Conditionally reprogrammed cells (CRC) methodology does not allow the in vitro expansion of patient-derived primary and metastatic lung cancer cells

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Novelty and impact:

Conditionally reprogrammed cells (CRC) methodology could represent a potential model for expansion of tumor/non-tumor lung patient cells, promoting the development of lung cancer therapeutics. We found that CRC methodology efficiently established non-malignant lung cell cultures from either tumor and non-tumor samples, while primary or metastatic lung tumor cell growth was not promoted under CRC culture conditions. Thus, CRC approach cannot be exploited for lung cancer therapeutic testing, as established CRC cultures are composed exclusively of (non tumoral) airway basal cells.

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ABSTRACT

Availability of tumor and non-tumor patient-derived models would promote the development of more effective therapeutics for Non Small Cell Lung Cancer (NSCLC). Recently, conditionally reprogrammed cells (CRC) methodology demonstrated exceptional potential for the expansion of epithelial cells from patient tissues. However, the possibility to expand patient-derived lung cancer cells using CRC protocols is controversial. Here, we used CRC approach to expand cells from non-tumoral and tumor biopsies of patients with primary or metastatic NSCLC as well as pulmonary metastases of colorectal or breast cancers. CRC cultures were obtained from both tumor and non-malignant tissues with extraordinary high efficiency. Tumor cells were tracked *in vitro* through tumorigenicity assay, monitoring of tumor-specific genetic alterations and marker expression. Cultures were composed of EpCAM+ lung epithelial cells lacking tumorigenic potential. NSCLC biopsies-derived cultures rapidly lost patient-specific genetic mutations or tumor antigens. Similarly, pulmonary metastases of colon or breast cancer generated CRC cultures of lung epithelial cells. All CRC cultures examined displayed epithelial lung stem cell phenotype and function. In contrast, brain metastatic lung cancer biopsies failed to generate CRC cultures. In conclusion, patient-derived primary and metastatic lung cancer cells were negatively selected under CRC conditions, limiting the expansion to non-malignant lung epithelial stem cells either from tumor and non-tumor tissue sources. Thus, CRC approach cannot be applied for direct therapeutic testing of patient lung tumor cells, as the tumor-derived CRC cultures are composed of (non tumoral) airway basal cells.

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INTRODUCTION

Lung cancer is one of the most common cancers worldwide and the leading cause of cancer-related death for both men and women [1].

Targeted therapies and immune checkpoint inhibitors have significantly improved responses in a fraction of patients with non-small cell lung cancer (NSCLC) [2]. Nevertheless, a majority of patients with lung cancer continue to have very poor outcomes. Current therapeutic options are still unsatisfactory due to intrinsic tumor resistance, short term antitumor efficacy or drug-toxicity. In fact, even though an encouraging initial benefit can occur, it is invariably followed by tumor evolution and progression toward resistant disease[3-5]. The ability to comprehensively characterize the genomic alterations in various subtypes of lung cancer at occurrence or at relapse has the potential to transform cancer care, by facilitating the identification of novel treatment strategies [6, 7].

Innovative personalized therapeutic approaches, in the field of deep molecular analysis-guided targeted therapy and immunotherapy or, more likely, combinations of multi-disciplinary approaches may represent a promising strategy to improve future lung cancer treatment [8]. Preclinical models of lung cancer may provide great hints to develop, test and validate potentially efficient and durable therapeutic strategies, thus generating advances in personalized medicine [9]. The establishment of cancer and non-malignant cell *in vitro* models from the same patient would be particularly useful in order to identify and validate new genetic anomalies, neo-oncoantigens exploitable for immunotherapy, or to test tumor-specificity of targeted compounds or therapeutic monoclonal antibodies. Thus, particular effort has been recently employed toward the establishment of patient derived xenografts (PDX) together with the *in vitro* expansion of patient material [9]. Both approaches need to be carefully validated to avoid experimental pitfalls, as demonstrated for the requirement of specific mouse background for optimal PDX obtainment, whose patient-like nature needs to be always verified [10, 11]. Conditionally reprogrammed cells (CRC) methodology has been recently described as an efficient system to expand tumor and non-tumor epithelial cells from various tissues *in vitro*, particularly prostate and breast cancers [12-14]. In co-culture with irradiated mouse fibroblasts as feeder layer and in the presence of the Rho kinase (ROCK)-inhibitor Y-27632 adult epithelial cells and cancer cells from epithelial tumors have been shown to rapidly generate exponentially growing cultures and might represent an excellent model for preclinical testing of anticancer compounds [14, 15]. These

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conditions that induce reprogramming of differentiated epithelial cells associated to active cell proliferation have been shown to generate long-term cultures of epithelial "stem like" cells [13]. Airway epithelial cells have been expanded from multiple districts of healthy or pathologic human airway epithelium including lung and trachea, and displayed stem cell properties, suggesting the possibility to exploit the CRC procedures also to obtain lung cancer cell cultures [16-19]. However, controversial results have recently emerged concerning the possibility to obtain tumor cell cultures from patient tumor biopsies under CRC conditions, including from lung tumors, as some studies reported the obtainment of cultures composed by tumor cells or non-tumoral cells from the tumor and healthy tissue, respectively, while others reported the selective isolation of non-tumoral cells from either sources and others suggested that mixed cultures could be generated [12-14, 20].

Here, we applied the CRC system to determine whether it may represent a reliable *in vitro* culture model to obtain matched epithelial stem cells from tumor and non-tumoral tissues of lung cancer patients. CRC conditions generated long term-cultures of non-malignant epithelial cells with high efficiency, from both healthy tissue and tumor biopsies. Similarly to primary lung tumors, pulmonary metastasis derived from colon or breast cancers failed to generate tumor cell cultures, as lung resident non-tumoral epithelial cells preferentially grew under CRC conditions, generating lung epithelial cell cultures. Lung cancer cell expansion was not simply overwhelmed by the fast-proliferating non-tumoral cells, as also lung cancer brain metastases or patient-derived xenografts (PDX), that are devoid of non-tumor epithelial cells, failed to generate tumor cell cultures under CRC conditions. In contrast, CRC cultures of non-malignant lung cells were obtained with high efficiency and displayed lung epithelial stem cell phenotype and properties, compatible with basal cells, including the ability to differentiate into various mature respiratory cells.

Thus, CRC approach, cannot be exploited for the establishment of matched tumor and non-tumoral cells from lung cancer biopsies for the development of antitumor therapeutics, as this system exclusively allows the long-term expansion of non-malignant lung epithelial stem cells.

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MATERIALS AND METHODS

In vitro expansion of epithelial cells from tumor and non-tumoral lung tissues.

