using the Transcriptome Analysis Console (TAC) software.

### RT-qPCR

Complementary DNA (cDNA) was obtained by reverse transcription (RT) by using the SuperScript III First-Strand Synthesis Super Mix kit (18080, Invitrogen) according to the manufacturer's instructions. One  $\mu\text{L}$  of each RT product was added to 9  $\mu\text{L}$  of mix containing the two primers (6  $\mu\text{M}$  each) and Brilliant III Ultra-Fast SYBR Green Master Mix (600882, Agilent Tech.). The primer sequences are shown in the **Table EV1**. PCR reactions were performed on a QuantStudio 5 real-time PCR instrument (Thermo Fischer Sci.) as follows: 95 °C for 3 min, followed by 40 cycles of 95 °C for 5 s, 60 °C for 10 s, and then 1 cycle at 95 °C for 1 min, 55 °C for 5 s, 95 °C for 30 s, 37 °C for 30 s. In all experiments,  $\beta$ 2-microglobulin (B2M) was used as the reference housekeeping gene and results were analyzed using the QuantStudio Design and Analysis Software.

### Bioinformatics and Statistical Analysis

The gene expression data used by Israel cohort, consisting of 390 arrays from primary colon adenocarcinomas, adenomas, metastasis and corresponding normal mucosae. The expression of GRHL2 mRNA level obtained from in GEO database with accession number GSE41258. Comparison of between different groups including normal colorectal epithelium, primary CRC, and metastatic CRC were tested with Mann–Whitney test.

Statistical significance for the gene expression data was analyzed using one-way analysis of variance (ANOVA) test. A value of  $P < 0.05$  was considered to be statistically significant. Each experiment was performed at least three times and data are shown as the mean ( $\pm$ ) SD.

**Table EV1.** The sequence of the forward and reverse primers used for qRT-PCR analysis.

Gene	Oligo Name	Sequence 5' to 3'	
SIX1	Forward Sequence	AAAGGGAAGGAGAACAAGGATAG	NM_005982.4
	Reverse Sequence	AGCCTACATGATTACTGGGATTT	
EYA2	Forward Sequence	CAGAGTGGATTTCCTCAGCTATG	NM_005244.5

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	Reverse Sequence	CCTCCTTGATAGAACCCTGTTG	
c-MYC	Forward Sequence	GCTGCTTAGACGCTGGATTT	NM_002467.4
	Reverse Sequence	GAGTCGTAGTCGAGGTCATAGTT	
EZRIN	Forward Sequence	CCGTGGGATGCTCAAAGATAA	NM_003379.5
	Reverse Sequence	TCCAAGCCAAAGGTCTGTTC	
GRHL2	Forward Sequence	CGTCTGGGTGCAGTAGTTATAG	NM_024915.4
	Reverse Sequence	GGGAACCCTGGAGGTATTTAG	
EPCAM	Forward Sequence	ACAAGGACACTGAAATAACCTGC	NM_002354
	Reverse Sequence	TTTTGAGAAGAATTTTGAACCAGAT	
Rab25	Forward Sequence	GGGAATGGAACTGAGGAAGATTA	NM_020387.4
	Reverse Sequence	CGTGAATCGGGAGAGTAGATTG	
CD133	Forward Sequence	TTGAATGAAACTCCAGAGCAAA	NM_001145847
	Reverse Sequence	CCTCCTAGCACTGAATTGATACTG	
ELF3	Forward Sequence	TCAACGAGGGCCTCATGAA	NM_004433.5
	Reverse Sequence	TCGGAGCGCAGGAACTTG	

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ZEB1	Forward Sequence	TGCACTGAGTGTGGAAAAGC	NM_001128128.3
	Reverse Sequence	TGGTGATGCTGAAAGAGACG	
B2M	Forward Sequence	GTCTTTCAGCAAGGACTGGTCT	NM_004048
	Reverse	TTACATGTCTCGATCCCACTTAAC	

### **Data availability**

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

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### **Author contributions**

P.B-E analyzed and interpreted the data. P.B-E, LC and CAP were major contributors in the conception of the study and the writing of the manuscript. All authors read and approved the final manuscript.

### **Conflict of interest**

The authors declare that they have no conflict of interests.

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## Figure Legends

**Figure 1. Expression pattern of *SIX1* and its co-activator *EYA2* under normal and TGF- $\beta$  activation conditions in colorectal cell lines.** **A.** Microarray transcriptomic analysis of *SIX1* expression and comparison between colon CTC lines, colorectal primary and metastatic cancer cell lines. **B.** RT-qPCR analysis of *SIX1* expression in CTC lines compared to SW620. **C.** RT-qPCR analysis of *SIX1* expression with and without TGF- $\beta$  activation. **D.** *EYA2* expression profile in TGF- $\beta$  activated and unactivated cells. ‘a’ indicates P value:2.73E-09, ‘b’ indicates P value: 9.80E-11, \*, \*\* indicates P<0.05 and P<0.001, respectively.

**Figure 2. Increased *MYC* and *EZRIN* gene expression in CTC-MCC lines compared to HT-29 cells.** **A.** Comparison of *MYC* transcriptomic analyses between CTC lines and HT-29 cells. **B.** RT-qPCR analyses of *MYC* expression in each CTC line and HT-29 cells. **C.** Comparison of *EZRIN* transcriptomic analyses between CTC lines and HT-29 cells. **D.** *EZRIN* expression pattern in each CTC line and HT-29 cells. ‘c’ and ‘d’ indicate P values c:1.00E-04, d: 6.41E-07\*\*P value< 0.01, \*\*\*P value< 0.001.

**Figure 3. Up-regulation of the MET marker transcription factor *GRHL2* in colorectal cancer CTC-MCC lines and its association with colorectal cancer.** **A.** The analysis of MET transcription factors expression profile and statistics analysis. **B.** The highest expression was detected in *GRHL2* MET marker. **C.** Statistics of the same patient (n=27) *GRHL2* mRNA level in the normal colorectal epithelium and primary CRC. The mRNA levels were obtained from the Israeli cohort (GSE41258). **D.** The expression of *GRHL2* mRNA level in the same cohort as in C, and compared to the normal colorectal epithelium (n=41), primary CRC (n=41), and lung & liver metastatic (n=66) CRC samples. **E.** *GRHL2* relative gene expression in SW620 and in each CTC line. ‘d’, ‘e’ and ‘f’ indicate P values, d: 2.71E-09, e: 1.80E-17, f: 1.54E-12. \* P value< 0.05,\*\*P value< 0.01,\*\*\* P value< 0.001.

**Figure 4. *GRHL2* direct and indirect targets *EpCAM*, *RAB25*, and *CD133* are differentially expressed in each CTC-MCC line.** **A.** Transcriptomic analyses of *EpCAM* in CTC lines and SW620 cells. **B.** Relative gene expression of *EpCAM* in each CTC lines and

SW620 cells. **C.** Transcriptomic analyses of *RAB25* in CTC lines and SW620 cells. **D.** *RAB25* gene expression profile in each CTC line and SW620 cells. **E.** Transcriptomic analyses of *CD133* in CTC lines and SW620 cells. **F.** *CD133* expression pattern in CTC lines and SW620 cells. ‘g’ and ‘h’ indicates P values, h: 1.57E-23, i: 1.34E-18. All the RT-qPCR results were normalized to the expression level of  $\beta$ 2-microglobulin in each sample. CTC expression statistically compared with SW620 values\* P value< 0.05,\*\*P value< 0.01,\*\*\* P value< 0.001.

**Figure 5. *ELF3* and *ZEB1* expression assays.** **A.** *ELF3* mRNA level was significantly up-regulated in all CTC-MCC lines compared to SW620 cells in GeneChip assay. **B.** RT-qPCR analysis of *ELF3* on CTC lines and SW620 cells. **C.** *ZEB1* expression pattern and statistical analyses in all CTC lines and SW620 cells. **D.** *ZEB1* expression analysis by RT-qPCR in CTC lines and SW620 cells. ‘f’ and ‘j’ indicates P values, f:1.54E-12, j: 1.20E-17. CTC lines expression statistically compared to SW620 values \* P value< 0.05,\*\*P value< 0.01,\*\*\* P value< 0.001.

**Figure 6. Schematic presentation of EMP markers expression network and their regulation in the serial CTC lines.** The significantly up-regulated genes are shown in red color (greater than 2 fold change in  $\log_2$ ), green color indicates up-regulated genes but without significant difference, blue-colored genes were significantly down-regulated.

## Expanded View and Legend

**Figure EV1. GeneChip assay of *SIX1* mRNA level in HT-29, SW620 and CTC cell clones.**

**Figure EV2. *EZRIN* gene expression profile by GeneChIP assay.**

**Figure EV3. GeneChip assay of *GRHL2* mRNA level in CTC-MCC lines and SW620 cells.**

**Fig EV4. *RAB25* gene expression profile by GeneChIP assay.**

**Table EV1.** The sequence of the forward and reverse primers used for qRT-PCR analysis.



### PARTIE 3 – Lignées CTC coliques : outils pour l'étude de la sensibilité aux traitements

Comme déjà évoqué dans l'introduction, les cellules tumorales *ex vivo* reflète la tumeur et sa réaction (sensibilité/résistance) face aux divers traitements donnés aux patients. Les lignées CTCs représentent donc un moyen idéal pour l'étude de nouveaux traitements ou l'application de traitement existant dans de nouveaux cas pathologiques.

