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ORGANOID MODELS TO STUDY THE CROSSTALK BETWEEN TUMOR CELLS AND TUMOR INFILITRATING LYMPHOCYTES

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SUMMARY

ABSTRACT

Recent advances in 3D culture technology allow embryonic and adult mammalian stem cells to generate organoids *in vitro*, which reflect key structural and functional properties of organs they originate (Clevers H., Cell, 2016). For this reason, they represent a powerful tool to study human physiological and pathological processes, in particular to investigate complex processes like tumorigenesis and tumor growth, resembling the *in vivo* mechanisms.

In particular, in tumor contest neoplastic cells activate several strategies to escape from immunesurveillance, such as the recruitment of immune cells with immunosuppressive functions. In this regard, $CD4^+$ T regulatory cells (Tregs), physiologically engaged in the maintenance of immunological self-tolerance and immune homeostasis, are potent suppressors of effector cells and found at high frequencies in various types of cancer. A recent transcriptome analysis performed in our lab (De Simone M. et al., Immunity, 2016) revealed that tumor-infiltrating Tregs, isolated from CRC (colorectal cancer) and NSCLC (non-small cell lung cancer) patients, expressed a unique and specific gene signature, correlated with patients' survival. In line with our findings, non-lymphoid tissue infiltrating Tregs can exhibit specific phenotypes and transcriptional profile involved in glucose metabolism, tissue repair and muscle regeneration, far from their well-established suppressive roles (Cipolletta D. et al., 2012; Arpaia N. et al., 2015).

Thus, our work wants to evaluate the immune dependent and independent function of tissue-infiltrating Tregs, exploiting a co-culture model with normal and colon cancer- derived organoids. This approach could be suitable to recapitulate primary tumorigenesis, cancer microenvironment effect on Tregs recruitment and phenotype and, vice versa, infiltrating Tregs influence on tumor onset, growth and tissue homeostasis.

In order to answer our biological questions by using organoid model, we first of all derived organoids starting from CRC patients' biopsies, according to protocol published by Sato T. et al. (2011). We generated a biobank of 20 and 28 human- normal and tumoral colon- derived organoid lines, respectively, from tumoral biopsies and the adjacent normal mucosa of patients affected by colorectal cancer. In particular, these organoid lines can be propagated, frozen and defrosted like any tumour cell line, morphologically recapitulating the cellular composition and architecture of colon primary tissue and, importantly, representative of all CRC molecular subtypes.

Moreover, our RNA-seq bulk analysis on our organoid lines unveiled that they are very stable from the transcriptional point of view during passages and preserve the inter-individual heterogeneity.

Furthermore, our ChIP-seq data showed that our library resembled the same epigenetic landscape of colorectal primary tumour; in this context our study represents an innovative approach to investigate epigenetic processes leading CRC development and progression.

Considering that Tumor-infiltrating- Tregs (TI- Tregs) were found to express a peculiar gene signature (De Simone et al., 2016; Plitas et al., 2016), we decided to exploit organoid model to better elucidate processes leading the development and the recruitment of these cells at the tumour site.

To this end, we proceeded with the establishment of Tregs- PDO co-culture, assessing the feasibility of this system and evaluating Tregs viability in organoid culture conditions, without observing any significant differences in their survival. Moreover, we evaluated their ability to migrate into 3D organoid structure, revealing their capacity to overcome firstly the obstacle represented by Matrigel (which mimics extracellular matrix) and then creeping into the 3D architecture of organoids, recapitulating the same behaviour they have when recruited at the tumour site.

An important evidence about the possible influence of tumoroids on Treg phenotype came from our preliminary co-culture experiment, revealing that PDO-Tregs co-culture upregulated the expression of PDL1 (one of the genes belonging to TI-Treg signature (De Simone et al., 2016)) specifically on Treg but not on Tconv cells.

On the other hand, with our co-culture preliminary experiment we observed that expanded TI-Tregs enhanced PDL1 expression on tumoroid cells, which not happened when organoids were co-cultivated with Tconv cells, suggesting the possible influence of Tregs on the expression of specific molecules on cancer cell surface.

In conclusion, the exploitation of TI-Treg-PDO co-culture could shed light on the interplay between tumor and TI-Tregs, giving the opportunity to potentially interfere in this crosstalk and design a peculiar anticancer therapy targeting TI-Tregs in a personalized manner.

INTRODUCTION

1. COLORECTAL CANCER (CRC)

The term colorectal carcinoma refers to a heterogeneous group of malignant epithelial tumour arising in the colon or rectum. Only tumours penetrating through *muscularis mucosae* into submucosa are defined malignant at this site. The presence of scattered Paneth cells, neuroendocrine cells or small *foci* of squamous cell differentiation is compatible with the diagnosis of adenocarcinoma. CRC is the most common malignant cancer affecting the gastrointestinal tract, representing about 13% of all malignancies affecting this anatomic site. In many patients, its development is anticipated by presence of benign neoplastic lesions: either adenomatous or serrated polyps (Bosman F. and Yan P., 2014).

1.1 Epidemiology of CRC

Colorectal cancer is one of the most spread malignancies worldwide, being the third in men and the second in women for its frequency and ranking fourth and third for tumour-associated deaths among these two groups, respectively (reviewed by Testa U. et al., 2018; Rougier P. and Mitry E., 2003; Torre LA. et al., 2015). In particular, it's the second most common cause of cancer-related death in Europe. Indeed, more than one million individuals have been estimated to develop worldwide colorectal cancer each year, with a mortality corresponding to about 33% in the developed world. The National Cancer Institute estimated 135,430 new cases of CRC in USA in 2017, corresponding to 8% of all new cancer cases; the estimated number of deaths in 2017 in the same country was 50,260, about 8.4% of all cancer deaths. Based on the 2012–2014 data, it was estimated that 4.3% of people (males and females) will be diagnosed with CRC at certain point during their lifetime. However, in the period 1992-2014 a significant reduction of incidence of new cases of CRC occurred, probably thanks to early cancer detection through colonoscopy and subsequent removal of precancerous lesions in adults from 50 to 75 years of age (Cress RD. et al., 2006; Siegel RL. et al., 2012) Indeed, introduction of colonoscopy increased from 19.1% to 54% in 2013 and from data recorded between 2007 and 2013 it was estimated that about 65% of patients survive at least five years or more after CRC diagnosis.

In most of cases (70%) the CRC occurrence is sporadic, due to accumulation of somatic mutations; however, about 20-30% of cases are familiar CRC, encompassing a group of diseases in which patients don't have a Mendelian inheritance, but only a familiar predisposition to develop CRC. Finally, about 5% of cases arises like a Mendelian inheritance syndrome (Burt RW, 2000).

Germline minor variant and/or single‐ nucleotide polymorphisms (SNPs) in oncogenes or tumour suppressor genes are responsible for the familial CRC, whereas inactivating mutations in the same genes are typical of hereditary CRC (Jasperson KW. et al., 2010; Duraturo F. et al., 2011). The main hereditary CRC syndromes are hereditary nonpolyposis colorectal cancer (HNPCC) and adenomatous polyposis syndrome (Rustgi AK., 2007).

1.2 Aetiology of CRC

The onset of CRC pathology is mainly determined by both environmental and genetic factors, as it occurres for almost all solid tumours, generally considered complex diseases. One of this factors is represented by diet and lifestyle; indeed, a high incidence of colorectal carcinomas is observed in subjects following a high caloric diet, rich in animal fats (typical of western countries) in combination with a sedentary lifestyle. Several data evidences that meat (in particular red one), smoking and alcohol consumption are risk factors, whereas vegetable-rich diet, prolonged use of non-steroidal anti-inflammatory drugs, oestrogen replacement therapy, and physical activity are inversely correlated.

One of the most relevant risk factors for CRC is chronic inflammatory bowel disease. The risk increases after 8-10 years and is highest in patients with early-onset and widespread manifestation (pancolitis).

Another chronic disorder, the ulcerative colitis, is also considered a pre-malignant condition, representing one of the major risk factors, up to an increase of 4 fold in mortality from colorectal carcinoma. An increased incidence up to 20 fold was observed in clinical studies.

Furthermore, the risk of CRC onset is 3 fold increased in patient with Crohn disease, another inflammatory disease often affecting different areas of digestive tract. Long duration and early onset of this pathology are risk factors for CRC (Bosman, 2014).

Among other important factors, molecules like non-steroidal anti-inflammatory drugs are known to inhibit the biochemical abnormalities in prostaglandin homeostasis in colorectal cancer. Some of these agents induced a significant involution of adenomas but their role in the CRC- prevention is less clear (Bosman, 2014; Thorat M. and Cuzick J., 2013). Polymorphisms in key enzymes regulating important metabolic pathway (i.e. N acetyltransferases, glutathione- S-transferases, aldehyde dehydrogenase and cytochrome P-450) could explain individual susceptibility or predisposition among populations to CRC (Crous-Bou M. et al., 2013; Bozina N. et al., 2009).

1.3 Pathogenesis of CRC

1.3.1 Normal physiology of human intestinal epithelium

The small intestinal epithelium is organized into anatomical and functional units of self-renewing crypt-villus (Fig. 1). The villi are finger-like protrusions of the gut covered by post-mitotic epithelium and highly maximizing the surface of the absorptive area. Each villus is surrounded by several epithelial invaginations, called crypts, and represents the site of actively proliferating progenitor cells, responsible for self-renewal of the intestinal epithelium.

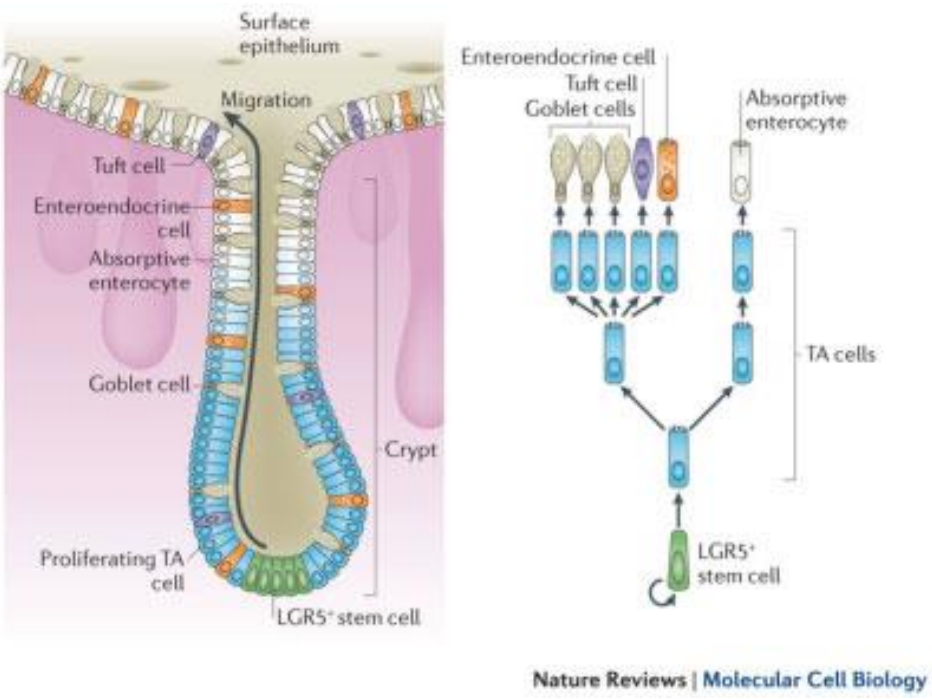


Fig. 1: Schematic representation of intestinal crypt cell population (adapted from Barker et al., 2014).