Primary lung cancer and metastatic colon and breast tumors as well as healthy lung patient tissues were obtained in accordance with consent procedures approved by the Internal Review Board of Department of Laboratory Medicine and Pathology, Sant'Andrea Hospital, University La Sapienza, Rome. Lung cancer brain metastases were obtained in accordance with consent procedures approved by the Internal Review Board of Department of Neurosurgery, Catholic University, Rome, Italy. Tissue dissociation was performed as previously described [21, 22]. Recovered cells were cultured in conditionally reprogrammed cells (CRC) methodology according to Liu et al [13]. Briefly epithelial cells were co-cultivated with irradiated murine J2 Swiss 3T3 fibroblasts (Kerafast) in the presence of 10μM Rock inhibitor Y-27632 (Selleck), in Fmedium (3:1 v/v F-12 Nutrient Mixture Ham: DMEM) supplemented with 5% FCS, 0.4μg/ml hydrocortisone, 5μg/ml insulin, 24μg/ml adenine, 8.4ng/ml cholera toxin, 10ng/ml EGF. Fibroblasts were cultured in 10% Characterized HyClone™ Fetal Bovine Serum (U.S.) and irradiated when reached 70% confluence. All cells were maintained at 37°C in a humidified incubator, with 5% CO2.

Flow cytometry, Immunofluorescence and immunohistochemistry

Cells and tissues were stained as described in Supplementary Information.

In vitro differentiation of CRC

To induce differentiation, CRC were deprived of feeder layer and grown in Bronchial Epithelial Cell Growth Medium (BEGM, Lonza) for 2 weeks before analysis.

Ali-liquid interface (ALI) cultures

To induce differentiation, $2x10^5$ CRC were cultured for 1 week in PneumaCult–Ex Medium (Stem Cell Technologies), until confluence was reached. Then, $1,5x10^5$ cells were plated in Corning 3460 inserts, and cultured in PneumaCult –Ex Medium in both basal and apical chambers for 4 days, afterwards medium was replaced with PneumaCult –ALI Medium in the basal chamber, leaving the apical chamber empty for 28days.

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Proliferation assays of CRC

To determine cell growth rate of CRC cultures 100000 cells were plated on feeder layer in complete growth medium and counted by trypan blue exclusion every 6 days. Proliferation index was calculated as population doublings (PD) using the following formula: PD=3.32(log cell number counted /cell number plated at day0).

Generation of subcutaneous xenografts in NSG (NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ) *mice*

Five hundred thousand cells were mixed 1:1 with matrigel and injected in the flank of NSG mice. Three injections were performed for each sample of cultured cells, while a single injection was performed for freshly dissociated tumor samples.

DNA extraction and mutational analysis

DNA was extracted from patient tissue or from 100000 cultured cells with DNA Easy Blood and Tissue kit (Qiagen) and subjected to next generation sequencing using Ion AmpliSeq™ Colon and Lung Cancer Research Panel v2 (Life Technologies).

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RESULTS

Conditionally reprogrammed cells (CRC) cultures generated from healthy and tumor lung tissue are composed of lung epithelial cells lacking tumorigenic potential

The aim of this study was to determine whether the CRC methodology may represent a reliable *in vitro* culture model to obtain matched tumoral and non malignant epithelial cell cultures, respectively from tumor and non-tumoral lung patient tissues. Paired primary lung tumor and healthy lung tissue specimens were obtained from 19 non small cell lung cancer (NSCLC) patients that underwent therapeutic surgical resection (Table 1). In the attempt to establish tumor and non-tumoral cultures *in vitro*, freshly dissociated cells from squamous cell carcinoma (SCC), adenocarcinoma (AC) large cell neuroendocrine carcinoma (LCNEC), carcinoid tumors (CT) and from their corresponding non-malignant tissues, collected distant from the tumor, were seeded in CRC conditions, medium was replaced twice a week until the cells reached confluence (approximately 2 weeks, passage1) and then passaged twice a week with new feeder layer as previously described [13]. Initial phases of tumor sample-derived cultures generated highly heterogeneous cell populations followed by more homogeneous cell populations in the next weeks. A morphologically homogeneous culture was completely reached already at passage 2 (3-4 weeks) (Figure 1A). Evaluation of marker expression was performed at passage 2 and showed that cultured cells displayed epithelial phenotype (approximately 90% of $EpCAM^+$ cells) and were almost free of human fibroblast contamination (<2% of $CD90^+$ cells), since growth of these cells was not favored under CRC conditions (Figure 1B top panels). Feeder cells represented a minuscule fraction (<3% murine MHC^+ cells), thus not affecting subsequent analyses (Figure 1B bottom panels). CRC displayed prominent and prolonged growth ability as shown for the 30 population doublings measured in approximately 30 days of cell proliferation monitoring, proving that CRC system may represent optimal growth conditions for prolonged expansion of epithelial cells from patient samples (Figure1C). Some cultures were monitored for at least 4 months and their growth rate did not decrease over time. Moreover, non malignant tissue- and tumor-derived cells displayed a highly comparable growth rate. These results suggested an apparent morphological, phenotypical and functional similarity among the tumor and non tumor-derived CRC cultures.

Given that non-tumoral and tumor tissues were obtained from different portions of the same surgical specimen, the possibility that rare tumor cells were disseminated within non-

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tumoral surrounding tissue, could not be excluded. If present they could potentially overgrow in culture due to proliferative advantage expected for their malignant phenotype. On the other hand, previous reports showed that proliferation of non-malignant epithelial cells is promoted under these culture conditions [20]. In order to determine the malignant or non-malignant nature of CRC cultures, their tumorigenic potential was evaluated following subcutaneous injection in NSG mice. In view of the fact that the efficiency of PDX obtainment from fresh samples was approximately 40%, tumorigenicity assay was evaluated only with CRC corresponding to patient samples that had shown the ability to generate PDX when injected under the same conditions. In contrast to $5x10^5$ fresh tumor cells, that generated patient-like tumors within 1-2 months post-injection, cultured CRC (up to $1x10^6$ cells) either originating from tumor and non-tumor tissue, were never able to generate a tumor *in vivo* (up to 5 months monitoring), suggesting that CRC cultures were composed of non-tumorigenic lung epithelial cells (Figure 1D). These results implied that tumorigenic cells originally present within the patient tumor sample were unable to proliferate and establish a culture under CRC conditions.

CRC-cultures generated from different types of lung cancers rapidly lose patient-specific genetic mutations or tumor specific antigens due to selective growth of healthy pulmonary cells

To clarify whether the tumor tissue-derived cultures were composed of tumor cells that had lost their tumorigenicity upon *in vitro* expansion or derived from the selective proliferation of non-malignant epithelial cells dispersed within the tumor tissue, we identified genetic marks of tumor cells, allowing to discriminate among paired tumor and non-tumoral cells and to track tumor cells within CRC cultures. Ion torrent next generation sequencing (Ion AmpliSeq™ Colon and Lung Cancer Research Panel V2 , Life Technologies) of tumor and non-tumor patient tissues and their corresponding CRC at passage-2 and -4 showed that most patient tumor samples contained 2 of the 22 hotspot gene mutations, representing a suitable genetic signature exploitable to track cancer cells in culture at early and late passages (Table 2). No mutations were detected in the corresponding non tumoral tissue (except for a MET mutation present in both tumor and non-tumor patient tissue, in line with a germline heterozygous mutation) thus validating the nature of patient samples (Table2). Tumor-derived passage-2 CRC displayed barely detectable/lack of genetic mutations, that completely disappeared at passage 4 in all samples, thus confirming the hypothesis that

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tumor cells were negatively selected and rapidly lost during CRC culture, in favor of an efficient expansion of non-malignant (mutation negative) cells (Table 2).