Nous avons pu observer lors de l'étude du transcriptome une dérégulation importante de la voie de signalisation PI3K/AKT/mTOR. C'est une voie intracellulaire qui régule des fonctions essentielles, y compris la synthèse des protéines, la croissance cellulaire, le contrôle du cycle cellulaire, la survie et la migration. De plus, l'activation et l'amplification de mutations oncogènes dans cette voie ont fréquemment été observées dans un grand nombre de types différents de cancers, soulignant l'importance de cette voie de signalisation dans le processus tumoral. En conséquence, de nouvelles thérapies ciblant cette voie ont été développées.

Dans cette étude, il a été observé que la première lignée CTC 'CTC-MCC-41' (obtenue avant traitement), exprime fortement les formes activées d'AKT (pAKT) et de S6 (pS6), kinase régulée par mTOR. Nous avons donc évalué l'action des inhibiteurs d'AKT : MK2206 et de mTOR : RAD001, ainsi que la combinaison des deux sur cette lignée CTC. Nous avons pu démontrer une inhibition des formes phosphorylées de ces protéines par leurs inhibiteurs respectifs ainsi qu'un effet synergique de la combinaison des 2 molécules. Les mêmes résultats ont été obtenus lors des tests de prolifération cellulaire. Cela suggère que les thérapies ciblant AKT et mTOR auraient peut-être été bénéfiques à ce patient qui a permis l'expansion de cette lignée CTC. Pour l'heure, seule la thérapie anti-mTOR a été testée dans le CCR et n'a pas montré de bénéfice à bloquer la progression tumorale. Ces résultats pourraient être améliorés par la combinaison de cette molécule avec d'autres inhibiteurs de la voie métabolique afin d'éviter une boucle rétroactive négative.

De plus, la forme activée d'AKT a montré une hétérogénéité d'expression en fonction des conditions de culture de cellules en suspension vs cellules adhérentes. En effet, ces lignées CTCs sont normalement cultivées en conditions *non adhérente* mais, si on les place en flasques adhérentes, on peut observer l'attachement de certaines d'entre elles alors que d'autres restent en suspension. De manière intéressante, si on sépare et cultive à part ces deux fractions (adhérentes et non adhérentes), on obtient à nouveau les 2 sous-populations cellulaires. Ces résultats montrent clairement la plasticité de ces cellules et leur capacité à adhérer à un support dès qu'elles le peuvent pour former un tapis cellulaire *in vitro*, une propriété qui pourrait être transposée chez le patient à la capacité de former des métastases. Ainsi, la différence d'expression de la forme active d'AKT reflète les différents états des

cellules et leur plasticité EMT car pAKT a été décrit comme sous régulé lors de l'induction de cette transition EMT.





Article 6

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High sensitivity of circulating tumor cells derived from a colorectal cancer patient for dual inhibition with AKT and mTOR inhibitors.

Daniel J. Smit, **Laure Cayrefourcq**, Marie-Therese-Haider, Nico Hinz, Klaus Pantel, Catherine Alix-Panabières and Manfred Jücker.

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Article

# High Sensitivity of Circulating Tumor Cells Derived from a Colorectal Cancer Patient for Dual Inhibition with AKT and mTOR Inhibitors

Daniel J. Smit <sup>1</sup> , Laure Cayrefourcq <sup>2</sup>, Marie-Therese Haider <sup>3</sup>, Nico Hinz <sup>1</sup>, Klaus Pantel <sup>4</sup> , Catherine Alix-Panabières <sup>2</sup> and Manfred Jücker <sup>1,\*</sup>

<sup>1</sup> Institute of Biochemistry and Signal Transduction, University Medical Center Hamburg-Eppendorf, Martinistraße 52, 20246 Hamburg, Germany; d.smit@uke.de (D.J.S.); hinz\_nico@gmx.de (N.H.)

<sup>2</sup> Laboratory of Rare Human Circulating Cells (LCCRH), University Medical Center of Montpellier, 34093 Montpellier, France; l-cayrefourcq@chu-montpellier.fr (L.C.); c-panabieres@chu-montpellier.fr (C.A.-P.)

<sup>3</sup> Molecular Skeletal Biology Laboratory, Department of Trauma, Hand and Reconstructive Surgery, University Medical Center Hamburg-Eppendorf, 20246 Hamburg, Germany; m.haider@uke.de

<sup>4</sup> Department of Tumor Biology, University Medical Center Hamburg-Eppendorf, Martinistraße 52, 20246 Hamburg, Germany; pantel@uke.de

\* Correspondence: juecker@uke.de; Tel.: +49-(0)-40-7410-56339

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**Abstract:** Circulating tumor cells (CTCs) are cells shed from the primary tumor into the bloodstream. While many studies on solid tumor cells exist, data on CTCs are scarce. The mortality of cancer is mostly associated with metastasis and recent research identified CTCs as initiators of metastasis. The PI3K/AKT/mTOR signaling pathway is an intracellular pathway that regulates essential functions including protein biosynthesis, cell growth, cell cycle control, survival and migration. Importantly, activating oncogenic mutations and amplifications in this pathway are frequently observed in a wide variety of cancer entities, underlining the significance of this signaling pathway. In this study, we analyzed the functional role of the PI3K/AKT/mTOR signaling pathway in the CTC-MCC-41 line, derived from a patient with metastatic colorectal cancer. One striking finding in our study was the strong sensitivity of this CTC line against AKT inhibition using MK2206 and mTOR inhibition using RAD001 within the nanomolar range. This suggests that therapies targeting AKT and mTOR could have been beneficial for the patient from which the CTC line was isolated. Additionally, a dual targeting approach of AKT/mTOR inside the PI3K/AKT/mTOR signaling pathway in the colorectal CTCs showed synergistic effects *in vitro*. Depending on the phenotypical behavior of CTC-MCC-41 in cell culture (adherent vs. suspension), we identified altered phosphorylation levels inside the PI3K/AKT/mTOR pathway. We observed a downregulation of the PI3K/AKT/mTOR signaling pathway, but not of the RAS/RAF/MAPK pathway, in CTCs growing in suspension in comparison to adherent CTCs. Our results highlight distinct functions of AKT isoforms in CTC-MCC-41 cells with respect to cell proliferation. Knockdown of AKT1 and AKT2 leads to significantly impaired proliferation of CTC-MCC-41 cells *in vitro*. Therefore, our data demonstrate that the PI3K/AKT/mTOR signaling pathway plays a key role in the proliferation of CTC-MCC-41.

**Keywords:** circulating tumor cells; CTCs; drug sensitivity assays; PI3K/AKT/mTOR pathway; AKT; MK2206; RAD001; dual targeting; colorectal carcinoma; targeted therapy; personalized medicine

## 1. Introduction

Most deaths in cancer are associated with metastasis of cells from the primary tumor [1]. During the metastatic process malignant cancer cells have the ability to access the lymphatic system, blood

vessels or surrounding tissue [2]. Although the importance of cancer cell metastasis has been shown in the past [3], the entire process is not completely understood yet.

Recently, circulating tumor cells (CTCs), which are shed from the primary tumor into the blood were successfully isolated and taken into cell culture, promising a valuable and robust model to study cancer cell metastasis [4,5]. The CTC cell culture allows insights into the cells responsible for metastasis and opens up a new field in the cancer research area. However, CTC isolation is still challenging due to the low number of cells in the patients' bloodstream [6,7]. Nevertheless, the possible clinical applications of CTCs are broad and include therapy monitoring [8–10], studies on resistance mechanisms [11–13], risk stratification for relapse [14–16] and potential targets for personalized medicine [17,18]. In some cancer entities, including pancreatic cancer, breast cancer and colorectal cancer, CTC counts are linked to an unfavorable prognosis [14,19,20], highlighting the impact of CTCs in disease progression.

Colorectal cancer (CRC) accounted for over one million cases and half a million death in 2018, making the disease the third most commonly diagnosed cancer entity worldwide [21]. Despite the extensive effort for early detection of CRC in developed countries [21], one out of five patients suffers from metastatic CRC at first diagnosis [22]. In colon cancer metastases can be frequently detected in different organs including the liver, lung, bone and brain. Treatment for metastatic colon cancer remains palliative and only around 14% of these patients survive the disease for more than five years after diagnosis [23].

In the past, the correlation of clinicopathological parameters and presence of CTCs could be established [24]. Furthermore, the presence of CTCs was linked to poor survival in a meta-analysis and CTCs were described as an independent prognosticator in CRC [25]. However, further characterization of CTCs is still limited by the low number of CTCs [26]. The recent establishment of cell lines from CTCs of cancer patients has therefore opened a new avenue for functional analyses of CTCs [4,27,28]. The CTC-MCC-41 colon CTC line that we analyzed in this study was the first long-term stable CTC line established from a patient with colon cancer. The CTC-MCC-41 line was derived from the blood sample of a 57-year-old male patient after first diagnosis prior to treatment of the metastatic colon cancer. In addition to various cancer infiltrated abdominal and mediastinal lymph nodes, the patient suffered from a solitary synchronous liver metastasis at first presentation.

