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Title: Theratyping cystic fibrosis *in vitro* in ALI-culture and organoid models generated from patient-derived nasal epithelial Conditionally Reprogrammed Stem Cells

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ABSTRACT

Question: Cystic Fibrosis (CF) is due to pathogenic variants in the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) gene. Recent improvement enabled pharmacologic therapy aiming at restoring mutated CFTR expression and function. CFTR "modulators" have revolutionized the CF therapeutic landscape, particularly the last approved Trikafta. This drug-combination is indicated by FDA and very recently by EMA for genotypes carrying at least one copy of CFTR with F508del pathogenic variant. However, several genotypes, are not eligible for Trikafta treatment, yet.

Materials/patients and methods: We exploited an innovative cellular approach allowing highly efficient *in vitro*-expansion of airway epithelial stem cells (AESC) through conditional reprogramming (CRC) from nasal brushing of CF patients. This approach, coupled to development of AESC-derived personalized disease models, as organoids and air liquid interface (ALI) cultures, revealed highly suitable for CFTR pharmacological-testing.

Results and answer to the question: We fully validated the experimental models and implemented the CFTR functional assays and biochemical CFTR protein characterization, that allowed to evaluate the efficacy of clinically available modulators in restoring CFTR maturation and function of each patient-derived "avatar" (theratyping). F508del homozygous genotypes, used as controls, confirmed the higher clinical activity of Trikafta in comparison with older modulators. Trikafta showed its efficacy also on three rare genotypes previously not eligible for modulators-treatment, opening the way to clinical translation. Finally, encouraging results for innovative drug combinations were also obtained.

INTRODUCTION

Cystic fibrosis (CF) is caused by pathogenic variants of the cystic fibrosis transmembrane conductance regulator (CFTR) gene (1). In the lungs of CF patients, defective CFTR protein results in a dehydrated surface liquid and compromised mucociliary clearance. The resulting thick and dense mucus makes the airway prone to chronic infection and inflammation, which consequently leads to airway structure damage and, eventually, respiratory failure. In past years, therapies have been mainly symptomatic(2). More than 2100 different variants (CFTR1 database) of CFTR have been described and, initially, grouped into six classes based on their phenotypic effects(3). Class II pathogenic variants cause protein trafficking defects, which may result in premature degradation of CFTR or accumulation of immature non-functional protein in the cytoplasm. The most common pathogenic variant, F508del, belongs to this class. Recently, advances in the understanding of the molecular genetics of CF have led to the development of novel mutation-specific therapies(4). For example, drugs targeting class II pathogenic variants would correct protein folding and endoplasmic reticulum export defect, while those targeting class III pathogenic variants would restore correct gating. Current therapies approved for specific CFTR variants, all from Vertex Pharmaceuticals, consist in the CFTR potentiator (Ivacaftor/VX770, commercial name Kalydeco) or combination of potentiator Ivacaftor with the correctors Lumacaftor/VX809 (Orkambi) or Tezacaftor/VX661 (Symdeko). Last year, after highly promising clinical trials, the triple combination Trikafta, consisting in Ivacaftor-Tezacaftor-Elexacaftor/VX445 (an innovative corrector), has been approved by FDA for genotypes bearing at least one copy of F508del and is proving highly encouraging so far(5, 6). In the last months this drug-combination has been approved for commercialization in EU by EMA (with the name Kaftrio) for the same genotypes. Nevertheless, a large number of pathogenic genotypes, are not included in the list of genotypes eligible for Trikafta treatment and the drug is not provided by the National Health Systems of EU states, yet.

Drugs allowing fully improved restoration of CFTR function are still needed, and development of more effective ones for all patients with CF is challenging. This is especially true for those with rare pathogenic variants, whose clinical trials are hampered by the scarcity of patients itself. The success of personalized therapy of CF has been hindered by poor functional characterization of CFTR variants and from the limited number of mutational classes studied, so far. Moreover, the great majority of CFTR pathogenic variants show multiple biochemical defects and seems to belong to multiple mutational classes. For this reason, a re-classification of CFTR pathogenic variants in a wider number of mutational types each with a more specific multi-classes functional description, is underway(7). These enhanced classification is aimed at identifying specific mutational types responding to specific therapy, an approach called “theratyping”(8) (a methodology approved in USA by the FDA).

Various cellular *in vitro* CF models have been used to validate personalized experimental strategies, including immortalized human bronchial epithelial cell lines and primary airway epithelial cells. The first ones with the limitations of immortalized cells and the latter with the limitation of low efficiency of culture establishment and limited supply of experimental material. Based on their intrinsic properties, stem cells would allow generating large amounts of functionally differentiated cells, representing the best cellular candidates for experimental CF models. The possibility to obtain with high efficiency large amounts of primary airway epithelial

stem cells (AESC) from CF patients with various gene defects, would represent a pivotal advancement for CF research, particularly for the investigation of treatment response. Of note, CF patients with rare genetic variants, being their low frequency a major obstacle for the accomplishment of valuable clinical trials, would particularly benefit from improved and personalized *in vitro* disease models(8-10).

The so-called "culture reprogramming condition" (CRC), consisting of co-culture of primary epithelial cells with irradiated mouse fibroblasts as feeder layer, in the presence of the Rock inhibitor Y-27632, induces reprogramming of differentiated epithelial cells associated to active cell proliferation, generating long-term cultures of stem-like epithelial cells from several tissues, including respiratory epithelium (pulmonary, bronchial, tracheal)(11, 12). We, and others, have demonstrated that CRC cultures can be obtained with very high efficiency from patient respiratory tissue, generating large amounts of AESC endowed with the ability to differentiate into mature mucociliary cells(13-16). The possibility to expand CRC stem-like cells from lung tissue of CF patients and their ability to generate differentiated cells *in vitro* have been demonstrated(15). The availability of large sources of AESC cells, from CF patients with different genotypes, would represent a great hint for improving both pharmacologic and genetic therapeutic approaches for CF. However, this approach has been limited so far by the low availability of patient lung tissue or invasiveness of procedures for bronchial brushes biopsies. Viable nasal epithelial cells can be obtained through a much less invasive procedure, as superficial nasal brushing. The ease to obtain AESC allows the availability of experimental material from each patient, supporting the investigation of virtually any genotype(17). Nasal brushing is feasible, in both adults and infants, allows harvesting of respiratory cells suitable for cell culture, although the yield of primary nasal cells has been very low and establishment of long-term cultures highly inefficient, so far(17, 18). Here, we generated large amounts of AESC using the CRC method from nasal brushing samples obtained from CF patients. We used these stem cells (CF-CRC) to model CF variants *in vitro*, through generating both respiratory tissue in air liquid interface (ALI) conditions and 3D organoids, mimicking the diseased respiratory tissue. These patient-specific models resulted highly suitable for testing basal activity and drug response of various genotypes not previously eligible for approved drugs, with the concrete possibility of a clinical translation.

MATERIALS AND METHODS

A detailed description of materials and methods can be found in Supplementary Information.

RESULTS

Establishment and validation of CF patient-derived nasal epithelial stem cell cultures under conditionally reprogrammed cell (CRC) conditions (CF CRC)

Nasal epithelial cells were obtained from affected CF patients with variable CFTR gene defects (two mutated CFTR genes) and from carrier individuals (heterozygous for CFTR gene pathogenic variants) through cytology brushing. Nasal brushing (NB) cells were cultured under CRC conditions as described in Supplementary Information and based on our previously established protocols(13). Although the number of freshly collected cells varied in different samples (generally approximately $5x10^4$, but ranging from 1 $x10^3$, to 2.75 $x10^5$ cells), all samples gave rise to cell clones in culture, leading to the generation of monolayers of adherent cells with epithelial morphology (100% efficiency of culture establishment) (Supplementary Figure 1S A). They proliferated with high to moderate growth rate for prolonged time, guaranteeing a long duration of cultures and generating huge amounts of cells (Supplementary Figure1S B). The calculated mean yield of total cells expanded in a time frame of 2 months from the brushing collection of culture has been estimated as higher than $2.5x10^8$, moreover this number was certainly increased when more abundant starting material was dedicated to culture (not shown), in line with other's results(19, 20).

CRC cells displayed epithelial cell phenotype, high viability (Supplementary Figure 2S A) and high clonogenic potential with 96 ± 3% cloning forming efficiency under CRC-conditions (see Supplementary Information). They expressed antigens of airway (basal) stem cells (NGFR: mean 82.5 ± 5%; ITGA6: mean 96,4 ± 2%; TROP2: mean 91.8 ± 5% positive cells), in agreements with our and other's previous results on healthy subject- or cystic fibrosis patient-derived cells, expanded from bronchoalveolar or nasal epithelia (Figure1 A and Supplementary Figure 2S B) (13, 15, 16). Stem cell antigen expression was heavily reduced, when cells were grown under culture conditions used for primary airway cells (Bronchial Epithelial Growth Medium, BEGM) (Supplementary Figure 2S C). We optimized two alternative CRC-differentiation protocols: cultivation of CRC in Air Liquid interface (ALI) culture (a condition mimicking the respiratory tissue microenvironment with epithelial cells lining at the surface of tissue in contact with air on top of specific transwell membranes) and a simplified differentiation protocol with cell cultured in ALI medium, in standard plates, as monolayers of submerged cultures. Both growth conditions determined decreased expression of the basal stem cell-related antigens (NGFR, ITGα6, CK14) and up-regulation of mature respiratory cell-markers, confirming the possibility to obtain full *in vitro* differentiation of the CRC cells (Figure1 B). Specifically, ALI-culture condition generated a respiratory tissue (visible at low magnification in the picture of Figure 1C middle) containing mature cellular elements such as acetylated α-tubulin-positive and FoxJ-positive ciliated cells as well as Muc5B-positive mucus-producing secretory cells. (Figure1 C left and right). Full functional differentiation and polarization obtained by culturing the basal cells under ALI-culture conditions is additionally proved by the movement of cilia at the air-exposed side of the tissue (Movie 1) and cilia and mucin-granules distribution at the apical side visible in Figure 1C right (Z) and Movie 2.