Time course analysis of DNA samples confirmed the rapid reduction of the mutation-positive tumor cell fraction within cultures, in agreement with their counter-selection due to concomitant overgrowth of non-tumoral cells. Mutation-bearing cells were undetectable in passage 4-CRC cultures expanded from originally-mutated patient tumor samples, proving that the initially highly heterogeneous cell culture rapidly originated a virtually pure population of non-malignant cells.

In addition to patient-specific genetic lesions, CRC cultures generated from neuroendocrine lung tumors lacked the diagnostic marker CD56, found in the tumor of origin, further confirming that CRC system, did not allow the growth of tumor cells (Figure 2A). Similar results were found for another neuroendocrine lung tumor, a CD56+ carcinoid that generated CD56-negative CRC cultures. Time course cytofluorimetric analysis showed a rapid decrease of CD56+ cells (5.8% at passage 1 and 0.2% at passage 2) (Figure 2B) in line with a tumor cell-depleting culture. Tumor cells survived for several weeks as adherent clones, that appeared less vital after few weeks and were gradually lost due to the growth of surrounding non-tumoral epithelial lung cells (Supplementary Figure 1A).

CRC cultures derived from pulmonary metastases of colon or breast cancers are composed by lung epithelial cells

Finally, CRC obtained from three samples of pulmonary metastases of $CK20^+$ colorectal cancers (Table 1) lacked the colon cancer-specific marker CK20 while expressed lung tissue antigen CK7, as expected for cells of lung origin and not for colon derived-cells (Figure 2C right panels), as the two antigens may contribute to differential diagnosis of tumors of colon or lung origin [23-25] and in line with immunohistochemistry analysis of the corresponding patient tissues where, clearly, colorectal cancer metastasis is $CK20^+/CK7^-$, while the surrounding patient lung tissue is $CK20^-/CK7^+$ (Figure 2C left panels). Supplementary Figure 1B shows the expected positivity of control colon cancer cells for CK20 and lung cancer cells for CK7 antibody [21, 22, 26]. Time course flow cytometric analysis of CK20 and CK7 expression revealed a rapid and progressive reduction of $CK20^+$ cells at passage 2, that completely disappeared at passage 4 and a concomitant massive increase

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of $CK7^{+}$ cells, constituting the predominant cell population at passage 2 and more than 90% of cells at passage 4 (Supplementary Figure 2C). These results showed that pulmonary metastases of colorectal cancers, generated CRC-cultures composed of pulmonary epithelial cells, presumably derived from the proliferation of lung cells resident in the metastasized respiratory tissue. Expression of CK5 and CK14 confirmed that these CRC cultures were composed of cells compatible with airway basal cells [16] (Supplementary Figure 1D). In line with these results, we found that mutation-positive colon cancer cells were progressively lost with culture as shown by low (p4) and undetectable (p8) fraction of the p53/NRAS/ErbB4 mutations-bearing cells observed through next generation sequencing (Table 2B). These results enforce the assumption that tumor cells are lost under CRC culture conditions, excluding the possibility that CRC cultures may be composed of tumor cells lacking the genetic mutations and endowed with a possible growth advantage over mutation-positive tumor cells. Similarly, CRC obtained from pulmonary metastasis of c-erbB2^{+++} breast cancer lacked the specific diagnostic marker (Figure 2D).

The final evidence that CRC methodology is suitable for the growth of non-malignant epithelial respiratory cells was that epithelial CRC cultures could be obtained from healthy lung tissue derived from non oncologic patients (Figure 2E).

Lung cancer cell cultures cannot be established from metastatic lung cancers or from patient-derived xenografts, under CRC conditions

In order to test whether the lack of tumor cells in passaged CRC cultures was due to the predominant growth of non-tumoral epithelial cells or to inability of lung tumor cells to survive and proliferate under CRC conditions, we evaluated the possibility to generate CRC cultures from lung tumor samples devoid of non-tumoral pulmonary epithelial cells, i.e. from lung cancer metastasis in non-epithelial tissues (brain) and from lung cancer patient derived xenografts (PDX) as non-tumoral human epithelial cells are lost following *in vivo* tumor passage. Two lung cancer brain metastases and five PDX (3 AC and 2 SCC subtype) samples were analyzed in these experiments. Metastatic lung tumor cells formed adherent clones and survived for several weeks under CRC conditions, however they did not actively grow and appeared gradually less healthy, in contrast to a fraction of the same cells cultured under standard conditions for cancer stem cells (CSC), demonstrating high

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viability and proliferative capacity of the tumor cells, when cultured under appropriate conditions [21, 22]. The fraction of CEA^+ tumor cells, highly abundant in the patient tumor (Figure 3A top panels) as well as in the CSC cultures, gradually decreased with CRC culture passages (in the first weeks) and after 5 weeks CRC cultures completely lacked CEA-positive tumor cells and the exon 19 EGFR deletion originally detected in the patient tumor (as shown in Supplementary Figure 2 murine feeder layer cells represented the only cell population detectable), and retained under CSC culture conditions (Figure 3A lower panels and *Supplementary Information*). Similarly, the human epithelial antigen EpCAM, marking freshly dissociated PDX cells, was gradually lost in culture, and after prolonged time (5 weeks) the cell population resulted composed exclusively of murine cells (human HLA negative) (Figure 3B). Thus, CRC methodology did not allow the expansion of lung tumor cells even in the absence of the growth-competitors fast-proliferating lung epithelial cells, demonstrating that CRC methodology does not represent a suitable model for supplying tumor cells from lung cancer patients suitable for research and therapeutic purposes.

CRC cultures are composed of lung epithelial stem-like cells with multiple differentiation potential

Other studies have shown that conditionally reprogrammed cells obtained from epithelial tissues represent a stem-like state of adult epithelial cells [27]. To determine if lung tissue-derived CRC cultures were composed of stem-like cells we compared them with the same cells cultured under standard conditions for pulmonary cells (BEGM). CRC displayed nuclear retention of β-catenin, higher expression of antigens associated with adult epithelial stem cells such as Integrin α6, Integrin β1 and NGFR, confirming a stem-like phenotype of CRC cultures, in agreement with previous results (Figure 4A-B) [17, 27]. Prolonged culture of CRC under Air-Liquid Interface culture conditions, allowed their differentiation into mature respiratory cells, as shown by the expression of lineage specific markers for ciliated cells (acetylated α-tubulin) and goblet cells (mucin 5B) after 4 weeks of differentiation (Figure 4C). In the orthogonal projection image acetylated tubulin and mucin 5B antigens are visible at the apical surface of the monolayer-forming cells as compared with basal nuclei staining, compatible with the expected cilia and mucin 5B localization.

