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Cetuximab effects on human colon cancer stem cells

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Abstract

Cancer Stem cells (CSCs), recently identified in the majority of solid tumors, are thought to drive tumor growth, giving rise to a cascade of differentiated cells composing the bulk of the tumor. Tumor relapse that most often follows treatment with anti-proliferative and cytotoxic drugs would be explained by selective resistance of CSC to these agents. Colon cancer stem cells (cCSCs), first isolated in the host laboratory from surgical specimens, can be grown *in vitro* as clusters called tumor spheres that maintain an undifferentiated state and are able, upon injection in immunodeficient mice, to generate a xenograft identical to the parental tumor, in terms of both antigen expression and histological tissue organization. Because of all these features cCSCs may represent predictive tools for patient's therapeutic response.

Cetuximab (Erbitux), currently in use for metastatic colorectal cancer, is a recombinant chimeric human:murine immunoglobulin IgG1 that binds to EGFR displacing its natural ligands. Cetuximab also induces receptor internalization and degradation. Mutations in signaling pathway mediators acting downstream of EGFR, including KRAS, BRAF, NRAS, or PIK3CA are believed to determine resistance to the drug. In particular, KRAS-mutated patients are currently excluded from treatment.

In order to verify whether Cetuximab treatment affects the stem cell compartment within tumors, in this study I analyzed its effect in a panel of cCSCs generated by individual patients, both *in vitro* and in xenografts. The data show that the effect of Cetuximab on individual cCSCs reflects the known clinical data on individual tumor mutations in the EGFR signaling pathway molecules. The study therefore confirms that panels of cCSCs generated by individual patients represent good predictive tools for the preclinical screening of pathway-oriented, cancer stem cell-directed therapeutics.

Most importantly, the analysis of stem cell content in Cetuximab-treated xenografts by cytofluorimetry, agarose assay, and serial re-transplantation into secondary hosts clearly demonstrate that Cetuximab, differently than the classical chemotherapeutics currently in use for colon cancer, is able to effectively hit cCSC populations included in the tumors.

1 Introduction

1.1 Colorectal cancer

Colorectal cancer (CRC) is the second most commonly diagnosed cancer in females and the third in males [1]. Survival is directly related to early detection, and outcome closely depends on degree of local tissue invasion, infiltration of neighboring organs and presence of metastases. Prognosis may be formulated according to different staging systems, that correlate with 5-year survival. Dukes' classification, already in use since 1932, focuses on tissue infiltration, lymph node involvement and presence of distant metastases [2]. TNM (Tumor-Node-Metastasis) classification, more recent, conforms to an international classification common to all types of cancer [3] .

Survival rates for early stages is about 5 times that of late stage cancers. For example, patients with a tumor that has not breached the muscularis mucosa (TNM stage Tis, N0, M0) have an average 5-year survival of 100%, while those with an invasive cancer, i.e. T1 (within the sub-mucosal layer) or T2 (within the muscular layer) cancer have an average 5-year survival of approximately 90%. Those with a more invasive tumor, yet without node involvement (T3-4, N0, M0) have an average 5-year survival of approximately 70%. Patients with positive regional lymph nodes (any T, N1-3, M0) have an average 5-year survival of approximately 40%, while those with distant metastases (any T, any N, M1) have an average 5-year survival of approximately 5%.

The main cause of death for CRC is development of metastasis. According to the American Cancer Society statistics in 2006 [4], over 20% of patients have stage IV metastatic colorectal cancer at the time of diagnosis. Up to 25% of this group will have isolated, potentially resectable liver metastases. Lesions which undergo curative resection have demonstrated 5-year survival outcomes now exceeding 50%.

In addition to surgery, metastatic CRC patients are treated with radio- and/or chemotherapeutic agents, that include 5-Fluorouracil (5-FU) alone or in combination with Oxaliplatin or Irinotecan [5]. These three compounds have different mechanisms of action. 5-FU is a nucleoside analog that blocks the enzyme thymidylate synthase impairing the synthesis of

thymine nucleotides needed for DNA replication [6]. Oxaliplatin is a platinum-based compound that prevents DNA replication and transcription trough the formation of cross-linking DNA adducts [7]. Irinotecan inhibits topoisomerase I, the nuclear enzyme which enables the uncoiling of the DNA during replication [8].

The combinatorial treatment, usually administered as FOLFOX (5-FU/ Leucovorin / Oxaliplatin) or FOLFRI (5-FU / Leucovorin / Irinotecan), has improved the response-rates for advanced CRC from 10-20% to 40-50% [9, 10].

Regardless of the nature and mechanism of action, however, resistance to chemotherapy eventually arises in almost all patients, so that improvement of overall five-year survival for colorectal cancer, now reaching 60% in Europe, is to be ascribed to the development and diffusion of early diagnostic methods, rather than to pharmacological efficacy.

For this reason, and given the increasing availability and reliability of high throughput technologies for genomic, transcriptomic and proteomic screening, a big effort is being put in recent years to the aim of identifying new agents, specifically directed to cancer-specific pathways, promising to lead to full and lasting tumor eradication.

1.2 Colon carcinogenesis

According to the model proposed by Fearon and Vogelstein in 1990 [11], colon cancer development is driven by accumulation of mutations in oncogenes and tumor suppressor genes, whose sequence often reflects tumor clinical progression (Fig.1). Indeed, genetic mutations observed in sporadic colorectal cancers are the same that characterize the hereditary forms, all mostly beginning with the deregulation of Wnt canonical pathway.

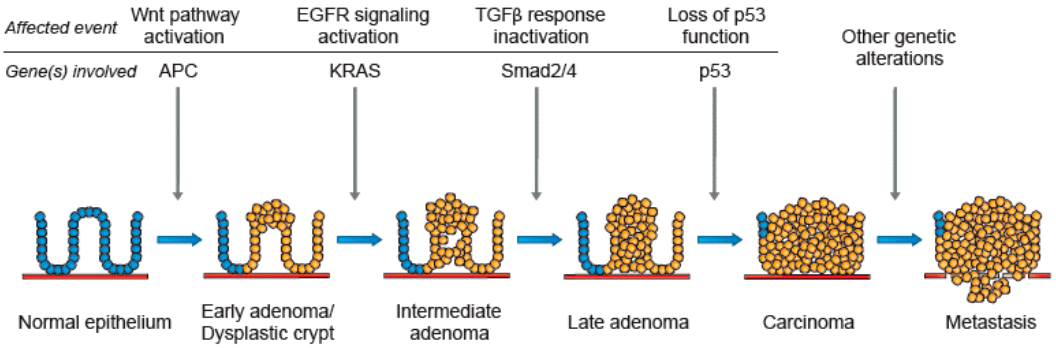

Fig.1 Fearon and Vogelstein model for colorectal carcinogenesis. Patients with FAP inherit a mutation in APC and develop numerous dysplastic foci in colon crypts. Progression towards a more malignant phenotype is then driven by the acquisition of other mutations (e.g. KRAS, SMAD4/2 and p53) and increased genomic instability.

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Activation of the APC/B-catenin pathway is the most common early event associated to adenoma formation, being altered in 80% of sporadic CRC cases. In line with this, germline mutations in APC (adenomatous polyposis coli) gene characterize individual carrying FAP (familial adenomatous polyposis), an inheritable genetic disease leading to development of noninvasive colonic adenomas (polyps).

APC is a negative regulator of Wnt signaling, in fact it regulates the intracellular levels of β-catenin [12]. In the absence of Wnt signaling, cytoplasmatic ß-catenin is captured by a destruction complex composed by axin, adenomatous polyposis coli (APC), and GSK3β. Captured ß-catenin is phosphorylated, ubiquitinated and degraded, preventing it from being translocated to the nucleus and interaction with members of LEF/TCF family . Binding of Wnt to the cysteine-rich domain of Frizzled receptors results in the disassembly of the destruction complex and the stabilization of β-catenin. APC mutations impair the ability of the protein to interact with the destruction complex, leading to ß-catenin stabilization and accumulation in the nucleus where it induces the transcription of Wnt target genes, among which c-MYC and Cyclin D1 (CCND1).

Activation of the Wnt pathway does not, by itself, cause cancer, but it is important for the initiation of the carcinogenic process, by inducing the development of noninvasive colonic adenomas (polyps). Presumably, this is caused directly by the over-expression of growth-

promoting genes driven by β-catenin-LEF/TCFs through canonical Wnt signaling. In fact, mouse models of FAP, such as the $Apc^{min/+}$ mouse, are characterized by innumerable intestinal (in this case, primarily small bowel) tumors, typically non-invasive and mostly analogous to human colonic adenomas [13].

Acquisition of new mutations leading to additional control gene loss is necessary for carcinogenesis progression. A second frequent genetic alteration occurring early in the adenoma-carcinoma sequence is the mutation of KRAS, that causes hyper-activation of the EGFR pathway [14]. Activating KRAS mutations characterize the 35-42% of CRCs, and are frequently observed already in adenomas, confirming that they represents an early event in tumor development.

Other mutations identified in CRC affect tumor suppressor gene such as SMAD2 and SMAD4 (Small Mother Against Decapentaplegic 2-4), whose protein products act as mediators of intracellular TGF-ß (transforming growth factor) signal. TGF-β transduction pathway regulates various processes including cell growth, differentiation, extracellular matrix production and apoptosis. The deletion of SMAD4 is known to abolish anti-proliferative TGF-ß signals [14].

Finally, the short arm region of chromosome 17 is frequently subject to deletion in CRC [14]. This event that involves TP53 gene lost. p53 protein plays a major function as a transcription factor regulating physiological processes such as stress response, DNA damage repair, cell cycle regulation and apoptosis [15].

1.3 Colonic crypt organization

The colon wall is composed of several layers: the mucosa, sub-mucosa, muscularis propria and serosa. Colon epithelium is formed by a columnar cell monolayer organized into functional units called crypts of Lieberkühn. Crypt-based, resident multipotent stem cells generate actively proliferating progenitors which, in turns, give rise to three main terminally differentiated colonic cell types: enterocytes, goblet cells and enteroendocrine cells [16, 17](Fig.2). Indeed, adult stem cells are defined by the two fundamental capabilities of i) self-renewal and ii) generate all the cyto-types of the specific tissue. These stem cells are responsible for the perpetual turn-over of the colonic epithelial cells during the whole lifetime of an individual.

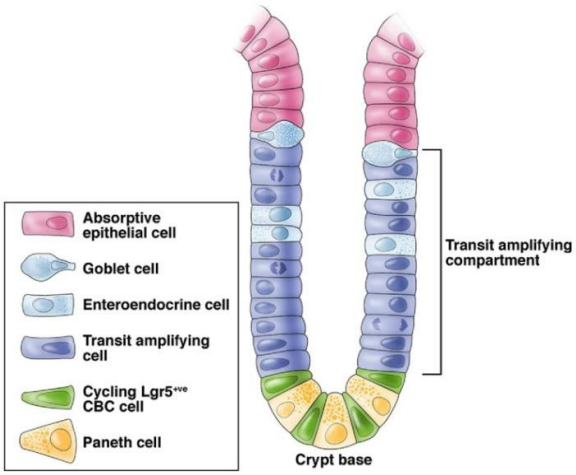


Fig.2 Schematic representation of the crypt of Lieberkühn.

From: [17]

The complexity of the crypt structure has long been an obstacle in understanding the key mechanisms that lead to the formation of the crypt from a single stem cell. Over the last 30 years, many studies have been undertaken, to indirectly localize intestinal stem cells (ISCs) within the colonic crypts through DNA label retention experiments (e.g. bromodeoxyuridine or tritiated thymidine)[18, 19]. More recently, the expression of specific molecular markers has been used as a tool to identify and locate cells provided with stem features within intestinal crypts.

The two main models about the identity and positioning of the intestinal stem cells (ISCs) currently debated are i) the "+4 position" model, and ii) the "stem cell zone" model (Fig.3).

According to the first, ISCs would be located at the +4 position above the Paneth cells, at the base of the crypt. These cells are marked by the expression of the polycomb group protein Bmi1 [20]. These cells are actively cycling and, through asymmetric division, give rise to a differentiated progeny of all four epithelial lineages.

The more recent "stem cell zone" model proposes that ISCs correspond to small undifferentiated cycling cells, termed crypt base columnar (CBC) cells, interspersed between Paneth cells in the small intestine, or located at the very bottom of the crypt in the colon. Barker and colleagues identified Lgr5 (leucine-rich-repeat containing G-protein-coupled

receptor 5) as a marker for ISCs [21]. Lgr5-positive CBC cells are actively cycling and have the capacity to generate *in vitro* all epithelial lineages [22]. According to Zhu L et al., LGR5-positive stem cells of the small intestine also co-express CD133 and are sensitive to neoplastic transformation [23].

These two models may not be impossible to conciliate, in fact recent studies have shown that complete ablation of the LGR5-expressing cells do not perturb homeostasis of the epithelium. Indeed, following LGR5-expressing cell ablation, Bmi1-expressing cells increasingly generate progeny cells, compensating LGR5-expressing cell loss [24].