Through inclusion in matrigel and after 3 weeks-culture in ALI medium, CRC cells generated organoids, functionally differentiated respiratory tissue, resembling mini-airways with 3D structure and morphology. They gradually exhibited the formation of an internal lumen and contained specialized functionally mature cellular elements (i.e. the ciliated cells endowed with actively-moving cilia oriented towards the lumen of organoid are visible by optical microscopy examination) (Figure1 D and Movie 3).

CRC cultures maintained the stem cell phenotype after repeated passages *in vitro,* even after cryopreservation, guaranteeing the possibility to largely expand and store these cells for subsequent studies. Basal stem marker expression, although decreasing gradually after prolonged cell passages (suggesting the progressive propensity of cells to partially differentiate after prolonged time in culture), remained moderate and much higher than that observed in cells differentiated in ALI culture (Supplementary Figure 2S). These results demonstrated a partial but low extent of cell differentiation under prolonged CRC conditions in line with the reduction of proliferation rate described for late passages cells (Supplementary Figure 1S B). However, cells maintained stem cell properties after several passages, such as ability to generate differentiated organoids (functional organoids with lumen and beating cilia obtained from CRC cells at passage 6 are visible in Movie 4).

Validation of CFTR gene/transcript diagnostic alterations in CRC

CFTR genetic defects, previously identified during diagnostic analysis of patient blood cells and patient brushing genetic material, were assessed in DNA and RNA extracted from CRC cultures, based on previously used protocols(21),(22, 23). CFTR DNA and RNA sequence analysis allowed to confirm the genetic alteration previously detected in affected patients, as well as, in some cases, to refine genetic diagnosis through identification of previously undetected mutations due to scarcity of biological material (Table 1). Subsequent DNA or RNA analysis for the newly identified alterations confirmed their presence in the carrier parent's genotype, delineating their relevance for the specific CF phenotype and proving the mechanism of disease inheritance. Genetic defects were maintained during repeated cell passages of CF-CRC cells (analyses of CFTR sequence at 2^{nd} or 6^{th} passage of cultured cells provided identical results) and no other genetic alterations arose, demonstrating that cultured cells maintaining genetic integrity during prolonged culture and at the same time guaranteeing the reliability of diagnostic material obtained after cell expansion. Thus, the CRC approach represents a precious tool to contribute to improve diagnostic procedures by providing large amounts of patient-derived material.

The analyzed genotypes included four F508del homozygotes (Table 1, CRC-CF#1, 2, 10 and 13, two of them with another CFTR variation in *cis* with one of the F508del), four compound heterozygotes for the F508del and another CFTR pathogenic variant (Table 1, CRC-CF#3, 5, 7 and 12), one compound heterozygote for non-F508del pathogenic variants (Table1, CRC-CF#8) and five carriers (Table1, CRC-CF#4, 6, 9, 11 and 14). Two out of the three large nucleotide insertions (Table1, CRC-CF#5, 7) are known to produce anomalous splicing of CFTR mRNA with the complete skipping of exon 10 (legacy name exon 9) according to our previous results(24). The third large nucleotide insertion (Table1, CRC-CF#12) is a novel CFTR pathogenic variant, similar to those already described, but with a different number of inserted nucleotides. It produces the same effect of a complete exon 10 skipping (our unpublished results). The skipping of exon 10 of CFTR is known to produce a non-functional protein for the failure of full glycosylation,

misfolding and clearance in the endoplasmic reticulum, with a pattern resembling that of F508del*(25),(26).* Consequently, these three large insertions are CFTR pathogenic variants probably of class II.

TABLE 1 - **Genotypes of patients included in the study.** The identification of each preparation of CRC cells (ID) is indicated, as used everywhere in the text. Genotypes are indicated in old (legacy name) and new (Human Genome Variation Society, HGVS name) nomenclature at both DNA and, whenever possible, protein level. For recently discovered disease-causing variants, only HGVS name is used. wt = wild-type allele.

ID	**Legacy name** (old nomenclature)	**HGVS name**	
		DNA level	**protein level**
CRC-CF#1	[F508del;I1027T] / F508del	c.[1521_1523delCTT;3080T>C];[1521_1523delCTT]	p.[(Phe508del;Ile1027Thr)];[(Phe508del)]
CRC-CF#2	F508del / F508del	c.[1521_1523delCTT];[1521_1523delCTT]	p.[(Phe508del)];[(Phe508del)]
CRC-CF#3	F508del / L558S	c.[1521_1523delCTT];[1673T>C]	p.[(Phe508del)];[(Leu558Ser)]
CRC-CF#4	[G576A;R668C] / wt	c.[1727G>C;2002C>T];[=]	p.[(Gly576Ala;Arg668Cys)];[(=)]
CRC-CF#5	-	c.[1521_1523delCTT];[1210-34TG[10]_1210-34TG[4]ins317]	-
CRC-CF#6	G85E / wt	c.[254G>A];[=]	p.[(Gly85Glu)];[(=)]
CRC-CF#7	-	c.[1521_1523delCTT];[1210-34TG[10]_1210-34TG[4]ins358]	-
CRC-CF#8	$(TG)_{11}T_5$ / L1065P	c.[1210-34TG[11];1210-12T[5]];[3194T>C]	-
CRC-CF#9	$(TG)_{12}T_5$ / wt	c.[1210-34TG[12];1210-12T[5]];[=]	-
CRC-CF#10	[F508del;I1027T] / F508del	c.[1521_1523delCTT;3080T>C];[1521_1523delCTT]	p.[(Phe508del;Ile1027Thr)];[(Phe508del)]
CRC-CF#11	-	c.[1210-34TG[10]_1210-34TG[4]ins354];[=]	-
CRC-CF#12	-	c.[1521_1523delCTT];[1210-34TG[10]_1210-34TG[4]ins354]	-
CRC-CF#13	F508del/ F508del	c.[1521_1523delCTT];[1521_1523delCTT]	p.[(Phe508del)];[(Phe508del)]
CRC-CF#14	[621+3A>G;F1052V] / wt	c.[489+3A>G;3154T>G];[=]	-

CFTR expression in fresh brushings, CRC cells, standard culture cells, ALI-differentiated cells

We next evaluated CFTR expression in CF-CRC and CF-CRC-derived differentiated cells with the aim to determine which culture condition could provide high CFTR-expressing cells for functional studies of its basal/residual or pharmacologically-restored activity. Real-time PCR analysis showed that CFTR was highly transcribed in all brushing samples, its expression was limited in CRC-cultured cells, as expected for non-specialized stem cells and slightly increased under standard BEGM culture. Finally, CFTR was strongly expressed in CRC-derived ALI-culture differentiated cells (Figure 2 A-B). In line with transcript expression, immunoblot analysis revealed very low amounts of CFTR protein in CRC or standard-culture cells (BEGM) and prominent amounts in ALI-medium differentiated cells (Figure 2 C-D). Therefore, ALI-culture seems the more suitable approach to model CF *in vitro* for CFTR studies. Parallel evaluation of differentiated ciliated cell marker (FOXJ) (Figure 2 D) in the same samples proved the occurrence of cell differentiation.

Wild type and F508del mutated CFTR proteins could be distinguished in immunoblot, based on their different molecular weights (high molecular weight band C of wild type functional CFTR and low molecular weight band B of defective misfolded protein) (Figure 2 C and D).

Pharmacologic Rescue of CFTR conformation: biochemical assay (immunoblot)

We next evaluated the possibility to use CRC-derived CF models for assessment of CFTR basal/residual function as well as for determination of the ability of pharmacological agents to increase/rescue CFTR activity. At biochemical level this was accomplished through detection of the basal or drug-enhanced relative amount of band-C, associated with correct conformation of CFTR.

We compared the efficacy of clinically approved CFTR correctors Lumacaftor, Tezacaftor and Elexacaftor, each indicated for a specific patient subgroup. Correctors were used as single agents or in combinations, including the Trikafta corrector-combination Elexacaftor/Tezacaftor. Initially, we tested a homozygous F508del patient, in order to verify the increment of CFTR maturation expected from Trikafta in comparison with previously approved drugs as proved in clinical trials and to validate the ALI-culture model approach (Figure 3 A). Next, we used this biochemical assay to determine the efficacy of drugs in the 3 unexplored rare compound heterozygous complex genotypes bearing the F508del and the 3 large nucleotidic insertions (F508del/ins), probably belonging to mutational class II (Figure 3 B-D).