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DISCUSSION

Although targeted therapy has improved lung cancer patient clinical management and course, wide effort is still required for the long term outcome improvement, as most tumors initially responding to targeted agents, invariably develop resistance mechanisms[6]. New targets need to be identified both in treatment-naive patients as well as to escape treatment-induced tumor progression and resistance. The possibility to expand patient tumor material *in vitro* associated with paired non-malignant samples of the same patient would represent an outstanding tool to identify tumor specific drug-targetable mutations or new tumor antigens for immunotherapy and to identify treatment escape mechanisms that concur to tumor resistance, in order to exploit them as new targets to circumvent drug-resistance. Recent studies reported the possibility to establish *in vitro* culture of tumor and non-tumor patient-derived cells with high efficiency [12, 13]. In co-culture with irradiated mouse fibroblasts as feeder layer and in the presence of the Rho kinase (ROCK)-inhibitor Y-27632 adult epithelial cells and cancer cells from epithelial tumors have been shown to generate exponentially growing cultures [14, 15]. CRC cultures of airway epithelial cells have been obtained from multiple districts of the human airway epithelium including lung and trachea, and displayed stem cell properties [16, 17]. However, controversial results have recently been reported concerning the possibility to obtain tumoral and non-tumoral cell cultures from lung cancer patient biopsies under CRC protocols [12, 14, 20]. In fact, while some studies have reported the possibility to expand both non tumor and tumoral cells under CRC protocols, others have clearly demonstrated that only non-malignant cell cultures can be obtained both from healthy lung tissue as well as from lung cancer specimens [20]. Thus, the possibility that only non-malignant cells contained within patient tumor biopsies are able to proliferate under CRC conditions has been reported and very recently the possibility that mixed cultures can grow under these conditions for some tumor types has been taken into consideration [12, 20]. Here, we applied the CRC methodology with the aim to deeply investigate and determine whether it may represent a reliable *in vitro* culture model to obtain matched epithelial stem cells from non-tumoral and tumor tissue from lung cancer patients.

The CRC methodology proved to be exceptionally efficient in the establishment of cell cultures, in fact approximately 100% patient samples yielded exponentially growing long term cultures of epithelial cells (EpCAM positive) (Figure 1). However, our results clearly

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demonstrated that cell cultures established from primary lung tumors were not composed of tumor cells but, they were virtually pure populations of non-malignant cells originated by the preferential overgrowth of non-malignant epithelial cells, originally contained within the tumor. Rapid growth of pulmonary epithelial cells present within tumors determined tumor cell fraction decrease and eventually loss after few passages in culture, non only in the case of lung cancers, but even in pulmonary metastases of colon or breast cancers, suggesting that CRC approach is highly suitable for the growth of respiratory cells that predominate over tumor cell types of various origin and aggressiveness. Finally, we analyzed whether in the absence of lung epithelial "competitor" cells, the establishment of a tumor cell culture could be obtained, although at lower proliferation rates. However, even in the epithelial cell-free lung cancer brain metastases or in the case of lung cancer PDX (that after *in vivo* passage are devoid of non-tumor cells), malignant cells survived but did not proliferate to form CRC cultures, and after several weeks, only murine cells (murine MHC^+/human HLA^-) cells were detectable in cultures. In contrast, a fraction of tumor cells obtained from the same sample was able to grow under conditions suitable for malignant cells, proving their viability and capacity to proliferate when appropriate methodologies are applied.

The presence of lung cancer-associated genetic defects was exploited to track tumor cells *in vitro*. Multiple mutations were considered in order to exclude the possibility that a single mutation present in a small fraction of tumor cells could be lost due to the possible growth advantages of other mutation-negative tumor cell fractions. Moreover, the detection of tumor-restricted markers expressed on the majority of tumor cells and not on non-malignant lung cells were used as additional tracking system, as in the case of $CD56^+$ neuroendocrine lung tumors, or pulmonary metastases of $CK20^+$ colon cancers and HER2-overexpressing breast cancers. Similarly, metastatic lung cancer could be easily tracked within the epithelial cell marker-negative brain microenvironment or, in the case of lung cancer PDX, human antigens-positive cancer cells could be distinguished from murine cells.

In agreement with other reports showing that CRC represent a stem-like state of adult epithelial cells we found that the expression of antigens related to the stemness of lung epithelial cells, as Integrin $\alpha 6$, Integrin $\beta 1$ and NGFR was increased in these cultures compared to the same cells grown under standard conditions (Figure 4). Finally, Air-Liquid Interface culture methodology revealed the multiple differentiation potential of CRC

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cultures, in fact CRC cultivated for a prolonged time under these conditions expressed markers of mature ciliated and goblet cells, in agreement with previous reports (Figure 4) [20].

In conclusion, our results showed that CRC system cannot be exploited to expand primary or metastatic lung tumor cells for the direct improvement/testing of anti-cancer strategies with great relevance for preclinical oncologic experimentation. However, it remains possible that tumor cells of different origin might be manageable to culture under these conditions (preferentially if not localized in the lung tissue). On the other hand, we found that, this methodology can provide a powerful unprecedented *in vitro* model of lung stem cell expansion, possibly providing new hints to understand cellular transformation, The exceptional success in culture establishment of stem cell with multiple differential potential from patient material would greatly imply CRC methodology applications for personalized therapy of various non oncologic pulmonary pathologies, possibly constituting suitable *in vitro* models or models for cellular therapy for cystic fibrosis, idiopathic pulmonary fibrosis or other severe lung diseases [19, 28].

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FIGURE LEGENDS

Figure 1. *Phenotype and functional analysis of conditionally reprogrammed cells (CRC) obtained from tumor and non-tumoral lung tissue.* A) Images showing the morphology of CRC cultures obtained from tumor (T) and non tumoral (N) tissues at passage 2 (3 weeks culture). B) Flow cytometric analysis of CRC for the expression of epithelial (EpCAM), human fibroblasts (CD90) or murine stroma (mMHC) markers, as indicated. The flow cytometry analysis is representative of 15 samples analyzed. C) Population doublings of CRC obtained from one matched tumor (T) / non tumoral (N) tissue sample monitored from passage 2 for 30 days. Standard error bars are relative to three wells replicates counted at each time point. Paired growth curves are representative of 3 patient-derived matched samples. D) Tumorigenic ability of freshly-obtained patient tumor cells (T1) or CRC expanded from tumor (T1-CRC) or non-tumoral (N1-CRC) tissue derived cells. Tumor growth was monitored over 13 weeks, as indicated. The assay is representative of 5 paired CRC samples analyzed.

Figure 2 *CRC-cultures rapidly lose tumor-specific antigens within few passages due to selective growth of healthy pulmonary cells*. A) Hematoxilyn and Eosin (HE) or CD56 immunoistochemistry of a $CD56^+$ Large Cell Neuroendocrine Carcinoma (T6-pt) and CD56 flow cytometric analysis of CRC obtained from tumoral (T6-CRC) and non-tumoral (N6-CRC) tissue of the same patient. Glioblastoma stem cells were used as positive control for CD56 expression. B) Diagnostic CD56 immunoistochemical analysis of a Carcinoid Tumor (T32-pt) and CD56 flow cytometric analysis of the corresponding CRC (T32-CRC) obtained at passage 1(p1) or 2 (p2). C) Hematoxilyn and Eosin (HE), or diagnostic CK20 and CK7 immunoistochemistry of pulmonary Colon Cancer metastasis (T4-pt) and CK20 and CK7 flow cytometric analysis of CRC obtained from tumoral (T4-CRC) and non-tumoral (N4-CRC) tissues of the same sample. D) c-ErbB2 immunoistochemistry of pulmonary breast cancer metastasis (T28-pt) and immunocitochemistry of tumoral (T28-CRC) and non-tumoral (N28-CRC) CRC-derived Cell Blocks obtained from the same patient. E) Pan-CK and CK7 flow cytometric analyses of CRC obtained from non oncologic patient tissues (representative of 3 samples).