A heterogeneous group of cells constitutes intestinal crypts. Differentiated cells constituting colon epithelium originate from multipotent stem cells, localized at basis of crypts (historically known as crypt base columnar cells, CBCs), better known as intestinal stem cells (ISCs), responsible for self-renewal in the intestinal epithelia. This cell population gives rise to daughter or progenitor cells, which can subsequently differentiate into the mature cell types required for normal gut function. In particular, this differentiating process happens according to a hierarchical pattern, starting from a stem cell population located at the bottom of crypts at the level of a position called +4 (Barker N. et al., 2008). Interestingly, Sangiorgi et al., 2008 described $Bmi1^{+}$ cells, located just above the base of the crypt (+4 position), as a population acting as ISCs, able to expand, self-renew and to give rise to all the differentiated intestinal cell subsets. Subsequent studies defined $LGR5^{+}$ cells as a sensitive

population to WNT activators, contributing to homeostatic regeneration and ablated by irradiation, whereas Bmi1^{+} cells as quiescent, apparently insensitive to WNT signalling cells, contributing weakly to homeostatic regeneration and resistant to radiation-induced injury (Yan et al., 2012). According to all these observations, a two-stem cell model is proposed involving the existence of an actively proliferating, but injury-sensitive ISC and a rare, injury-resistant pool of quiescent ISC. In this model, proliferating LGR5^{+} cells would promote a rapid regeneration of epithelial colon cells in homeostatic conditions and tissue damage, whereas quiescent Bmi1^{+} stem cells would represent the subset with high resistance to DNA damage, resistant to Wnt pathway stimulation, involved in the maintenance of the intestinal epithelium in the basal state and in epithelium regeneration after injury (Yousefi et al., 2017), recapitulating for some aspect stem/progenitor organization of hematopoietic compartment.

The immediate daughter cells of the stem cells proliferate a finite number of cycles and form a group of transit amplifying cells (TA cells) situated directly above the stem cells. In particular, Leucine-rich repeat-containing G-protein coupled receptor 5 (LGR5) $^{+}$ ISCs divide every 24 h and generate TA cells, which move upward to differentiate into absorptive enterocytes or goblet and tuft cells, other specialized intestinal subpopulations.

Paneth cells reside at the basis of crypts, intermingled with LGR5^{+} ISCs, since they provide important factors for survival and proliferation of stem niche, like Wnt ligand and EGF, and secrete bactericidal proteins. Moreover, also from metabolic point of view, Paneth cells and LGR5^{+} cells cooperate: indeed, Paneth cells support stem cell function by providing the lactate necessary to sustain the enhanced mitochondrial oxidative phosphorylation in LGR5^{+} ISCs, which are characterized by a high mitochondrial activity. Though, Paneth cells are typical of small intestine, however in colon tissue regenerating islet-derived family member 4 (REG4)- positive deep crypt secretory cells are present near to LGR5^{+} cells and act as Paneth cells substitutes to support stem niche survival and proliferation (Clevers, H., 2013).

The most frequent cell population is represented by enterocytes, highly polarized epithelial cells with absorptive functions. Other intestinal cell subtypes include: goblet cells, which secrete mucins; enteroendocrine cells, involved in the release of several hormones; finally, tuft cells which are involved in the sensing of the luminal content. All of these cell types can be localized both in villi and crypts. Moreover, microfold cells are specialized cells which have a very peculiar localization at the level of the epithelium recovering the Peyer's patches. These cells are known to initiate mucosal immunity responses in the intestinal mucosa, thanks to their ability to take up antigens from the lumen of small intestine via endocytosis, phagocytosis or transcytosis (Mabbott NA. et al.,

2013). Interestingly, in the crypt, most of cells are short-lived and only few specialized cells, like Tuft cell and Paneth cells, are long-lived.

1.3.2 Main signalling pathways regulating ISC niche

The WNT pathway plays an essential role in the maintenance, proliferation and differentiation of ISCs. The Wnt/β-catenin target genes show their highest expression at the crypt bottom. The LGR5 and LGR4 proteins expressed on the surface of intestinal stem cells are used to bind secreted R-spondin proteins, acting as local enhancers of Wnt/β-catenin signalling.

In the absence of Wnt ligand, β-catenin, the key intracellular mediator of Wnt signalling, is inhibited by binding to APC/GSK3β/Axin complex, which is responsible for its degradation. However, when Wnt ligands bind Frizzled-LRP5/6 receptors, the complex is disassembled and stabilized β-catenin can migrate and accumulate into the nucleus, acting as a co-factor for the transcriptional complex TCF (T-cell factor)/ LEF (lymphoid enhancer factor). Beta- catenin and TCF/LEF complex will induce the expression of Wnt target genes, including Axin2, Rnf43, Znrf3 e Lgr5, with a mechanism of positive and negative feedback loop (Clevers and Nusse, 2012) (Fig. 2). Moreover, Wnt is also required to induce the expression of transcription factor Ascl2, that binding to the LGR5 promoter, contributes to a self-perpetuating state of stemness in $LGR5^+$ cells (Schuijers, J. et al., 2015).

Beyond positive regulation mechanisms there are also inhibitory pathways which have to be silenced for a correct ISC homeostasis. In line with this concept, Barry and colleagues (2013) demonstrated that the expression of YAP1 in the cells at the bottom of crypts had a growth-suppressive function, restricting Wnt signalling during intestinal regeneration. Indeed, the loss of YAP resulted in a hypersensitivity to Wnt, with a subsequent expansion of ISCs and niche cells (Koo et al., 2012).

$LGR5^+$ cells are characterized by specific molecular mechanisms protecting them from hyperactivation of Wnt signalling. Indeed, a recent study evidenced how the $LGR5^+$ ISCs can express the tumour suppressor RNF43, a stem cell E3-ligase, that induces the endocytosis and subsequent degradation of Wnt receptors.

Moreover, R-spondin ligands bind different LGR4–6 receptors and can amplify the WNT/β-catenin signalling which represents a differentiation signal for Paneth cells and controls their localization at the level of crypts.

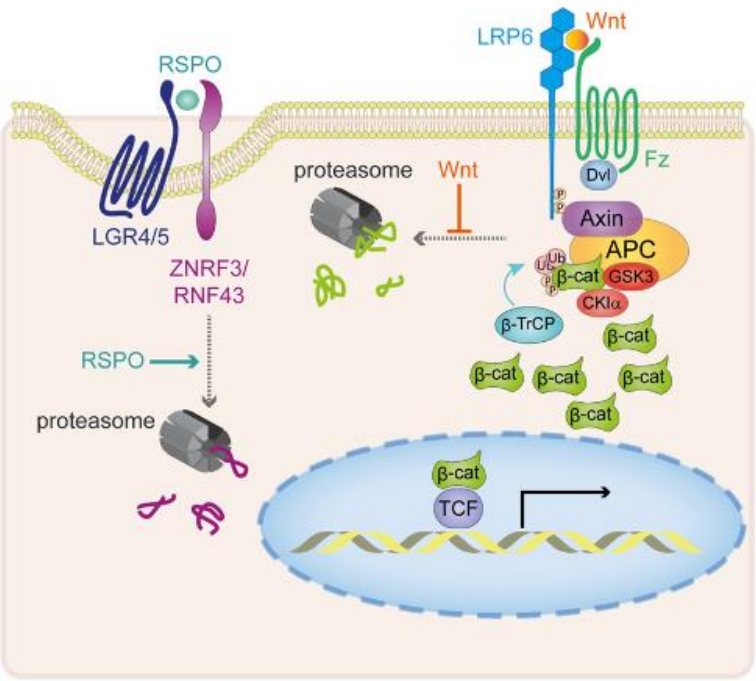


Fig. 2 Schematic representation of canonical Wnt signaling. In the absence of the Wnt signal, cytosolicβ-catenin is bound by a multimeric destruction complex that includes Axin and APC. Beta-catenin is then phos-phorylated by serine/threonine kinases CK1αand GSK3α/β. The presence of Wnt ligand bridges the Frizzled-LRP proteins, leading to recruitment of the destruction complex to cytosolic tails of clustered LRP receptors). Consequently, newly synthesizedβ-catenin molecules accumulate in the cytoplasm and shuttle to the cell nucleus to transactivate expression of TCF-dependent target genes. R-SPO forms a ternary complex with theirLGR4/5 receptor and transmembrane E3 ubiquitinligase proteinsZNRF3 and RNF43, thereby inhibiting turnover of the Wnt (Krausova M. et Korinek V., 2014)

BMP (Bone Morphogenic Protein) signalling pathway is an important pathway that mediates the interaction between intestinal epithelial cells and mesenchymal stroma, contributing to the intestinal growth, morphogenesis, differentiation and homeostasis (Batts LE. et al., 2006; de Santa Barbara P. et al., 2005). In particular, mesenchymal component in direct contact with the basis of the crypts secretes BMP-pathway inhibitors, supporting ISC maintenance; whereas BMP ligands expressed in villi create a microenvironment promoting differentiation (Fig. 3). A recent work showed how BMP inhibitors, like noggin or gremlin induced an ectopic villi formation in crypts, in parallel with an increased number of ISCs (Davis H. et al., 2015).