The ability of CFTR correctors to restore CFTR maturation was evaluated in ALI medium-differentiated CF-CRC cells exposed to correctors for 48 hours, through immunoblot quantification of CFTR band-C relative proportion in control or drug-treated cells. The F508del homozygous sample, used as positive control, showed a marked relative increase of band-C, following Tezacaftor/Elexacaftor drug treatment (Figure 3 A). These findings were in line with the higher Trikafta activity found *in vitro* and in clinical trials(6). Single-correctors as well as the Lumacaftor/Tezacaftor combination treatments induced a limited or non-significant increase in Band-C level. In contrast, Elexacaftor/Lumacaftor treatment resulted in robust correction of the CFTR protein, even superior to the Tezacaftor/Elexacaftor (Figure 3).

In F508del/ins samples (CF#5, 7 and 12 in Table1) CFTR protein drug-correction occurred at similar extent compared to homozygous F508del/F508del genotypes. Also in F508del/ins genotypes the efficacy of drug combinations was superior than that observed by single correctors, and corrector combinations appeared particularly effective when Elexacaftor was used, suggesting

higher synergy of this corrector. These results imply a promising therapeutic efficacy of Trikafta for the tested F508del/ins genotypes. In effect, the efficacy of Trikafta in a phase 3 study in patients with a single F508del allele has been demonstrated(5). However, it is likely that in F508del/ins genotypes here tested, there is also an additional effect of correctors on the allele with the large insertion (probably of mutational class II). We found a general highly encouraging efficacy of the Trikafta correctors Elexacaftor/Tezacaftor in all variants analyzed. In addition, a particularly high effectiveness was observed in cells treated with Elexacaftor/Lumacaftor combination, suggesting that further investigation in this direction might be indicated.

Pharmacologic Rescue of CFTR function: Forskolin-induced swelling of organoids (FIS) and Fluid re-absorption assay

Although highly indicative of CFTR protein folding/maturation, biochemical quantification of CFTR band-C may not be sufficient to determine whether a specific compound is able to restore also CFTR surface localization and gating function. Thus, in order to substantiate biochemical results, we evaluated the efficacy of CFTR modulators through the Forskolin-induced swelling (FIS) assay of organoids, based on the measurement of CFTR activity, as the direct effect of chloride passage through the channel and fluids movement (27). Following CFTR modulators exposure, organoids generated from CF-CRC bearing specific genotypes responded to drugs with variable measurable swellings (Figure 4), whose extents were consistent with the relative Band-C increase, observed in immunoblot of the corresponding genotypes (Figure 3). The homozygote F508del responded to an approximately similar extent than the genotypes with a single F508del allele associated to the other alleles with large insertions, in line with immunoblot assays. Combined drugs proved higher activity than single drugs with a markedly encouraging efficacy of Trikafta (Figure 4 A). The triple combination Elexacaftor/Lumacaftor/Ivacaftor induced a robust swelling of organoids, comparable (and in some cases superior) to Trikafta, in line with biochemical analysis (Figure 4 A).
In addition, we used ALI-culture model to set up the Fluid-reabsorption assay, to measure basal or drug-potentiated CFTR activity (induction of chloride ions and water efflux in the apical chamber of ALI-culture transwells that counteracts the physiologic sodium channel-mediated fluid movement in the opposite direction toward the basal chamber, usually indicated as fluid re-absorption)(28). The extent of decrease of liquid re-absorption from the apical compartments in specific drug-treated samples compared to control samples indicated that, both in F508del/F508del and F508del/ins genotypes, Trikafta showed high efficacy in restoring CFTR protein function (decreased liquid re-absorption) and its activity was markedly superior to singularly used drugs (Figure 4 B).
Thus, the different biochemical and functional CFTR assays produced comparable results enforcing reliability of 2D and 3D CF-CRC models and of the different assays, providing confirmatory results of the efficacy of the newly introduced Trikafta triple combination and pushing for further researches to possibly extend its indication to a larger number of CFTR variants.

DISCUSSION

A huge research effort in the attempt to increase disease knowledge, identify innovative therapeutic targets and more effective CFTR modulators, together with improvements of symptomatic therapies, have led to markedly increased life expectancy and better quality of life for CF patients, in the last years. Innovative specific therapies have been developed and approved for the most frequent genetic variants, however most rare-variant patients remain orphan and CF remains a progressively devastating disease, for most of them(29). Therapeutic advancements in the direction of personalized treatment have been dramatically hampered by lack of valuable *in vitro* disease models suitable for experimental therapeutic testing of the huge number of CFTR variants. In fact, rare genetic variants have not been research-available, neither in the preclinical experimental setting nor for clinical trials, due to their scarcity and to the inefficiency of *in vitro* primary cultures establishment. Here, we used the Conditionally Reprogrammed Cell (CRC) approach to generate with high efficiency CF models *in vitro*. We fully validated and used these models for the evaluation of response to currently used, also recently approved, drugs in rare disease variants in comparison with the most studied/most frequent F508del homozygous variant.

In agreement with our previous data on bronchial/pulmonary healthy tissue, CRC methodology proved highly efficient in establishment of long-term cultures of AESC from nasal epithelia of CF patients. CRC cultures were established with 100% efficiency, in spite of the limited amount of nasal brushing sample and proved able to supply huge amounts of cells(13). The unprecedented potency of the CRC approach coupled with the fact that nasal epithelium represents an easily accessible source of samples, and that nasal brushing is a relatively low-invasive procedure, might allow preclinical experimentation of, virtually, each CF variant, with strong implications for personalized therapy.

CF-CRC cells proved endowed with airway epithelial stem cell (basal) phenotype and function, being able to extensively proliferate and generate mature respiratory cells of various type when cultured under differentiating conditions. Three different models of differentiation were developed: 2D monolayers of respiratory cells, ALI-cultures in transwells generating polarized respiratory tissue and 3D airway organoids recapitulating the airway tissue architecture. These models were fully validated for differentiation and CFTR expression, before use for experimental assays.

CFTR genetic defects were maintained after cell expansion and patient genotypes did not undergo culture-linked alterations, proving that the CRC approach of cell expansion from CF brushing material may represents a precious tool to contribute improving diagnostic procedures by providing large amounts of patient-derived material.

CFTR was abundantly detected in fresh nasal brushing samples, its expression decreased dramatically in (CRC) culture-expanded cells and reached very high levels in ALI medium-differentiated airway models, proving their suitability for CFTR studies. Biochemical evaluation of CFTR protein folding and maturation was achieved using 2D monolayers through immunoblot, CFTR functional activity was evaluated in 3D organoids (Forskolin induced swelling, FIS) and in airway tissue generated by standard ALI-culture (fluid re-adsorption assay).

Response to the drugs currently used for specific variants, Orkambi, Symdeko, Kalideko and Trikafta was assessed in F508del homozygous genotypes as validation of the experimental approach and in heterozygous compound genotypes F508del/ins (see text for explanation) to assess and compare efficacy of different drugs also on these variants.

Trikafta showed markedly higher activity compared to other therapies. Moreover, similar efficacy of each treatment was observed in genotypes bearing either two copies (F508del/F508del homozygous) or a single copy (F508del/ins) of F508del. Considering that in F508del/ins genotypes, the ins CFTR allele probably acts as a mutational class II variant, a therapeutical effect also on the ins allele is to be considered. These results provide support for the forthcoming treatment of specifically tested genotypes with Trikafta, following the US template of potential guide of personalized patient treatment based on results derived from "*in vitro* trials".
F508del alteration results in lower molecular mass of the immature CFTR protein, detectable in immunoblot, confirming the use of this approach as indicator of non-functional CFTR. Based on this assumption, determination of CFTR molecular weight in immunoblot may contribute to characterize at protein level unexplored variants identified through genetic analysis guiding their diagnostic classification, as well as to determine the ability of pharmacological agents to rescue CFTR function (as is the case for the 3 large insertions here analyzed).
The different biochemical and functional CFTR assays used in this study, produced comparable results, enforcing reliability of all assays based on AESC-derived models and results.
Of note, the new corrector Elexacaftor displayed remarkable efficacy in the enhancement of CFTR activity, compared to Lumacaftor or Tezacaftor. This might be due to a potential stronger ability to correct CFTR protein, but also to its dual activities, as corrector and potentiator, recently described (30). Moreover, besides the strong potential of the combination Texcaftor/Elexacaftor, as expected by these two Trikafta correctors, Elexacaftor displayed marked synergy when associated with Lumacaftor, as well. Thus, the innovative combination of Lumacaftor/Elexacaftor might warrant further investigation. At the same time the possible efficacy of Elexacaftor used as single agent, or its potential synergy with other compounds should be explored and might lead to more effective, more tolerated or less expensive treatment options.
Our studies demonstrated that the CRC approach enables massive expansion of AESC generating highly suitable CF-models for testing CFTR modulators. The extraordinary efficiency of CRC culture establishment and their massive expansion *in vitro*, coupled with the stability of cultures allow the use also of late passage-CRC cells, all aspects missing in other cellular approaches, thus further increasing the cell amount available for experimentation. In fact, CF-CRC cells showed to maintain the differentiation ability and stable antigen expression over several cellular passages (at least up to P6). On the contrary, primary airway epithelial cells appeared to be able to maintain the specific airway expression pattern no longer than P3 (31).