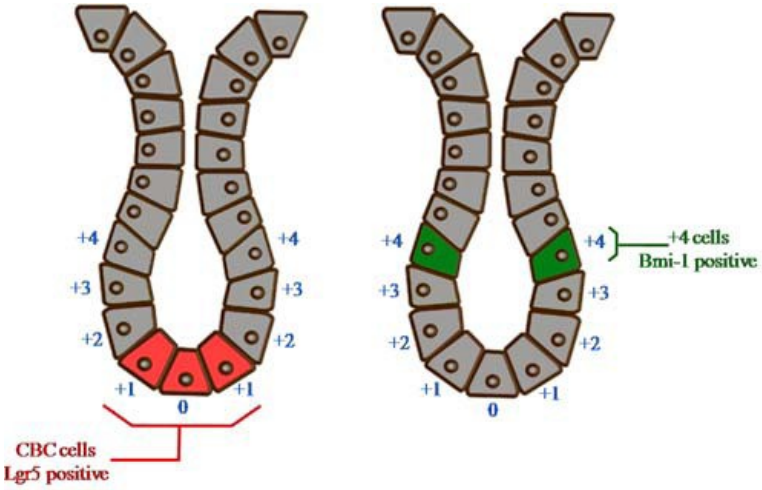


Fig.3 Models for stem cells location in intestinal crypts. On the left the "+4 position" model suggests that intestinal stem cells are located just above the Paneth cells at position +4 relative to the crypt bottom (green). The most important marker that identifies these cells is Bmi-1. On the right the "stem cell zone" model assumes that small, undifferentiated, cycling cells, so-called crypt base columnar (CBC) cells (red), interspersed with Paneth cells, are the true intestinal stem cells. The most important marker to identify these cells is Lgr5.
From: [25]

1.4 Cancer stem cells

It has long been known that many types of tumors are composed of a heterogeneous population of cells that differ in morphology, proliferative capability and tumorigenic potential [26]. According to traditional models of carcinogenesis, this heterogeneity was explained by stochastic genetic events that, under the influence of the microenvironment, would generate a series of events of clonal selection. The Cancer Stem Cells (CSCs) hypothesis, formulated in

recent years, instead proposes that the development of the tumor is driven by a small population of cells, that similarly to normal stem cells are able to both self-renewal and to generate a differentiated progeny [27]. CSCs would share a number of characteristics with normal stem cells, including i) the ability of asymmetric division, ii) the low rate of replication, iii) the expression of genes related to stem cell, iv) the resistance to chemotherapeutic drugs and radiation.

CSCs existence was first shown in 1997 by John Dick et al., who first isolated human leukemic CSCs by the same method used for normal hematopoietic stem cells i.e. cell sorting for $CD34^+/CD38^-$ cell population. This fraction, isolated from bone marrow of AML patients, proofed to be able to regenerate the leukemic compartment into immunodeficient mice (NOD/SCID). Conversely, the $CD34^+/CD38^+$, more differentiated population, lacked this ability [28-30]

The study of CSCs in solid cancers was delayed by technical difficulties posed by tissue dissociation, separation of cellular sub-populations and a poor knowledge of membrane markers. In the following years, however, breast CSCs were identified as the $CD44^+CD24^+$ subpopulation able to induce tumors reproducing patients histology upon transplantation in the mammary fat pad of immunodeficient mice [31], and glioblastoma CSCs, able to initiate tumors upon intracranial injection were separated on the bases of the expression of CD133 [32].

Finally, many reports have now described CSCs in several malignancies including head and neck [33], pancreas [34, 35], melanoma [36], mesenchymal [37], liver [38], lung [39], prostate [40], and ovarian [41] tumors.

1.4.1 Colon Cancer stem cells (cCSCs)

cCSCs were first described by ours and another research group in human surgical samples of CRC, as $CD133^+$ cells able to initiate xenografts that faithfully reproduced parental tumor in immunodeficient mice [42, 43]. Serial transplantation in mice demonstrated self-renewal activity after several *in vivo* passages. CD133, also known as Prominin-1, is a glycoprotein expressed in neural, hematopoietic, epithelial and endothelial cells. $CD133^+$ primary cells do not

express cytokeratin 20 (CK20), a component of intermediate filaments expressed in differentiated intestinal epithelial cells. $CD133^+$ cCSCs can be expanded in culture, in a selective serum free medium supplemented with EGF and bFGF and can be induced to differentiate *in vitro* by removing growth factors and adding bovine serum to the culture medium. Differentiation involves morphological changes, increased expression of CK20 with concomitant decrease of CD133, and loss of tumor-initiating capability.

In the intestinal epithelium, in consideration of its rapid turnover and of the short lifespan of committed-differentiated cells, the idea that the accumulating oncogenic events leading to carcinoma development should necessarily occur at the level of stem cell stem cells appears particularly convincing. In normal stem cells, being of long life, oncogenic mutations can accumulate over years or decades. Once transformed, CSCs would divide both symmetrically and asymmetrically, giving rise both to other CSCs and to committed/differentiated bulk tumor cells. In the end, the whole niche would be colonized by mutant cells, and the crypt will be filled by their offspring, an event called "clonal conversion". The proliferating tumor cells may be subject to further changes which affect the progression of cancer .

several observations support this assumption, in fact $CD133^+$/LGR5+ intestinal stem cells have been found to be sensitive to neoplastic transformation [23]. Recently, by lineage-tracing has been also demonstrated that Lgr5 also marks a subpopulation of adenoma cells that fuel the growth of established intestinal adenomas. This subpopulations (about 5-10%) generates additional Lgr5+ cells as well as all other adenoma cell types [44].

1.4.2 CSCs Assays (markers, spheroids, clonogenesis, xenografts)

Not surprisingly, research on CSCs relies on a series of assays inherited and modified from normal cell stem methods.

These include i) membrane marker selection ii) spheroid formation iii) clonogenicity in soft agar and iv) tumor initiating capability into immunodeficient mice.

The first membrane marker used for cCSCs selection, as discussed in the previous paragraph, was CD133. Different marker combinations have been proposed from other groups in the following years. Among these, Dalerba et al. showed that $EpCAM^{high}CD44^+$ tumor cells are able

to engraft in immunodeficient mice, whereas $EpCAM^{low}CD44^{-}$ populations do not induce tumors. Co-selection for CD166 together with CD44 further enriches the fraction for TIC content [45].

Aldehyde dehydrogenase 1 (ALDH1) has also been suggested to mark CRC stem cells. Huang et al. identified ALDH1 expression in the bottom of the crypts where the ISCs are located, and transplantation assay revealed that only ALDH1 positive cells were able to develop into a tumor, whereas ALDH1 negative cells failed [46].

The most recent molecule reported as a robust marker for TIC and metastasis-initiating cells is CD44V6 [47].

The specificity of these different selection systems, and overlap among the different CSCs populations described, still remains to be investigated.

Spheroid formation and colony formation assay are two *in vitro* assays commonly accepted as indicators of self-renewal capability of CSCs.

As described in the previous paragraph, culture of freshly dissociated primary tumors in the appropriate serum-free medium in the presence of EGF and bFGF allows the selection of a self-renewing population of cCSCs, able to maintain for long periods the capability to initiate xenografts into immunodeficient mice. In these cultures, cells grow in suspension as cellular clusters called 'tumor spheres'[42]. Ability of single cells to give raise to spheroids is taken as an indicator of CSCs presence in a cell population.

The clonogenic assay was originally introduced in 1956 by Puck et.al [48] to assess the effects of radiation on clone-forming ability of single mammalian cells plated *in vitro* on feeder layer. Later, this assay, that determines the ability of a cell to clone and generate colonies of large size, has been widely applied for the identification of normal and cancer stem populations, using different types of substrates, in particular agarose.

Finally, similarly to normal stem cell systems such as hematopoietic and skin, the golden standard for CSCs is represented by the capability to initiate tumors *in vivo*, that i) give rise to all the differentiated cellular populations of the primary tumor, and ii) can be serially re-transplanted without loss of tumorigenic potential, demonstrating self-renewal [49].

1.4.3 Cancer stem cell therapy

One main point in the CSCs hypothesis is its strong implications for tumor therapy. In fact, CSCs have been shown to exhibit relative resistance to anti-proliferative and cytotoxic drugs [50-52]. This can be explained by different mechanisms, among which high expression of multidrug resistance and multidrug efflux genes, enhanced checkpoint activation and DNA damage repair activity, increased expression of anti-apoptotic proteins, and increased Wnt/β-catenin and Notch signaling [53]. In colon cancer, in particular, $CD133^+$ cCSCs have been reported to resist to Oxaliplatin-induced apoptosis by IL-4 production both *in vitro* and in a xenograft model [50]. Furthermore, Ciclophosphamide (CPA) or Irinotecan treatment has been shown to increase the frequency of Tumor Initiating Cells (TIC) within cCSCs xenografts [51]. Interestingly, Dylla and collaborators also showed that ALDH1 enzymatic activity, which is generally highest in cCSCs, appears to play a direct role in mediated resistance to CPA, in fact its inhibition *in vitro*, or its reduced expression *in vivo*, sensitize cCSCs to the bioactive metabolite of CPA [51].

The resistance of cCSCs to standard chemotherapy may underlie to the relapse that in almost all cases occurs in treated patients, and cytotoxic drugs may not be the therapy of election if it turns out they are not hitting the cancer-sustaining population.

Conversely, new therapeutics able to kill cCSCs may constitute the treatment allowing to permanently eradicate the tumor. For this reason, CSCs represent a new concept in cancer biology that promises to offer new approaches in pathway-targeted therapies in the next future. The identification of agents able to target CSCs is currently a major challenge for research.

1.5 EGFR (Epidermal growth factor receptor) biology

Several different pathways are deregulated in colon carcinogenesis, including Wnt, transforming growth factor β (TGF-β) and EGFR [54-56]. While a number of trials for inhibitors of these pathways are currently ongoing, anti EGFR inhibitors are the only agents already approved for clinical use in CRC patients.

EGFR is a member of the EGFR tyrosine kinase family, which consists of EGFR (ErbB1/HER1), HER2/neu (ErbB2), HER3 (ErbB3) and HER4 (ErbB4). All family members contain an extracellular ligand-binding domain, a single membrane-spanning region, a juxtamembrane nuclear

localization signal, and a cytoplasmatic tyrosine kinase domain. These receptors are expressed in several cell types, but primarily in those of epithelial and neuronal origin and their activation is tightly regulated by the availability of ligands, belonging to the EGF family. This family includes EGF, transforming growth factor alpha (TGF-α), amphiregulin, betacellulin, heparin-binding EGF, epiregulin and neuregulins (NRG1-4). Once these ligands bind to the extracellular domain, EGFR forms homo- or heterodimers with its family members ErbB2/Neu, ErbB3/HER3 and ErbB4/HER4, and induces autophosphorylation of the intracellular domain through intrinsic tyrosine kinase activity and subsequent activation of downstream signaling [57].

EGFR activation triggers the activation of a multitude of intracellular signaling pathways, including the RAS/RAF mitogen activated protein kinase (MAPK), the PI3K/AKT and the JAK2/STAT3 pathways, responsible for cancer cell proliferation, survival, invasion, metastasis formation and neo-angiogenesis [58, 59] (Fig.4).