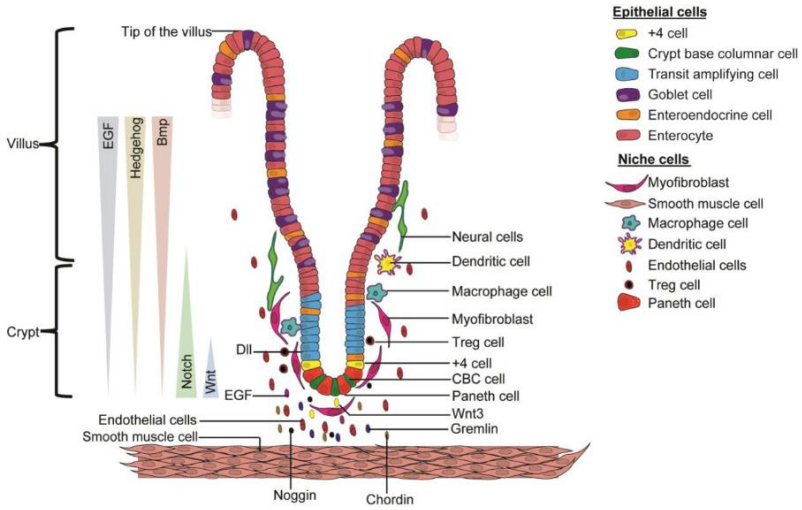


Fig. 3: Schematic representation of signalling pathways regulating ISC niche. While Wnt and Notch pathways promote ISC self- renewal, BMP pathway acts in opposite direction, inducing stem cell differentiation (Adapted from Sailaja BS. et al., 2016).

Notch pathway represents another important signalling pathway in intestinal homeostasis of ISC niche. In particular, it determines lineage decisions between enterocyte and secretory cell differentiation. When Notch receptor is engaged from one of its five ligands (Dll1-4) this induces the release of Notch intracellular domain (NCID), which translocates into the nucleus and binds to the transcription factor Rbp-J, inducing expression of Notch target genes. Among these genes, it is important to underline the activation of Hes transcription factor family, which inhibits Atoh1, one of the principal TF determining secretory lineage (Ueo T. et al., 2012). Notch inhibition determines a rapid conversion of crypt proliferation cells (also ISCs) into goblet cells, analogously Atoh1 induction (Kazanjian A. and Shroyer NF., 2011).

Beyond Wnt, BMP and Notch pathways, TFG-β pathway seems to have a relevant role in the generation of secretory cell progenitors from ISCs, controlling intestinal stem cell differentiation. Moreover, EGF and its orthologs (TGFα and ephiregulin) are involved in signalling pathway RAS/ERK/MAPK mediated and PI3K/Akt mediated, which control cellular proliferation and migration processes, in particular for ISCs (Wong et al., 2012).

1.3.3 Oncogenic transformation of ISCs

Tumorigenesis represents a multistep process, involving an initial mutagenic event, known as tumour initiation. This first genetic mutation induces a malignant phenotype, conferring only a limited growth advantage over the normal counterpart. However, this event is followed by other genetic alterations and/or epigenetic modifications (generally called tumour promotion), determining tumour growth and high proliferation (West N.R. et al., 2015; Easwaran H., 2014). This process culminates with clinically detectable tumour development and is known as tumour progression.

Firstly, Fearon and Vogelstein proposed a model, called the adenoma → carcinoma sequence model, in which specific mutations were directly related to different stages of tumour development. In particular, they supported the idea that APC gene mutations triggered tumour initiation, inducing adenoma formation and the development of "dysplastic crypts". After that, other mutations on K-RAS, p53 and SMAD4 induced tumour promotion and progression, determining an increased growth of adenoma and expansion of specific malignant clones, leading to a subsequent tumour invasion and metastasis. As previously mentioned, APC negatively regulates Wnt, by inducing β-catenin proteosomal degradation in normal colon. Therefore, cells with mutated APC gene show an accumulation of β- catenin into the nucleus, acting as a co-activator of T-cell factor (TCF)-lymphocyte enhancer factor (LEF). Then, the β-catenin/TCF-LEF complex activates key cell-cycle regulatory genes like cyclin D1 and c-Myc. Thus, according to adenoma → carcinoma sequence model, APC loss induces an increased β-catenin accumulation and subsequent increased Wnt target gene expression, including LGR5, c-Myc, Axin2 and cyclin D1.

However, other studies, like that proposed by Dow and colleagues (2015), observed how APC loss could induce intestinal differentiation but not proliferation defects, which also required the additional activation of KRAS.

Germ-line mutations in the APC gene cause a hereditary cancer syndrome known as familial adenomatous polyposis (FAP). FAP patients carry heterozygous APC mutations. The second allele is frequently lost in cells growing into colon adenomas and polyps (Plawski A. et al., 2013).

Murine models with conditional suppression of APC showed adenoma formations both in small and large intestine which, in presence of mutated p53 and KRAS genes, induced the progression to colon cancers. In these mouse models, the restoration of a functional APC protein determined cancer cell differentiation, with a normal crypt-villus tissue organization and tumour regression, without tumour relapse (Hashimoto et al., 2017).

While APC mutation represents the first “hit” for initial adenoma, KRAS and BRAF mutations are responsible for adenoma enlargement. Other mutations occurred in cancer transition affected TGF-β, PI3KCA and p53 genes. In particular, mutation in p53 in intestinal epithelial cells in mouse models alone was insufficient to generate CRC initiation, but was relevant in determining a major tumour invasiveness and metastasis (Nakayama M. et al., 2017).

Several studies suggest a role of PIK3CA mutations in CRC carcinogenesis: indeed, constitutive PI3K activity seemed to be related to tissue hyperplasia and the formation of invasive mucinous adenocarcinomas (Leystra et al., 2012).

Another model for CRC oncogenesis has been recently proposed, called “Big Bang model”, which implies that most of genomic alterations accumulate during the early stages of carcinogenesis, before the development of a big tumoral mass (Davis et al., 2017). In this way, small colorectal polyps can have different fates, with some growing (because of accumulated genetic mutations) and some regressing in size and others remaining stable in their size.

In general, the large majority of CRCs is characterized by somatic mutations on APC gene, whereas germ-line mutations of the APC gene occur in the FAP, characterized by the formation of a very large number of polyps in the colorectum, with a high risk of cancer transformation.

Individuals with classic FAP start developing polyps in late childhood to teen years. FAP represents about 1% of all CRCs. Most of the germline mutations of APC gene observed in FAP are nonsense or frameshift mutations leading to the synthesis of a truncated protein (Nieuwenhuis, M. and Vasen N., 2007). APC-truncating mutations occurred in 69.4% of CRC patients; loss of heterozygosity (LOH) at APC was detected in 32.1% of colorectal cancers, mostly caused by chromosomal deletion; and 74.8% of CRCs displayed either a LOH or at least one APC truncating mutation (Nieuwenhuis, M. and Vasen N., 2007).

In conclusion, in about 60-65% of total CRC cases, APC mutations represent the first event, followed by sequential accumulation of genetic mutations in other genes (like p53 and KRAS and BRAF) and chromosomal instability (CIN), causing microsatellite stable (MSS) tumours.

About 2-5% of CRC cases are caused by germline mutations in mismatch repair (MMR) genes, typical of Lynch syndrome (hereditary non-polyposis coli, also known as hereditary nonpolyposis colorectal cancer, HNPCC) an autosomal, dominantly inherited syndrome caused by mutations on MLH1, MSH2, MSH6 and PMS2 or the EPCAM genes). Hence, these individuals are microsatellite instability (MSI-H).

Finally, 10–15% of colorectal cancers are believed to initiate via activating mutations in the BRAF oncogene, which amplifies MAPK signalling and drives the serrated neoplastic pathway to colorectal cancer (Landau, M.S. et al., 2014; Carragher, L.A. et al., 2010).

1.4 Epigenetics of CRC

Not only genetic mutations characterize CRC onset, but also epigenetic mechanisms are involved in tumour progression, including DNA methylation, histone modifications, chromatin remodelers and non- coding RNAs. In particular, in neoplastic cells epigenetic events occur determining activation of oncogenes and silencing of tumour suppressor genes and loss of imprinting (LOI) (Choong MK. et al., 2012; Dawson MA. et al., 2012; Roy S. et al., 2012). For instance, global genomic hypomethylation was observed even in early stages of CRC (Matsubara N., 2012), affecting mainly CpG dinucleotides within mobile and repetitive genome elements, like satellite DNA, LINE (Long Interspersed Nuclear Elements) repeats and retrotransposons (Van Engeland M. et al., 2011). In particular, since DNA hypomethylation could affect chromosomal stability, it was often found to be associated to CIN CRCs (Matsubara N., 2012). Another mechanism of CRC development and progression due to hypomethylation is oncogene positive transcriptional regulation (You JS. and Jones PA., 2012; Roy S. et al., 2012; Tost J., 2010). Interestingly, APC mutation, a driving event in colorectal tumorigenesis, seems to be able to control DNA methyltransferase (DNMT) expression and activity, determining the demethylation of a number of genes and forces intestinal cells to remain in a more undifferentiated state (Hammoud SS. et al., 2013).

Loss of imprinting (LOI) is defined as the impairment of the epigenetically regulated in a parent-of-origin manner, monoallelic, selective expression of certain genes, which could lead to developmental abnormalities. LOI is present in about 40% of CRC cases in early processes of CRC tumorigenesis (Roy S. et al., 2012; You JS. and Jones PA., 2012; Tost J, 2010; Suzuki K. et al., 2006; Matsunoki A. et al., 2012; Schnekenburger M. and Diederich M, 2012). Interestingly, LOI of the insulin-like growth factor-2 gene (IGF2), leads to aberrant expression of the normally epigenetically-repressed maternally-inherited copy in CRC tissues and cell lines, enhancing the activation of IGF1 receptor (IGF1R) and its downstream signalling pathways (PI3K)/ Akt and GRB2/Ras/extracellular signal-regulated kinase (ERK) (Jelinic P. and Shaw P., 2007).

As previously mentioned, another epigenetic modification in CRC is represented by DNA hypermethylation. Interestingly, inactivating hypermethylation was observed on promoters of APC, Cadherin-1 (CDH1), runt-related transcription factor 3 (RUNX3), mutL homolog 1 (MLH1), O-6-methylguanine- DNA methyltransferase (MGMT), cyclin- dependent kinase inhibitor 2A (CDKN2A), and RASSF1A genes in CRC patients (Nilsson TK. et al., 2013; Kim JG. et al., 2013). Moreover, APC promoter hypermethylation correlated with metastatic CRC, whereas promoter inactivation by hypermethylation of the mismatch repair gene MLH1 was often observed in sporadic CRC with MSI (Dawson MA. et al., 2012; Roy S. et al., 2012).