The presented cellular approach of CRC-derived ALI cultures, is quite novel as applied to CF. However, another similar approach has been recently described for the expansion of human nasal epithelial stem cells, based on dual SMAD inhibition and feeder-free (32). A comparative study has shown that respiratory cells generated in ALI-culture from these SMAD inhibited/feeder free cells did not substantially differ from ALI-cultures generated from standard CRC cells, in terms of structural morphology or baseline global proteomics profile. However, standard CRC-derived ALI-cultures displayed increased cilia and CFTR activity, further enhancing the reliability of the standard CRC-approach, used here, for CFTR studies (33).

Finally, given the relative inaccessibility of primary bronchial epithelial cell cultures, our findings support the use of patient-derived nasal epithelial cell cultures for preclinical studies of therapeutic interventions. In this context, the approach of "theratyping" is of enormous translational impact,

allowing the identification of specific pathogenetic variants responding to a specific therapy. This methodology has been approved in US by the FDA as the unique preliminary step necessary and sufficient for the treatment of patients with the specific responding genotype, by drugs clinically approved for other genotypes(10). Moreover, the use of personalized organoid technology to guide treatment decisions would be widely accepted by patient community as reported in a recent Australian report(34). We are confident that this approach might be assumed as a sort of *in vitro* personalized clinical trial in the near future, and might contribute facilitating the access to more beneficial cure for CF patients, with particular benefit for orphan patients.

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AUTHOR CONTRIBUTION

GS, SLC, AE and ML designed the experiments; GS, SLC, GB, SMB, SP and VS performed the experiments and analyzed the data. ML, GCi and BF selected patients, provided patient brushings and patient clinical diagnosis; GS, SLC, VS and GCa expanded and cultured patient samples; MF supported the microscopy analyses; AE and MB supervised cell biobanking. AE and ML wrote the paper; RDM and MB revised the manuscript.

BIBLIOGRAPHY

1. Elborn JS. Cystic fibrosis. Lancet. 2016 Nov 19;388(10059):2519-31.
2. Cohen-Cymberknoh M, Shoseyov D, Kerem E. Managing cystic fibrosis: strategies that increase life expectancy and improve quality of life. Am J Respir Crit Care Med. 2011 Jun 01;183(11):1463-71.
3. Amaral MD. Novel personalized therapies for cystic fibrosis: treating the basic defect in all patients. J Intern Med. 2015 Feb;277(2):155-66.
4. Corvol H, Thompson KE, Tabary O, le Rouzic P, Guillot L. Translating the genetics of cystic fibrosis to personalized medicine. Transl Res. 2016 Feb;168:40-9.
5. Middleton PG, Mall MA, Drevinek P, Lands LC, McKone EF, Polineni D, et al. Elexacaftor-Tezacaftor-Ivacaftor for Cystic Fibrosis with a Single Phe508del Allele. N Engl J Med. 2019 Nov 7;381(19):1809-19.
6. Keating D, Marigowda G, Burr L, Daines C, Mall MA, McKone EF, et al. VX-445-Tezacaftor-Ivacaftor in Patients with Cystic Fibrosis and One or Two Phe508del Alleles. N Engl J Med. 2018 Oct 25;379(17):1612-20.
7. Veit G, Avramescu RG, Chiang AN, Houck SA, Cai Z, Peters KW, et al. From CFTR biology toward combinatorial pharmacotherapy: expanded classification of cystic fibrosis mutations. Mol Biol Cell. 2016;27(3):424-33.
8. Cutting GR. Cystic fibrosis genetics: from molecular understanding to clinical application. Nature reviews Genetics. 2015;16(1):45-56.
9. Dekkers JF, Berkers G, Kruisselbrink E, Vonk A, de Jonge HR, Janssens HM, et al. Characterizing responses to CFTR-modulating drugs using rectal organoids derived from subjects with cystic fibrosis. Sci Transl Med. 2016;8(344):344ra84.
10. Durmowicz AG, Lim R, Rogers H, Rosebraugh CJ, Chowdhury BA. The U.S. Food and Drug Administration's Experience with Ivacaftor in Cystic Fibrosis. Establishing Efficacy Using In Vitro Data in Lieu of a Clinical Trial. Ann Am Thorac Soc. 2018;15(1):1-2.
11. Liu X, Krawczyk E, Suprynowicz FA, Palechor-Ceron N, Yuan H, Dakic A, et al. Conditional reprogramming and long-term expansion of normal and tumor cells from human biospecimens. Nat Protoc. 2017 Feb;12(2):439-51.
12. Liu X, Ory V, Chapman S, Yuan H, Albanese C, Kallakury B, et al. ROCK inhibitor and feeder cells induce the conditional reprogramming of epithelial cells. Am J Pathol. 2012 Feb;180(2):599-607.
13. Sette G, Salvati V, Giordani I, Pilozzi E, Quacquarini D, Duranti E, et al. Conditionally reprogrammed cells (CRC) methodology does not allow the in vitro expansion of patient-derived primary and metastatic lung cancer cells. Int J Cancer. 2018 Jan 17.
14. Gao B, Huang C, Kernstine K, Pelekanou V, Kluger Y, Jiang T, et al. Non-malignant respiratory epithelial cells preferentially proliferate from resected non-small cell lung cancer specimens cultured under conditionally reprogrammed conditions. Oncotarget. 2016 Dec 29.
15. Gentzsch M, Boyles SE, Cheluvaraju C, Chaudhry IG, Quinney NL, Cho C, et al. Pharmacological Rescue of Conditionally Reprogrammed Cystic Fibrosis Bronchial Epithelial Cells. Am J Respir Cell Mol Biol. 2016 Dec 16.
16. Butler CR, Hynds RE, Gowers KH, Lee Ddo H, Brown JM, Crowley C, et al. Rapid Expansion of Human Epithelial Stem Cells Suitable for Airway Tissue Engineering. Am J Respir Crit Care Med. 2016 Jul 15;194(2):156-68.
17. Muller L, Brighton LE, Carson JL, Fischer WA, 2nd, Jaspers I. Culturing of human nasal epithelial cells at the air liquid interface. J Vis Exp. 2013 Oct 08(80).
18. Mosler K, Coraux C, Fragaki K, Zahm JM, Bajolet O, Bessaci-Kabouya K, et al. Feasibility of nasal epithelial brushing for the study of airway epithelial functions in CF infants. J Cyst Fibros. 2008 Jan;7(1):44-53.
19. Brewington JJ, Filbrandt ET, LaRosa FJ, 3rd, Moncivaiz JD, Ostmann AJ, Strecker LM, et al. Brushed nasal epithelial cells are a surrogate for bronchial epithelial CFTR studies. JCI Insight. 2018 Jul 12;3(13).
20. Hayes D, Jr., Kopp BT, Hill CL, Lallier SW, Schwartz CM, Tadesse M, et al. Cell Therapy for Cystic Fibrosis Lung Disease: Regenerative Basal Cell Amplification. Stem cells translational medicine. 2019;8(3):225-35.

21. Lucarelli M, Narzi L, Piergentili R, Ferraguti G, Grandoni F, Quattrucci S, et al. A 96-well formatted method for exon and exon/intron boundary full sequencing of the CFTR gene. Anal Biochem. 2006 Jun 15;353(2):226-35.
22. Ferraguti G, Pierandrei S, Bruno SM, Ceci F, Strom R, Lucarelli M. A template for mutational data analysis of the CFTR gene. Clin Chem Lab Med. 2011 Sep;49(9):1447-51.
23. Auriche C, Di Domenico EG, Pierandrei S, Lucarelli M, Castellani S, Conese M, et al. CFTR expression and activity from the human CFTR locus in BAC vectors, with regulatory regions, isolated by a single-step procedure. Gene Ther. 2010 Nov;17(11):1341-54.
24. Pierandrei S, Blacona G, Fabrizzi B, Cimino G, Cirilli N, Caporelli N, et al. Two novel and correlated CF-causing insertions in the (TG)mTn tract of the CFTR gene. PLoS One. 2019;14(10):e0222838.
25. Strong TV, Wilkinson DJ, Mansoura MK, Devor DC, Henze K, Yang Y, et al. Expression of an abundant alternatively spliced form of the cystic fibrosis transmembrane conductance regulator (CFTR) gene is not associated with a cAMP-activated chloride conductance. Hum Mol Genet. 1993 Mar;2(3):225-30.
26. Delaney SJ, Rich DP, Thomson SA, Hargrave MR, Lovelock PK, Welsh MJ, et al. Cystic fibrosis transmembrane conductance regulator splice variants are not conserved and fail to produce chloride channels. Nat Genet. 1993 Aug;4(4):426-31.
27. Liu Z, Anderson JD, Deng L, Mackay S, Bailey J, Kersh L, et al. Human Nasal Epithelial Organoids for Therapeutic Development in Cystic Fibrosis. Genes (Basel). 2020 May 29;11(6).
28. Aarbiou J, Copreni E, Buijs-Offerman RM, van der Wegen P, Castellani S, Carbone A, et al. Lentiviral small hairpin RNA delivery reduces apical sodium channel activity in differentiated human airway epithelial cells. J Gene Med. 2012 Dec;14(12):733-45.
29. Bardin E, Pastor A, Semeraro M, Golec A, Hayes K, Chevalier B, et al. Modulators of CFTR. Updates on clinical development and future directions. Eur J Med Chem. 2021 Mar 5;213:113195.
30. Laselva O, Bartlett C, Gunawardena TNA, Ouyang H, Eckford PDW, Moraes TJ, et al. Rescue of multiple class II CFTR mutations by elexacaftor+ tezacaftor+ivacaftor mediated in part by the dual activities of Elexacaftor as both corrector and potentiator. Eur Respir J. 2020 Dec 10.
31. Reeves SR, Barrow KA, White MP, Rich LM, Naushab M, Debley JS. Stability of gene expression by primary bronchial epithelial cells over increasing passage number. BMC Pulm Med. 2018 May 29;18(1):91.
32. Mou H, Vinarsky V, Tata PR, Brazauskas K, Choi SH, Crooke AK, et al. Dual SMAD Signaling Inhibition Enables Long-Term Expansion of Diverse Epithelial Basal Cells. Cell Stem Cell. 2016 Aug 4;19(2):217-31.
33. Awatade NT, Wong SL, Capraro A, Pandzic E, Slapetova I, Zhong L, et al. Significant functional differences in differentiated Conditionally Reprogrammed (CRC)- and Feeder-free Dual SMAD inhibited-expanded human nasal epithelial cells. J Cyst Fibros. 2021 Jan 4.
34. Fawcett LK, Wakefield CE, Sivam S, Middleton PG, Wark P, Widger J, et al. Avatar acceptability: views from the Australian Cystic Fibrosis community on the use of personalised organoid technology to guide treatment decisions. ERJ Open Res. 2021 Jan;7(1).