The cell line features an intermediate epithelial-mesenchymal phenotype with stem cell-like characteristics and the ability to induce endothelial cell tube formation in vitro [4]. Genomic analysis of CTC-MCC-41 cells revealed that *PIK3CA*, *AKT*, *PTEN*, *KRAS* and *NRAS* were wild type, but the cell line harbors a *BRAF*<sup>V600E</sup> mutation with a stable allele frequency of 50% [29]. The *BRAF*<sup>V600E</sup> mutation detected in CTC-MCC-41 cells matches the mutational status of the primary tumor and the lymph node metastasis [4,29]. Notably, CTC-MCC-41 has the capacity to form xenograft tumors in mice after subcutaneous injection [4] with matching mutational profile underlining the tumorigenic potential of CTCs as reported in the past [30]. Moreover, in-depth transcriptional analysis has revealed that this CTC line has a distinct transcriptomic signature, not observed in established CRC lines (e.g., HT29) of other sources [4,31], suggesting that CTCs have unique properties that enable them to survive in the bloodstream.

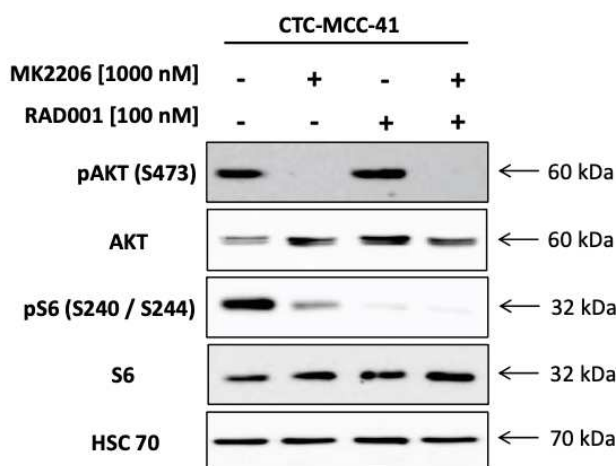
The PI3K/AKT/mTOR signaling pathway not only plays a crucial role in physiologically cell biology, but also in cancer as it regulates cell proliferation, survival, migration, angiogenesis and the utilization of nutrition [32]. Disruption of the tightly regulated PI3K/AKT/mTOR signaling induced by genetic alternations in cancer leads to growth advantages and common cancer-associated patterns [33] such as accelerated cell growth without negative feedback, increased survival and metastatic potential [34]. Therefore, the PI3K/AKT/mTOR signaling pathway became a popular target for new drugs in cancer [35–37]. For full activation of AKT, phosphorylation at serine residue 473 is required [38]. Upon full activation, AKT further regulates various downstream substrates inside the PI3K/AKT/mTOR pathway [38,39]. In CRC, a phosphorylation at this site could be detected in nearly half of the CRC samples and was correlated with a poor disease outcome [40].

In the present study, we assessed the functional role of the PI3K/AKT/mTOR signaling pathway in the permanent CTC line CTC-MCC-41 [4]. Further understanding of the tumor cells that are said to initiate metastasis and their signaling is of high importance in order to develop new treatment strategies. Here, we report that the PI3K/AKT/mTOR signaling pathway, which is frequently hyperactivated in cancer, also exhibits an important role in CTCs and may be a valuable target for anti-cancer therapies. We demonstrate strong synergistic effects on the growth of CTCs in vitro by dual targeting AKT and mTOR with MK2206 [41] and RAD001 [42] within the nanomolar range compared to single agent treatment, suggesting a novel therapeutic strategy. Moreover, we could identify differential functions of AKT isoforms on the growth of CTC-MCC-41 line after shRNA mediated knockdown, indicating a crucial role of AKT in this subset of more aggressive tumor cells.

## 2. Results

### 2.1. Strong Activation of the PI3K/AKT/mTOR Signaling Pathway in CTC-MCC-41

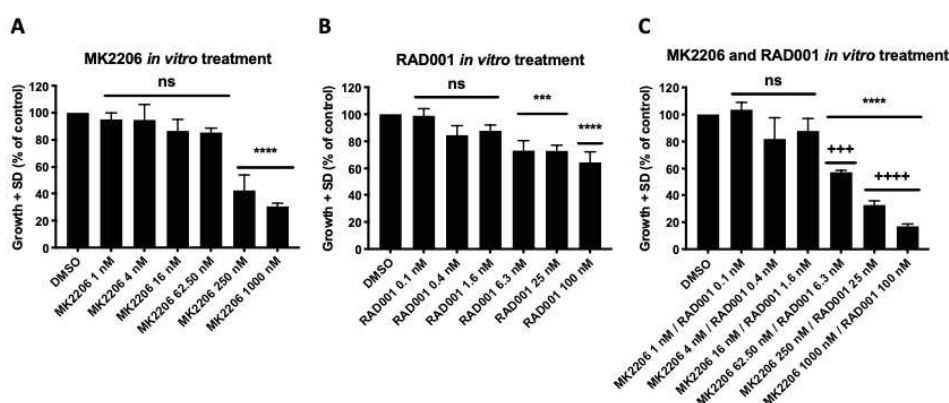
We examined the activation of the PI3K/AKT/mTOR signaling pathway by western blotting (Figure 1). The CTC-MCC-41 line shows a strong phosphorylation of AKT (S473) and of the mTOR downstream substrate S6 (S240/S244). In the next step, we treated the cells with the allosteric pan-AKT inhibitor MK2206, the mTOR inhibitor RAD001 or a combination of both drugs in vitro. The CTC line showed an impaired activation of AKT (S473) and mTOR downstream substrate S6 (S240/S244) after treatment, indicating a potential treatment target. Inhibition of AKT alone by MK2206 completely suppressed phosphorylation of AKT (S473) and diminishes further downstream signaling as measured by pS6 (S240/S244) in CTC-MCC-41 cells. A dual targeting approach of AKT/mTOR was able to further decrease pS6 (S240/S244) level in the CTCs compared to single treatment with MK2206. The treatment with RAD001, either as a single treatment or as a dual targeting approach by additionally targeting upstream AKT, resulted in a strong decrease of pS6 (S240/S244) levels. However, neither the single admission of RAD001 nor the combination therapy was able to completely suppress pS6 (S240/S244) signaling in CTC-MCC-41 line. When targeting mTOR by using RAD001, we did not detect an increase in phosphorylated AKT (S473) as propagated in the past due to feedback loops.



**Figure 1.** Activity of the PI3K/AKT/mTOR signaling pathway in CTC-MCC-41 and the inhibition of AKT/mTOR axis. The whole cell population of CTC-MCC-41 line (i.e., adherent and suspension cells) was treated for 24 h with AKT inhibitor MK2206 [1000 nM], mTOR inhibitor RAD001 [100 nM], the combination of both [MK2206 1000 nM/RAD001 100 nM] or solvent DMSO. Primary specific antibodies against pAKT (S473), pS6 (S240/S244), pan AKT and S6 were used to access the activity of the pathway and confirm the inhibition of the respective target. Housekeeping protein HSC70 was used as a loading control.

Next, the effect of MK2206 and RAD001 on proliferation of the colon CTC line was measured under varying concentrations of allosteric AKT inhibitor MK2206 and mTOR inhibitor RAD001 ranging from 1 nM to 1000 nM (MK2206) and from 0.1 nM to 100 nM (RAD001), respectively (Figure 2). A dose-dependent inhibition of CTC growth was observed in comparison to untreated cells with  $IC_{50}$  values within the nanomolar range. The results indicate a high sensitivity of the colorectal CTC line CTC-MCC-41 for inhibition of the PI3K/AKT/mTOR signaling pathway by MK2206 and RAD001.

Single targeting of either AKT or mTOR by MK2206 ( $IC_{50}$ : 186 nM) or RAD001 ( $IC_{50}$ : 2.6 nM) in CTC-MCC-41 (Figure 2A,B) showed a high sensitivity for the inhibitor. However, dual targeting of the AKT/mTOR axis was superior to single inhibition and could further inhibit the colon CTC line growth in the combinatory treatment. The analysis of combination indices, according to the Chou and Talalay method [43], revealed synergistic (+++) to strong synergistic (+++++) effects in CTC-MCC-41 cells in concentrations ranging from 62.50 nM/6.3 nM (MK2206/RAD001) to 1000 nM/100 nM (MK2206/RAD001) ( $p \leq 0.0001$ ) (Figure 2C).



**Figure 2.** Inhibition of the PI3K/AKT/mTOR signaling pathway inhibits tumor cell growth of CTC-MCC-41 in vitro. The whole cell population of CTC-MCC-41 line (i.e., adherent and suspension cells) was seeded in triplicates in a density of 3000 cells per well into a 96 well flat bottom cell culture plate. The next day MK2206 (A), RAD001 (B) or the combination of both inhibitors (C) was added to the cells and the plate transferred into the IncuCyte® Zoom Live Cell Analysis System. Cell confluence was measured every two hours. Analysis of data was performed based on cell confluence using the provided manufacturers IncuCyte® Zoom Software. The data points were extracted from one representative time point during the exponential growth phase ( $t = 110$  h).  $p$  values were calculated using one-way ANOVA with Dunnett’s multiple comparisons test (ns  $p > 0.05$ ; \*\*\*  $p \leq 0.001$ ; \*\*\*\*  $p \leq 0.0001$ ). Combination indices (CI) were calculated according to the Chou and Talalay method (++++ strong synergism CI 0.1–0.3; +++ synergism CI 0.3–0.7). The mean values ( $n = 3$ ) with standard deviation are shown.