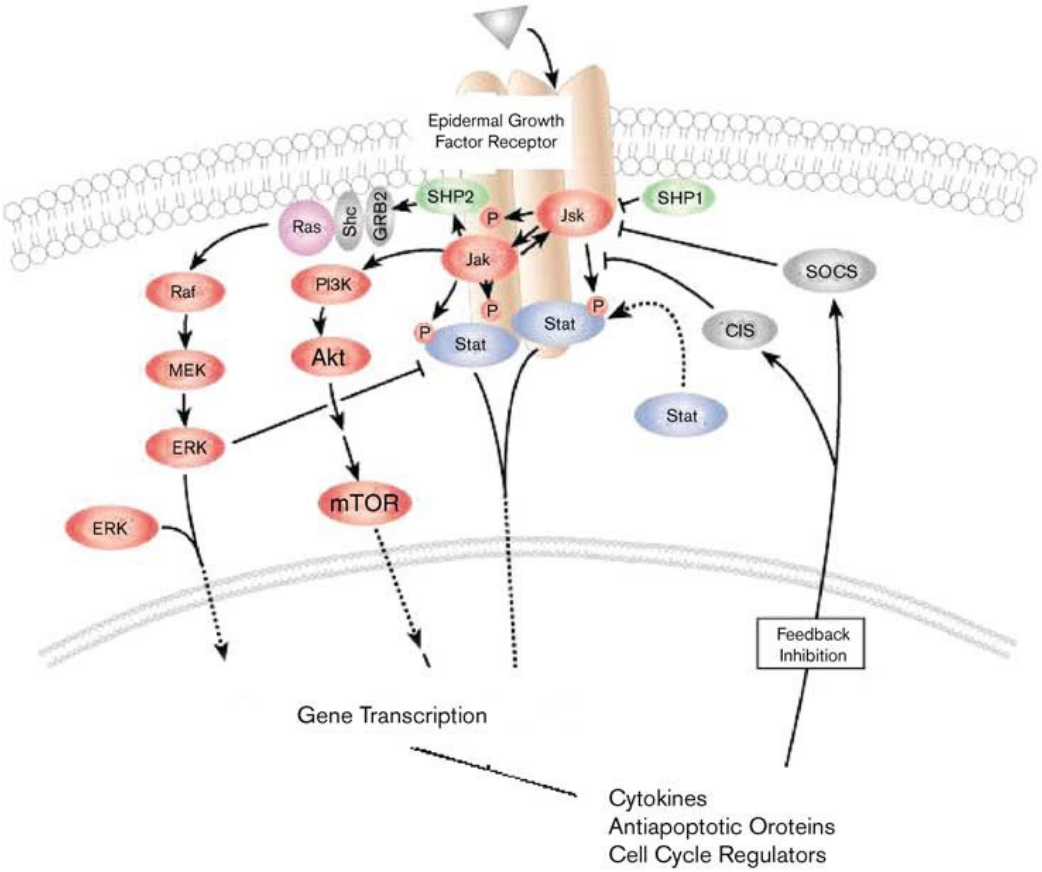


Fig.4 The EGFR signaling pathways. After ligand activation, the EGFR phosphorylates and activates the RAS-Raf-MAP kinase, PI3K/AKT, and STAT/JAK pathways. This in turn results in activation of transcription factors and modulation of the cell cycle, growth, apoptosis, and angiogenesis processes. **From**: **[60]**

EGFR has been linked to the growth of many human epithelial malignancies including lung, neuronal, colon and pancreatic cancer.

Targeting of EGFR has been intensely pursued over the last three decades as a strategy for cancer treatment. From these efforts, two fundamental approaches have proven useful (Fig.5). One approach involves the use of small molecule tyrosine kinase inhibitors (TKIs) that bind to the ATP-binding site in the tyrosine kinase domain (TKD) of EGFR. To date, three anti-EGFR TKIs, erlotinib (OSI-774, Tarceva), gefitinib (ZD1839, Iressa) and lapatinib (GW572016, Tykerb) are FDA-approved for use in oncology.

A second approach uses monoclonal antibodies (mAbs) against the extracellular domain of EGFR to block natural ligand binding: Cetuximab (C225, Erbitux) and Panitumumab (Vectibix).

Cetuximab is an immunoglobulin G1 chimeric mouse-human monoclonal antibody that specifically targets the extracellular domain of EGFR blocking endogenous ligand binding, enhancing receptor internalization and finally reducing cellular proliferation in a variety of cancer models [61].

Cetuximab induces arrest in the G1 phase of the cell cycle by increasing levels of p27kip1 [62, 63]. *In vivo* experiments confirmed this finding and identified a reduction in PCNA in tumor xenografts [64]. Cetuximab also decreases cancer cell metastasis via down-regulation of pro-angiogenic factors and matrix metalloproteinase responsible for cell adhesion [65, 66], induces the up-regulation of various pro-apoptotic factors such as Bax, in addition to the down-regulation of Bcl2, leading to the activation of caspases [67]. Finally it induces antibody-dependent cellular cytotoxicity *in vivo* by recruiting immune cells to tumor cells [68, 69].

Collectively, Cetuximab results in several biological effects that have impact on the growth and spread of multiple human tumors.

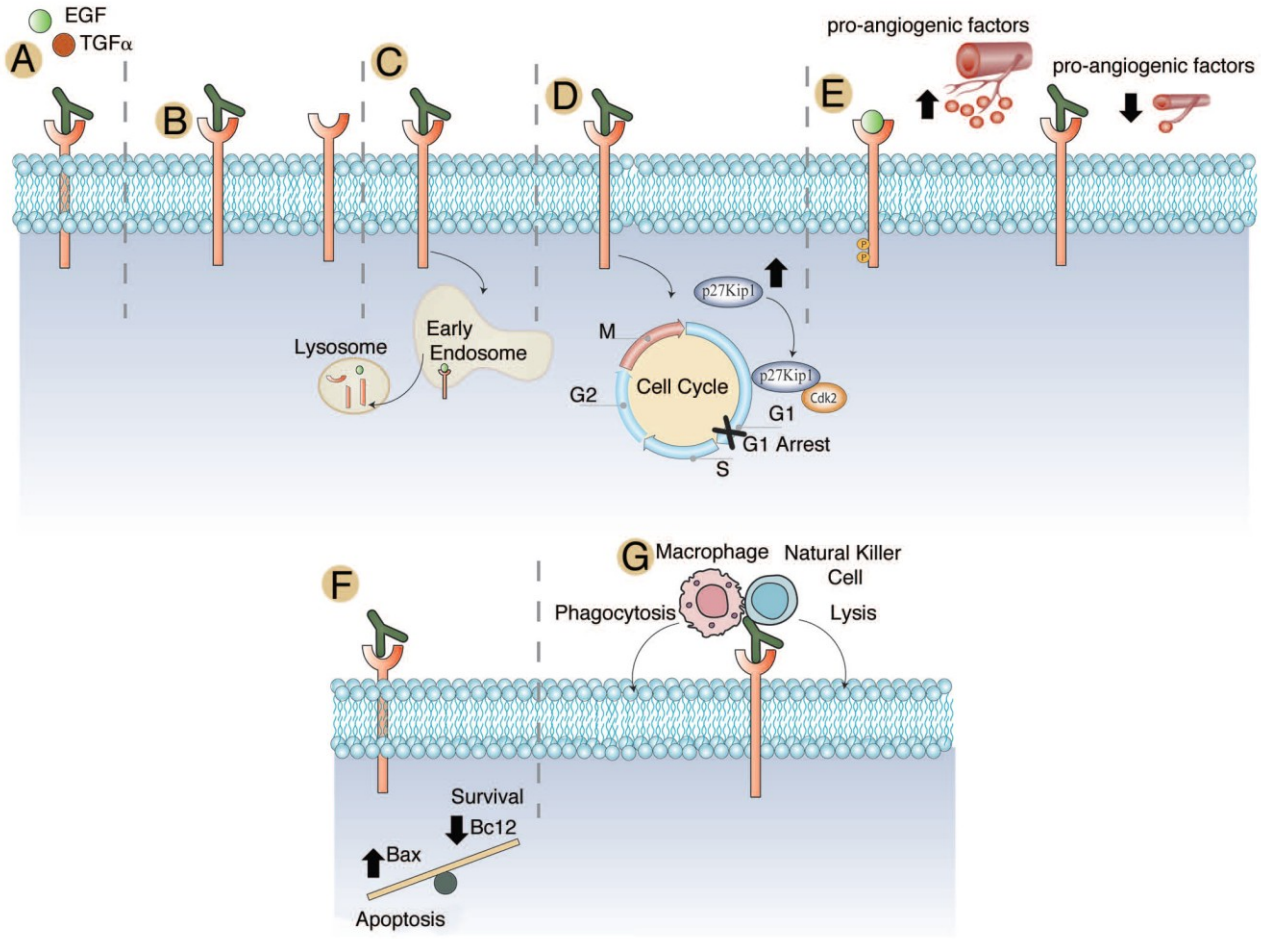


Fig.5 Mechanisms of action of Cetuximab. (A) Cetuximab has a higher affinity for the EGFR than either ligands and effectively blocks ligand binding and ligand induced EGFR phosphorylation. (B) Cetuximab has been noted to sterically hinder the binding of EGFR to other HER family members. (C) Cetuximab promotes the internalization and degradation of the EGFR. (D) Cetuximab treatment induces cell cycle arrest in the G1 phase of the cell cycle. (E) Treatment with Cetuximab has been shown to dramatically decrease the expression of pro-angiogenic factors. (F) Cetuximab treatment has also been noted to influence the balance of apoptosis and cell survival through modulation of the expression of Bax, which promotes apoptosis and Bcl2, which promotes survival. (G) Antibody-dependent cellular cytotoxicity mediated by Cetuximab has also been noted in several studies.

From: [70]

1.6 Predictive Biomarkers of Cetuximab Response

Since the FDA approval of Cetuximab and its associated clinical successes, intense investigations have been made to find markers in patient tumors that could predict individual responses to Cetuximab therapy and positive clinical benefit.

1.6.1 KRAS mutations

The most predictive biomarkers of response to Cetuximab is the mutational status of the KRAS gene. Retrospective analysis of single arm studies in heavily pretreated mCRC patients [71], [72], [73], [74], [75] strongly supported the hypothesis that the KRAS mutations are associated with the lack of response to Cetuximab in chemorefractory mCRC patients, leading the American and European health authorities to restrict the use of Cetuximab only to patients with KRAS wild-type (WT) tumors.

Recently, two prospective trials, the OPUS (Oxaliplatin and Cetuximab in First-Line Treatment of Metastatic Colorectal Cancer) [76], and the CRYSTAL (Cetuximab Combined With Irinotecan in First-Line Therapy for Metastatic Colorectal Cancer) [77], confirmed these data: when administered in first line, Cetuximab in combination with either Oxaliplatin-based or with Irinotecan-based chemotherapy, resulted effective only in a fraction of KRAS WT mCRC patients.

Mutations of KRAS occur in approximately 40% of CRC. They represent an early event in the colorectal carcinogenesis [78, 79]. They mostly consist in single nucleotide point mutations occurring in codons 12 and 13 of exon 2, while less frequent mutations are located on codons 61, 146 and 154. KRAS is a molecular switch activated by tyrosine kinase receptors. When mutated, it becomes constitutively active, and render the cells independent from the EGFR signaling activation, in fact the protein accumulates in a constitutively active GTP-bound status, impairing intrinsic and GTPase Activated Protein (GAP) mediated hydrolysis of GTP to GDP [80]. This general rule, however, is being recently challenged for the KRAS G13D mutation by new data: as recently reported by De Roock et al., chemotherapy-refractory mCRC patients which carry a KRAS mutation in codon 13 may have longer OS (overall survival) (median, 10.6 [95% confidence interval, 5,7-24.6] months vs 7.4 [95% confidence interval, 5.5-9] months) and longer PFS (progression free survival) (median, 4,1 [95% confidence interval, 2,8-6,9] months vs

2,8 [95% confidence interval, 2,5-3,7] months) compared with patients harboring other KRAS mutations, when treated with Cetuximab plus chemotherapy [81]. The molecular mechanism behind this discrepancy has not yet been identified. Nevertheless, it introduces the concept that different mutations occurring in the same gene can play different roles in terms of response to treatment.

Even though selection for KRAS WT patients enriches treatment groups more likely to benefit from anti-EGFR therapy, it is to note that only 20–40% of KRAS WT patients will actually respond to Cetuximab. Therefore, new selection markers for responsiveness are still needed to further select the subset of CRC responsive patients, avoiding unnecessary treatment.

1.6.2 BRAF, PI3K and NRAS mutations

The idea that mutations in molecules involved in downstream pathways of the EGFR signaling could be responsible for the lack of sensitivity to EGFR targeting agents has directed the search to BRAF, PI3K and NRAS mutations.

BRAF is one of the primary downstream effectors of KRAS signaling [82]. The V600E is the most common point mutation involving the BRAF gene and it is present in approximately 10% of mCRC. Since KRAS and BRAF belong to the same pathway of EGFR and mutations in these genes are mutually exclusive, it is speculated that the presence of an active mutation in only of these two molecules is sufficient to drive constitutive activation of the pathway.

At least three early studies [83], [84] have shown that BRAF V600E mCRC tumors do not respond to Cetuximab and have lower progression free survival and overall survival, as compared with BRAF wild type patients. However, these studies investigated the role of BRAF mutations in response to anti-EGFR agents used as monotherapy or in combination with Irinotecan-based regimens in chemorefractory patients, so it was speculated that these results might be mostly driven by a baseline prognostic effect of BRAF mutations rather than held an actual predictive effect [85]. Recent results from first line randomized studies (OPUS [76] and CRYSTAL [77]) have shown a trend in favor to Cetuximab treatment for BRAF V600E patients, not reaching statistical significance. This point thus awaits further investigations.

One of the main pathways activated by EGFR is the PI3K/PTEN/AKT signaling cascade. PIK3CA mutations mainly occur in exons 9 and 20, with exon 9 showing the highest incidence (68.5%

approximately). These mutations can be found in the same tumor together with KRAS and BRAF mutations, and this makes difficult to evaluate their specific role in defining the sensitivity to anti-EGFR mAbs. The correlation between PIK3CA mutations and Cetuximab response is still not clear, and several conflicting data have been reported [86, 87]. Patients with mutations in KRAS or BRAF and PIK3CA seem not to respond to Cetuximab, while patients WT for KRAS and BRAF, but mutant for PIK3CA, may have different sensitivity, depending on the site of the mutation they harbor: while PIK3CA exon 20 mutations are associated with resistance to Cetuximab, PIK3CA exon 9 variants have no significant effect on response [84]. Again, this underlines the paradigm of different mutations affecting the same gene but playing different roles in terms of drug sensitivity.