LEGENDS TO FIGURES AND TABLES

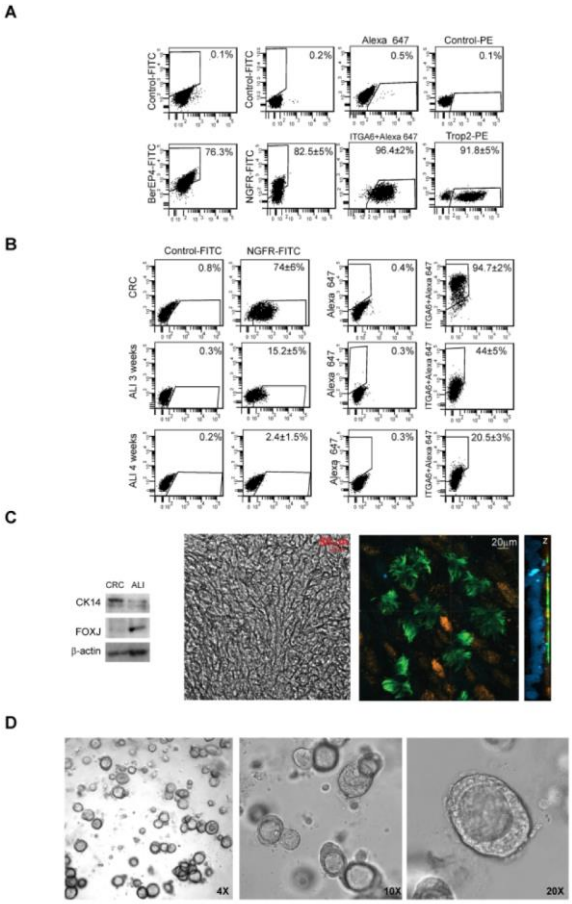

Figure 1. CF-CRC characterization and generation of differentiated respiratory cell models. Results from one representative preparation (CF2 according to Table 1) are reported. **A)** Flow cytometric analysis of CF-CRC for epithelial/basal-like antigens (EpCAM/BerEP4, ITGA6, TROP2, NGFR). Passage 2 cells have been used for these experiments.
B) Flow cytometry analysis of the basal cell markers NGFR and ITGA6 in CF-CRC (passage 3) and 3-4 weeks differentiated cells (ALI culture condition). C) (Left) Immunoblot showing the acquisition of the differentiation marker of mature ciliated cells FOXJ and the loss of basal stem cell marker CK14, after ALI differentiation; β-actin is shown for equal loading. (Middle) Picture of ALI-cultured cells showing the tissue appearance at 10X magnification. (Right) Immunofluorescence and confocal analysis for differentiation markers acetylated α-tubulin (green) and mucin5B (orange) after ALI-culture differentiation. Magnification is 60X and orthogonal projection (Z) shows tissue thickness with nuclei (stained in blue with DAPI) at the bottom and differentiation antigens at the apical side. **D)** Representative images of CRC-derived organoids showing their density, distribution in the well and morphology (4X magnification) and the 3D structure with the organoid wall and the presence of the internal lumen visible at higher magnification (10X and 20X). All experiments described have been performed in triplicate.

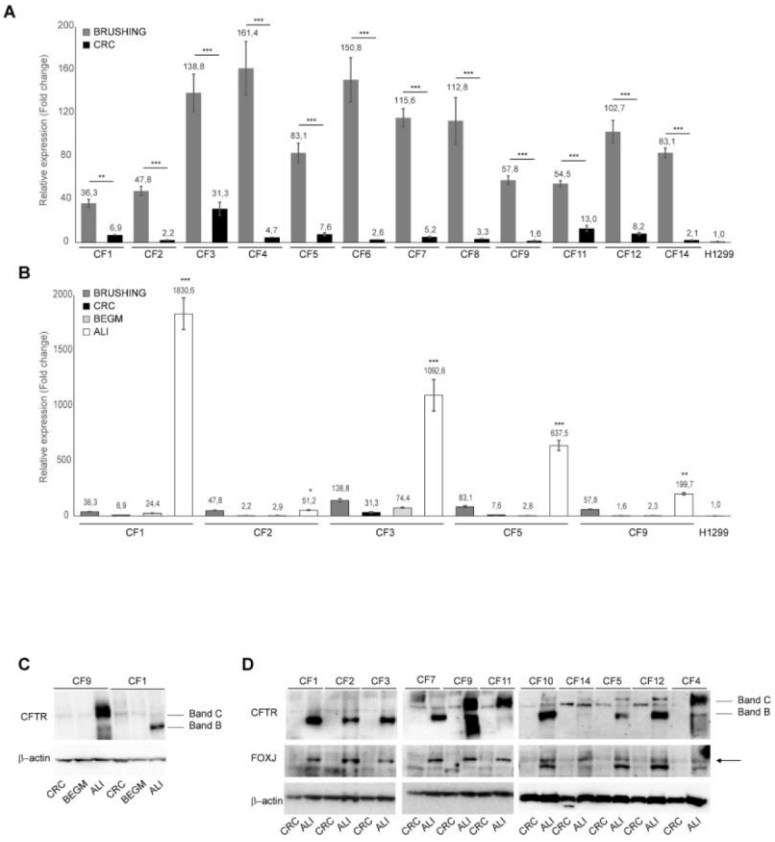

Figure 2. CFTR expression analysis in CF-CRC. Preparations from CF1 to CF14 are according to Table 1. **A)** CFTR mRNA expression levels (average ± DS) measured by real-time PCR comparing fresh brushing samples with CF-CRC cultured in CRC conditions (CRC); ANOVA $p<0.0001$, Bonferroni's post test ***$p<0.0001$ for each brushing *vs* CRC, with the exception of CF1 for which **$p<0.01$. **B)** CFTR mRNA expression levels (average ± DS) measured by real-time PCR comparing CF-CRC undifferentiated (CRC) with differentiated in BEGM and air-liquid interface (ALI) conditions; ANOVA $p<0.0001$, Bonferroni's post test ***$p<0.0001$ for each ALI *vs* all the other conditions of the same preparation, with the exception of CF2 for which ALI *vs* brushing is n.s. and for the other differences *$p<0.05$ and CF9 for which ALI *vs* brushing **$p<0.01$. In both A) and B) the relative expression (fold change) (calculated as described in Materials and Methods, CFTR expression analysis) is referred to the expression in the H1299 cells". **C)** Immunoblot showing CFTR protein levels in CF-CRC cultured in CRC conditions (CRC), standard primary culture conditions (BEGM) and air-liquid interface conditions (ALI) (according to Table 1: CF9 is a carrier, CF1 is a homozygote F508del); β-actin is shown for equal loading. **D)** Immunoblot showing CFTR protein levels in CF-CRC cultured in CRC (CRC) or air-liquid interface (ALI) conditions (according to Table 1: CF1, CF2, CF3, CF5, CF7, CF10 and CF12 are homozygotes or compound heterozygotes whereas CF4, CF9, CF11 and CF14 are carriers); FOXJ immunoblot is shown for validation of proper *in vitro* differentiation. For both C) and D) the mature (Band C) and immature (Band B) CFTR protein are shown; β-actin is shown for equal loading. All experiments have been performed in triplicate.