### 2.2. Differential PI3K/AKT/mTOR Signaling in Suspension and Adherent Phenotype of CTC-MCC-41 Cells

To further investigate the activity of the PI3K/AKT/mTOR signaling pathway and other pathways that frequently interact with PI3K/AKT/mTOR signaling, such as the RAS/RAF/MEK/ERK signaling pathway, we conducted further western blot analysis on the CTC-MCC-41 cells (Figure 3). As the cells show a biphasic phenotype in cell culture (suspension vs. adherent), we separated the suspension and adherent fraction particularly. Comparing the whole population, the adherent and the suspension cell fraction, we detected differences restricted to the pAKT (S473) levels (Figure 3A). While the adherent cells show a strong activation of AKT (S473) and therefore matching the whole cell population, the suspension fraction shows significantly decreased pAKT (S473) levels compared to all cells ( $p = 0.0005$ ) and the adherent fraction ( $p = 0.0055$ ) (Figure 3B). No significant differences could be observed in pmTOR (S2448), pERK1/2 (T202/Y204) and pS6 (S240/S244) with respect to the fractions and the whole population. However, we found that CTC-MCC-41 in general showed a strong activity of mTOR, AKT,

ERK1/2 and S6. Comparing the whole cell lysate to another solid colorectal cancer cell line, namely HT29 cells, we detected significant higher levels of pAKT (S473) ( $p = 0.0017$ ) and pS6 (S240/S244) ( $p = 0.0082$ ), but not of pmTOR (S2448) ( $p = 0.8729$ ) in the CTCs. Interestingly, pERK1/2 (T202/Y204) expression was significantly higher ( $p = 0.0005$ ) in HT29 control and lower among the whole population, as well as the suspension and adherent fraction of CTC-MCC-41.

**A**

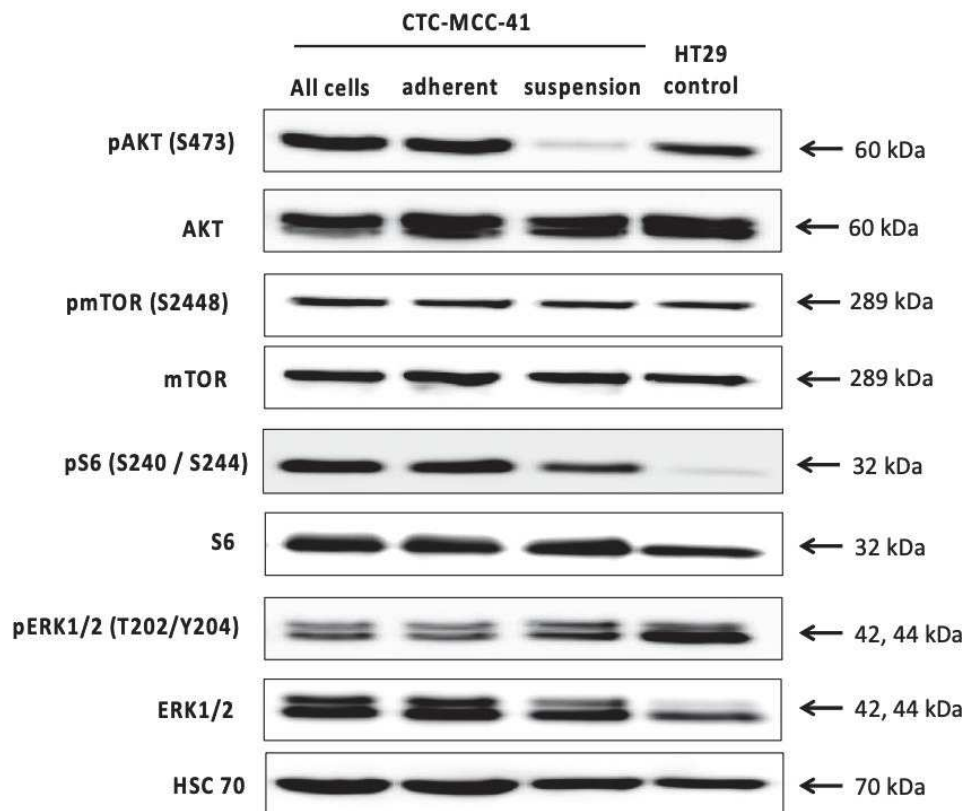
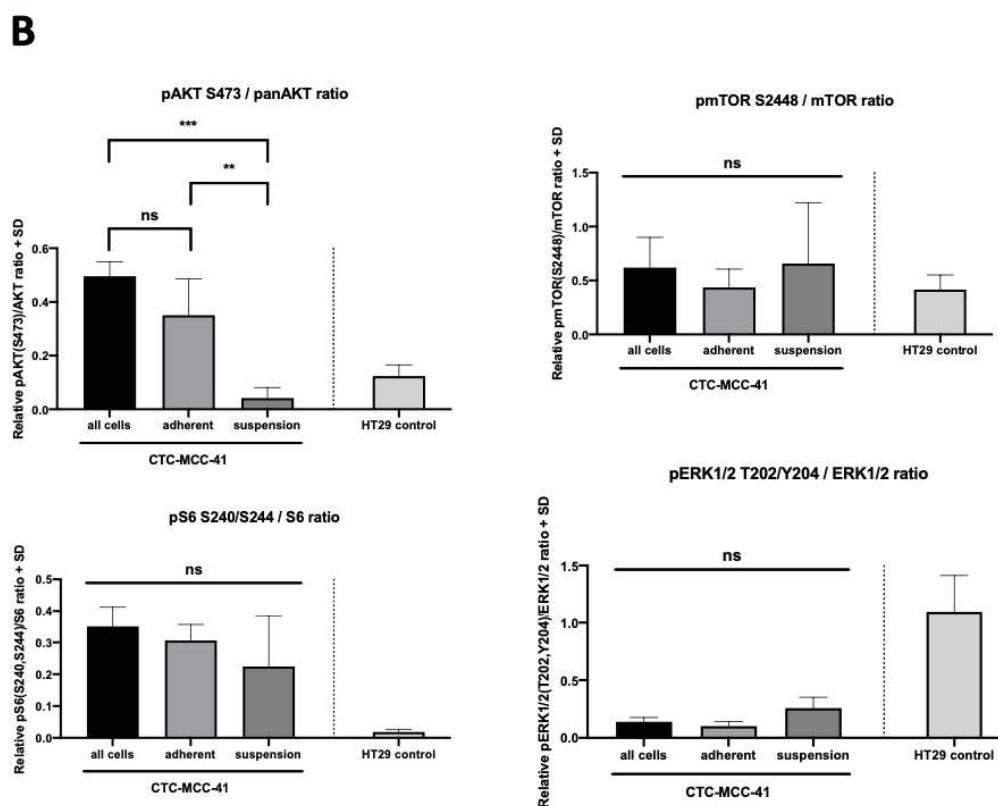


Figure 3. Cont.



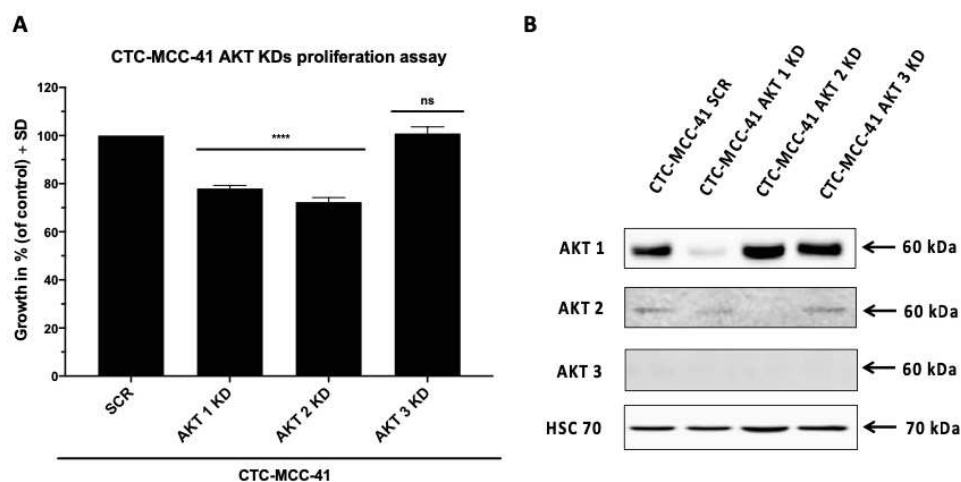
**Figure 3.** Differential activity of the PI3K/AKT/mTOR signaling pathway in suspension and adherent phenotype of CTC-MCC-41. (A) CTC-MCC-41 adherent and suspension cells were separated in the cell culture and subjected to western blot analysis. Whole cell lysates (also referred to as whole population) of CTC-MCC-41 and colorectal cancer cell line HT29 cells were used as control. Primary antibodies against mTOR, pmTOR (S2448), AKT, pAKT (S473), ERK1/2, pERK1/2 (T202/Y204), S6 and pS6 (S240/244) were used to analyze the activity of the RAS/RAF/MEK/ERK and the PI3K/AKT/mTOR signaling pathway. HSC70 was used as a loading control for equal protein loading. (B) Densitometric quantification of the western blot analysis as shown in (A). The mean values of relative protein activity ( $n = 3$ ) normalized to HSC70 loading control with SD are shown.  $p$  values were calculated using one-way ANOVA with Tukey’s multiple comparisons test (ns  $p > 0.05$ ; \*\*  $p \leq 0.01$ ; \*\*\*  $p \leq 0.001$ ).