Depending on aberrant hypermethylation CRC can be classified as CpG island methylator phenotype (CIMP) positive (+) and CIMP negative (−) CRCs. In particular, the first ones are frequent in high- grade mucinous carcinomas, located in proximal colon, and characterized by MSI, increased incidence of kRAS or BRAF mutation, but *wild type* p53. These tumors are most frequently associated with older ages and the female gender (Matsubara N., 2012; Choong MK. et al., 2012).

Other epigenetic aberrations include histone acetylation and methylation on specific residues, whose type of modification and location can define CRC outcome. In this context, LSD1, a histone methyl- transferase (HMT) can interact with several molecules, like p53 and DNMT1, an association that seems to enhance CRC proliferation, invasiveness and metastatic potential (Jin L. et al., 2013; Ding J. et al., 2013). Moreover, LSD1 has been positively correlated with TNM stages, lymph node infiltration and metastatic disease in CRC patients (Jie D. et al., 2013).

The Polycomb-group protein family, consisting of PRC1 and PRC2 complexes, mediates epigenetic silencing via histone methylation. *In vitro* evidences from CRC cells indicated that EZH2, a member of Polycomb complex, could be involved in tumour proliferation and growth (Fussbroich B. et al., 2011). Moreover, members of the Polycomb group family, such as Bmi1, was found to be overexpressed in CRC patients and correlated with advanced stages and aggressive types of CRC (Du J. et al., 2010).

Overexpression or mutated forms of several members of the chromatin remodelers were identified in several hematological and solid tumours (Dawson MA. et al., 2012; Tost J., 2010; Vaiopoulos AG. et al., 2012). For instance, the nucleosome remodelling and histone deacetylase (NuRD) was found to be partially responsible for inhibition of transcriptional activity of AP-1 transcription factor, which play an important role in intestinal proliferation and promotion of tumorigenesis (Aguilera C. et al., 2011). Moreover, NuRD is also involved in the silencing of tumour suppressor genes, such as acting on a negative regulator of Wnt, often in synergy with DNMTs (Cai Y. et al., 2013).

However, most epigenetic events involved in CRC onset and progression are still unknown and our knowledge about these mechanisms derived mainly by CRC primary tissue or cell lines. The development of reliable system to study these processes will allow their better elucidation and offer new insights in the molecular interactions involved in the pathogenesis of CRC.

1.5 Classification of CRC

Depending on tumour features, CRC can be classified in different ways.

- **Clinical classification**

CRC can be subdivided as proximal or right-sided when originated from colon sections proximal to splenic flexure (i.e. caecum, ascending and transverse colon), whereas distal or left-sided colon, when tumours generate distally with respect to this site (descending colon and sigmoid colon).

Moreover, colon cancers can be classified as rectal cancers when they arise within 15 cm of the anal sphincter. In particular, this class of tumour shows higher rates of loco-regional relapse and lung metastases, whereas colon cancers have a higher tropism for liver spread and usually have a moderately better prognosis.

Most colon cancers are classified as adenocarcinomas, further subdivided into low-grade and high-grade. Rare histological subtypes include mucinous adenocarcinoma, adeno-squamous carcinoma, signet-cell carcinoma and medullary carcinoma. In this context, medullary colon cancer seems to be associated with microsatellite instability (MSI) and a better prognosis, whereas signet-ring cell carcinomas have a poor prognosis (Nitsche U. et al., 2013).

- **TNM classification**

In particular, for CRC staging (the process that allows to assign a stage to the disease), clinicians generally used the so-called TNM system. The T parameter describes the size of the primitive tumour (that is, it describes how deeply the primary tumour has grown into the bowel lining), the N parameter takes into consideration the possible involvement of the lymph nodes and finally the parameter M refers to the presence or not of distant metastases. Each category, in turn, is divided into subgroups, depending on the progressively increasing size of the tumour, the number of lymph nodes involved, and the presence or not of distant metastases. According to the dimensions, five degrees are distinguished, starting from T0 to T4. With regard to the lymph nodes, N0 is defined as a condition in which the regional lymph nodes are not affected, and with a growing abbreviation from N1 to N3 the progressive involvement of a greater number of lymph node stations. The presence of metastases is characterized by the indication M1, while M0 indicates their absence. Besides TNM system, often information about grade (G) is reported. It represents a parameter that considers how much cancer cells look like healthy cells when viewed under a microscope and, according to this, four degrees are distinguished: form G1 (characterized by well differentiated cells) to G4 (very undifferentiated cells) (Amin MB. et al., 2017).

Overall, a tumour is considered to be as advanced as it is bulky and extended beyond the affected organ. In addition to the prognosis formulation, tumour staging is crucial for establishing the most effective treatment plan.

- **Molecular classification**

Genomic analysis revealed the presence of activating mutations in KRAS, NRAS and BRAF, which are mutually exclusive, with an occurrence of 55-60% of CRC. The presence of one of this genetic alterations is important for the selection of CRC patients to be treated with anti-EGFR monoclonal antibodies, like Cetuximab or Panitumumab (Bardelli A and Siena S., 2010).

From the molecular point of view, CRCs are a very heterogeneous group of diseases. About 85% sporadic CRC is characterized by chromosomal instability (CIN), principally due to dsDNA damages in multiple *loci*, responsible for widespread chromosomal aberrations, amplifications and loss of heterozygosity (LOH). In particular, the most frequent cytogenetic abnormality is the LOH at the long arm of chromosome 18. One of the negative consequences of CIN consists of the loss of tumour suppressor genes, like APC, KRAS, PI3KCA, SMAD4 and TP53. Moreover, CIN seems to be associated with a worse prognosis (Walther, A. et al., 2008).

Multigene sequencing for the molecular profiling of CRC allow to distinguish between microsatellite stability (MSS) and MSI. MSI-high (MSI-H) CRCs originate from mutations in mismatch repair (MMR) genes that determine a multifunctioning gene product or from promoter methylation causing the epigenetic silencing of MMR protein expression (MMR-deficient). MSI-H or MMR-deficient CRCs could be potentially treated through administration of some immunological agents. In particular, MSI is involved in about 15% of CRCs and is characterized by variable, altered lengths through insertions or deletions of repeated nucleotide sequences (microsatellites). In this tumour subtype two mutations are frequently observed TGFbR2 and of the activin receptor type 2 genes (Testa U. et al., 2018). Deficiencies in the mismatch repair mechanism represent the key mechanism responsible for MSI: the inactivation of the MMR mechanism results from germline mutations (about 20% of cases) or epigenetic silencing (about 80% of cases) of components (MLH1, MSH2, PMS2, MSH6) of the MMR machinery. Moreover, MSI patients usually have a better prognosis than MSS patients (Sepulveda, A.R. et al., 2017).

Epigenetic regulation plays an important role in determining CpG island methylator phenotype (CIMP), which results in abnormal transcriptional repression of genes usually expressed and viceversa, due to presence (or absence) of methylated CpG clusters at level of specific promoters. Methylation of these CpG islands, usually at the level of tumour suppressor gene promoter, occurs in several cases of CRC (Ogino, S. et al., 2008). In particular, we can distinguish between: low-level of methylation increasing with age and high-level of methylation of a peculiar subset of CpG

islands, resulting in gene silencing. CIMP CRC, and particularly CIMP high tumours, form a distinct subgroup characterized by specific pathologic features, like wild-type (WT) P53, MSI and mutated BRAF (Testa U. et al., 2018).

In recent years, Guinney and colleagues (2015) proposed another molecular classification, based on bio-informatic analysis of gene expression profiles from thousands of CRC patients, subdividing CRC into four different molecular subtypes (CMS) (Guinney J. et al., 2015; Fig. 4):

- CMS1: consists in hypermutated phenotypes (14% of all cases of CRC), which are MSI-H, CIMP-H, CIN, BRAF-mutated (in about 40% of cases) and not very frequently mutated at the level of APC (about 35%), TP53 (about 30%) and KRAS (about 25%). CMS1 are hypermutated and have low prevalence of somatic copy number alterations (SCNAs). This subtype is also defined "immunological" because in these tumours there is an increase of immune infiltrate (in particular Th1 lymphocytes and CTLs) and expression of immune evasion- associated genes. These tumours originate mainly from serrated lesions, located in the proximal regions of the colon and their prognosis is intermediate, but poor after relapse, CMS1 tumours were frequently diagnosed in females with right-sided lesions and presented with higher histopathological grade.
- CMS2: this group encompasses about 40% of all cases, corresponding to the canonical subtype, characterized by $CIMP^-$, MS stable, CIN-H, with frequent APC mutations (75%) and with Wnt and Myc pathway activation. BRAF mutations are absent, while TP53 mutations are frequent (about 70%) and KRAS mutations are moderately frequent (about 30%). For this reason, CMS2 is general considered the "canonical subtype" of CRC. these tumours predominantly originate from tubular lesions located in the distal region of colon.
- CMS3: this category groups about 10% of all cases of CRC, characterized by low frequency of $CIMP^+$ (20%), MSI (about 15% of cases) and TP53 and BRAF mutations, CIN-H in about 54% of cases and hypermutated in 30% of cases, with a gene expression pattern characterized by the upregulation of multiple metabolic signatures, with frequent KRAS and APC mutations. For this reason, this CMS is also defined "metabolic subtype". These tumours are equally distributed in the proximal and distal segments of the colon and are associated with an intermediate prognosis.
- CMS4: it represents about 25% of all CRC cases, which are rarely hypermutated, $CIMP^+$, very frequent CIN and SCNAs. This subtype is characterized by upregulation of genes involved in epithelial-to-mesenchymal transition (EMT) and in TGF-β signalling, with frequent mutation on PC, TP53 and KRAS genes, but rare BRAF mutations and a major stromal infiltration. For this reason, CMS4 is also defined "mesenchymal subtype". these tumours originate from serrated

precursor lesions, localized in the distal segments of colon and are associated with a worse relapse-free and overall survival.