Figure 3. *Tumor cell cultures cannot be established from metastatic lung cancers and from PDX under CRC methodology*. A) (left) Diagnostic Hematoxylin and Eosin staining (HE)

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and immunoistochemistry for CEA of Lung Cancer brain Metastasis (T31). (Middle) CEA flow cytometry of patient tumor cells cultured in CSC or CRC conditions as indicated. (Right) Detection of EGFR Ex19 deletion in patient tumor cells cultured in CSC or CRC conditions. Comparison of database EGFR sequence with sequence of PCR-amplified EGFR cDNA of CSC or CRC. B) Flow cytometric time course (week 0 to 5) analysis of EpCAM expression in CRC cultures obtained from T7 patient-derived xenograft cells. HLA flow cytometric expression on the same cells performed after 5 weeks culture.

Figure 4. *CRC display stem cell phenotype*. A) β-Catenin immunofluorescence of CRC and the same cells (BEGM) cultured in standard conditions for primary bronchial epithelial cells. Nuclei in immunofluorescence images are fluorescently blue-labeled with DAPI and β-catenin with green Alexa Fuo 488. B) Flow cytometry for integrin-α6, -β1 and NGFR expression on CRC (red) and BEGM cells (light gray). Negative control antibody staining is indicated in black. C) Confocal microscopy of CRC differentiated in Air-liquid interface culture for 28 days, fixed and fluorescently labeled with DAPI (nuclei), or antibodies for α-tubulin-Alexa Fluor 488 (ciliated cells) or MUC5B-Alexa Fluor 647 (goblet cells). The upper stripe (XZ) represents an orthogonal projection image where nuclei are visible at the bottom and stained antigens at the apical surface of the monolayer-forming cells.

Table 1. *Clinical staging, classification and diagnostic markers of primary lung tumors, pulmonary metastases of colon and breast tumors and lung cancer brain metastases.*

Table 2. *CRC-cultures rapidly lose patient-specific genetic mutations within few passages in culture due to selective growth of healthy pulmonary cells*. Next generation sequencing of 22 lung and colon cancer associated genes (Ion AmpliSeq™ Colon and Lung Cancer Research Panel v2) performed on DNA isolated from tumor (T) and healthy (N) lung (A) or colon (B) patient tissues (pt) and from corresponding CRC at passage (p) 1, 2, 4 or 8, as indicated.

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A

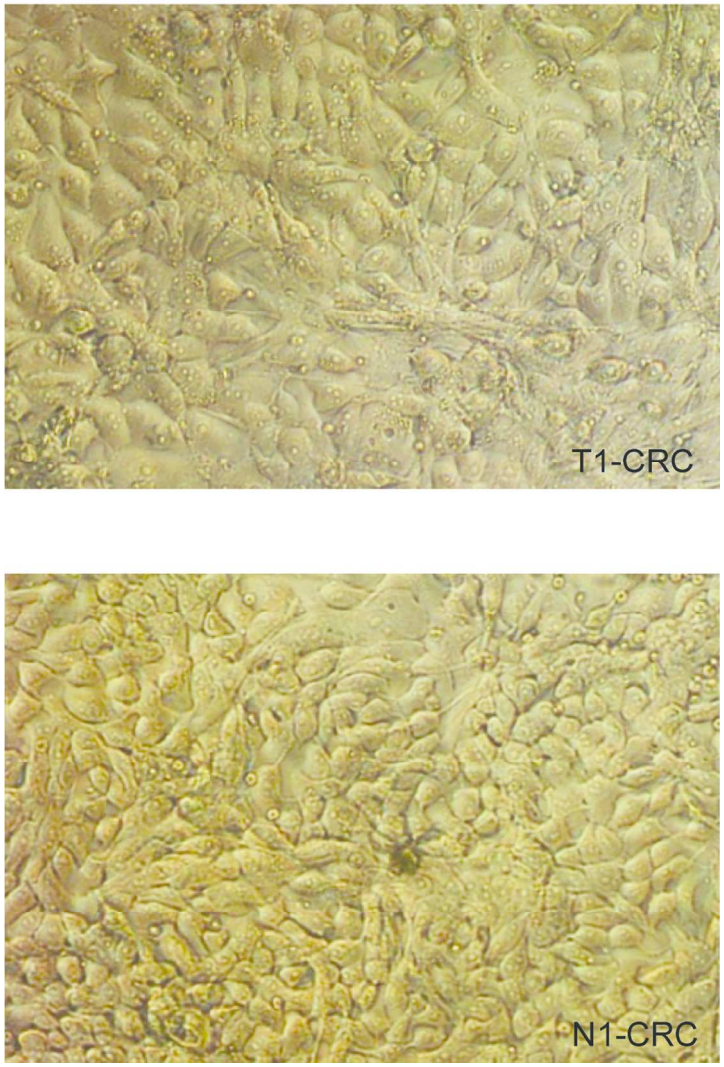

Figure_1A

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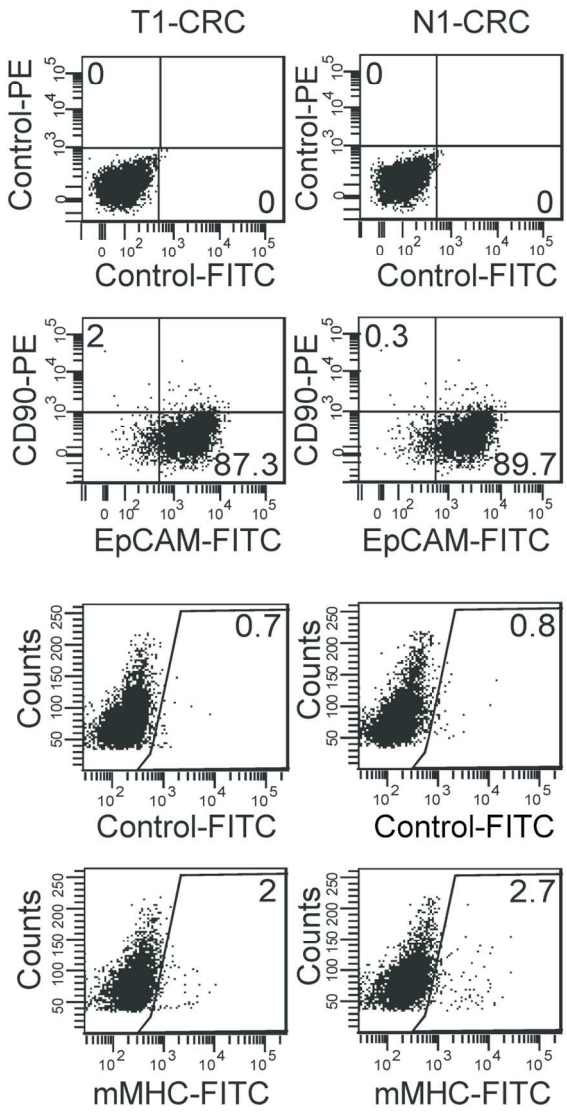