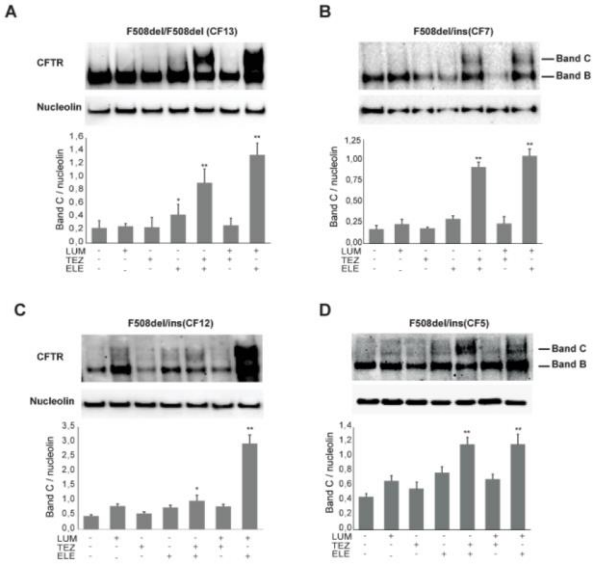

Figure 3. Evaluation of CFTR protein maturation rescue induced by modulators. Immunoblot analysis of expression levels and molecular mass of CFTR protein, in the different control or drug-treated CF-CRC derived ALI-differentiated samples, are shown. Nucleolin is shown for equal loading. **A)** F508del/F508del homozygous genotype; **B)**, **C)** and **D)** F508del / ins (ins = one of the rare alleles with the insertion of variable length) compound heterozygotes, with genotypes according to Table 1. For each panel, the densitometric quantification (average $\pm$ DS) is shown below the immunoblot. The treatments by the drugs are indicated at bottom: LUM = Lumacaftor; TEZ = Tezacaftor; ELE = Elexacaftor; Band C = mature CFTR; Band B = immature CFTR. Mean ± S.D. of three independent experiments is shown. *P<0.05 **P<0.01.

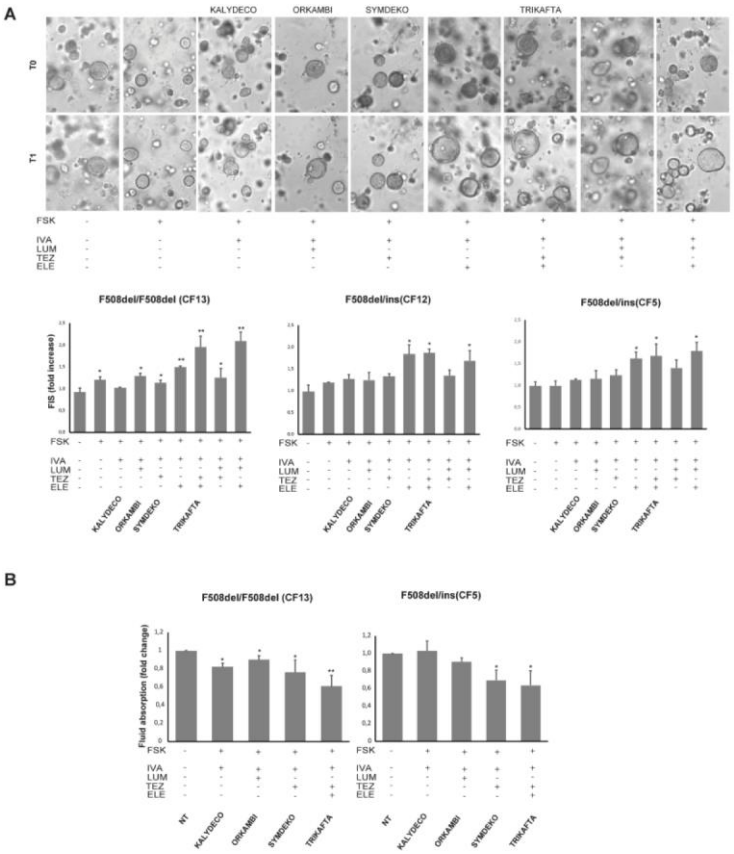

Figure 4. Evaluation of CFTR function recovery induced by modulators. A) Forskolin-induced swelling (FIS) in CF-CRC-derived 3D organoids. In the images some representative examples of FIS are reported relative to F508del / F508del homozygous cells (CF13). The same organoids, monitored and measured before (T0) and after (T1) treatments, are shown. In the histograms (lower panels), the response of F508del / F508del homozygous genoptypes (mean of three independent experiments including that corresponding to the images above) and two F508del / ins (ins = one of the rare alleles with the insertion of variable length) compound heterozygous genotypes (according to Table 1) is shown. Measures are relative to the increase of organoid area (mean of at least 5 organoids / sample) after stimulation with the drugs indicated below. FSK = Forskolin, IVA = Ivacaftor, LUM = Lumacaftor, TEZ = Tezacaftor, ELE = Elexacaftor. Commercial names of clinically used drugs are indicated above corresponding images and below histograms. **B)** Fluid re-absorption assay in CF-CRC-derived ALI-cultures. The results of a F508del / F508del homozygous and a F508del / ins (ins = one of the rare alleles with the insertion of variable length) compound heterozygous genotypes (according to Table 1) are shown. The absorbed fluid is calculated as the input volume in the apical chamber detracted of the remaining volume / transwell membrane area in cm^2 (1.12 cm^2) / time in hours (48 h). The ratio corresponding to values of sample treated with the indicated drug *vs* the value of untreated sample (NT) is reported. Mean ± S.D. of three independent experiments is shown. *P<0.05 **P<0.01. Commercial names of clinically used drugs are indicated below.

SUPPLEMENTARY INFORMATION

MATERIALS AND METHODS

Nasal brushing processing and CRC culture. Human nasal epithelial samples were provided in accordance with consent procedures approved by the Internal Review Board of Regional (Lazio) Reference Center for Cystic Fibrosis, Policlinico Umberto I Hospital, Sapienza University of Rome (Ethics committee ref. 5660 prot 983/19 December 18th 2019). Nasal epithelial cells were obtained through cytology brushing (Doctor Brush, AIESI) of the inferior turbinates from both nostrils, pooled into a single 15-ml conical tube filled with DMEM/F12 (Euroclone) and 5X antibiotics (Penicillin/Streptomycin and Amphotericin B). Samples were repeatedly washed, and recovered cells cultured under the Conditionally Reprogrammed Cell (CRC) methodology, according to our previous protocols (*Sette et al., 2018*). Briefly, epithelial cells were co-cultivated with irradiated (30 gray) murine J2 Swiss 3T3 fibroblasts (Kerafast, Boston, MA, USA) in the presence of 10 μM Rock inhibitor Y-27632 (Selleck, Munich, Germany), in F medium (3:1 v/v F-12 Nutrient Mixture Ham: DMEM) supplemented with 5% FCS, 0.4 μg/ml hydrocortisone, 5 μg/ml insulin, 24 μg/ml adenine, 8.4 ng/ml cholera toxin, 10 ng/ml EGF. Fibroblasts were cultured in 10% characterized HyClone™ Fetal Bovine Serum (South Logan, Utah, USA) and irradiated when reached 80% confluence. All cells were maintained at 37°C in a humidified incubator, with 5% CO_2. For standard culture conditions (those usually used for primary airway cells), CRC cells were deprived of feeder layer and grown in Bronchial Epithelial Cell Growth Medium (BEGM, Lonza) for 2 weeks before analysis.

Differentiation of CRC in Ali-liquid interface (ALI) culture conditions. To induce differentiation, 1.1x10^5 cells were plated in Corning 3460 inserts, and cultured in CRC complete Medium in both basal and apical chambers until confluence was reached (5-7 days), afterwards medium was replaced with PneumaCult – ALI Medium (Voden) in the basal chamber, leaving the apical chamber empty for 28 days, with medium replacement every other day. Alternatively, ALI differentiation was achieved through culture in standard tissue culture-treated plates as 2D monolayers in ALI medium for 3 weeks.

Growth curve of CRC. To determine cell growth rate of CRC cultures $1x10^5$ cells were seeded on feeder layer in complete growth medium and counted by trypan blue exclusion every 4 to 8 days. Proliferation index was calculated as population doublings (PD) using the following formula: PD=3.32 x log(cell number counted / cell number plated at day 0) (Figure 1S).

Clonogenic assay of CRC. CRC cells were plated at single cell density in 96 well plates under CRC conditions. After overnight culture, wells with a single cell were counted and irradiated fibroblasts were added to the wells to support the growth of single cells. Epithelial cell clones rapidly appeared and positive-wells were counted to calculate the percentage of colony forming cells after 5-7 days. Clones exponentially grew for at least 2 weeks proving their extended growth ability. The mean clonogenic efficiency was calculated as mean of three different samples.

CFTR mutational analysis. Genomic DNA was extracted from the CF-CRC by the QIAamp DNA Blood midi kit (Qiagen, Hilden, Germany) and quantified using a fluorimeter (Qubit, Invitrogen, CA, USA). The CFTR (RefSeq NM_000492.4, NG_016465.4) genotype of each preparation of CF-CRC was confirmed by sequencing. The proximal 5'-flanking, all exons and adjacent intronic zones, as well as the 3'-UTR were PCR-amplified and sequenced by a Sanger cycle sequencing protocol (ThermoFisher Scientific, Waltham, MA, USA) in a 96-well format as previously described (*Lucarelli et al., 2006*), using a genetic analyzer (ABI PRISM 3130*xl*; Applied Biosystems, Foster City, CA, USA). For data analysis, a specific template for SeqScape software version 4.0 (Applied Biosystems) was used (*Ferraguti et al., 2011*). Genotype confirmation was completed by multiplex ligation-dependent probe amplification (SALSA MLPA probemix P091 CFTR, MRC Holland, Amsterdam, The Netherlands).