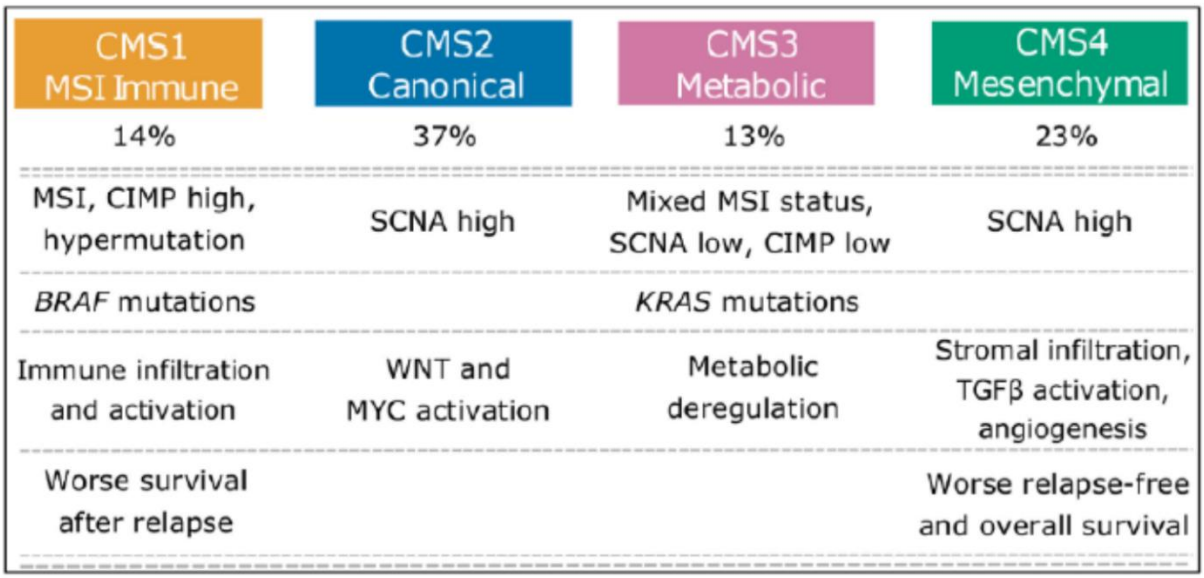


Fig. 4: schematic representation of proposed taxonomy of colorectal cancer, reflecting significant biological differences in the gene expression-based molecular subtypes (Guinney J. et al., 2015).

Although CMS classification is generally accepted in scientific community as a good instrument to subdivide different colorectal cancers, the contribution of the stromal compartment should not be overlooked. Indeed, this component has a relevant role in influencing tumoral gene expression, since cancer cells communicate and influence the surrounded cells, but, on the other hand, they are also profoundly conditioned by factors released by tumour microenviroment. In line with this, Isella C. and colleagues (2017) underlain the strong impact of stromal cells on the transcriptional classification of CRC and proposed another molecular taxonomy. Based on patient-derived xenografts (PDXs) models they could discriminate between stroma and tumour, since stromal cells constituted by murine cells, whereas cancer cells were human, allowing to assess cancer-cell intrinsic transcriptional features. Through this approach they identified five CRC intrinsic subtypes (CRIS):

- CRIS-A: it included mucinous, glycolytic phenotypes, enriched for MSI or KRAS mutations;
- CRIS-B: it characterized by activation of TGF-β pathway, EMT and poor prognosis:
- CRIS-C: it encompassed CRC with high EGFR signalling and sensitivity to EGFR inhibitors;
- CRIS-D: it included CRC with WNT activation, IGF2 gene overexpression and amplification;
- CRIS-E: it characterized by a Paneth cell-like phenotype and TP53 mutations.

Interestingly, the correspondence between CMS and CRIS subtypes showed that CMS1 mainly corresponds to CRIS-A and, in part, also to CRIS-B; CMS2 mainly corresponds to CRIS-C, but also to CRIS-D and CRIS-E; CMS3 mainly corresponds to CRIS-A; CMS4 corresponds to the five CRIS subtypes.

All of these approached highlight the heterogeneous and complex genetic background behind CRC tumorigenesis and progression and the difficulty to have a reliable classification which takes in consideration each aspect of the pathology.

2. ORGANOID MODEL TO STUDY CRC

As previously mentioned, CRC represents a group of heterogeneous and malignant epithelial cancers, with an extremely genetic complexity. In order to understand the pathological processes underlain CRC onset and progression, researchers sought more and more instruments able to recapitulate *in vitro* the *in vivo* situation.

The first approaches used to uncover molecular basis of tumorigenesis were represented by 2D culture technique. This model relies on adherence to a flat artificial surface (i.e. petri dish, glass of polystyrene) providing a mechanical support for the cells. Moreover, 2D culture allows cells, cultivated as a monolayer, to have access to analogous amount of nutrients, growth factors and oxygen for a homogeneous growth and proliferation. For this reason, scientists started to use 2D platforms in biological and clinical research.

Although these approaches are well- accepted and have significantly advanced our understanding of biological processes, 2D cell cultures also present great limitations: first of all, growth on plastic supports induces significant modifications both in morphology and in gene expression of these cells. Indeed, long-term culture can accumulate further genetic mutation during passages, a variable which cannot be easily controlled, but able to affect *in vitro* studies on signalling pathways in physiological and pathological contexts (see tumorigenesis studies). Another important issue is intrinsic in the simplicity of 2D model: the single monolayer of cells completely loses the cellular heterogeneity of the original primary tissues, and all mechanisms based on cell-microenvironment interactions and functional organization.

To overcome these great limitations, scientist developed 3D cell culture systems to better recapitulate the complexity of organisms *in vivo*. Among these 3D platforms, organoids represent an important bridge between traditional 2D cultures and *in vivo* models, maintaining the same cellular heterogeneity and architecture of original tissue and being more amenable for the manipulation of niche components, signalling pathways and genome editing compared to *in vivo* models.

Organoids represent a 3D culture which, starting from stem cells, is able to self- organize and recapitulate *in vitro* the architecture, cellular heterogeneity and the functioning of the original tissue in *vivo*, in suitable culture conditions.

Organoids can be derived from different type of stem cells:

- Pluripotent embryonic stem cells (ESC) and their synthetic induced pluripotent stem cell (iPSCs) counterparts (collectively we refer to both of them with pluripotent stem cells, PSCs)
- Multipotent organ- restricted adult stem cells (aSCs).

Both techniques exploit the almost infinite expansion potential of normal stem cells in culture. For PSCs this feature has been an essential prerequisite for their discovery, but for several years aSCs, instead, have been considered incapable to proliferate *in vitro*. In presence of specific growth factor cocktails, mimicking organ stem cell niches, both PSCs and aSCs are able to differentiate and self-organize *in vitro* into structures that reflect the most important features of the tissues to which they are fated.

While for PSC-derived organoids developmental processes were exploited for their *in vitro* establishment, organoids can be generated from ASCs through manipulation of culture conditions in order to reproduce the stem cell niche environment, present during homeostatic or after damage tissue repair.

The most important contribution in the establishment of aSC-derived organoids comes from knowledge in field of intestine morphogenesis and its constitution.

2.1 Human normal intestinal-derived organoid model

Gastrointestinal tract represents the most important field in which organoid model has been the wider application, due to rapid turnover which characterized its epithelium.

Intestinal stem cells (ISCs) are anatomically localized at the basis of crypts, the functional unit of intestine and give origin to the other cell types present. The identification of Lgr5 as an ISCs specific marker allows the isolation and characterization of these stem cells (Barker et al., 2007). The ability of these proliferating and indefinitely long live cells opened the possibility to use the isolated $Lgr5^+$ stem cells as putative source for intestinal organoids. $LGR5^+$ stem cells proliferation and self-renewal both *in vitro* and *in vivo* are closely dependent on direct cell contact between these cells and Paneth cells, making ISCs incapable to grow *in vitro* in the absence of Paneth cells (Durand et al., 2012; Farin et al., 2012). In this context, 2D culture systems are not suitable to recapitulate the situation *in vivo*, and thus 3D models become necessary for this purpose. Indeed, when cultured as organoids in Matrigel drops, intestinal stem cells spontaneously differentiate into all epithelial cell types, with stem cells maintained only at the bottom of crypts. Intestinal organoids represent one of the first 3D model to be well established and used to study intestinal homeostatic processes and CRC tumorigenesis. In particular, Sato and colleagues (2009) were the pioneers in the definition of a culture system to generate organoids starting from ISCs of small intestine of mouse, in which single stem cells $LGR5^+$ were able to create structures similar to crypts and with a self-renewal activity for more than a year. This system is based on a serum-free medium without mesenchymal component, but in presence of a well-defined set of factors (added to a basal medium), necessary for the maintenance of the stem niche (like EGF, Noggin and R-spondin), and

Matrigel or analogous synthetic supports (like hydrogels or hybrid PEG, see Gjorevski et al., 2016), mimicking extracellular matrix (ECM) function. Matrigel is a reconstituted basement membrane preparation that is extracted from the Engelbreth-Holm-Swarm (EHS) mouse sarcoma, a tumour rich in extracellular matrix proteins. It is rich in collagen IV, laminin, entactin and presents traces of some growth factors, like PDGF, VEGF, IGF and TGF-β. In particular, it forms a scaffold, resembling extracellular matrix, capable of supporting morphogenesis, cell differentiation and proliferation (Kleinman and Martin, 2005). Indeed, a strong adhesion between epithelium and basal membrane is essential for epithelial cell survival and it is maintained through integrin signalling; the loss of this strict adhesion induces apoptosis due to detachment, defined anoikis. In this context, ROCK kinase play an important role in regulating cytoskeleton during apoptosis. Its inhibition through Y-27632 determinates a great efficiency in generating organoids from colon stem cells.

In these conditions, it is possible to generate intestinal organoids resembling their normal physiological counterpart both in their cellular content (generation of various cell lineages), multicellular architecture and functional properties. In particular, each cellular component has a specific localization, mimicking *in vivo* organization: ISC and Paneth cells are located in the lower part of the buds (which give rise to crypt-like structures) while mature enterocytes migrate to central of the cystic structure (which generate villus-like structures). Thus, the basal side of the cells is oriented towards the outside, in contact with the matrix, while the enterocytes constitute the luminal surface and Paneth cells and goblets secrete towards the lumen (Grün et al., 2015; Sato T. et al., 2009).

Moreover, intestinal organoids also recapitulate the digestive and absorption functions along the axis proximal-distal. Subsequent studies lead by Sato and colleagues (2011) established the essential factors to grow human intestinal organoids: besides previously mentioned factors, p38 and TGF-β inhibitors and the addition of Wnt3a, a ligand relevant for Wnt pathway activation. In summary, stem niche factor necessary for human colon organoid culture establishment, starting from isolated ISCs or crypts, are:

1. Wnt3a/R-spondin. As previously mentioned, Wnt signaling plays a crucial role in maintenance of stemness, in the regulation of proliferation and differentiation of Paneth cells. Wnt ligands (Wnt3, Wnt6 and Wnt9b secreted by cells Paneth, and Wnt2b secreted by mesenchymal cells *in vivo* (Farin HF. et al., 2012)), following the link with the surface receptors Frizzled and LRP5/ 6, trigger intracellular mechanisms leading to accumulation of β- catenin in the cytoplasm and its translocation to the nucleus; βcatenin is associated with the family of TCF transcription factors and activates a transcriptional program that keeps stem cells in an undifferentiated state.