Figure_1B

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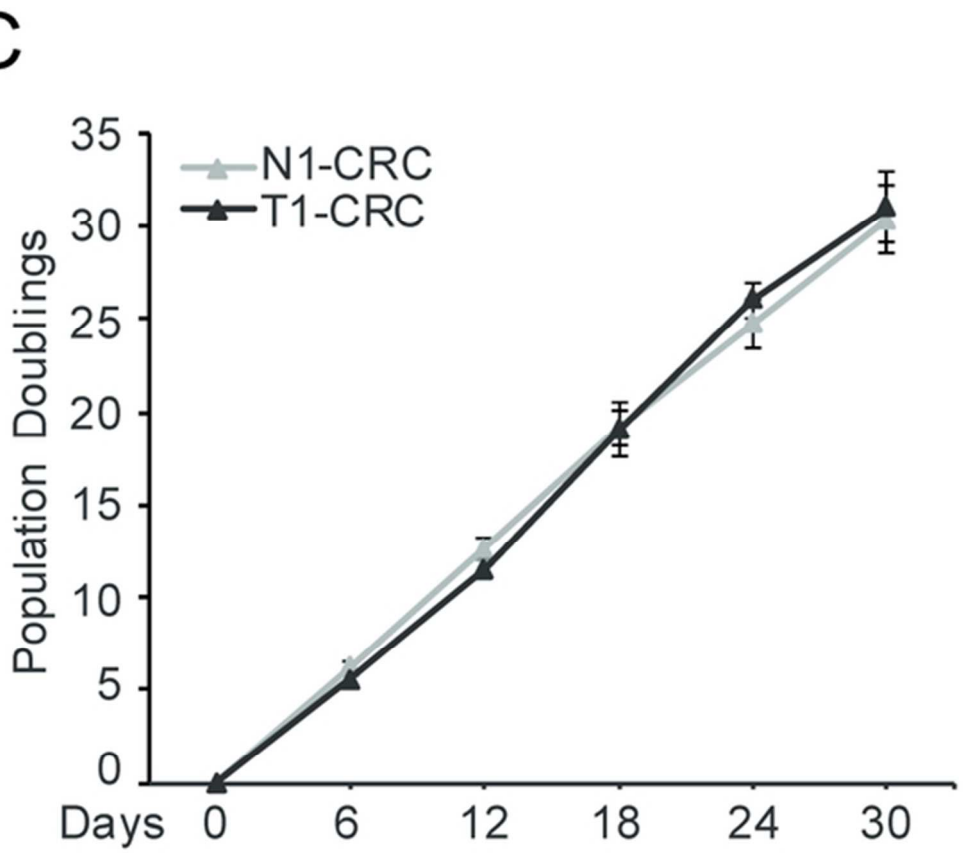

Figure_1C

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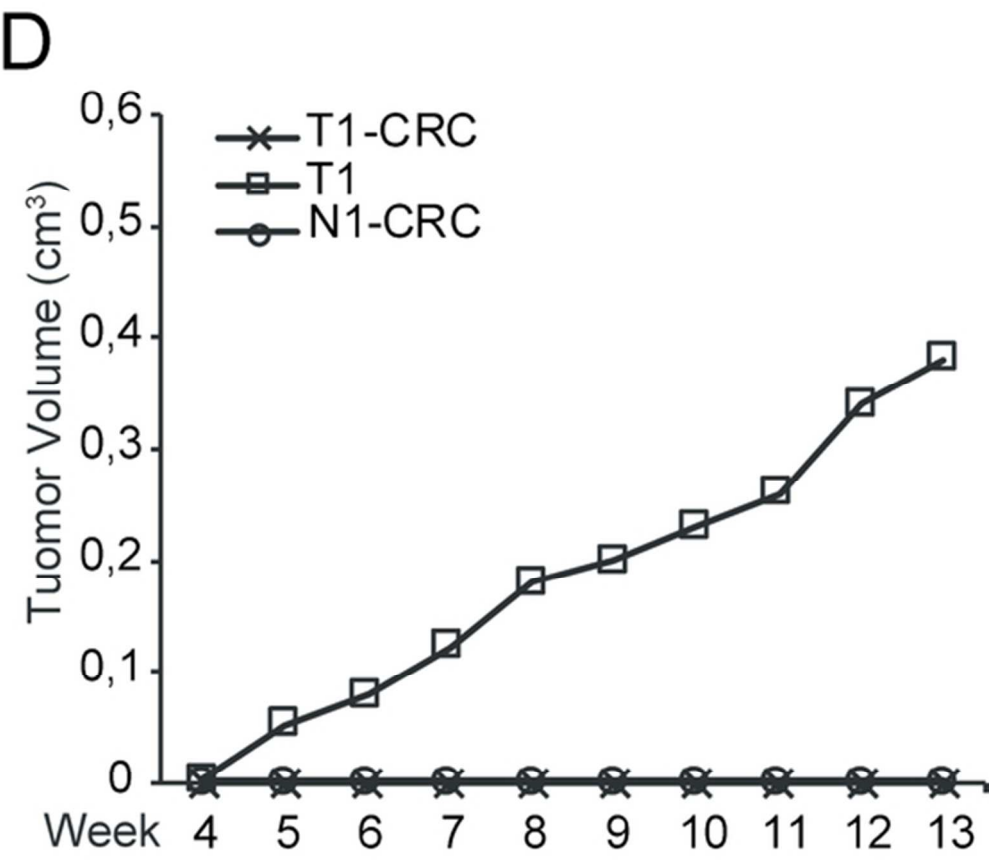

Figure_1D

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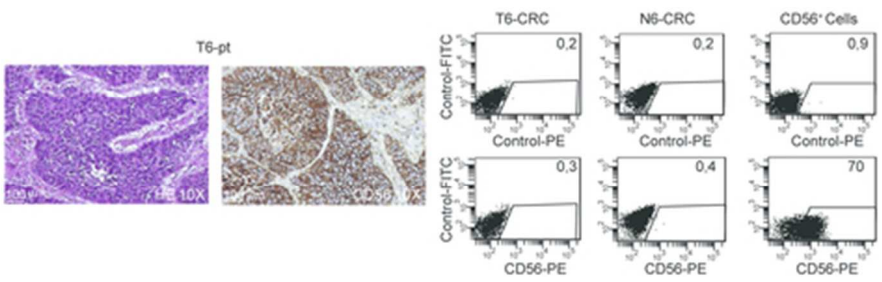

Figure_2A

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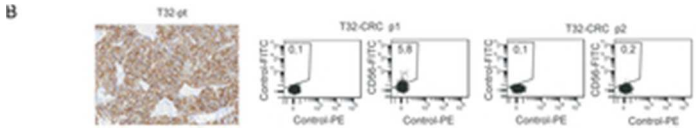