### 2.3. AKT Isoform Specific Signaling in CTC-MCC-41 Cells

To further verify the hypothesis of a distinct role of AKT isoforms in the CTC-MCC-41 line we generated stable lentiviral isoform specific AKT knockdowns of AKT1, AKT2 and AKT3 in CTC-MCC-41 cells. In proliferation assays we could detect an impaired growth of CTC-MCC-41 cells harboring AKT1 ( $p < 0.0001$ ) and AKT2 knockdown ( $p < 0.0001$ ) compared to scrambled control (Figure 4A). The efficacy of the AKT knockdown was 89% and 91% for AKT1 and AKT2, respectively (Figure 4B). We were not able to detect AKT3 expression in CTC-MCC-41 cells by western blotting. Matching the findings from the western blot analysis (Figure 4B), no significant difference on the proliferation in cells, stably expressing the AKT3 knockdown vector ( $p = 0.9754$ ) compared to control was observed (Figure 4A). To analyze the possibility that growth inhibition of CTC-MCC-41 cells after AKT knockdown was due to increased apoptosis, we conducted FACS analysis and PI/Annexin V FITC staining (Supplementary Figure S1). The apoptosis rates did not significantly differ between the AKT isoform specific knockdowns. Additionally, we could detect an equal viability among the



cells. Therefore, we conclude that growth inhibition of CTC-MCC-41 cells was not due to increased apoptosis after AKT isoform specific knockdown.



**Figure 4.** AKT1 and AKT2 knockdown significantly decreases CTC growth in vitro. **(A)** AKT isoform specific shRNA mediated knockdowns (KD) of the whole cell population (i.e., adherent and suspension cells) of CTC-MCC-41 and scrambled (SCR) control were seeded in triplicates in a density of 3000 cells per well into a 96 well flat bottom cell culture plate and placed into the IncuCyte® Zoom Live Cell Analysis System. Cell confluence was measured every two hours. Analysis of data was performed based on cell confluence using the provided manufacturers IncuCyte® Zoom Software. The data points were extracted from one representative time point during the exponential growth phase ( $t = 90$  h).  $p$  values were calculated using one-way ANOVA with Dunnett’s multiple comparisons test (ns  $p > 0.05$ ; \*\*\*\*  $p \leq 0.0001$ ). The mean values ( $n = 3$ ) with standard deviation are shown. **(B)** Confirmation of stable AKT isoform specific shRNA mediated knockdown of AKT1, AKT2 and AKT3 in CTC-MCC-41 using western blot analysis. A scrambled (SCR) vector served as control. AKT3 signal could not be detected in CTC-MCC-41 cells.

### 3. Discussion

In this work, we provide first evidence on a crucial role of PI3K/AKT/mTOR signaling in CTC-MCC-41 cells. The sensitivity towards the allosteric AKT inhibitor MK2206 and the synergistic effects on the proliferation by additional targeting mTOR using RAD001 in the colon CTC line provides a rationale for dual targeting the AKT and mTOR axis as postulated earlier [44–48]. The complexity of signaling pathway networks including the PI3K/AKT/mTOR and the RAS/RAF/MEK/ERK pathway makes it difficult to choose the right treatment without triggering crosstalk [49]. Aberrant activation of the PI3K/AKT/mTOR pathway in CRC has been associated with carcinogenesis, early metastatic formation as well as maintenance of established CRC [50]. High levels of phosphorylated AKT (S473) have been reported in 46% of CRC samples and could be linked to increased proliferation, reduced apoptosis and poor clinicopathological outcome including lymph node metastasis and venous vessel invasion [40]. Additionally, a study by Johnson et al., revealed a significantly upregulation of Akt1, Akt2, pmTOR (S2448) and pS6 (T389) in colorectal cancer samples compared to normal tissue of the same patient [50].

Accounting for the high prevalence of *PIK3CA* mutations in one third of all CRCs [51,52], combined targeting of AKT and downstream substrate mTOR will be necessary to maintain the inhibition of the signaling pathway [53].

Interestingly, mTOR is not only a downstream substrate of AKT, but also obligatory for full activation of the AKT activity by catalyzing the phosphorylation at serine residue 473 [32]. Dual targeting

of both AKT and mTOR therefore provides a stronger inhibition of the PI3K/AKT/mTOR signaling pathway and fewer possible escape mechanisms than single drug therapy. The efficacy of RAD001 was previously examined in a phase II clinical trial (NCT00419159) as a single agent therapy for CRC patients who relapsed on conventional treatment regimens. Although the high frequency of PI3K/AKT/mTOR pathway mutations in CRC and the results from a phase I study promised a robust anti-tumor activity, RAD001 failed to prevent disease progression in a phase II clinical trial [54]. The authors emphasized that a combination therapy may be beneficial as mTOR inhibition alone may trigger negative feedback loops inside the pathway leading to increased upstream signaling [49,54]. Therefore, the efficacy of combinatorial inhibition of CTCs *in vitro* using MK2206 and RAD001 should be further examined and complemented by *in vivo* data in xenograft models.

Drug sensitivity screening using CTCs has been conducted in the past [27,28,55]. In 2014, Yu et al. were the first to establish drug sensitivity testing on six stable CTC lines *in vitro* as well as in a xenograft mouse model *in vivo* [27]. Pearl et al. were able to successfully predict the sensitivity and response to treatment using CTCs derived from patients suffering from ovarian cancer [55]. Moreover, the authors could successfully confirm the prediction for 13 out of 13 patients in a retrospective analysis [55]. Recently, Koch et al. established a permanent CTC line, namely CTC-ITB-01, derived from a metastatic breast cancer patient. CTC-ITB-01 line exhibited tumorigenic and metastatic capacity in a xenograft mouse model *in vivo* and remained estrogen receptor positive in culture. Interestingly, the CTC-ITB-01 conserved the same characteristics as the CTCs in the patient. In an *in vitro* drug sensitivity assay the authors detected a high sensitivity of CTC-ITB-01 for CDK4/6 inhibition using Palbociclib. The high sensitivity of Palbociclib, which is now routinely used in patients who relapsed on prior endocrine therapy, may implicate that the patient the CTC were derived from may could have benefitted from treatment with Palbociclib [28]. Hence, these studies and the model established by Koch et al. indicate the potential of preclinical drug screening with CTCs which should be extensively performed and validated to be implemented into the clinics.

In addition to the drug sensitivity assays concerning the PI3K/AKT/mTOR pathway, we successfully established stable lentiviral knockdowns of AKT1, AKT2 and AKT3 to investigate the functional role of these AKT isoforms in the CTC-MCC-41 line. In the past years, the distinct functions of AKT isoforms were part of extensive research [56–58]. In agreement with previously published findings on the effect of AKT1 on the proliferation of solid tumor cells [56,59], as well as on disseminated tumor cells [60], we observed a decrease in cell proliferation as a consequence of AKT1 knockdown. Although mutations in AKT are rare events in primary CRC samples [61], an oncogenic effect of the AKT1 missense mutation E17K leading to increased signaling through constitutive activation has been reported in CRC [62]. A study on breast cancer CTCs conducted by Aktas et al. was recently able to identify AKT2 expression as a marker of epithelial-mesenchymal-transition (EMT) in more than 60% of CTC positive samples analyzed [63]. The functional role of AKT2 in the physiologically state and in cancer cells has been reported as opposing compared to AKT1 [64,65]. While AKT1 activation directly stimulates cell growth by phosphorylation of p21 and therefore disinhibiting G1-S-transition, AKT2 activation promotes cell cycle exit through p21 binding, therefore competing with AKT1 phosphorylation [64]. However, in our experiments, we observed a significant reduction of CTC-MCC-41 proliferation *in vitro* after AKT2 specific knockdown, suggesting a crucial role of the kinase in these metastases-competent CTCs. In line with our data, other groups reported increased cell death and suppressed proliferation in CRC cell lines [66,67] and further entities of cancer after AKT2 specific knockdown [68,69]. As Grottko et al. reported, knockdown of AKT3 promoted migration and metastasis in breast cancer cells [70]. Therefore, a reduced or even undetectable expression of AKT3 may be interpreted as a result of downregulation in metastasizing CTCs.

The CTC-MCC-41 line has been described to express ALDH1 and CD133, two common markers of stem cells [4,29,71]. Additionally, expression of EMT inducer SNAIL1 indicates an intermediate epithelial/mesenchymal phenotype which is in line with the phenotype of the cell line in culture reversing from adherent to suspension cells and vice versa [4]. Moreover, separate cultivation of

one fraction, either the suspension or adherent CTCs alone, gives rise to the respective counterpart. One possible explanation in the context of CTCs for this morphology *in vitro* may be that it reflects the situation in the bloodstream of metastatic patients. The suspension phenotype may reflect those cells who have gone through EMT, while the adherent cells potentially mimic the cells after mesenchymal–epithelial transition. In our experiments we observed diminished phosphorylated AKT (S473) levels exclusively in the suspension fraction of CTC-MCC-41 compared to the adherent fraction and whole cell population. Our data are in line with results from Salt et al. [72] who induced EMT in non-small cell lung cancer cells and also could detect lower pAKT levels after EMT. This may support the hypothesis of a crucial role of EMT in CTCs that has been frequently discussed in the past [73]. Additionally, the lower levels of phosphorylated AKT (S473) in the suspension fraction may implicate a dormant state, as this fraction seems to mimic the properties of CTC in the patient's bloodstream. The idea of a dormant state with only basic metabolism rate without proliferation has already been proposed in the past [74]. However, as only one permanent CTC line was used in this study, the crucial role of the AKT/mTOR axis in colorectal cancer CTCs should be confirmed in the future when more long-term stable CRC CTC lines are available.