R-spondin is a secreted protein expressed by subepithelial intestinal fibroblasts which, after binding to the Lgr5 receptor, suppresses Wnt receptor degradation, inducing a sustained activation of Wnt pathway. Colon organoids, unlike those of small intestine that, having Wnt-producing Paneth cells grow even in the absence of the latter, require exogenous administration of Wnt ligands. GSK3β inhibitors (essential component of the degradation complex of β-catenin), like CHIR99021, can further amplify Wnt signalling (Yin X. et al., 2014).

2. EGF. Intestinal organoids require activation of EGFR signalling pathway to proliferate. Indeed, treatment with an EGFR inhibitor slows down considerably their growth. This factor can be replaced with HB-EGF or IGF-1, since these molecules also activate the KRAS and PI3K / Akt pathways (Reynolds et al., 2014).
3. Notch signalling. Inhibition of Notch induces upregulation of Notch ligands (Dll1 and Dll4) on Paneth cells and on progenitors of the secretory lineage, which, in turn, activate adjacent Notch^{+} cells. Therefore, the ISCs constantly require contact with cells expressing ligands of Notch in order to generate organoids *in vitro* (Sato et al., 2011b).
4. TGF-β/ BMP signalling. Noggin is a secreted homodimeric glycoprotein that antagonizes the BMP family of proteins and is essential for maintaining the organoids in culture for long periods. Indeed, the lack of this factor determinates a decreased Lgr5 expression and proliferation in few days. Besides noggin, also gremlin is able to block BMP pathway, stimulating organoid proliferation (Scoville DH. et al., 2008).

   TGF-β blocks the proliferation of organoids, and its inhibition, obtained by adding small inhibitory molecules of ALK4/ 5/7 (activin receptor-like kinase 4/5/7, that is TGF-β type I receptors) like A83-01, is therefore necessary for the cultivation of human intestinal organoids, more difficult to keep in culture. Moreover, also p38 inhibitor (SB202190) is another key factor for human colon organoid generation *in vitro*. This means that Alk and p38 signalling negatively regulate long-term maintenance of human ISCs. Indeed, it has been reported that the inhibition of p38 blocks the differentiation of goblet cells and increases epithelial proliferation intestinal *in vivo* (Otsuka et al., 2010).

Finally, Sato et al. also identified a hormone, gastrin, that can be added to the basal medium for the cultivation of human colonoids; although its effects are only marginal, this molecule supports the growth of the gastrointestinal mucosa (Sato et al., 2011).

Recent studies demonstrated the genetic stability of mouse and human intestinal organoid cultures, through exome sequencing analysis on organoids at early and late passages (Fujii et al., 2016). Moreover, analysis of gene expression of intestinal organoids demonstrated the effective cellular

heterogeneity of these cultures, resembling the cellular composition of primary tissue *in vivo* (Grün et al., 2015).

During stem cell culture, the formation of a small symmetrical cyst is initially observed; subsequently, a bud is formed around the Paneth cell which, within 2-3 days, develops into a crypt-like structure consisting of stem and Paneth cells. The proliferative niche consisting of CBC and Paneth cells pushes outwards from the central cyst, a process driven by repulsive interactions between EphB (Ephrin type- B receptor) and the respective ligands of surface EphrinB (Sato and Clevers, 2013). EphB2 and EphB3 are target genes of Wnt expressed mainly by Paneth cells and by ISCs (the cell populations nearest to Wnt source) in the lower part of the crypt, and their expression guides the local production of Wnt; EphrinB is instead expressed by differentiated cells, following an inverse gradient.

In organoids, since in the central cyst the cells do not come into contact with the Wnt signals and express Ephrin, $EphB2^{+}$ cells are ejected from cyst forming new buds: therefore, the gradient of Wnt comes automatically recreated along axis of the crypt. The proliferating TA cells are mechanically pushed towards the lumen by new younger TA cells, and, in this way, they are subjected to a rapid decrease in Wnt levels: this guides their differentiation into a well-defined cell type (Sato and Clevers, 2013) (Fig. 5).

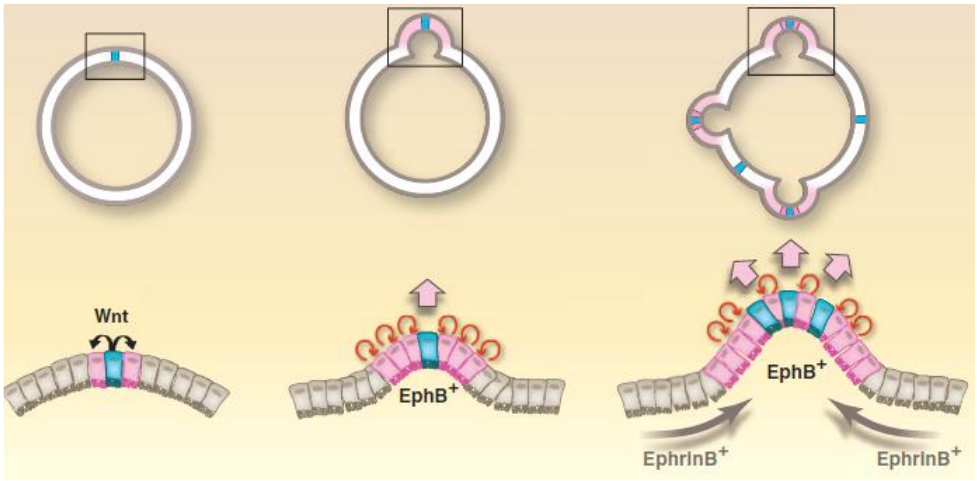


Fig. 5: schematic representation of self-organizing mechanisms in intestinal organoid culture (Sato and Clevers, 2013). In red and in blue activating Wnt cells and Paneth cells are indicated respectively.

2.2 CRC-derived organoid models

Organoids can represent a powerful tool to recapitulate tumour-related cellular and genetic heterogeneity. Several works demonstrated the feasibility to generate organoids from different type of tumours, like prostate, glioblastoma, pancreatic and colon cancers (Boj et al., 2015; Hubert et al., 2016; Gao et al., 2014; Sato et al., 2011; van de Wetering et al., 2015). Moreover, the possibility to derived organoids from different patients with the same neoplasia provides a powerful tool to study how the same tumour, different in terms of genetic origin/background, evolves and responds to specific chemotherapeutic treatments *in vitro*, predicting the possible tumour evolution *in vivo*.

The advances in the long-term growth of intestinal $LGR5^{+}$ cells gave the great opportunity to develop a 3D culture system able to recapitulate *in vitro* the mechanisms relative to tumorigenesis and tumour growth in primary intestinal cells (Onuma K. et al., 2013).

Analogously to normal intestinal protocol, isolated tumoral altered crypts are included in a Matrigel drop and cultivated in the presence of a medium supplemented with the factors necessary for the maintenance of the stem niche (EGF, noggin, A83-01 and SB212090). However, in this case Wnt and R-spondin are dispensable, since mutations on APC/Wnt pathway are the most frequent alteration responsible for tumour initiation (Sato et al., 2011).

This method allows the production of tumoral organoids, constituted by all cellular types observed in the original primary tumour, maintaining functional regions similar to crypts and depending on the tumoral grade a migratory capacity of differentiated cells along the crypt-villus axis. This makes the organoid system functionally relevant for studying *in vivo* responses to cancer, compared to traditional 2D culture methods, providing a powerful link between *in vivo* and *in vitro* studies.

One of the most intriguing issue is represented by the possibility to study tumour onset, reproducing step by step each event leading to tumour initiation *in vitro*. In line with this, two independent studies developed a model of adenoma- carcinoma progression by introducing four sequential mutations into human colon organoid stem cells using CRISPR/CAS9 technology. In the first one, Drost et al. (2015) induced firstly an inactivating mutation on APC gene and, subsequently, mutations on TP53, KRAS and SMAD4 genes. Analogously, Matano et al (2015) generated CRC organoids starting from normal human colon organoids by introducing APC, TP53, KRAS and SMAD4 mutations. In both experiments, these mutated organoids grew independently from the presence of stem niche factors and, moreover, xenotransplantation of these organoids into immunodeficient mice revealed the progressive transformation of stem cells, inducing the formation of invasive tumours *in vivo* (Drost J. et al., 2015; Matano M. et al., 2012).

2.3 Potential application of organoid models

The ability to recapitulate 3D structures and cellular heterogeneity of the original tissues, even from low amount of biological material, put the organoid model in a privileged position for the *in vitro* study of many physiological and pathological processes. Indeed, they represent an intermediate system between 2D cell lines and *in vivo* approaches, providing infinite possibilities to exploit organoid technology in several different branches of research (Fig. 6).

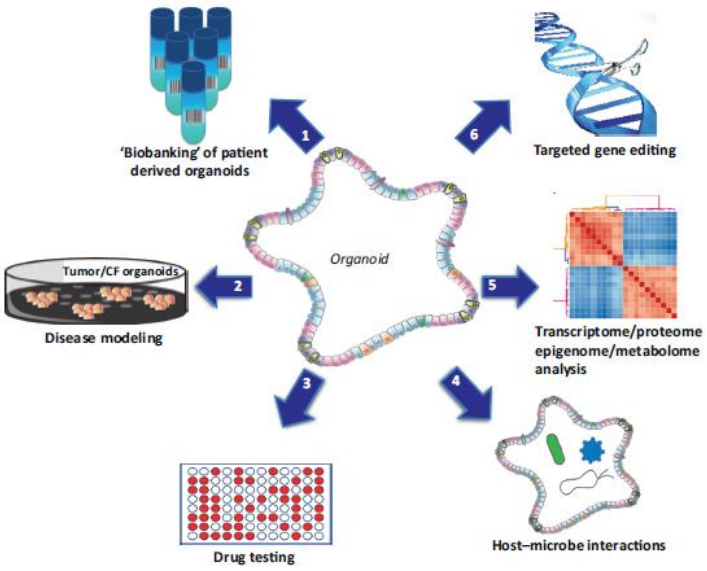


Fig.6: Schematic representation of all possible clinical applications of stem cell-derived organoids technology, like organoid biobanking, disease modeling, drug toxicity testing, personalized therapy, host–microbe interaction studies, and omics analysis (transcriptomics, proteomics, epigenomics, and metabolomics) of healthy and diseased organoids. Moreover, targeted gene therapy using the CRISPR/Cas9 system can be utilized on organoids derived from disease tissue (Dutta et al., 2017).