Figure_2B

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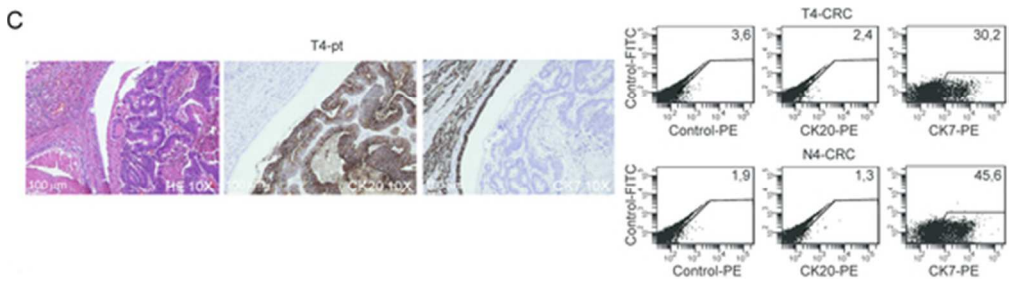

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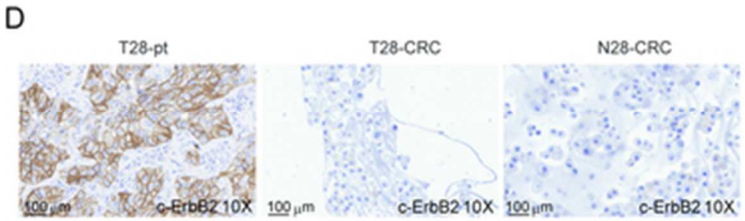

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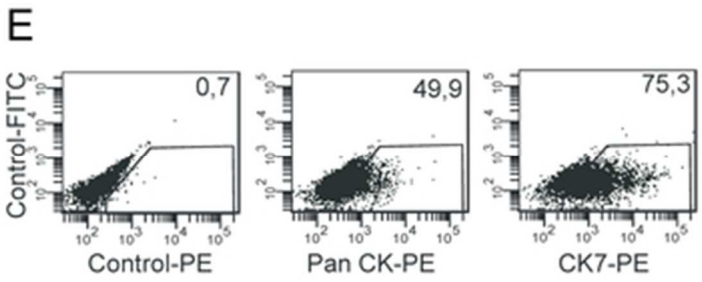

Figure_2E

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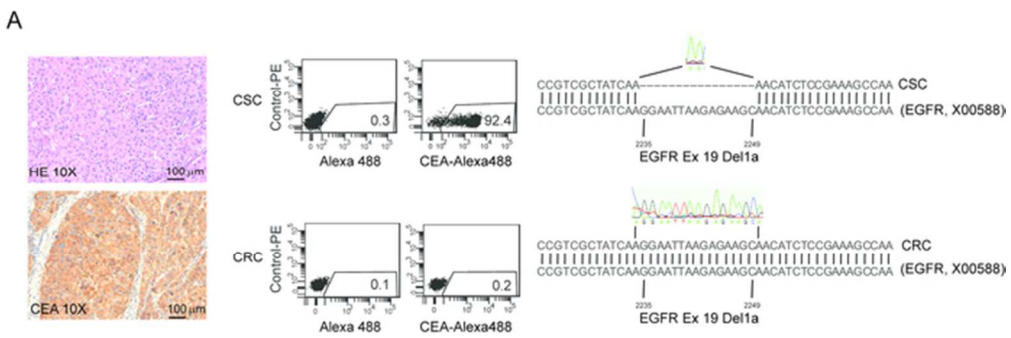

Figure_3A

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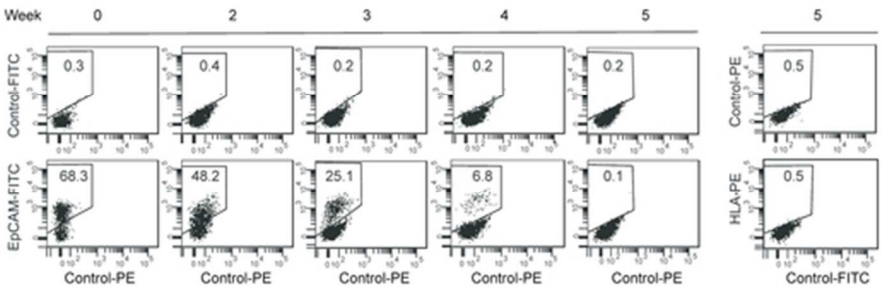

Figure_3B

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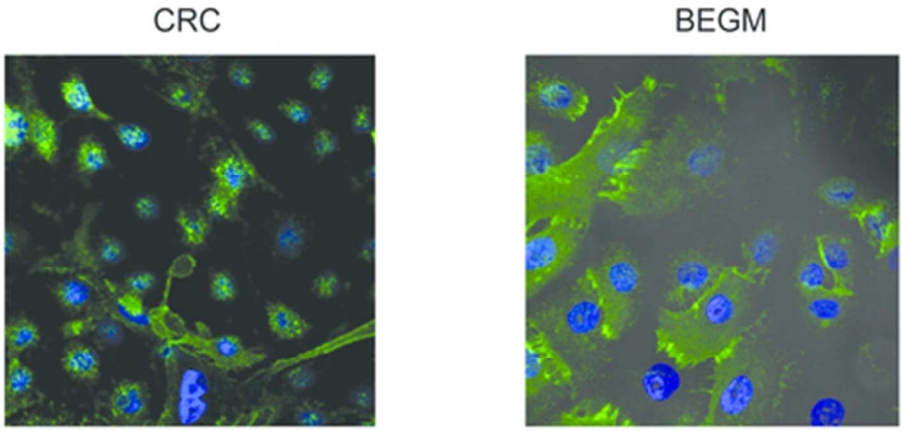

Figure_4A

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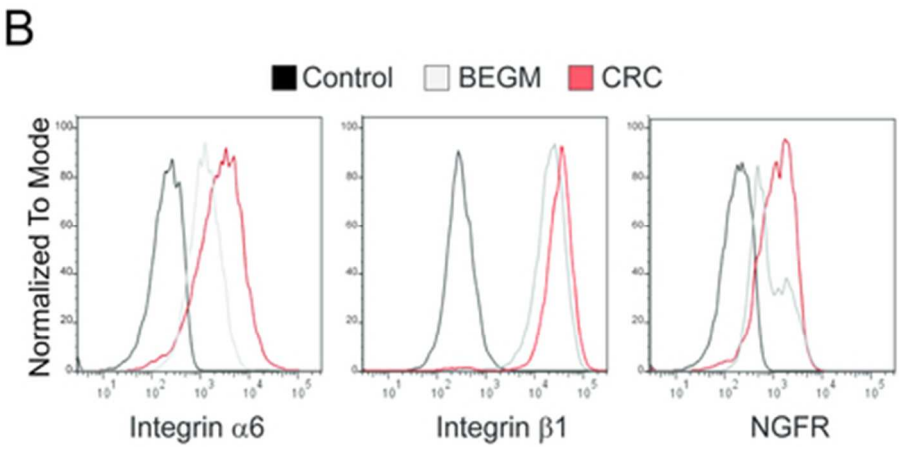