In conclusion, we functionally assessed the PI3K/AKT/mTOR pathway in a permanent CTC line, namely CTC-MCC-41, derived from a patient with colorectal cancer. In our AKT isoform specific knockdown we demonstrated that AKT1 and AKT2 play a crucial role with respect to proliferation in this colon CTC line. Additionally, we could identify the inhibition of AKT and mTOR in CTCs as a novel druggable target in colorectal cancer. Especially the dual inhibition of AKT and mTOR led to strong decrease of CTC-MCC-41 cell growth *in vitro*. Although further validation of drug sensitivity screening and comparison with the primary tumor site are necessary before implementation into the clinical routine, the inhibition of CTCs may be a valuable future target to block metastatic progression in CRC patients.

#### 4. Materials and Methods

##### 4.1. Standard Cell Culture

The colon CTC-MCC-41 line was established in the laboratory of rare human circulating cells at the University Medical Center of Montpellier, France [4] and transferred to University Medical Center Hamburg-Eppendorf, Germany. For CTC cultivation, Roswell Park Memorial Institute (RPMI)-1640 media (#11875-093, Thermo Fisher Scientific Inc., Waltham, MA, USA) supplied with 10% fetal calf serum (FCS) (#26140-079, Thermo Fisher Scientific Inc., Waltham, MA, USA) 1% penicillin/streptomycin mix (#15140-122, Thermo Fisher Scientific Inc., Waltham, MA, USA) 1% L-glutamine (#25030-081, Thermo Fisher Scientific Inc., Waltham, MA, USA), 1% insulin-transferrin-selenium-A supplement (100×) liquid (#51300-044, Thermo Fisher Scientific Inc., Waltham, MA, USA), 10 ng/mL human FGF2 (#130-093-839, Miltenyi Biotec, Bergisch Gladbach, Germany), 50 ng/mL human EGF (#130-097-749, Miltenyi Biotec, Bergisch Gladbach, Germany), 0.1 µg/mL hydrocortisone (#74142, Stemcell Technologies Inc., Vancouver, Canada) was used. CTCs were routinely cultivated in non-adherent cell culture flasks (Greiner Bio-One, Kremsmünster, Austria) and passaged by separating the cells growing adherent from the cells growing in suspension. The supernatant was transferred into a falcon tube and centrifuged at 650 g for 5 min at room temperature (RT). In the meanwhile, the cells adherent to the flask were washed with 5 mL PBS and then incubated with Accutase® solution (#A6964, Sigma Aldrich, St. Louis, MO, USA) for 5 min at 37 °C, 5% CO<sub>2</sub> in the incubator. Suspension fraction cells were washed with PBS and afterwards merged with adherent fraction, centrifuged again at 650 g for 5 min at RT and then resuspended in fresh cell culture medium. The CTC-MCC-41 medium was changed every week. HT29 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (#41965-039, Thermo Fisher Scientific Inc., Waltham, MA, USA) supplied with 10% FCS and 1% penicillin/streptomycin mix. Cell culture media were changed twice a week. HEK293T cells were cultivated in DMEM supplied with 10% FCS, 1% penicillin/streptomycin mix, 2 mM L-glutamine, 1 mM sodium-pyruvate (#S8636, Sigma

Aldrich, St. Louis, MO, USA) and 20 mM HEPES (#15630, Thermo Fisher Scientific Inc., Waltham, MA, USA). Cultivation medium was refreshed two to three times a week. For transduced cell lines selection antibiotics in an appropriate concentration were maintained in standard cell media to prevent growth of not transduced cells. All cells were grown at 37 °C, 5% CO<sub>2</sub>. All used cell lines were regularly tested for mycoplasma contamination.

#### 4.2. Stable AKT Isoform Specific Knockdown

HEK293T cells were transfected with specific shRNAs purchased from Sigma-Aldrich (St. Louis, MO, USA) targeting either AKT isoforms (pLKO.1-AKT1-puromycin, pLKO.1-AKT2-puromycin, pLKO.1-AKT3-puromycin (2 µg)) or scrambled control (pLKO.1-shRNA-puromycin (2 µg)). Packaging plasmids for third generation lentiviral transfection (pCMV-VSV-G (2 µg), pMDLg/PRE (10 µg), pRSV-REV (5 µg)) were used. The transfection rate was enhanced using the Lipofectamine 3000 transfection reagent according to the manufacturer's instructions (#L3000-008, Thermo Fisher Scientific Inc., Waltham, MA, USA). The target cell line was seeded in a density of  $2 \times 10^5$  cells into six well culture plates the day before. Every 24 h the viral particles were collected, filtered through a 0.22 µm filter and 2 mL of the supernatant was added to the target cell line CTC-MCC-41. This step was subsequently repeated three times. Transduction was enhanced using 16 µg hexadimethrine bromide (#H9268, Sigma Aldrich, St. Louis, MO, USA). On the fourth day of transduction the cells were selected by adding 1.5 µg/mL Puromycin to the cell culture media for at least two weeks before cells were used for experiments. Successful isoform specific knockdown of the AKT isoforms was confirmed in western blot analysis prior to further experiments.

#### 4.3. Proliferation Assay and Therapeutic Drug Sensitivity Testing

The CTC-MCC-41 line was treated as described above and seeded in technical triplicates in a density of 3000 cells per well into a 96 well flat bottom cell culture plate (Greiner Bio-One, Kremsmünster, Austria). The cells were allowed to settle down at 37 °C, 5% CO<sub>2</sub> overnight. The next day, the plate was placed into the IncuCyte® Zoom Live Cell Analysis System (Essen Bioscience, Ann Arbor, MI, USA) and cell confluence was measured every 2 hours. Analysis of data was performed based on cell confluence using the provided manufacturer's IncuCyte® Zoom Software. Statistical analysis of acquired data was carried out using GraphPad Prism 8 (GraphPad Software Inc., San Diego, CA, USA). For the therapeutic drug sensitivity testing the CTC-MCC-41 line was prepared as written above for the proliferation assay. The next day, freshly prepared serial dilutions of either AKT inhibitor MK2206 (#S1078, Selleck Chemicals, Houston, TX, USA), mTOR inhibitor RAD001 (#S1120, Selleck Chemicals, Houston, TX, USA), the combination of both drugs or vehicle dimethyl sulfoxide (DMSO) (#D5879, Sigma-Aldrich, St. Louis, MO, USA) were added to the cells. Measurement and calculation using the IncuCyte® Zoom system was carried out as described in the proliferation section above.

#### 4.4. Apoptosis Assay

$5 \times 10^5$  cells of either CTC-MCC-41 scrambled (SCR) control, CTC-MCC-41 AKT1 knockdown, CTC-MCC-41 AKT2 knockdown or CTC-MCC-41 stably expressing the AKT3 knockdown vector were plated in 6 well culture plates in biological quadruplicates two days before analysis. 2 µM of Staurosporin (#S5921, Sigma-Aldrich, St. Louis, MO, USA) was added as a positive control 48 h prior to measurement. Unstained CTC-MCC-41 scrambled was used as negative control. The cells were then transferred to the incubator at 37 °C, 5% CO<sub>2</sub> for 48 h. After 48 h, the suspension cells were removed, adherent cells carefully washed with PBS and detached with 0.05% trypsin-EDTA (#25300-054, Thermo Fisher Scientific Inc., Waltham, MA, USA). Afterwards the adherent and suspension cells were centrifuged at 1900 rpm for 5 min at room temperature. The cells were resuspended in 100 µL 1× binding buffer supplied within the FITC Annexin V Apoptosis Detection Kit (#556547, BD Biosciences, Franklin Lakes, NJ, USA) and transferred to Falcon® round-bottom polystyrene tubes (Corning Inc., New York, NY, USA). Cells were stained with propidium iodide, FITC Annexin V or the combination

of both dyes according to manufacturer's instructions. The FACS measurement was performed on BD LSRFortessa (BD Biosciences, Franklin Lakes, NJ, USA) and laser settings precisely improved for CTC-MCC-41 cells using the unstained control. Data was analyzed with FlowJo version 10.6.1 (BD Biosciences, Franklin Lakes, NJ, USA). Statistical analysis of the data was performed as described in the statistical analysis section.