2.3.1 Cancer

As previously mentioned, tumour heterogeneity resides also in the evidence that the same tumor can develop in multiple ways in each patient, accumulating mutations in different pathways. This variability has been observed also in organoid models.

In particular, Fuji et al. (2016) developed a colorectal tumour organoid library (CTOL), derived from a wide range of grades and subtypes of CRC, in which they confirmed the genetic stability of organoids compared to the primary colon tissue. Analogously Van der Watering et al. (2015) generated another CRC-derived organoid biobank from 20 different patients and demonstrated, beyond the genetic stability of their cultures, the evidence of a correlation between mutational background and the responsiveness to specific antitumor drugs. In line with this, in the last years,

big steps forward have been made in order to create an organoid biobank derived from patient affected by different types of tumour (colon, prostate, lung, pancreas, breast, and ovarian cancers). This huge biobank contains information of every organoid line about genetic, transcriptional, epigenetic and proteomic profiles together with the levels of responsiveness to specific drugs (http://hub4organoids.eu/living-biobanks/) (Dutta et al., 2017).
The availability of all this information will probably allow to give a great impulse to the use of organoids in the field of personalized cancer therapy, having the possibility to exploit a well- known and reliable tumour model able to recapitulate most of features of the primary neoplastic tissue.

Other potential application of organoid technology involved: infectious and hereditary diseases, toxicology, personalized medicine, regenerative medicine and gene therapy.
In this context, one of the most important example is represented by studies about cystic fibrosis (CF), a pathology whose onset is due to mutations in gene coding for cystic fibrosis transmembrane conductance regulator (CFTR), a chloride channel physiologically expressed in epithelial cells of different tissues. Interestingly, starting from iPSCs derived from patients with CF, Wong et al. (2012) managed to generate lung organoids lacking in CFTR expression, mimicking *in vitro* the same situation occurring *in vivo*. Moreover, the possibility to have a 3D model *in vitro* for this pathology allowed them to use it as a suitable platform to screen some drugs able to revert the effects of CFTR genetic alterations.
Another interesting work on this disease was published by Dekkers et al. (2013), who generated intestinal organoids from rectal biopsies of CF patients for this purpose. They found that Forskolin was able to induce a robust swelling of *wild type* organoids due to the correct CFTR functioning, determining fluid transport to the organoid lumen. Though this didn't occur in CF organoids, the situation could be reverted for the common, temperature-sensitive CFTR-F508del mutant by culturing at 27°C and also by the addition of experimental CFTR corrector compounds.
Moreover, Firth et al. (2015) established iPSC-derived lung organoids from CF patients and used this 3D platform to apply the CRISPR/Cas9 technique for correcting mutation. The corrected iPSCs were then converted to mature airway epithelial cells demonstrating recovery of normal CFTR function.

2.4 Advantages and limitations of organoid model

As above mentioned, organoids represent an important bridge between 2D culture systems and *in vivo* mouse/human models, as they are more physiologically relevant than monolayer cultures and allow an easier manipulation of stem cell niche, signalling pathways and genome editing than in other models. In particular organoid models offers different advantages:

- They represent suitable platform to study adult stem cells and tissues in multiple contexts, being nearer to physiological condition, thanks to the ability to recapitulate both architecture and cell composition of primary tissue.
- Adult stem cells can be propagated in organoids, and specific tissue lineages can be cultured in high purity with minimal contributions from other cell types (i.e. fibroblast or endothelial cells).
- They can be propagated for a long period of time in culture, without genomic alterations.
- They can be derived from several sources: aSCs and PCSs.
- They can generate different kind of tissues, starting also from limited biological material.
- They can be used as model for human diseases that are difficult to model in animals
- They can be used in order to generate isogenic adult tissue for transplantation in regenerative procedures.
- They can be used for drug screening to study patient-specific response to a specific treatment.

As *in vitro* models, this powerful technique also retains some limitations, mainly due to the lack of the surrounding stroma with all the processes mediated by its cellular components (i.e. inflammatory responses driven by immune cells, interactions between immune cells and stem cell niche, absence of vascularization, etc.), and to the rigid extracellular matrix (ECM), that could limit drug penetration.

However, some of the limitations can be overcome by the addition of stromal cellular components (i.e. immune cells, fibroblasts, endothelial cells) to the organoid culture, setting up co-culture models. In this context, a very recent work demonstrated how co-cultures of autologous tumoral organoids and peripheral blood lymphocytes could be exploited to enrich tumour-reactive T cells from peripheral blood of patients with mismatch repair-deficient CRC and NSCLC (Dijkstra et al., 2018). In this case, the authors proposed the organoid model as a suitable platform to study tumour-specific T cell responses to epithelial cancer cells in a personalized manner.

Moreover, Neal JT. and colleagues (2018) exploited organoid model to shape tumour immune microenvironment. In particular, cultivating organoids with stromal cells from the same patients, they observed that T cell receptor repertoire was highly conserved between tumour and organoids and were able to recapitulate the immune checkpoint blockade *in vitro*.

Regarding the second limiting point, the application of novel matrices and innovative microfluidic technologies can be helpful to provide a primary tissue-similar ECM and to generate concentration gradients for growth factors and nutrients.

3. $CD4^+$ T regulatory cells

$CD4^+$ T regulatory cells (Treg cells or Tregs) represent a subtype of $CD4^+$ T lymphocytes, known to have a pivotal role in the repression of immune responses and in maintenance of self-tolerance. Most of these cells expresses high level of α-chain of IL2R (also known as CD25), lacking any other markers typical of lymphocyte activation (Abbas A., 2012). The transcription factor FOXP3 (forkhead box P3) plays a crucial role in the development and function of Treg cells. Indeed, FOXP3 KO mice develop a multisystem autoimmune syndrome, characterized by the absence of $CD25^+$ T regulatory lymphocytes. In human, mutations on FOXP3 gene seems to be involved in determining a rare autoimmune disease called IPEX (Immune dysregulation, Polyendocrinopathy, Enteropathy, X-linked syndrome) (Brinkow et al., 2001; Fontenot et al., 2003; Ochs HD. et al., 2007).

Tregs are a population phenotypically distinct form other lymphocytes, expressing low levels of IL7R (also known as CD127) and using IL2 instead of IL7 as a growth factor for their survival. Interestingly, this profile is opposite to memory T cells, which express high levels of IL7R and low levels of IL2R, showing a strong dependence on IL7 for their survival. Moreover, IL2 signalling seems to be necessary for sustained expression of Foxp3 and CD25 in natural Tregs and enhance their suppression activity *in vitro* (Fontenot et al., 2003, Shevach et al., 2011).

Tregs were firstly characterized in 2001 as $CD4^+CD25^+$ T cell population by several groups (Taams LS. et al. (2002); Levings M. (2001); Ng, W. et al. (2001); Jonuleit H. et al. (2001); Dieckmann D. et al. (2001)), starting from observation of Sakaguchi, S. and colleagues (1995) that immunologic self‑tolerance was maintained by activated T cells expressing IL‑2 receptor α‑chains (CD25) in mouse models. Only in 2005 FOXP3 was identified as a specific marker for human Treg cells (Roncador, G. et al., 2005).

3.1 Origin and development of Tregs

Tregs can originate both in the thymus and secondary lymphoid organs upon self or non-self-antigens. In particular, Tregs can derive from $CD4^+$ T cells which recognize self-antigens in the thymus. These cells are also known as *natural Treg* cells. In peripheral lymphoid organs, antigen recognition without a strong innate immunity activation promotes the generation of regulatory cells derived from naïve $CD4^+$ T cells, although sometimes Tregs can originate in presence of inflammation. These type of Tregs are also called *inducible* or *adaptive Treg cells*. Tregs originated in the thymus are specific for self-antigens, whereas Tregs originated in periphery can be specific also for non- self –antigens (Fig. 7) (Kitagawa et al., 2013).

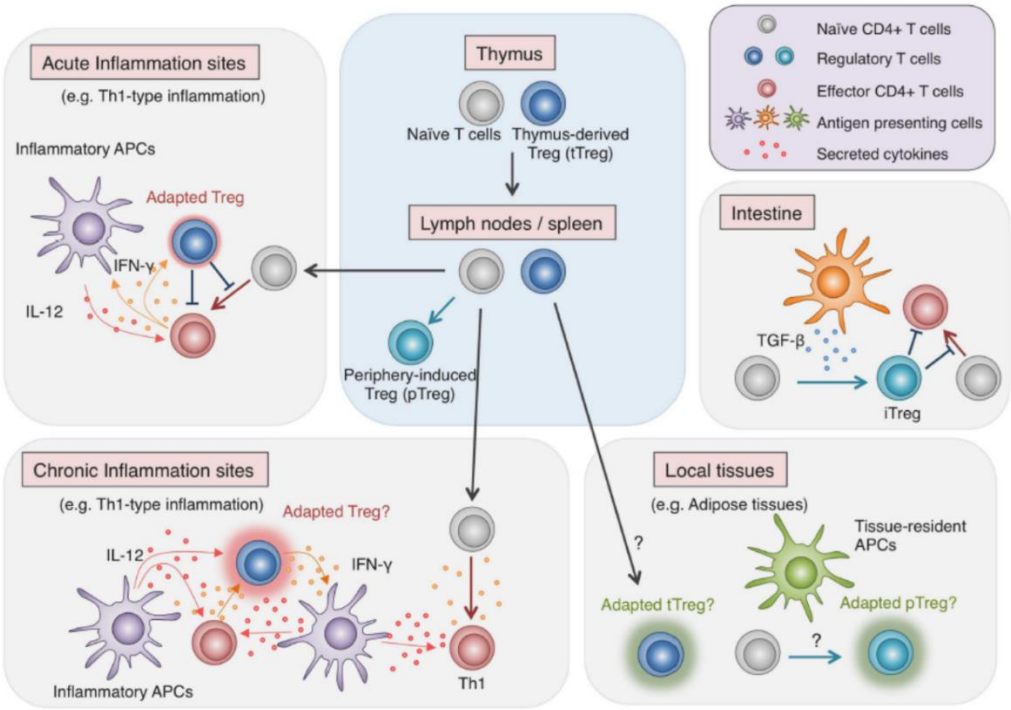


Fig. 7: Adaptability of Treg cells. Most Tregs derive from thymus, playing an important role in controlling immune self- tolerance. However, they can also originate in periphery from T conv cells, where they have important role in tissue homeostasis (Kitagawa et al., 2013).