Finally, the role of NRAS mutations, a member of the RAS oncogene family, has also been investigated. The frequency of these mutations in mCRC is very low (about 2.6%). Preliminary findings suggest that NRAS mutations may be associated with resistance to Cetuximab [84]. Further studies are needed to confirm these results.

1.6.3 EGFR expression

Following clinical data obtained in breast cancer, showing that woman with breast cancer with high HER2 receptor expression are more likely to respond to anti-HER2 therapy, it was hypothesized that expression levels of EGFR may serve as a simple predictive biomarkers for the likelihood of response to Cetuximab therapy. However, clinical studies did not confirm such a correlation in CRC. Chung et al. confirmed that several CRC patients exhibited a major objective response to Cetuximab despite the absence of measureable EGFR, therefore levels of the EGFR alone are not currently considered a reliable predictor of response to Cetuximab therapy [88].

1.6.4 EGFR gene copy number as a predictor of response

Although mutations within the EGFR do not predict response to Cetuximab therapy, increased copy number of the EGFR gene is associated with response in CRC [89]. Large CRC clinical cohorts confirmed a relationship between EGFR gene amplification and clinical response to

Cetuximab [90, 91]. Increased copy number of the EGFR gene does not lead to increased expression of EGFR in these patients [90, 91] therefore is recently unknown how EGFR gene copy number correlates with improved response.

1.6.5 HER-2 gene copy number

In addition to homodimerization, EGFR can heterodimerize with the other members of the HER family, which, if altered, may influence the response to anti-EGFR agents. A few works have investigated the role of HER2, the primary EGFR dimerization partner, in this process. In mCRC, three recent studies demonstrated that HER2 gene amplification allows for the activation of downstream signaling even when Cetuximab is bound to EGFR, thus leading to drug resistance [92], [93], [94].

1.6.6 EGFR ligand expression

In colorectal tumors that develop in absence of mutations in KRAS or BRAF, molecular alterations may be substituted by autocrine and/or paracrine loops that involve the EGFR and its ligands. These tumors may be addicted to the production of EGF-like growth factors and display high sensitivity to Cetuximab. Recent studies demonstrated that expression of EGFR ligands, in particular EREG (Epiregulin)and AREG (Amphiregulin), correlates with response to Cetuximab in mCRC WT for KRAS [95], [96], [97], [98] .

2 Aim of the thesis

Metastatic colon cancer therapy after resection mostly relies on chemotherapeutic agents, such as 5-Fluorouracil (5-FU) alone or in combination with Oxaliplatin or Irinotecan. However, resistance to chemotherapy eventually arises in almost all patients, so that improvement of overall five-year survival for colorectal cancer, now reaching 60% in Europe, is to be ascribed to the development and diffusion of early diagnostic methods, rather than to pharmacological efficacy.

A large number of clinical and experimental data show that in colon cancer tumor growth is sustained by a small subset of cells endowed with stem cell properties, able to both self-renewal and to give raise to a whole hierarchy of differentiated cells, that constitute the bulk of the tumor. cCSCs exhibit relative resistance to anti-proliferative and cytotoxic drugs: in colon cancer, in particular, $CD133^+$ cCSCs have been shown to resist to Oxaliplatin-induced apoptosis by IL-4 production in a xenograft model [50], and Ciclophosphamide or Irinotecan treatment was found to increase the frequency of Tumor Initiating Cells (TIC) within cCSC xenografts [51]. CSC resistance is possibly due to different mechanisms, among which high expression of multidrug resistance and multidrug efflux genes, enhanced checkpoint activation and DNA damage repair activity, increased expression of anti-apoptotic proteins, and increased Wnt/β-catenin and Notch signaling [53].

For these reasons, a big effort is being put in recent years to the aim of identifying and hitting CSCs specific, sensitive pathways. Several targeted inhibitors directed against Sonic Hedgehog, Notch and Wnt intermediates are presently in clinical trial [99, 100]. However, none of these treatment is approved for therapy yet.

Among the new, target-oriented drugs that recently entered clinical practice, the anti-EGFR humanized antibody Cetuximab is effective on metastatic colon cancer [77, 101]. Cetuximab binds to EGFR blocking the binding of the ligands, and enhancing EGFR internalization and degradation.

The main aim of this study has been to verify whether Cetuximab is active on cCSCs, by using a panel of cCSCs isolated by individual patients. A second aim of the study has been to verify the predictive value of the cCSCs model for the screening of target-oriented, cancer stem cell directed therapies. To this purpose, the experimental work has been directed to:

1) Generate a panel of cCSCs from primary CRC primary samples;
2) Develop a validation system allowing to assess the validation of cCSCs;
3) Analyze the effect of Cetuximab on a panel of cCSCs by different direct and indirect stem-cell methods, in comparison with a common chemotherapeutic agent;
4) Investigate the concordance between cCSCs sensitivity to Cetuximab observed on cCSCs and known clinical parameters of response in patients.

3 Material and Methods

3.1 Colon cancer stem cells generation and culture

Fresh human CRC tissues were obtained in accordance with the ethical standards of the institutional Committee on human experimentation (authorization no. CE5ISS 09/282) and processed within 24-48 hours after surgical resection, as previously described. Briefly, fresh CRC tissues were mechanically disaggregated and incubated 1 hour at 37°C in DMEM medium (Gibco-Invitrogen, Carlsbad, CA) supplemented with collagenase type II (1.5 mg/ml, Gibco-Invitrogen) and DNase (20 µg/ml, Roche Diagnostics, Indianapolis, IN). Cells were then washed in PBS, filtered trough100-µm nylon mesh and cultured in humidified atmosphere containing 5% CO^2 at 37°C, in ultra-low attachment tissue culture flask (Corning Costar, Cambridge, MA) in CSC medium, composed of DMEM-F12 (Gibco) supplemented with: 2 mM glutamine (Gibco), 0.6% glucose, 9.6 mg/ml putrescine, 6.3 ng/ml progesterone, 5.2 ng/ml sodium selenite, 0.025 mg/ml insulin, and 0,1 mg/ml transferrin (all from Sigma Aldrich, Boston, MA). h-EGF (20 ng/ml) and h-bFGF (10 ng/ml), both from PeproTech (London, UK) were also added to the medium. CSCs lines grow as a spheroid that are serially passaged by mechanical dissociation weekly. All experiments were performed within 10 cell passages.

3.2 Gene mutation analysis

Human KRAS exon 1 (codon 12,13), BRAF exon 15 (codon 600) and PI3K exon 9 (codon 542) and exon 20 (codon 1047)analysis were performed on genomic DNA following PCR amplification with the following set of intronic primers: KRAS forward 5'-GATACACGTCTGCAGTCAACTG-3', reverse 5'-AGAATGGTCCTGCACCAGTAA-3'; *BRAF* exon 15 (codon 582-620) forward 5'-CTAGTAACTCAGCAGCATCTCAG -3', reverse 5'- CTTCATAATGCTTGCTCTGATAG -3'; and PI3K exon 9 forward 5'-GAAAAATATGACAAAGAAAGC-3', reverse 5'-AAACATGCTGAGATCAGCCA-3'and exon 20 forward 5'-TGTCTACGAAAGCCTCTCTAA-3', reverse 5'-AGACCGATTGCATAGGAATTG-3'. PCR amplifications were carried out with the high-fidelity Optimase Polymerase (Transgenomic) at conditions indicated by Optimase Protocol Writer software (Transgenomic).

Mutation analysis of the amplimers was performed via denaturing high-performance liquid chromatography (DHPLC) with the Wave 2100 System (Transgenomic) at column temperatures recommended by Navigator software, version 1.6.4.12 (Transgenomic). Amplimers with abnormal denaturing profiles were purified (Microcon PCR [Millipore]) and then sequenced bidirectionally with the ABI Big Dye Terminator Sequencing Kit v.3.1

3.3 Proliferation assay

cCSCs viability upon treatment with Cetuximab was determined with the CellTiter-Glo assay (Promega, Madison, WI) according to the manufacturer's directions. Briefly, cCSCs (3-5x10^4/ml) were seeded in 96-well plate (6 replicates/experimental point), in CSC medium, in the presence of Cetuximab (100 µg/ml final concentration, Merck KGaA, Darmstad, Germany) and incubated in a humidified atmosphere, 5% C02, at 37°C. At different time-points, the incubation was blocked and sample processed according to manufacturer's instructions. Luminescence was detected by DTX880 multimode micro-plate reader (Beckman Coulter, Brea, CA).

3.4 Xenograft assays

All animal procedures were performed according to the national Animal Experimentation guidelines (D.L.116/92) upon approval of the experimental protocol by the Istitutional Animal Experimentation Committee. Female 5 weeks old NOD.Cg-Prkdc*scid* Il2rg*tm1Wjl*/SzJ (NSG) mice (The Jackson Laboratory, Bar Harbor, ME) were subcutaneously injected with 10^6 cells resuspended in 100 µl CSCs medium/Matrigel (BD Pharmingen San Jose, Ca) (1:1). Tumors were measured by using an external digital caliper twice a week, and volumes were calculated using the following formula: $\pi/6 \times d^2 \times D$. After tumor establishment (100-150 mm^3), mice were treated with 10 mg/ml Cetuximab i.v. twice a week for 4-16 weeks. Irinotecan (Pfizer) was administered i.p. weekly at the dose of 15 mg/Kg. Average tumor volume ± SEM are shown (n=10-12 mice for group). For secondary transplantation experiments, tumors were harvested from individual animals and dissociated into single cells by mechanic dissociation. Cells were injected at serial doses ranging from 10 to 3x10^3 cells.

3.5 Cytofluorimetric analysis

Tumor spheres or xenograft-derived cells were dissociated as single cells, washed with PBS and incubated with the appropriate dilution of control or specific antibody for 45' at room temperature. Fluorescence intensity of labeled cells was evaluated with a FACSCanto (Becton Dickinson, Franklin Lakes, NJ) instrument.

Antibodies used in the study were: anti-EpCAM (BD), anti-CD44v6 (R&D, Minneapolis, MN), anti-EGFR (BD), anti-CD133 (Miltenyi Biotech, Bergisch Gladbach, Germany), anti-IgG2b isotype control, anti-IgG1 isotype control and anti-IgG isotype control (Miltenyi Biotec).

7-aminoactinomycin D (10 µg/ml, BD) was added for dead cell exclusion.

3.6 Clonogenic assay

The clonogenic capacity of xenograft-derived cells was assessed by plating 500 cells/ml/well in triplicate in 24-well plates containing a soft agar bilayer (0.3% top and 0.4% bottom layer, SeaPlaque Agarose, Cambrex, East Rutherford, NJ). Cultures were incubated at 37°C in humidified atmosphere in the presence of 5% CO2, for 21 days. Colonies were stained with crystal violet (0.01% in 10% MetOH) and counted under a light microscope. Data represent the percentage of colonies normalized to the number of cells plated.

3.7 DNA extraction and STR loci profile

Genomic DNA was extracted with Dnasy mini kit (Qiagen) according to the manufacturer's instructions. AmpFlSTR Identifiler Plus uses multiplex PCR to simultaneously amplify fifteen STR loci plus amelogenin for gender determination. These loci are among the most informative polymorphic markers in the human genome (D8S1179, D21S11, D7S820, CSF1PO, D3S1358, TH01, D13S317, D16S539, D2S1338, D19S433, vWA, TPOX,D18S51, D5S818, FGA). A unique DNA pattern of repeating units is generated for each human cell line analyzed. STR analysis is critical today to verify the identity of human cell lines and is now routinely performed at the time of accessioning a new cell line and at the replenishment of each distribution stock to avoid misidentified cell lines to be distributed to the scientific community.

3.8 RNA extraction and quantitative real time PCR

Total RNA was extracted with Rnasy mini kit (Qiagen, Venlo, TheNetherlands) according to the manufacturer's instructions. 100 ng of RNA were reverse transcribed with M-MLV reverse transcriptase (Invitrogen) and cDNA was diluted 1:10 in the PCR reactions. Quantitative real-time PCR analysis was performed by SYBR green technology. Results were normalized against Glyceraldehydes 3-phosphate dehydrogenase (GAPDH) gene expression. Values are expressed in terms of $2^{-\Delta\Delta CT}$ where $\Delta\Delta CT = \Delta CT_{sample} - \Delta CT_{calibrator}$, ΔCT was the difference in threshold cycles between the target gene and GAPDH amplicons, and CT was a parameter given by ABI PRISM 7900 Sequence Detector software according to the manufacturer's instructions (Applied Biosystems, Foster City, CA). Each experiment was performed in duplicate for two-three times.