#### 4.5. Western Blot Analysis and Densitometric Quantification

Cell lysates were prepared in NP-40 lysis buffer containing 50 mM HEPES (#H3375, Sigma-Aldrich, St. Louis, MO, USA) at pH 7.5, 150 mM NaCl (#0278, J.T. Baker, Thermo Fisher Scientific Inc., Waltham, MA, USA), 1% NP-40 (#98379, Sigma-Aldrich, St. Louis, MO, USA), 2% Aprotinin (#A162.2, Carl Roth GmbH + Co. KG, Karlsruhe, Germany), 2 mM EDTA (#E5134, Sigma-Aldrich, St. Louis, MO, USA), 50 mM NaF (#S7920, Sigma-Aldrich, St. Louis, MO, USA), 10 mM NaPPI (#S6422, Sigma-Aldrich, St. Louis, MO, USA), 10% Glycine (#3908, Carl Roth GmbH + Co. KG, Karlsruhe, Germany), 1 mM Sodium orthovanadate (#S6508, Sigma-Aldrich, St. Louis, MO, USA) and 1 mM PMSF (#10837091001, Sigma-Aldrich, St. Louis, MO, USA). Protein determination was conducted using a lowry protein assay (#5000112, Bio-Rad Laboratories Inc., Hercules, CA, USA). Proteins were separated in SDS page gel electrophoresis and transferred to a nitrocellulose membrane. Afterwards the membranes were incubated with specific primary antibodies against mTOR, pmTOR (S2448), pAKT (S473), AKT (pan), AKT1, AKT2, AKT3, pERK1/2 (T202/Y204), ERK1/2, pS6 (S240/S244), S6 (all Cell Signaling Technology Inc., Danvers, MA, USA) or HSC70 (Santa Cruz Biotechnology Inc., Dallas, TX, USA). Species specific horseradish peroxidase linked secondary antibodies against anti-rabbit IgG or anti-mouse IgG were purchased from Santa Cruz Biotechnology Inc (Dallas, TX, USA). Equal protein loading was ensured by 0.2% Ponceau S staining (#33427, Serva Electrophoresis GmbH, Heidelberg, Germany) and housekeeping protein HSC70. For more information on antibodies used in the experiments please see the supplementary material (Supplementary Table S1). Western blot images were acquired using the ImageQuant LAS 4000 system (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA). Densitometric quantification of the western blots ( $n = 3$ ) was done with the AIDA Image Analysis software (Elysia-raytest GmbH, Straubenhardt, Germany). Statistical analysis of the densitometric quantification was performed in GraphPad Prism 8 (GraphPad Software Inc., San Diego, CA, USA) as described in the statistical analysis section below.

#### 4.6. Calculation of Combination Indices

Combination indices (CI) for drug combinations were calculated according to the Chou and Talalay method using CompuSyn software (ComboSyn Inc., Paramus, NJ, USA). CI values were encoded as recommended by Chou and Talalay [43]. Very strong synergism CI < 0.1; strong synergism CI 0.1–0.3; synergism CI 0.3–0.7; moderate synergism 0.7–0.85; slight synergism CI 0.85–0.90; nearly additive CI 0.9–1.10; CI values > 1.1 were considered as antagonism.

#### 4.7. Statistical Analysis

All statistical analyses were performed with implemented functions in GraphPad Prism 8.4.1 (GraphPad Software Inc., San Diego, CA, USA). A  $p$  value < 0.05 was considered as statistically significant.  $p$  values were encoded into asterisks as following: ns  $p > 0.05$ ; \*  $p \leq 0.05$ ; \*\*  $p \leq 0.01$ ; \*\*\*  $p \leq 0.001$ ; \*\*\*\*  $p \leq 0.0001$ .

## 5. Conclusions

The PI3K/AKT/mTOR axis plays a crucial role in colorectal carcinoma (CRC). Here, we demonstrate a pivot role of this signaling pathway in the CTC-MCC-41 line, which is derived from a patient with colorectal cancer. CTC-MCC-41 is highly sensitive for dual inhibition of AKT/mTOR using MK2206 and RAD001 in vitro and exhibits strong synergistic effects within the nanomolar range in therapeutic drug screening assays. Strong activation of the PI3K/AKT/mTOR pathway indicated by phosphorylation of

AKT and downstream substrates could be observed. Furthermore, AKT isoform specific knockdown of AKT1 and AKT2 significantly diminished proliferation of the cell line in comparison to control, suggesting a crucial role of the PI3K/AKT/mTOR pathway in CTC-MCC-41. Therefore, these data may indicate that the inhibition of the PI3K/AKT/mTOR pathway in CTCs may be a valuable target to block metastatic progression in CRC patients that should be confirmed in further xenograft mouse models *in vivo*.

**Supplementary Materials:** The following are available online at <http://www.mdpi.com/2073-4409/9/9/2129/s1>, Supplementary Figure S1: Effect of AKT isoform specific knockdowns on apoptosis of CTC-MCC-41 cells, Supplementary Table S1: Primary and secondary antibodies used for Western blot analysis.

**Author Contributions:** Conceptualization, D.J.S., K.P., C.A.-P. and M.J.; methodology, D.J.S. and M.J.; formal analysis, D.J.S., M.J.; investigation, D.J.S.; resources and material support, L.C., C.A.-P. and M.J.; data curation, D.J.S.; writing—original draft preparation, D.J.S.; writing—review and editing, D.J.S. and M.J.; writing—critical revision of the manuscript: D.J.S., L.C., M.-T.H., N.H., K.P., C.A.-P. and M.J.; visualization, D.J.S.; supervision, D.J.S. and M.J.; project administration, D.J.S., K.P. and M.J. All authors have read and agreed to the published version of the manuscript.

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# Discussion et Perspectives





Dans le cadre de ce projet de thèse, l'un des principaux objectifs était de mieux appréhender les mécanismes du processus métastatique. Pour ce faire, l'obtention de lignées cellulaires à partir de CTCs représente un enjeu majeur car ces cellules sont les éléments clés de la dissémination tumorale. La rareté des CTCs rend leur analyse difficile et l'obtention de lignée CTC représente un graal dans ce domaine de recherche par la profusion de matériel à disposition pour diverses analyses. Mais outre la quantité de cellules tumorales que nous pouvons générer avec l'obtention d'une lignée CTC, c'est surtout le phénotype et le génotype de cette sous-population cellulaire à propriété de 'cellule souche' qui sont cruciaux à analyser et caractériser.

Au cours de l'étude clinique COLOSPOT, qui avait pour but d'évaluer la valeur prédictive précoce du dénombrement de CTCs chez des patients ayant un CCRm d'emblée et traité par FOLFIRI-Avastin en première ligne de traitement, nous avons pu tenter la mise en culture de CTCs à chaque prélèvement. Cohen *et coll.* ont montré il y a plus de 10 ans que le nombre de CTCs avant et pendant le traitement est un marqueur prédictif indépendant de la SSP et de la SG chez les patients atteints d'un CCRm (Cohen, Punt et al. 2008). Depuis, un certain nombre d'études a confirmé ces données préliminaires (Tol, Koopman et al. 2010, Gazzaniga, Raimondi et al. 2013, KUBOKI, MATSUSAKA et al. 2013, Sastre, Vidaurreta et al. 2013) et d'autres ont étendu l'intérêt de la détection des CTCs aux cancers colorectaux non métastatiques (Bork, Rahbari et al. 2015, van Dalum, Stam et al. 2015). Cependant, il n'y a pas, pour le moment, d'utilisation des CTCs en pratique clinique pour les CCR car pour le moment seule la validité clinique est démontrée. L'utilité clinique du dénombrement de CTCs n'a pas encore été testé dans le cadre du CCR et à notre connaissance il n'y a pas d'étude en préparation.

Au sein du LCCRH, nous sommes parvenues à cultiver neuf lignées permanentes de CTCs à partir d'échantillons sanguins d'un seul et unique patient CCRm ayant un nombre élevé de CTCs (>300 CTCs), avant l'initiation de sa première ligne de traitement et au cours de la progression de son cancer et des traitements successifs qu'il a reçu jusqu'à son décès. Ces lignées CTCs représentant longitudinalement la maladie jusqu'au décès du patient sont un matériel unique ; en effet, il n'existe pas, à notre connaissance, d'autres lignées de CTC coliques permanentes, ni plusieurs lignées obtenues au cours de la maladie d'un même patient dans les cancers épithéliaux. L'obtention de ces lignées CTC coliques est une formidable opportunité d'étude pour identifier les mécanismes sous-jacents à la formation de métastases et les mécanismes de résistances aux traitements dans les cancers colorectaux. Malgré l'apport indéniable de ce matériel, il nous est souvent fait remarquer qu'il ne représente qu'un seul patient et que les mécanismes identifiés ne représenteront peut-être pas l'ensemble des cancers colorectaux. Cependant, nous possédons encore dans notre biobanque les cellules issues d'un

prélèvement fait au cours d'une progression clinique d'une patiente pour laquelle nous avons déjà réussi à cultiver les CTCs sur un temps court (#2 mois). Nous avons prévu de décongeler ces cellules dans un futur proche afin d'essayer d'obtenir une nouvelle lignée de CTCs coliques. Si nous parvenons à amplifier les cellules de cette patiente, nous pourrions ainsi comparer ces différentes lignées CTC coliques pour dessiner un profil commun de biomarqueurs ou propre à chaque patient.

La caractérisation de ces lignées CTCs coliques nous a permis de mettre en évidence qu'EpCAM, le marqueur spécifique des cellules épithéliales par excellence, était fortement exprimé sur les CTCs coliques initiatrices de métastases malgré la présence simultanée de marqueurs de l'EMT. A cette époque, il faut noter que l'utilisation d'EpCAM pour l'enrichissement des CTCs était fortement controversée avec des preuves grandissantes de l'existence de l'EMT. Aujourd'hui, le paradigme semble avoir changé et la sous-expression d'EpCAM ne semble plus être un impératif à l'EMT (Eslami, Cortés-Hernández et al. 2020). En effet, l'expression d'EpCAM est davantage tissu-dépendante et sa sous-expression resterait modérée durant les toutes premières étapes de la dissémination tumorale (Keller, Werner et al. 2019). De plus, EpCAM est également présent sur les cellules souches (Ng, Ang et al. 2010) et sa suppression par knockdown a montré un arrêt de prolifération cellulaire.