CFTR expression analysis. RNA was extracted from CF-CRC by the RNeasy mini kit (Qiagen, Hilden, Germany). It was reverse transcribed by the iScript cDNA Synthesis kit (Bio-Rad, Hercules, CA, USA) that includes a mix of oligo(dT) and random hexamers as a priming strategy. Retrotranscription was performed using 1 µg of total RNA in 5.5 µL, 4 µL of 5X iScript reaction mix, 1 U of iScript reverse transcriptase in 1 µL, 9.5 µL of H_2O, in a final volume of 20 µL, according to the manufacturer's instructions. The reactions

were incubated in a PTC 100 thermocycler (Bio-Rad), according to a program that allows the synthesis of the double stranded cDNA: 5′ 25 °C, 30′ 42 °C and 5′ 85 °C.

For sequence analysis of the CFTR cDNA, it was amplified by a protocol that, using an optimized set of primers, produced 6 amplicons spanning the entire CFTR mRNA, as previously described (*Auriche et al., 2010*). The PCR mix was in a final volume of 15 µL containing: 2.5 µL of cDNA mix, 175 µM of each dNTP (Fermentas, Waltham, MA, USA), 1.5 mM $MgCl_2$, 6 pmol of each primer and 0.5 U GoTaq hot start polymerase with 1X manufacturer's buffer (Promega, Madison, Wisconsin, USA). The PCR step was conducted in a PTC100 thermocycler (Bio-Rad, Hercules, CA, USA) with the following PCR cycle: 2′ 95 °C; 35 cycles of 45″ 94 °C, 1′ 30″ 60 °C, 2′ 30″ 72 °C followed by 7′ 72 °C. The amplicons were subsequently analyzed by electrophoresis on a 3% agarose gel, to possibly detect anomalous CFTR mRNAs. All the 6 cDNA amplicons were extracted from agarose and individually sequenced as described above.

For quantitative CFTR expression analysis, starting from the cDNA mix described above, a TaqMan gene expression assay (code 4331182, ID Hs00357011_m1; ThermoFisher Scientific, Waltham, MA, USA) was applied using a specific no-ROX Master Mix (FluoCycle™ II Master Mix for probe, EuroClone, Milan, Italy) according to the manufacturer's instructions. The β-glucoronidase (GUSB) gene was used as housekeeping by the TaqMan assay (code 4331182, ID Hs00939627_m1). Both TaqMan probes were FAM dye-labeled. The final reaction volume was 20 µL, using 1 µL of cDNA mix, 10 µL of 2X no-ROX master mix, 1 µL of specific TaqMan probe assay, 8 µL of H_2O, according to manufacturer instructions. The real time PCR instrument used was the MJ MiniOpticon (Bio-Rad), with the following program: 5′ 95 °C and 45 cycles of 15″ 95 °C followed by 1′ 60 °C. The threshold cycles of both CFTR and GUSB genes were acquired, in triplicate for each sample. The analysis was performed using the ΔCt, calculated as the difference between the average Ct of CFTR and the average Ct of GUSB, and then calculating the value of $2^{-\Delta\Delta Ct}$ referring to H1299 cells.

Flow cytometry and Immunofluorescence. For flow cytometry, $1x10^5$ cells were incubated with the following antibodies: FITC-conjugated monoclonal mouse anti-human Epithelial Antigen Clone Ber-EP4 (Dako), FITC-conjugated anti-mouse CD271/LNGFR (Miltenyi Biotech), PE-conjugated monoclonal mouse anti-human TROP-2 (R&D system) or corresponding isotype control antibodies. Staining for integrin α6 was performed with rat

monoclonal anti-integrin α6 Clone GoH3 (Abcam) and secondary goat anti rat Alexa Fluor 647 antibody (Invitrogen). Stained cells were analyzed with FACScan or LSRII flow cytometer (Becton Dickinson). For immunofluorescence, cells were fixed with 2% paraformaldehyde, permeabilized with 0.1% Triton X-100/PBS and incubated with antibodies to mucin-5B (Santa Cruz, H-300) to assess goblet cells differentiation, with acetylated α-tubulin (Sigma, clone 6-11B-1) for cilia detection. Nuclei were counter-stained with DAPI (Invitrogen). Images were acquired by a FV1000 confocal microscope (Olympus, Tokyo, Japan), using a (Olympus) planapo objective 60x oil A.N. 1.42. Excitation light was obtained by a 408 nm Laser for DAPI, an Argon Ion Laser (488 nm) for Alexa 488, and a Diode Laser HeNe (561 nm) for Alexa 568. DAPI emission was recorded from 415 to 485 nm, FITC emission was recorded from 495 to 550 nm, Alexa 568 from 583 to 628 nm. Images recorded have an optical thickness of 0.5 mm.

Immunoblot and analysis of pharmacologic rescue of mature CFTR protein. For immunoblotting studies, 20 µg of total lysate proteins from each sample were resolved on 3–8% polyacrylamide gel electrophoresis NuPAGE Tris-Acetate (Invitrogen, Carlsbad, CA, USA) and transferred to nitrocellulose membranes. The following primary antibodies were used: mouse monoclonal antibody CFTR-596 (CFTR Antibody Distribution Program (Cystic Fibrosis Foundation, UNC-Chapel Hill), mouse monoclonal FOXJ1 clone 2A5 (eBioscience™), rabbit polyclonal CK14 (Biolegend), mouse monoclonal nucleolin C-23 (Santa Cruz), mouse monoclonal DM1A alpha Tubulin (Novus Biologicals) and mouse monoclonal β-actin (Sigma-Aldrich) antibodies. Peroxidase-conjugated secondary antibodies were purchased from Amersham™.

For evaluation of the ability of drugs to rescue CFTR protein maturation CF-CRC cells were plated in 6 well plates ($2x10^5$ cells/well) and cultured in ALI medium for 3 weeks (medium replaced every other day) to allow cell differentiation and expression of higher levels of CFTR protein. For the last 48 hours cells were exposed to drugs (Lumacaftor 5μM, Tezacaftor 20μM, Elexacaftor 3μM or their combinations) before cell lysis and lysates processed as described above. Quantification of immunoblot bands intensity were performed through Image lab software (Chemidoc XRS+, Biorad). To quantify CFTR maturation, the relative amount of CFTR band-C protein was normalized to nucleolin or tubulin measured in the identical protein sample, and these levels were used for subsequent calculations.

CRC-derived organoid generation and forskolin-induced swelling assay. Cells were suspended at 50000 cells/100 μL in Growth Factor-Reduced matrigel (Corning), vigorously but carefully pipetting to generate a single-cell suspension while avoiding the generation of air bubbles. This mixture was seeded in 100 μL aliquots into 24 well plates, creating a spherical "drop" of matrigel. The plates were incubated at 37 °C and 5% CO_2 for 30 min, to allow matrigel setting. CRC medium was added to the wells to cover the matrigel drop. After 3-4 days cells were shifted in PneumaCult–ALI Medium until mature 3D structure was formed (typically after 21 days, with the presence of a lumen and a slightly thickened spheroid wall, suggesting a pseudostratified epithelium with motile cilia (Movie 1 and Movie 2), replacing medium every other day. For functional assays, organoids were pre-treated with VX809 (Lumacaftor), VX661 (Tezacaftor), VX445 (Elexacaftor) or their combinations (all drugs purchased from Selleck Chemicals), for 48 hours, at the same doses as described above. Spheroid images were captured (10X magnification) using Time-lapse imaging station (Olympus, Tokyo, Japan), at time 0 and after 2 days of subsequent stimulation with 5μM VX770 (Ivacaftor) and 20μM Forskolin (Selleck Chemicals), to monitor and assess spheroid swelling (n = 10 spheroids per condition for all experiments described were analyzed). Images were analyzed by manually delineating the area of each spheroid using ImageJ software. Staff performing the analysis were blinded to mutation or condition of each image. Spheroid area (basal and after stimulation) percent change was calculated for each individual spheroid.

Analysis of CFTR activity in Fluid Re-adsorption assay. After differentiation of CRC in ALI-culture conditions (as described above) cells were left untreated or treated with drugs or drug combinations (in triplicate) for 2 days (in the basolateral chamber). Medium with drugs was replenished in the basolateral chambers and cells were washed 3 times (apical chamber) with 0.5 ml PBS with Ca^{++}/Mg^{++}, then 200μl of FSK-containing PBS were added and overlayed with 300 μL mineral oil to avoid evaporation. Plates were put back into incubator for 2 days to allow liquid reabsorption in the epithelium. Liquid (PBS plus oil) was collected, separated through brief centrifugation and PBS was measured to determine the amount of volume re-adsorbed in each sample. Volume of liquid re-adsorbed (ΔV) was calculated as follows: initial volume of PBS - residual volume measured) / (membrane area (1.12 cm^2) x 48 hours and expressed as μl / (cm^2 x hr).

REFERENCES

Auriche C., Di Domenico E.G., Pierandrei S., Lucarelli M., Castellani S., Conese M., Melani R., Zegarra-Moran O., Ascenzioni F. CFTR expression and activity from the human CFTR locus in BAC vectors, with regulatory regions, isolated by a single step procedure. Gene Therapy (2010) 17(11):1341-1354.