3.9 Generation of lentiviral vectors and gene transfer

For TW-EGFP/KRAS and TW-EGFP/KRAS G12V generation, mRNA was extracted from tumor tissue and reverse transcribed (RT) to cDNA with MMLV reverse transcriptase and oligo (dT). Then, cDNA was amplified by PCR and sequenced by MWG (Ebersberg, Germany). The fragment was sub-cloned in the lentiviral vector TW under the control of the CMV promoter. An empty vector was used control. Cells were plated in a 6-well plate 24 hours prior to viral infection and incubated overnight with 2 ml of serum-free medium containing EGF and b-FGF. The day after the medium was removed and cells were infected adding 2 ml of complete medium with Polybrene (5µg/ml) and lentiviral particles described previously. Transduction efficiency was measured through EGFP expression analysis.

3.10 Statistical analysis

The statistical significance of the results was evaluated by ANOVA and Bonferroni post-tests. All statistical analyses were performed using GraphPad Prism v.4.0 for Windows (GraphPad Software, San Diego, CA, www.graphpad.com) and statistical significance was accepted up to 0.05. P values are displayed on the graphs using a single asterisk for significances ranging from

0.05 to 0.01, two asterisks for values between 0.001 and 0.01 and three when statistical differences produced significance below 0.001.

4 Results

4.1 Generation and validation of cCSC lines

A growing panel of cCSC lines was established from primary tumors of different CRC patients. Specifically, fresh CRC tissues are mechanically and enzymatically disaggregated into single cells and cultured at low density in serum-free medium containing epidermal growth factor (EGF) and basic fibroblast growth factor (b-FGF). After approximately 4-8 weeks, the cultures are exclusively composed by cellular clusters resembling the so-called "tumor spheres" or "spheroids". In order to validate these lines we established a procedure that includes two benchmarks (Fig.6):

1) Assessment of the identity of the lines by extracting DNA and matching their individual STR loci profile with those of the corresponding patient's normal mucosa. These data are then added to our profile database;

2) Assessment of cCSCs capability to generate tumors after subcutaneous injection into immunodeficient mice. Xenografts are finally validated for histological compatibility with the original, primary tumor.

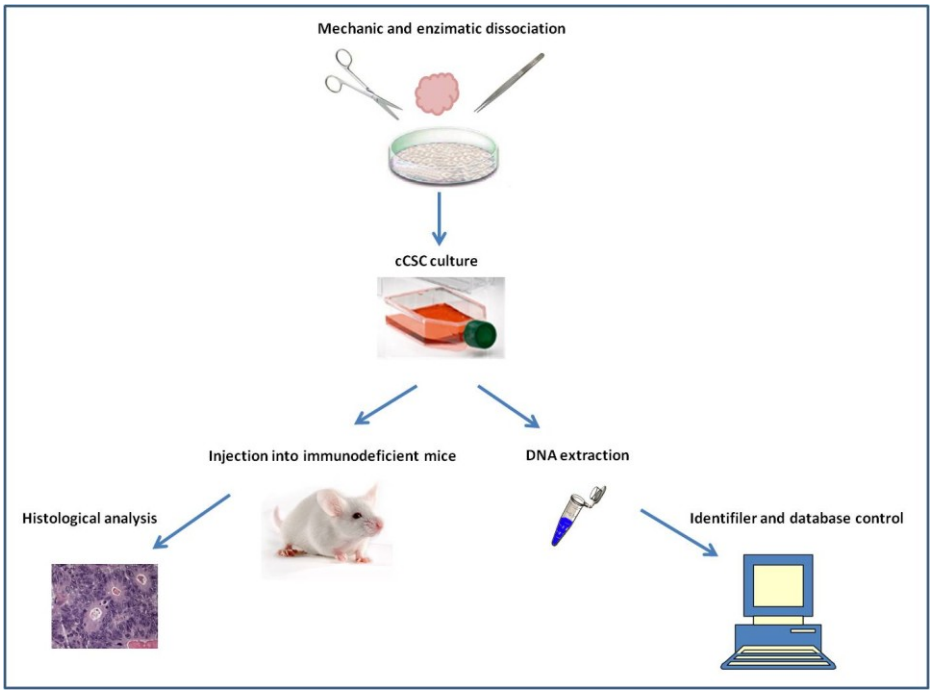


Fig.6 Schematic representation of sperimental procedure for the generation and validation of cCSC lines.

In figure 7 is shown an example of the two-step validation procedure. 7a: matching of STR loci profile between an individual cCSCs and its respective normal mucosa.

7b: matching of the histological analysis of an individual cCSCs xenograft with the respective patient's primary tumors specimen Ematoxylin-Eosin (H&E) staining. Images demonstrate that xenograft cCSCs effectively replicate the human disease in the mouse.

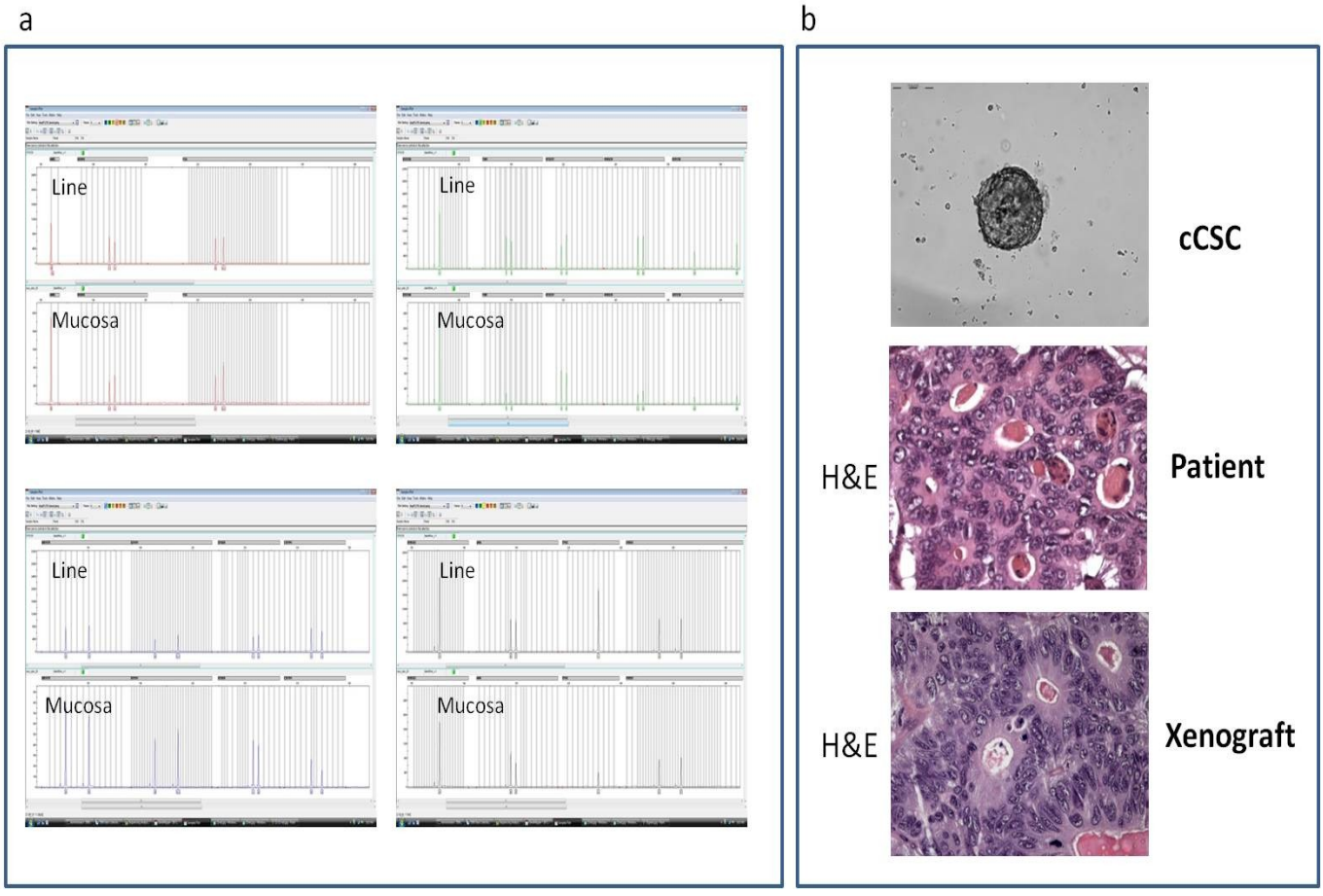


Fig.7 Validation of cCSCs.

a) STR loci profile of cCSCs and of its corresponding normal mucosa.

b) Microscopy images of cCSCs spheres, and Hematoxylin-Eosin (H&E) staining of tumor specimens derived from parental tumor (patient) and from tumors generated by subcutaneous injection of cCSCs in immunodeficient mice (xenograft).

4.2 Mutational charachterization of cCSCs

In a panel of cCSCs generated from different patients, I screened a panel of mutation related to Cetuximab resistance, including KRAS, BRAF, PI3K and NRAS. In fact constitutive activation of this EGFR pathway intermediates render the tumor insensitive to EGFR inhibition.

The results of PCR and sequencing are reported in Table 1.

	KRAS	BRAF	PI3K	NRAS
CTSC1	WT	WT	E542K	WT
CTSC2	WT	WT	WT	WT
CTSC3	WT	WT	WT	WT
CTSC4	WT	WT	WT	WT
CTSC5	G12V	WT	WT	WT
CTSC6	G13D	WT	WT	WT
CTSC7	WT	V600E	WT	WT
CTSC8	WT	V600E	WT	WT
CTSC9	WT	WT	WT	WT

Table 1: Mutational status of different patient-derived cCSC lines.

4.3 cCSCs responsiveness to Cetuximab vs Irinotecan

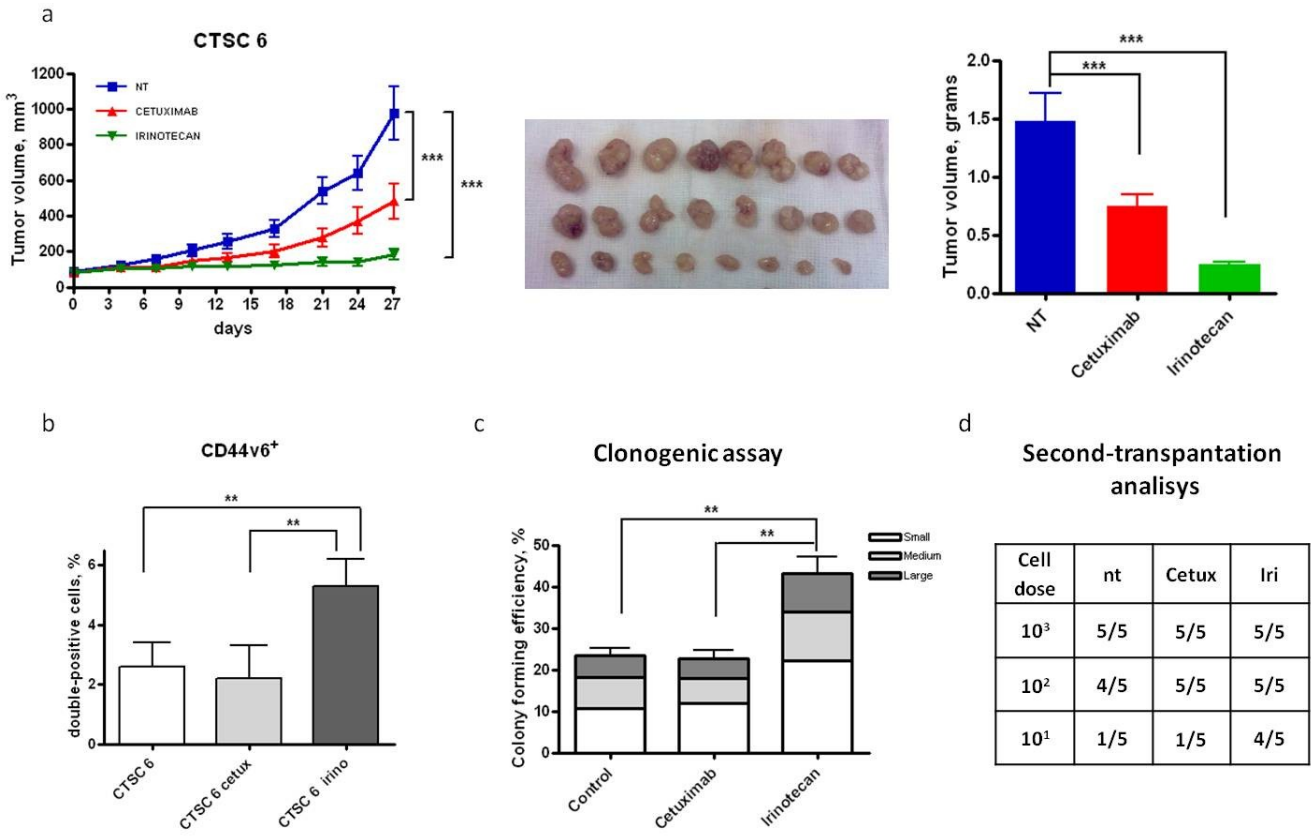
It has been previously shown by other authors that common chemotherapeutics selectively kill differentiated cells within the tumor, sparing cancer stem cells [51]. In order to verify whether such an effect could be observed also for the target-oriented agent Cetuximab, the drug was assayed in parallel with Irinotecan, on subcutaneous xenografts generated with cCSCs.