La capacité de ces CTCs à être très plastique (EMP) et à avoir des propriétés de cellules souches est une des caractéristiques cruciales (hallmarks) pour la prolifération des cellules métastatiques au sens strict du terme. Nous avons pu voir, dès la première caractérisation des lignées CTCs, la présence de certains marqueurs indiquant des compétences de cellules souches (ex : CD133, CD44v6, ALDH) ainsi que des marqueurs de plasticité (ex : SNAIL, BMP7, Fibronectine). Ces données nous ont conduit à analyser plus en détail les marqueurs de l'EMP (EMT et MET). De nombreuses études ont permis la compréhension du phénomène d'EMT mais la MET reste un processus moins renseigné et mal connu à ce jour car plus difficile à étudier (Pei, Shu et al. 2019). En cela, les lignées CTCs représentent, selon nous, un bon modèle pour commencer à appréhender ce processus. En effet, nous avons démontré que cette sous-population de CTCs, qui se sont retrouvées dans le compartiment circulant possédaient un phénotype tourné davantage vers la MET plutôt que vers l'EMT, suggérant une capacité certaine à former des tumeurs à distance chez le patient. Afin d'étudier plus en détails ces résultats, nous avons entrepris une analyse des capacités de transition de nos lignées CTCs en induisant *in vitro* l'EMT et la MET. Avec de telles expériences, nous voulions appréhender la plasticité de ces cellules. Est-elle totale ou existe-t-il une sorte de verrou empêchant les cellules d'aller trop vers un phénotype mésenchymateux afin de pouvoir faire le chemin inverse ?

Par ailleurs, l'analyse approfondie du transcriptome a mis en évidence des caractéristiques clés des mécanismes de progression tumorale, comme les modifications du système de réparation de l'ADN, la

possession de nombreuses caractéristiques de cellules souches, une stimulation du métabolisme notamment celui lié aux mitochondries et une sur-activation des mécanismes de détoxification cellulaire pour l'élimination des médicaments.

Le métabolisme du cancer est un des plus anciens sujets d'étude dans la pathologie cancéreuse. Le domaine est basé sur le principe que les activités métaboliques sont modifiées dans les cellules cancéreuses par rapport aux cellules normales, et que ces altérations favorisent l'acquisition et le maintien de propriétés tumorales. La reprogrammation du métabolisme est un « hallmark » du cancer. Depuis les découvertes de Warburg dans les années 1950-1960 (Warburg 1956, Warburg 1956), de nombreuses études ont pu montrer que les cellules cancéreuses puisent leur énergie essentiellement à partir du glucose, à travers la glycolyse et l'oxydation du glucose, dans la mitochondrie, afin de répondre à leur besoin en énergie, mais également à leur besoin en biomasse nécessaire à leur division cellulaire et réplication accrue (Cantor and Sabatini 2012, Weinberg and Chandel 2015, DeBerardinis and Chandel 2016). Il nous paraît donc essentiel d'observer le comportement de nos lignées CTCs en termes de glycolyse et d'utilisation de l'énergie générée par la mitochondrie. Pour cela, nous sommes en train d'utiliser la plateforme Seahorse (IRMB – INSERM) qui nous permet d'étudier la consommation de glucose et d'oxygène de nos lignées CTCs, toujours en comparaison avec les lignées coliques HT-29 et SW620 (primaire et métastatique, respectivement). Nous allons également tester le métabolisme du fructose grâce à cette plateforme afin d'observer le comportement du sous-groupe CTC41.5 [ABFG] originaire de métastase hépatique, ceci en comparaison avec une lignée de cellules cancéreuses de foie, organe du métabolisme du fructose.

Un autre objectif de cette thèse est la découverte de biomarqueurs potentiels pour une utilisation en clinique. La validation de certains gènes significativement exprimés de manière différentielle dans les lignées CTCs obtenues au cours de la maladie nous a permis de mettre en évidence (i) un biomarqueur de résistance aux traitements à base de 5FU, **CDA** ainsi qu' (ii) un marqueur de métastases hépatiques, **ALDOB**.

**CDA** est une enzyme qui intervient dans la régulation des niveaux de pyrimidine dans les cellules. Le patient, à partir duquel les 9 lignées CTCs ont été obtenues, a été traité par 2 lignes de chimiothérapie, FOLFIRI et FOLFOX, toutes les deux à base de 5FU, un analogue de la pyrimidine. La surexpression significative de cette enzyme dans la lignée obtenue directement après l'échec du second traitement, la lignée CTC-MCC-41.4, révèle une acquisition de résistance face à ce traitement. Ce postulat pourra être validé grâce à la plasmathèque générée lors de l'étude COLOSPOT. En effet, cette enzyme étant sécrétée, nous avons pu la détecter dans le surnageant de culture des lignées CTCs. Ainsi, nous souhaitons doser le CDA plasmatique dans les plasmas des 167 patients de cette étude clinique et

corrélés ces nouvelles données avec celles des données cliniques (progression du cancer sous traitement).

**ALDOB** est également une enzyme qui est principalement exprimée dans les cellules du foie pour participer au métabolisme du fructose. Bu *et coll* ont récemment démontré que les cellules tumorales coliques métastasées au foie acquéraient l'expression de cette enzyme pour s'adapter à leur nouvel environnement (Bu, Chen et al. 2018). L'expression de cette enzyme a été identifiée seulement dans certains clones des dernières lignées obtenues juste avant le décès du patient. Cette information démontre pour la première fois la recirculation de cellules tumorales provenant de métastases hépatiques chez un patient. Ainsi, la détection d'ALDOB sur les CTCs pourrait révéler la présence de métastases hépatiques chez des patients considérés comme ayant un cancer du côlon localisé mais qui aurait déjà des micrométastases non visibles avec l'imagerie médicale. Cependant, l'intérêt clinique d'un tel biomarqueur reste à démontrer dans une étude clinique du fait de la rareté des CTCs dans le cancer colorectal. Évidemment, on ne peut pas en faire une cible thérapeutique car la forte expression de cette protéine dans les cellules hépatiques entraînerait des défaillances hépatiques sévères.

Finalement, grâce à une collaboration avec l'équipe du Pr Manfred Jüncker d'Hambourg, nous avons pu aborder un aspect complémentaire à ceux initialement prévus dans mon sujet de thèse : l'utilisation des lignées CTCs coliques comme modèles pour l'étude de nouveaux traitements. Nous avons ainsi pu montrer la sensibilité de la première lignée CTC-MCC-41 aux inhibiteurs de la voie de signalisation PI3K/AKT/mTOR, et notamment l'effet synergique de l'utilisation d'inhibiteurs d'AKT et de mTOR, sur l'activation des effecteurs de cette voie ainsi que sur la prolifération cellulaire. Comme abordé dans l'introduction, il a déjà été rapporté que les expansions de CTCs *in vivo* (par injection de CTCs chez des souris immunodéficientes) reflètent la réponse au traitement chez les patients. Cependant, l'expansion des CTCs *in vivo* prend beaucoup de temps et reste une limitation sérieuse pour une utilisation en routine. Si nous parvenions à cultiver ces CTCs au moment du prélèvement sanguin, et surtout sur des temps relativement courts, nous pourrions proposer un *oncogramme* afin de tester les médicaments en temps réel et de proposer un traitement personnalisé.

*En conclusion*, au cours de cette thèse, je suis parvenue à atteindre tous les objectifs que nous étions fixés au départ. L'obtention et la caractérisation de ces lignées CTCs coliques nous a permis de comprendre certains des mécanismes intervenant dans le processus métastatique, mettant en avant une signature spécifique et propre aux CTCs, les différenciant totalement des tumeurs primaires et des métastases. Nous avons pu observer que le système de réparation de l'ADN, la plasticité cellulaire et différentes voies métaboliques (mitochondrie, xénobiotique) faisaient partie des processus clés de la



cascade métastatique et l'analyse approfondie de ces mécanismes nous a permis de faire ressortir 2 biomarqueurs potentiels, un marqueur de résistance au traitement (CDA) et un marqueur de métastases hépatiques (ALDOB).

Les possibilités d'études de ces lignées CTCs coliques sont encore énormes et pourront faire l'objet de nombreux autres sujets d'étude. A titre d'exemple et dans la continuité du travail présenté sur l'étude des ARNm, nous avons entrepris l'analyse du miRNome (population de microARN) de nos lignées CTCs coliques afin de comprendre les mécanismes régulateurs des processus cités précédemment. De plus, une étude sur les exosomes générés par ces cellules et leurs capacités à préparer les niches pré-métastatiques dans différents organes est le sujet de thèse de Luis Enrique Cortes Hernandez. Une autre étudiante du laboratoire, Zahra Eslami-Samarin, travaille sur l'aptitude des CTCs à éduquer les plaquettes et à les utiliser pour survivre dans la circulation sanguine. Enfin, de nombreuses collaborations sont en cours avec différentes équipes expertes dans leur propres domaines : étudier les modifications épigénétiques de ces lignées CTCs, quelles sont les informations que nous pouvons avoir par l'analyse des ARN longs non codants (LncRNAs).



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