Ferraguti G., Pierandrei S., Bruno S.M., Ceci F., Strom R., Lucarelli M. A template for mutational data analysis of the CFTR gene. Clinical Chemistry and Laboratory Medicine (2011) 49(9):1447-1451.

Lucarelli M., Narzi L., Piergentili R., Ferraguti G., Grandoni F., Quattrucci S., Strom R. A 96-well formatted method for exon and exon/intron boundary full sequencing of the CFTR gene. Analytical Biochemistry (2006) 353(2):226–235.

Sette G., Salvati V., Giordani I., Pilozzi E., Quacquarini D., Duranti E., De Nicola F., Pallocca M., Fanciulli M., Falchi M., Pallini R., De Maria R., Eramo A. Conditionally reprogrammed cells (CRC) methodology does not allow the in vitro expansion of patient-derived primary and metastatic lung cancer cells. International Journal of Cancer, (2018) 143(1):88-99.

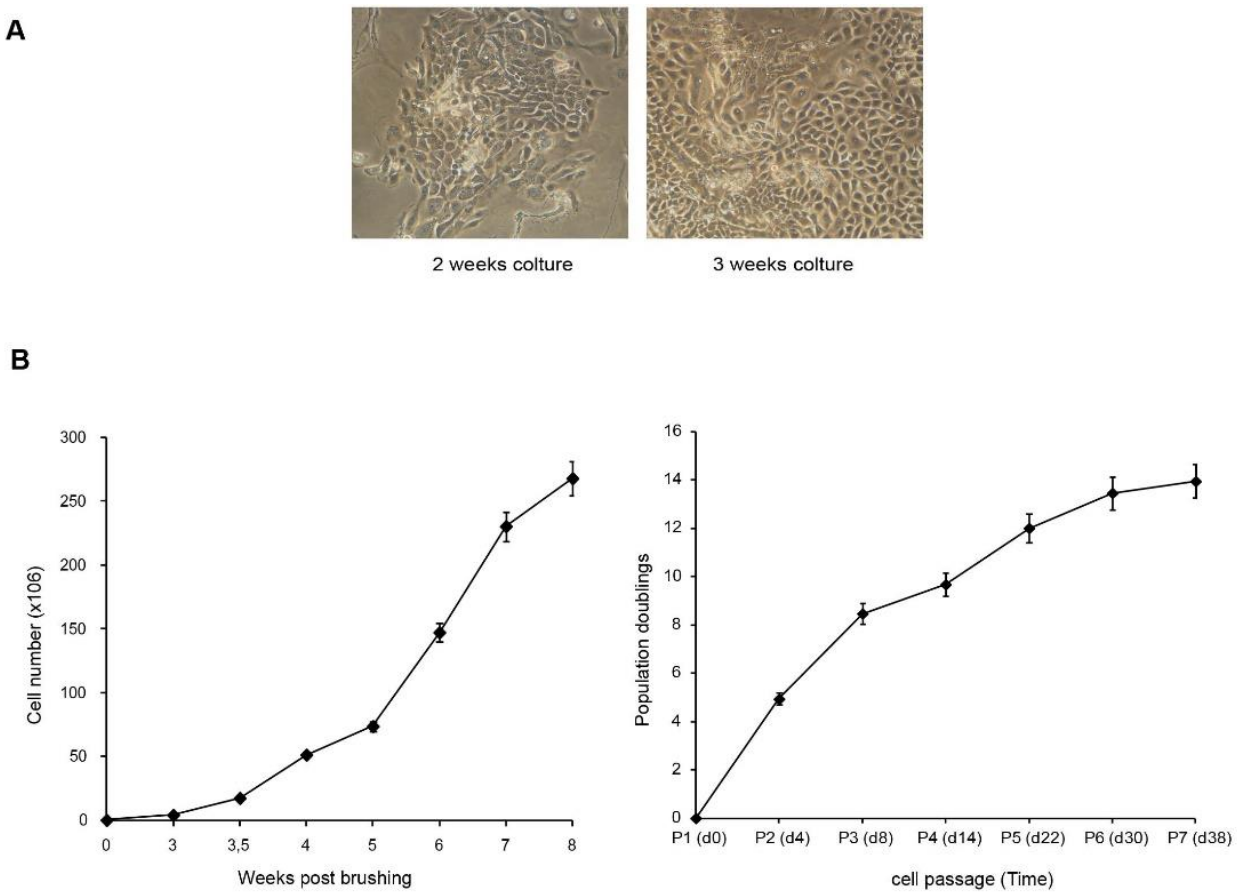

Figure 1S. Cells obtained from nasal brushing of CF patients are able to extensively proliferate under CRC culture conditions, generating long-term cultures of CF-CRC-AESC. ***A)*** *Images show that these cells grow as epithelial-like adherent clones (2 weeks) and form homogeneous monolayers (3 weeks).* ***B)*** *Growth curve (left) and population doublings (right) showing CRC methodology ability to generate huge amounts of cells with prolonged growth potential and active proliferation rates.*

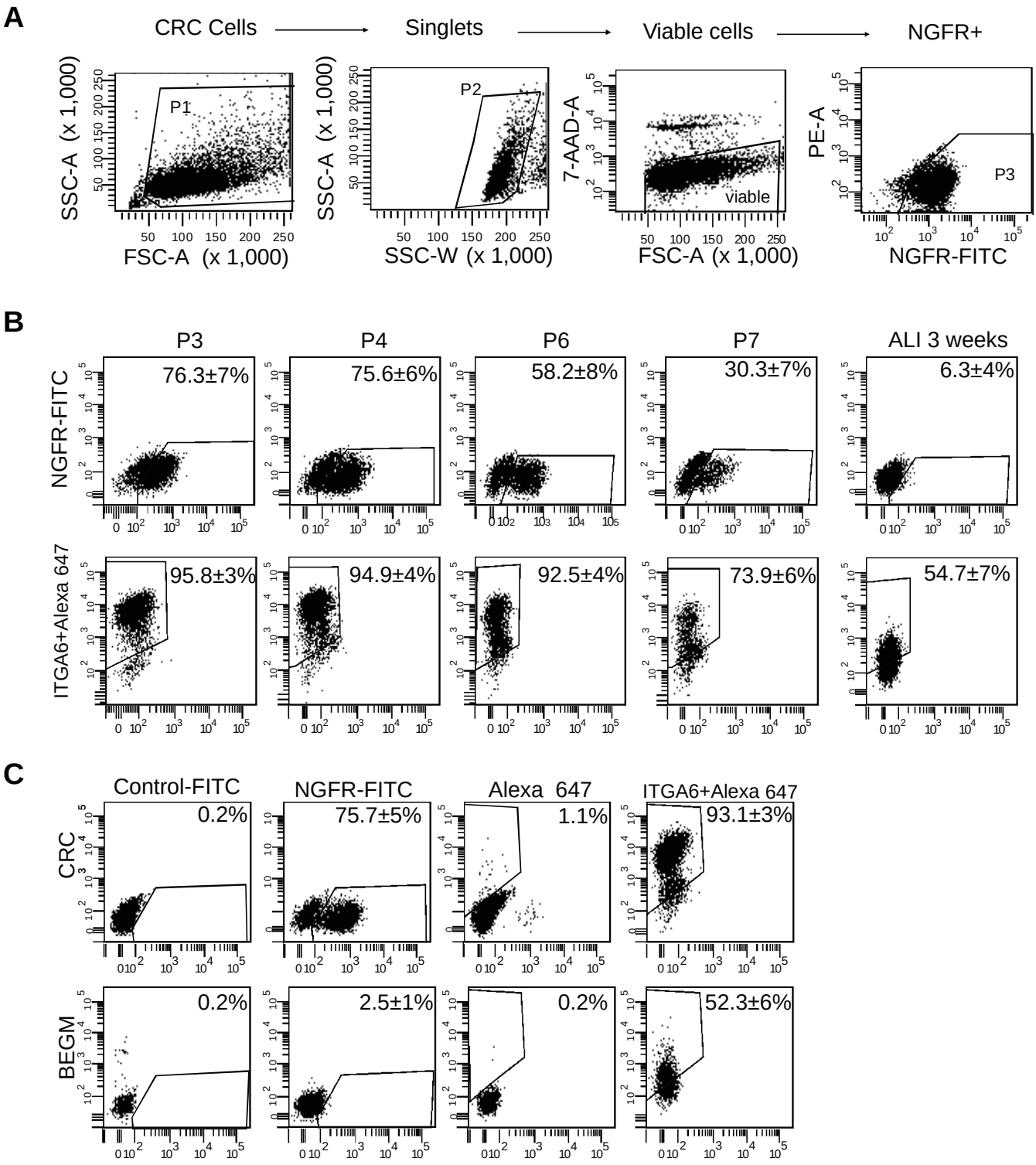

Figure 2S. A) Example of the complete gating strategy used for flow cytometry to evaluate cell viability and cell doublets for the acquisition and analysis of single viable cells. B) *Maintenance of basal stem cell-associated markers NGFR and ITGα6 during prolonged passaging in vitro. Flow cytometry analysis of passage 3 (P3) to passage 7 (P7) and ALI differentiated cells (3 weeks) is reported.* ***C) Flow cytometric comparison of basal***

stem cell-associated marker expression (NGFR, ITGA6) in CF-CRC cultures (upper panels) and standard growth condition (BEGM) cultures (lower panels).