Figure_4B

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C

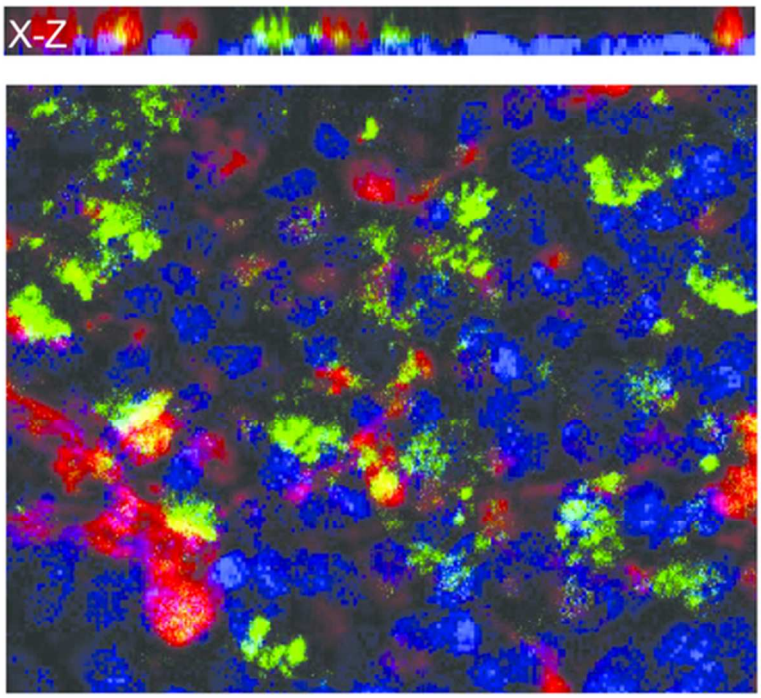

Figure_4C

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Primary tumors of the lung				
Sample	Patient (sex/age)	Tumor subtype	TNMstage/grading	Diagnostic markers expression
#1	M/67	AC	pT3 pN2-IIIA/G3	TTF1+, NapsinA+, p63-, ALK-, ROS1-
#2	M/69	SCC	pT1a pN0-IA/G2	p63+, CK7- ,TTF1-
#5	M/59	AC	pT2a pN2-III A/G3	ALK-, ROS1-
#6	M/71	LCNEC		pan-CK (AE1-AE3)+, CD56+, TTF-1-, CromograninA-, Synaptofisin-, p63-
#7	M/76	AC	pT2a pN2-III A/G1	TTF1+, ALK-, ROS1-
#8	F/60	AC	pT3 pN2-III A	TTF1+
#16	M/73	AC	pT2a pN0-IB/G3	TTF1+, ALK-, ROS1-
#18	M/66	SCC	pT3 pN0-IIB/G3	p63+, CK7- ,TTF1-
#19	M/55	AC	pT2a pN2-IIIA/G3	TTF1+, ALK-, ROS1-
#20	F/67	SCC	pT1b pN1-IIA/G2	p63+, CK7- ,TTF1-
#21	M/48	AC	pT1b pN0-IA/G1	TTF1+, NapsinA+, ALK-, ROS1-
#22	F/65	AC	pT2a pN2-IIIA/G2	TTF1+, ALK-, ROS1-
#23	M/63	AC	pT2a pN2-IIIA/G3	TTF1+, ALK-, ROS1-
#25	F/75	AC	IV	TTF1+, ALK-, ROS1-
#29	F/69	AC	pT2a pN0-IB	CK7+, TTF-1+, NapsinA+, p63+ CK5/6-
#30	F/73	AC	pT1b pNo-IA	TTF1+, NapsinA+
#32	M/76	Carcinoid	pT1a pN0-IA	CD56+, Synaptofisin+
#33	M/73	SCC	pT2a pN0-IB	p63+, CK7- ,TTF1-
#35	M/71	SCC	pT3 pN0-IIB	p63+, CK7- ,TTF1-
Pulmonary metastases				
Sample	Patient (sex/age)	Tumor type	stage	Diagnostic markers expression
#4	M/65	Colon cancer	IV	CK20+, CDX2+, TTF-1-, CK7-, NapsinA-
#26	M/76	Colon cancer	IV	CK20+, CDX2+, TTF-1-, CK7-, NapsinA-
#27	M/83	Colon cancer	IV	CK20+, CDX2+, TTF-1-, CK7-, NapsinA-
#28	F/51	Breast cancer	IV	CK7+, c-ErbB2+++, ER-, PGR-
Lung Cancer Brain Metastases				
Sample	Patient (sex/age)	Tumor type	stage	Diagnostic markers expression
#m34	M/63	SCC	IV	EGFR WT, ALK-
#m31	F/49	AC	IV	EGFR -exon 19 mutation (c.2235_2249del15)

Table 1

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A		
sample	**mutated gene (site of mutation): % of mutation**	
T1-pt	p53(p.Val173Leu/Met): 19%	KRAS(p.Gly12Cys/Arg/Ser): 30%
N1-pt	wt	wt
T1-CRCp2	wt	wt
N1-CRCp2	wt	wt
T6-pt	p53(p.Ala159Ser/Pro/Thr): 48%	MET (p.Arg359Gln): 72%
N6-pt	wt	MET (p.Arg359Gln): 52%
T6-CRCp2	p53(p.Ala159Ser/Pro/Thr): 4,8%	MET (p.Arg359Gln): 54%
N6-CRCp2	wt	MET (p.Arg359Gln): 52%
T19-pt	KRAS(p.Gly13Cys): 21,2%	PTEN (p.Asn334fs): 15%
N19-pt	wt	wt
T19-CRCp4	wt	wt
N19-CRCp4	wt	wt
T20-pt	p53(p.Glu285Val): 34,6%	PI3KA (p.Glu542Lys): 29,8%
N20-pt	wt	wt
T20-CRCp4	wt	wt
N20-CRCp4	wt	wt
T2-pt	p53(p.Lys132Met/Arg/Thr): 41%	
N2-pt	wt	
T2-CRCp2	wt	
N2-CRCp2	wt	
T16-pt	p53(p.Arg248Gln): 30%	
N16-pt	wt	
T16-CRCp1	p53(p.Arg248Gln): 4,4%	
T16-CRCp2	wt	
T16-CRCp4	wt	
N16-CRCp4	wt	
T18-pt	p53(p.Arg273Leu): 48%	
N18-pt	wt	
T18-CRCp1	wt	
T18-CRCp2	wt	
T18-CRCp4	wt	
N18-CRCp4	wt	

B			
sample	**mutated gene (site of mutation): % of mutation**		
T4-pt	p53(p.Arg175Leu/Pro/His): 14%	NRAS(p.Gln61Glu|/Lys): 7.4%	ErbB4 (p.Ala287Glu): 6.7%
N4-pt	wt	wt	wt
T4-CRC p4	p53(p.Arg175Leu/Pro/His): 6,8%	NRAS(p.Gln61Glu/Lys): 4.5%	ErbB4 (p.Ala287Glu): 4%
T4-CRC p8	wt	wt	wt
N4-CRC p4	wt	wt	wt

Table 2

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