As shown in 8a, both Cetuximab and Irinotecan are effective on xenograft growth, the first inducing a reduction of growth rate, and the second fully inhibiting tumor growth itself.

Xenografts from this experiment were dissociated, and single cells were analyzed by cytofluorimetry for the expression of the stem cell marker candidate CD44V6 (Fig.8b). As expected, the frequency of stem cells within tumors is increased following Irinotecan treatment. Conversely, the frequency of CD44V6-positive cells is comparable in Cetuximab-treated versus untreated tumors, indicating that Cetuximab equally affects differentiated and stem cells within the tumor.

The results of the Clonogenic assay, shown in Fig.8c, also demonstrates that clone initiating cells (CIC) frequency is about doubled in Irinotecan-treated xenografts as compared to untreated controls. Conversely, no significant variation is observed in CIC frequency in Cetuximab treated xenografts vs untreated controls. To further investigate the status of stem cells within the xenografts, dissociated cells were inoculated at different doses into secondary mice. The frequency of tumors developed from the grafts after 24 weeks is reported in Fig.8d, and shows that while, as expected, the frequency of Tumor Initiating Cells (TIC) is increased by treatment with Irinotecan, no significant variation is observed in Cetuximab-treated versus untreated primary xenograft.

Taken together, these data indicate that Cetuximab does not share the preferential toxicity against differentiated tumor cells held by Irinotecan, but that it efficiently kills cCSCs within tumors.



Cell dose	nt	Cetux	Iri
10^3	5/5	5/5	5/5
10^2	4/5	5/5	5/5
10^1	1/5	1/5	4/5

Fig.8 cCSCs responsiveness to Cetuximab vs Irinotecan. a) Inhibition of xenograft growth by Cetuximab and Irinotecan. Data represent the average of 10 tumors ± SEM, *** P<0.001; b) cytofluorimetric analysis for EpCAM/CD44v6 of cells dissociated from xenograft in b. Data represent the average of 6 xenograft for group, individually analyzed ± SD, **P<0.01; c) clonogenic assay of cells dissociated from xenografts in b. Data represent the average of 6 xenografts for group, individually analyzed ± SD, **P<0.01; d) Second transplantation of cells dissociated in b.

4.4 Responsiveness to Cetuximab of cCSCs with different KRAS and BRAF mutational status

To analyze the effect of EGFR-targeted therapy on a wider panel of cCSCs, I assayed the sensitivity to Cetuximab of seven cCSCs carrying different mutations (Tab.1), both *in vitro* (Fig.9a) and on subcutaneous xenografts in NSG mice (Fig.9b). Out of the seven lines tested, four were wild type both for KRAS and BRAF. Among these, two where fully inhibited by Cetuximab both *in vitro* and *in vivo* (CTSC1 and CTSC2), while two other where unresponsive to the drug in both settings (CTSC3 and CTSC4). The proliferation of CTSC1 and CTSC2, wild type for both KRAS and BRAF, is fully inhibited *in vitro* by the drug, and treatment *in vivo* induces persistent tumor regression even after treatment suspension. Conversely, the G12V KRAS-

mutated CTSC5 is insensitive to treatment *in vitro*, while only a modest delay in xenograft growth is apparent *in vivo*. Interestingly, G13D KRAS mutated (CTSC6) and V600E BRAF mutated (CTSC7) lines show intermediate, different degrees of response to Cetuximab.

a

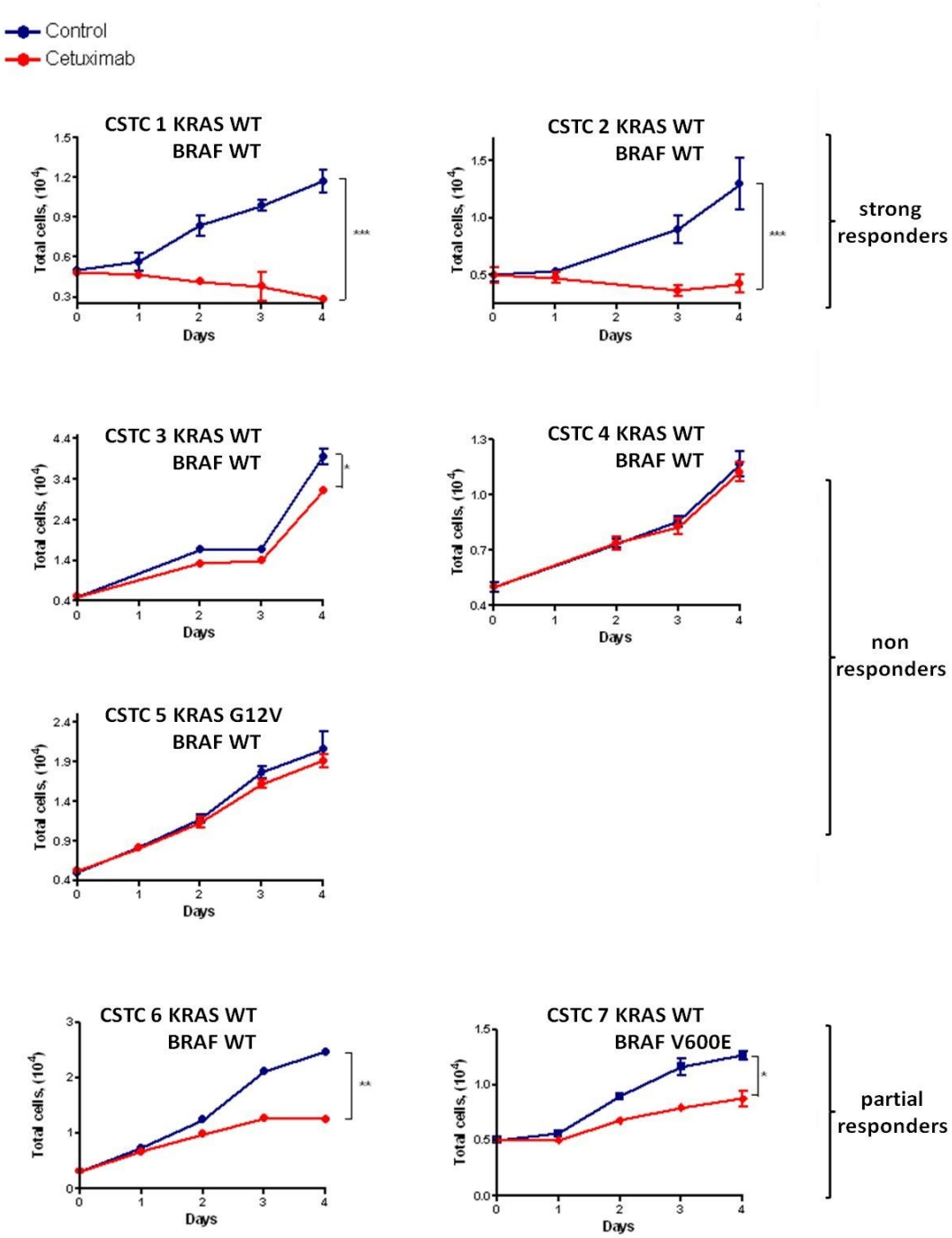


Fig.9a *In vitro* proliferation assay: responsiveness to Cetuximab of cCSCs with different KRAS and BRAF mutational status. Data represent the average of 6 replicates/experimental point ± SD, ***P<0.001, **P<0.01, *P<0.05.

b

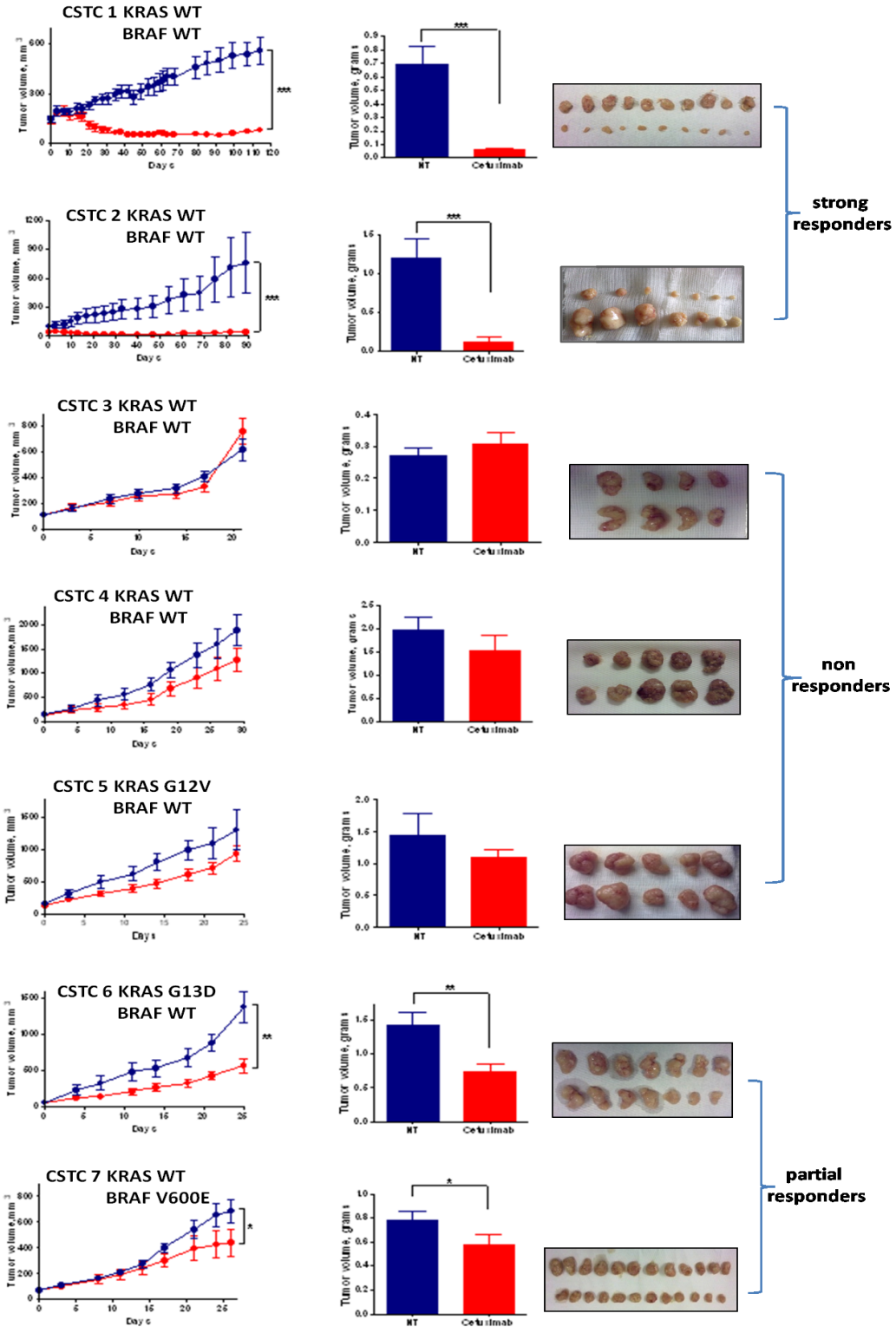


Fig.9b Cetuximab effect on cCSCs xenograft growth. Data represent the average of 10-12 tumors/group ± SEM, ***P<0.001, **P<0.01, *P<0.05.

4.5 EGFR expression as a predictor of response to Cetuximab

It has been proposed that EGFR expression may be predictive of Cetuximab responsiveness [89].

To verify this hypothesis, I analyzed EGFR expression in our cCSCs panel, both by cytofluorimetry and qRT-PCR. My data (Fig.10a-b) did not evidentiate any significant correlation between cCSCs responsiveness to Cetuximab and EGFR mRNA, nor with protein expression on cell membrane.

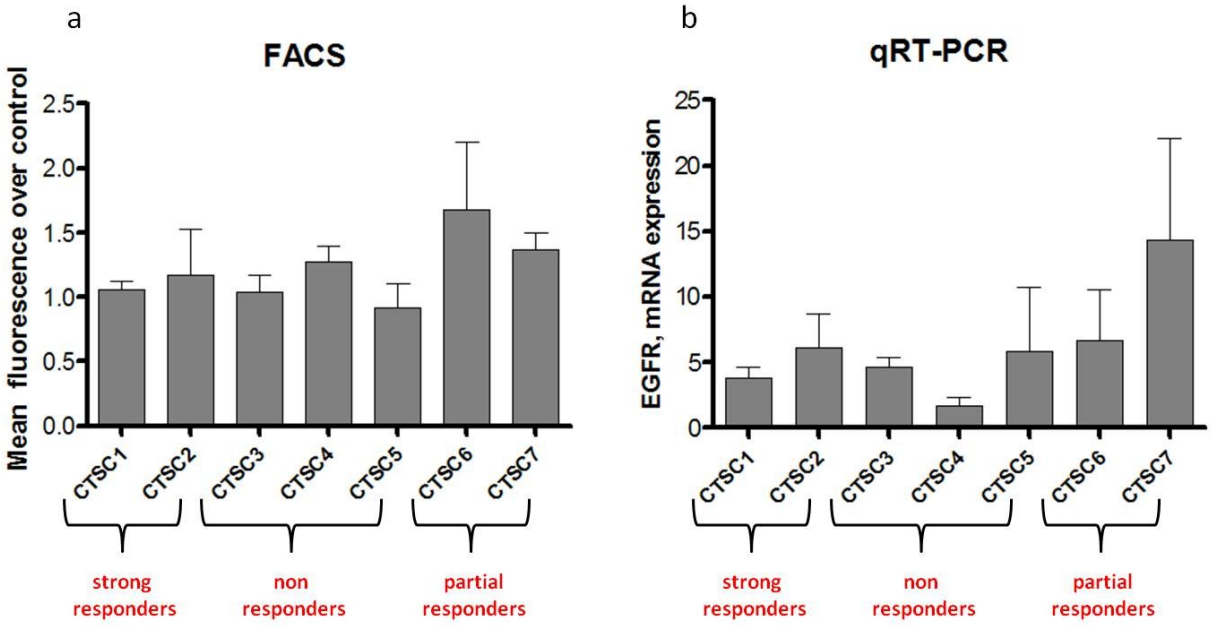


Fig.10 EGFR expression profile in cCSCs: a) cytofluorimetry; b) qRT-PCR, $\Delta CT_{calibrator}$ = CSTC4.

Average of two independent experiments ± SD is shown.

4.6 EGFR ligands expression as a predictor of response to Cetuximab

Clinical data have recently confirmed that expression levels of Epiregulin (EREG) and Amphiregulin (AREG) may predict progression free survival (PFS), overall survival (OS), and Cetuximab therapeutic response in patients WT for KRAS [95, 96, 102].

I assayed AREG and EREG mRNA expression by qRT-PCR analysis. My data (Fig.11a-b) confirm that lines responsive to Cetuximab (CTSC1 and CTSC2) express higher mRNA level of EREG and AREG respect to non-responsive lines (CTSC3 and CTSC4).

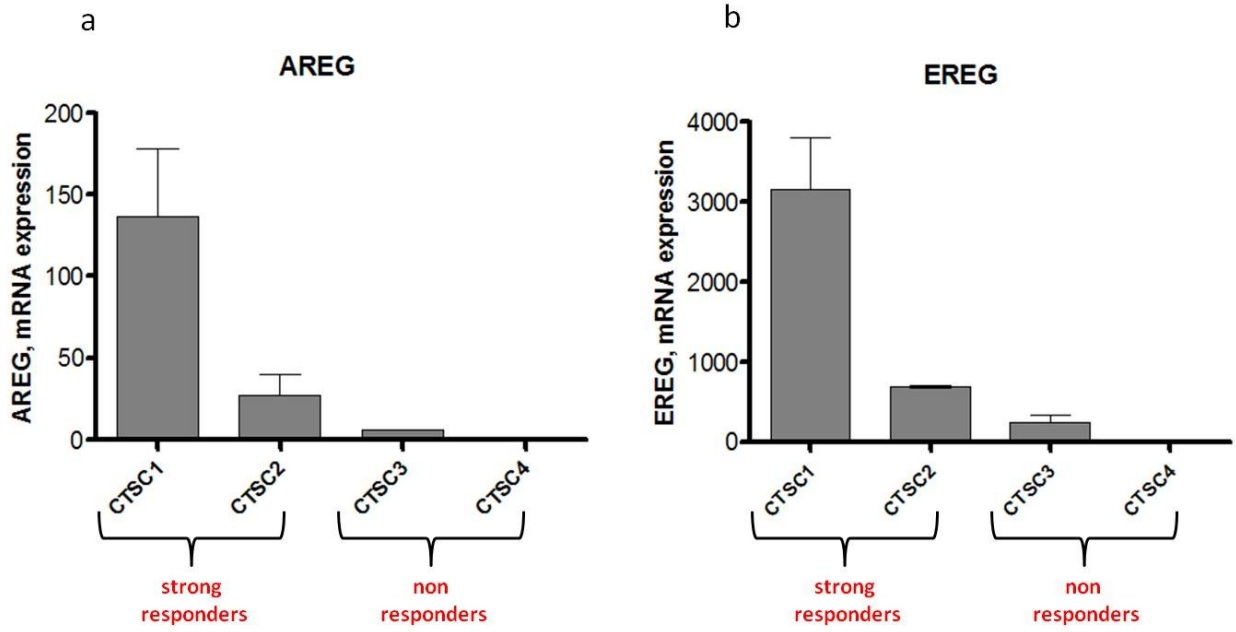


Fig.11 mRNA levels of EGFR ligands Amphiregulin (a) and Epiregulin (b) measured by qRT-PCR.

$\Delta CT_{calibrator}$ = CTSC 4. Average of 2 independent experiments ± SD is shown.

4.7 Content of stem cell markers positive cells and Clonogenic units in Cetuximab-treated versus untreated tumors

In order to verify a possible differential effect of Cetuximab on differentiated vs stem cells within the tumor, xenografts from the experiments shown in Figure 9 were harvested, dissociated into single cells and analyzed by cytofluorimetry for the expression of stem cell markers (Fig.12a-c) and by Clonogenic assay in agarose for their capability to generate colonies (Fig.12d). Data represent the mean of 5 tumors for each line.

No significant differences in the frequency of cells expressing the different stem cell marker combinations EpCAM$^+$/CD133$^+$, EpCAM$^+$/CD44$^+$/CD166$^+$ [45], nor EpCAM$^+$/CD44v6$^+$ is observed in Cetuximab-treated versus untreated xenografts, indicating that the drug equally affects stem cell marker-positive and -negative cells within the tumor.

Clonogenic assay in soft agar was then performed on three representative lines: Cetuximab-responsive CTSC1, Cetuximab-resistant CTSC3 and Cetuximab-partially responsive CTSC6. Data show that the frequency of Clonogenic unit within xenografts is comparable in treated versus untreated samples.

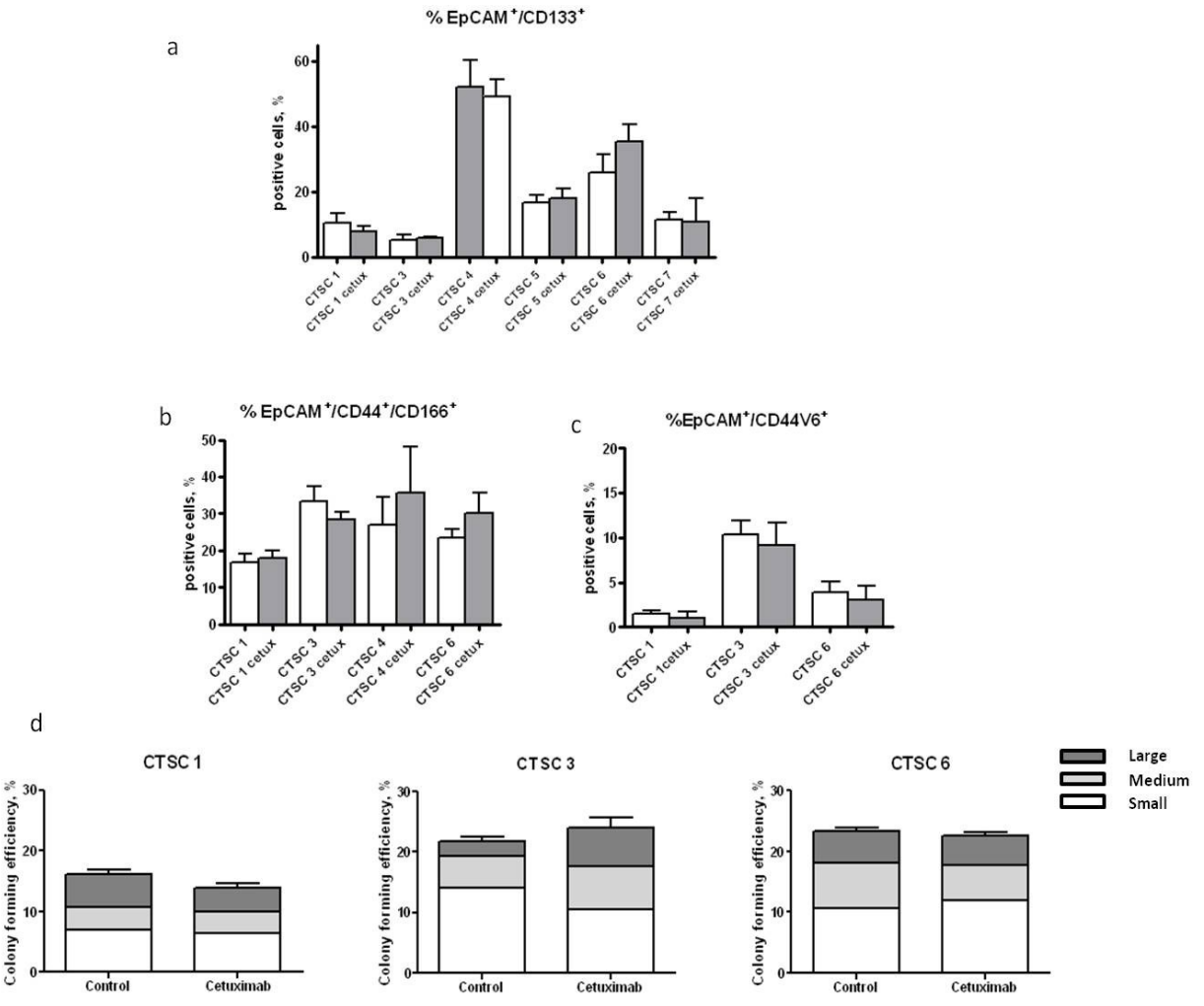


Fig.12 Content of stem cell markers positive cells and clonogenic capability in Cetuximab-treated versus untreated tumors. Cytofluorimetric analysis was performed on dissociated cells; a)EpCAM/CD133; b)EpCAM/CD44/CD166; c) EpCAM/CD44v6; d) Clonogenic assay . All data represent the average of 5 xenograft for group, individually analyzed ± SD.

4.8 Tumor-initiating-cell (TIC) content of Cetuximab-treated and untreated xenografts: second transplantation analysis

In order to verify how Cetuximab affects the content of stem cells *in vivo*, cells dissociated from individual tumors were re-injected sc into secondary recipient mice. For each individual tumor, different cell doses were tested as indicated in the table. Mice were recorded negative when no graft was observed after 24 weeks from the inoculum.

No significant reduction in TIC content is observed in Cetuximab-treated versus untreated xenografts.

Colon cancer cell	cell dose	positive/injected	
		Cetuximab	control
CTSC 1	$3x10^2$	4/8	4/8
	10^2	2/6	3/6
	10	0/6	0/6
CTSC 2	$3x10^2$	2/8	2/8
	10^2	0/6	0/6
	10	0/6	0/6
CTSC 3	$3x10^2$	8/8	8/8
	10^2	6/8	8/8
CTSC 4	$3x10^2$	6/8	6/8
	10^2	0/8	0/8
	10	0/8	0/8
CTSC 5	$3X10^3$	10/10	10/10
	10^3	0/10	2/10
CTSC 6	$3x10^2$	10/10	10/10
	10^2	8/10	8/10
CTSC 7	10^3	10/10	10/10
	$3x10^2$	8/10	8/10
	10^2	2/10	4/10

Table 2: TIC content of Cetuximab-treated and untreated xenografts: second transplantation analysis.

4.9 Cetuximab sensitivity and EGF-dependence of cCSCs

To clarify the relationship between Cetuximab resistance and dependence on EGF in cCSCs, I introduced a vector carrying the KRAS G12V in CTSC1 cells, that are strongly sensitive to Cetuximab inhibition. Control cells were infected with a vector carrying the KRAS WT gene. On both lines, dependence on EGF and response to Cetuximab were then analyzed. As shown in Fig.13, cells infected with KRAS WT are dependent on EGF medium for proliferation. Conversely, upon G12V infection, cells lose the dependence on EGF and became resistant to the drug, indicating that a strict relationship exist between cCSCs sensitivity to Cetuximab and an intact EGFR signaling pathway.

a b c

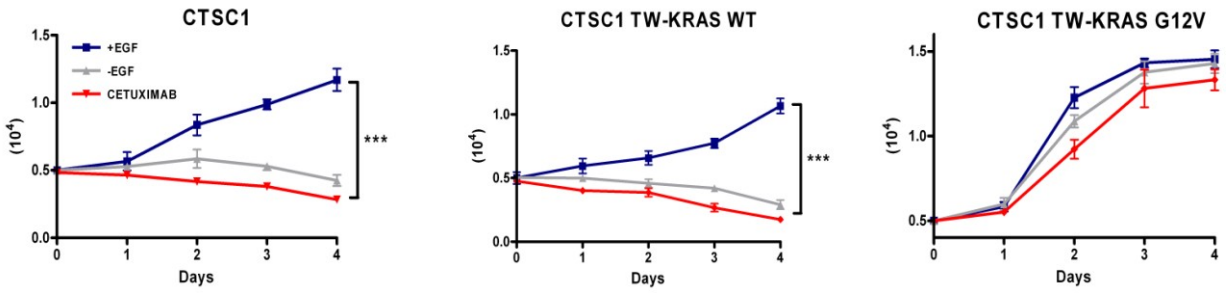


Fig.13 *In vitro* proliferation assay: Cetuximab and EGF-responsiveness of CTSC1 infected line. a)CTSC 1 control line; b)CTSC1 infected with a vector carrying the KRAS WT gene; c)CTSC1 infected with a vector carrying the KRAS G12V mutation. Data represent the average of 6 replicates/experimental point ± SD, ***P<0.001, **P<0.01, *P<0.05.

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5 Discussion

Relative resistance of CSCs to classic chemotherapeutic has opened a new insight into the problem of cancer cure. Relapse that in most cases follows treatment with conventional drugs is possibly driven by a minor population of resistant CSCs that are spared by these treatments. Conversely, agents directed against CSCs may lead to permanent tumor eradication. Therefore, a big effort is being put in recent years to the aim of identifying and hitting CSCs specific, sensitive pathways.

A series of recent reports have given a proof of principle for the efficacy of such an approach, showing that targeting of CSCs can be achieved by different strategies: in 2009 Gupta et al., by high-throughput screening of about 16,000 chemical compounds, identified the ionophor antibiotic Salinomycin, as active in markedly and selectively reduce the viability of breast cancer stem-like cells [103]. This drug has later been proved to effectively eliminate CSCs in preclinical models of different solid tumors including colon [104-107]. Salinomycin acts on CSCs through several different mechanisms, not fully dissected yet, that include induction of differentiation and inhibition of anti-apoptotic mediators [108-110].

Among the pathways selectively affecting cCSCs, inhibition of the Notch ligand DLL4 (delta like 4 ligand) has been shown to reduce growth and secondary TIC content of colon cancer xenografts, implying targeting of cCSCs self-renewal [111, 112]. Lombardo et al. demonstrated that BMP4 (Bone Morphogenetic Protein 4) induces differentiation of cCSCs, thus increasing response to chemotherapy in mice xenografts [113]. Benoit et al. showed that pharmacological inhibition of PRC2 (Polycomb repressive complex-2) induces apoptosis in human cCSCs by increasing PTEN expression, which in turn inhibits the PI3K/AKT pro-survival signaling pathway [114]. Several inhibitors of CSCs-related pathways, such as Sonic Hedgehog, Notch and Wnt, are presently in clinical trial [99, 100]. However, no cCSCs-directed treatment has been translated to therapy yet.

In colon cancer, the anti-EGFR antibody Cetuximab is presently approved for clinical use, alone or in combination with chemotherapy.

In the light of previous studies, that have shown that chemotherapeutics such as Irinotecan, Cyclophosphamide and 5-FU increase the frequency of cCSCs in treated xenografts, and therefore demonstrated that they preferentially hit differentiated cells within tumors [51], in this study we have asked the question whether Cetuximab affects cCSCs. To this aim, I have tested its effect on a panel of cCSCs generated by individual patients, carrying different mutations in signaling intermediates of the EGFR pathway.

The data of this study show that Cetuximab has a different efficacy on cCSCs from individual patients, in fact testing of seven individual cCSCs, both *in vitro* and in xenografts, allowed to divide them into three groups: strongly-responding, partially-responding and non-responding.

I observed that this differential sensitivity correlates well with known clinical data on the mutational state of EGFR pathway intermediates. In fact, two lines strongly responding to Cetuximab, both *in vitro* and *in vivo,* were WT for both KRAS and BRAF, and one KRAS G12V-mutated line showed completely resistant to the drug both *in vitro* and *in vivo.* On the other hand, two other cCSCs, also double KRAS/BRAF WT, were insensitive to the drug.

It is well known that wild-type KRAS/BRAF status is not sufficient to confer sensitivity to anti-EGFR monoclonal antibodies, in fact only about 50% of KRAS WT patients actually respond to Cetuximab. Mutational analysis of the two WT resistant cells in our panel, did not evidentiate mutations in either exon 20 PI3K, BRAF or NRAS. In order to verify whether I could identify a correspondence with other markers of Cetuximab resistance in our cell panel, I analyzed EGFR expression both by cytofluorimetry and qRT-PCR. Expression of EGFR was not correlated with Cetuximab sensitivity in our panel. Conversely, sensitivity broadly correlated with individual cCSCs expression of the EGFR ligands Epiregulin and Amphiregulin, confirming recent reports [115]. Interestingly, two cCSCs mutated, respectively, in BRAF V600E and in KRAS G13D showed partially responsive to treatment. The exact therapy-predictive efficacy of these mutations is still debated, because statistically significant clinical data have proven hard to collect, due to their low frequency.

Regarding the KRAS G13 mutation, in a pooled analysis of 579 patients from 7 different clinical trials (CO.17, BOND, MABEL, EMR202600, EVEREST, BABEL, and SALVAGE) Cetuximab improved OS and PFS in patients with KRAS G13D mutation as compared to patients harboring other KRAS mutations (median, 10.6 [95% confidence interval, 5,7-24.6] months vs 7.4 [95% confidence

interval, 5.5-9] months) and (median, 4,1 [95% confidence interval, 2,8-6,9] months vs 2,8 [95% confidence interval, 2,5-3,7] months) [81]. Most recent pooled data from the CRYSTAL and OPUS studies [76, 77], indicated that patients carrying the G13D mutation, treated with chemotherapy plus Cetuximab versus chemotherapy alone, have prolonged PFS (median 7.4, [95% confidence interval, 7,3-8,2] months vs 6 [95% confidence interval, 5,4-7,8] months) and increased OS (median 15.4, [95% confidence interval, 12,4-20,4] months vs 14.7, [95% confidence interval, 12,4-19,4] months) [116].

Conflicting results are reported on the therapeutic predictive value of V600E. In the CRYSTAL study, BRAF mutations were found associated with poor prognosis in patients with KRAS WT tumors. Even though the sample size was too small to draw statistically significant conclusion, the study suggested that BRAF mutants gain additional benefit by addition of Cetuximab to FOLFIRI [117].

Molecular studies on cCSCs panels may help to dissect the role of these mutations in cell response to Cetuximab.

Most importantly, I have shown that xenograft regression induced by Cetuximab is sustained by parallel decrease of both differentiated and stem cells. In fact, analysis of the frequency of stem cells by a panel of stem cell markers demonstrated no significant variation in the percentage of cell expressing CD133, or EpCAM CD44/CD166 and or in EpCAM/CD44v6 in treated versus untreated xenografts.

Frequency of clonogenic units in agarose did not vary either, in treated xenografts as compared to untreated tumors. Finally, the golden standard assay for cCSCs, i.e. re-transplantation assay into secondary recipient mice, demonstrated that there is no significant variation in the TIC frequency in Cetuximab-treated xenografts as compared to controls. Therefore, we conclude that Cetuximab do not share the preferential toxicity against differentiated cells held by chemotherapeutics such as Irinotecan, but kills stem and differentiated tumor cells with the same efficacy. This behavior is common both to strongly-responding and to partially-responding cCSCs in our panel. As expectable, no variation in stem cell content was observed in non-responding clones.

The observation that, in sensitive lines, all the cells along the hierarchical organization of the tumor are equally sensitive to Cetuximab fits with the model proposed by Rasheed et al., that

anticipated that drugs acting both on bulk and stem cells would induce fast shrinking of the tumor without the relapse observed with cytotoxic drugs (Fig.14) [118]. Indeed, xenografts generated with the two strongly-responding lines in our panel (CTSC1 and CSTC2), fastly and fully remitted upon treatment and did not relapse for at least 7 and 4 weeks respectively after treatment suspension.

It is easy to speculate that tumor sensitivity to Cetuximab is due to dependence on a functional signaling pathway of EGFR, and indeed the introduction of G12 mutated KRAS gene into a sensitive cCSC rendered the cells both independent on EGF and resistant to Cetuximab. cCSCs dependence on EGF is possibly inherited from their normal stem cell counterpart. Indeed, a series of data support the idea that tumorigenic mutation(s) responsible for colon cancer initiation hit and transform normal colon stem cells: for example, Zhu et al. demonstrated that $CD133^{+}$/LGR5+ intestinal stem cells are sensitive to neoplastic transformation by over-activation of Wnt signaling in an *in vivo* mouse model [23], and Schepers et al. showed by lineage-tracing that Lgr5 marks a subpopulation of adenoma-initiating cells [44].

On the other hand, dependence of normal colonic stem cells on EGFR ligand(s) is indicated by several reports. In fact, EGF is included among the minimal requirements necessary for gut mini-organ development from colon stem cells *in vitro* [119]. Furthermore, it has recently been shown that loss of lrig1, a negative feedback regulator of the ErbB receptor family, leads to a marked increase in crypt size in the small intestine, indicating that ErbB signaling is a strong mitotic signal for ISCs with a key role in intestinal development [120].

Taken together, our data support the hypothesis that, at least in some cases, EGF dependence is transmitted by normal colon stem cells to cCSCs, and propagated to all the later cellular stages included in the tumor, thus conferring Cetuximab sensitivity to all the cells composing the tumor itself. Events disrupting EGFR dependence, such as KRAS G12V mutation, can concurrently or later render cCSCs independent on EGFR and resistant to Cetuximab.

In summary, in this study I have shown that the target-oriented drug Cetuximab, differently than the classical chemotherapeutics currently in use for colon cancer, is able to effectively hit cCSCs population included in the tumors.

I have also shown that panels of cCSCs generated by individual patients do represent predictive tools for preclinical screening of new, pathway-oriented, cancer stem cell-directed therapeutics.

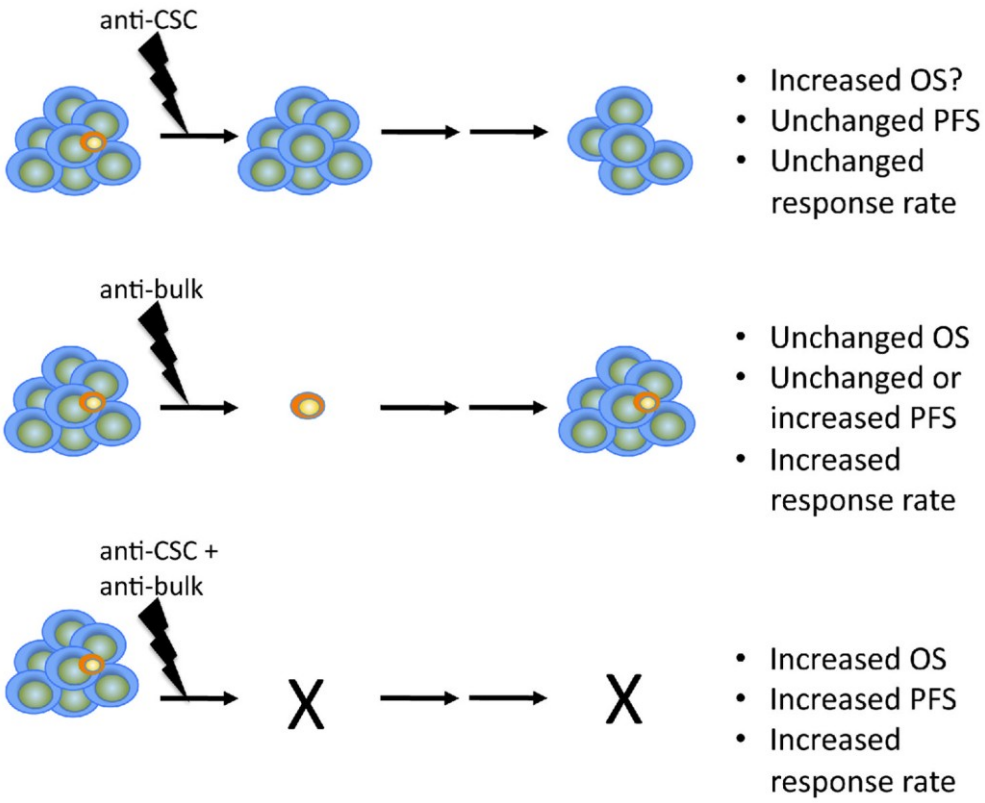

Fig.14 Anticipated clinical outcomes with agents targeting the cancer stem cell population (top), bulk tumor population (middle), or both (bottom). OS -overall survival, PFS –progression free survival.

From: [118]

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