Direct evidence of the thymic origin of Tregs and their persistence in the periphery was shown by pioneering experiments which showed the autoimmune response development after mouse neonatal thymectomy (about three days after birth) involving various organs (thyroid, stomach, ovaries and testis) (reviewed in Sakaguchi S., 2010). In line with this, other works illustrated how rat adult thymectomy followed by sublethal X-irradiations induced autoimmune thyroiditis and type I diabetes. Notably, adoptive transfer of $CD4^+$ thymocytes from untreated syngeneic animals resulted in the inhibition of autoimmune diseases (Sakaguchi, S. et al., 1982; Fowell and Mason, 1993).

Only in 1995 Sakaguchi et al. delineated $CD4^+CD25^+$ T cells as the subpopulation preventing autoimmunity, through an experiment in which they observed that transfer of depleted $CD25^+$ subset T cell suspension didn't resolve autoimmune diseases in athymic nude mice, whereas cotransfer of $CD4^+CD25^+$ T cells did.

As previously mentioned, FOXP3 represents a master regulator of Treg development and function (Hori et al., 2003; Khattri et al., 2003; Fontenot et al., 2003). The Foxp3 gene was identified for the first time as the defective gene in mouse strain Scurfy, a X-linked recessive mutant exhibiting excessive activation of $CD4^+$ T cells with consequent hyperproduction of proinflammatory cytokines (Brunkow et al., 2001). Mutations in human FOXP3 gene are responsible for a pathology, analogous to Scurfy, called IPEX (Ochs et al., 2007). The similarities between these two diseases pushed researchers to seek for a possible role of FOXP3 in Treg development and function.

FOXP3 protein is a transcription factor belonging to the forkhead/winged-helix family, known to act as a homo-oligomer and able to interact with the other transcription factors NFAT (nuclear factor or activated T cells), AML1 (acute myeloid leukemia-1)/Runx1 (runt-related transcription factor 1), the histone acetyl transferase (HAT)/ histone deacetyl transferase (HDAC) complex, and possibly NF-kB (reviewed by Sakaguchi S. et al., 2008). Several mechanisms of FOXP3-mediated transcriptional control have been described: sometimes it directly binds promoters or enhancers of its target genes, in other situations it is required an interaction with other transcription factors (Fig. 8) (Rudra et al., 2012; Floess et al., 2007; Schmidl et al., 2009; Zheng et al., 2010).

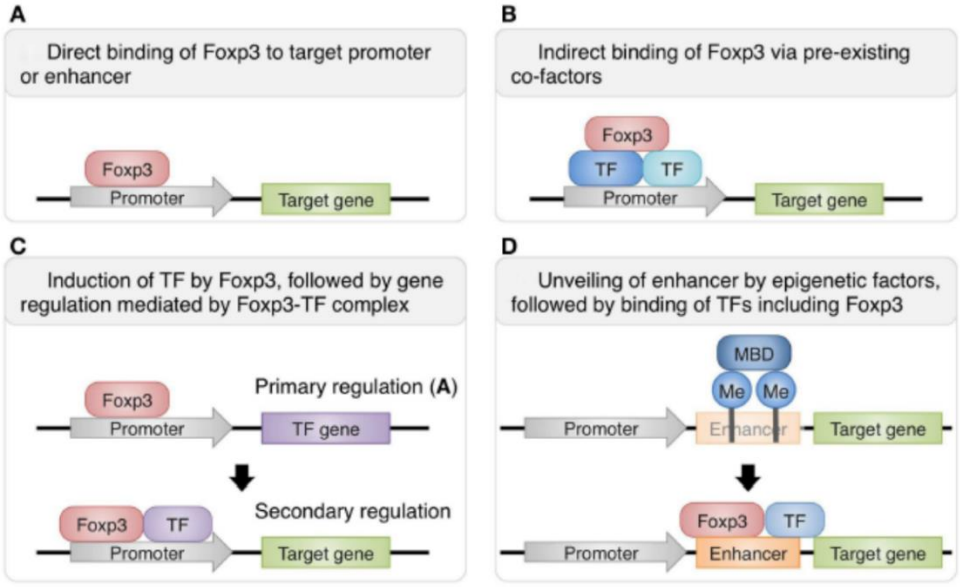


Fig. 8: Various mechanisms of Foxp3-dependent gene regulation in Treg cells (adopted from Kitagawa et al, 2013). Some genes are directly regulated by Foxp3 alone (A), while others require the protein complexes containing Foxp3 and its co-factors for transcriptional regulation. Foxp3 can interact with pre-existing transcription factors such as Runx1 and Ets-1 (B) or with direct targets of Foxp3-mediated gene regulation, such asGATA-3 (C) (Rudra et al., 2012).

Furthermore, there are also genes regulated by both Foxp3 and epigenetic changes (D). (Floess et al., 2007; Schmidl et al., 2009; Zheng et al., 2010).

One of these transcription factors is NFAT, whose activity is Ca^{2+}-dependent and controlled by calcineurin. Upon activation it forms a complex together with AP-1 and NF-kB and activates the transcription of genes like *Il2*, *Il4* and *Ctla4* and other conventional T cells typical genes. Mutation on *Foxp3* gene in the domain of interaction with NFAT inhibits FOXP3 from repressing *Il2* gene expression and activating *Ctla4* and *Cd25* transcription (Wu et al., 2006).

Several works identified about 700 genes directly or indirectly controlled by FOXP3, 10% of which bound directly by Foxp3 (Marson et al., 2007; Zheng et al., 2007). Among them, there are genes encoding for signal transduction molecules (i.e. *Zap70* and *Ptpn22*), transcription factors (i.e. *Crem*), cytokines (i.e. *Il2*), cell surface molecules (i.e. *Il2ra*, *Ctla4*), enzymes involved in cellular metabolism (i.e. *Pde3b*) and microRNAs (i.e. *miR-155*).

Another important aspect to consider is the epigenetic changes occurring during T cell differentiation involved in Treg phenotype establishment. Indeed, Tregs show a specific DNA methylation pattern which distinguishes them from naïve T cells. In particular, Treg cell lineage is associated with DNA hypomethylation at *Foxp3* conserved non coding sequence 2 (CNS2), located at the level of intron 1 of *Foxp3* gene, a modification necessary for stable expression of this transcription factor (Floess et al., 2007; Kim and Leonard, 2007). Consequently, this demethylation affects some of the genes regulated by FOXP3, like *Ctla4*, *Ikzf2* (*Helios*), *Ikzf4* (*Eos*) and *Tnfrsf18* (GITR), typical of Treg signature (Ohkura et al., 2012). In particular, these modifications are specific for Treg development and not induced by TCR or TGFβ stimulation (Ohkura et al., 2012; Polansky et al., 2008).

Thus, demethylation of CNS2 region of *Foxp3* gene act as an enhancer for its transcription, making it accessible to the binding of transcription factors like CREB and Ets-1, essential for Treg cell function (Kim and Leonard, 2007; Polansky et al., 2010).

TCR stimulation seems to play a relevant role in inducing Foxp3 expression and Treg cell epigenome. Indeed, *Foxp3* induction seems to depend mainly on the TCR stimulation intensity (Coutinho A. et al., 2005; Kawahata et al., 2002), while Treg epigenetic programme seems to rely on TCR stimulation duration (Ohkura N. et al., 2012) (Fig. 9). Probably, in the thymus only thymocytes which have acquired Treg- lineage epigenetic changes upon TCR stimulation become "poised" for Foxp3 expression and thus ready to develop into stable $Foxp3^+$ Tregs after receiving a proper TCR stimulation (Ohkura N. et al., 2012 and 2013).

In conclusion, Tregs development appears to be a complex mechanism, with multiple forces involved in determining *Foxp3* induction and Treg-specific epigenetic landscape.

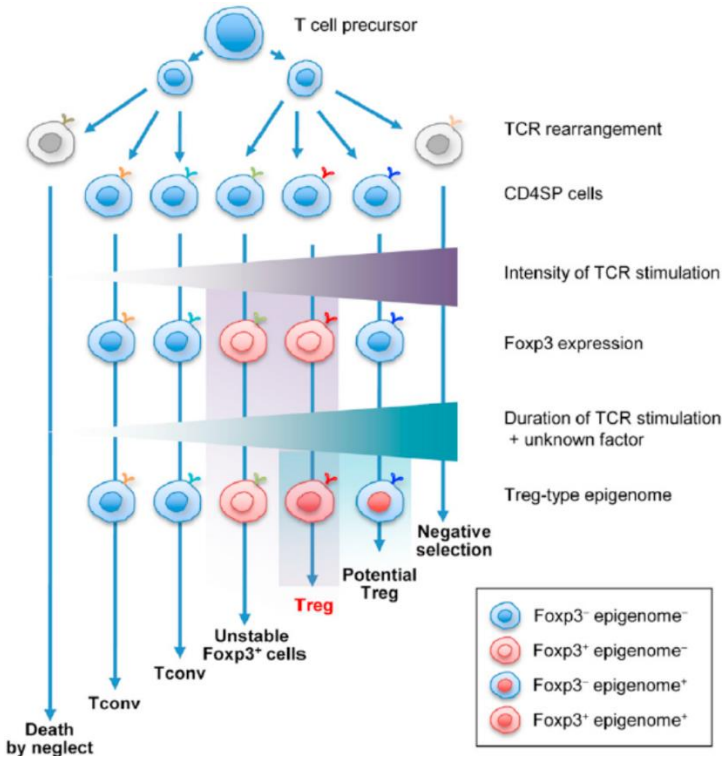


Fig. 9: Schematic representation of a model for Treg development in the thymus, showing how TCR stimulation can induce Foxp3 expression and Treg cell epigenome. Indeed, upon a proper TCR stimulation, only T cells Foxp3$^+$ and epigenome$^+$ T cells are driven toward a stable Treg cell lineage (Ohkura N. et al., 2013).

3.2 Tregs in immunological context

In our organism, the immune system represents the most important defence against external pathogens, thanks to its ability to discriminate self- antigens from non-self- ones. Therefore, on one hand its role focuses on the development of proper immune responses to eradicate infection, but on the other hand it is also important to control these responses, to prevent excessive inflammation, potentially dangerous for the host (the so-called immune homeostasis) (reviewed by Sakaguchi S. et al., 2008). In particular, there are two types of mechanisms for achieving self-tolerance and immune homeostasis: recessive (cell-intrinsic) and dominant (cell-extrinsic) mechanisms. The first ones involve that autoreactive lymphocytes could be fated to apoptosis when recognize self-antigens in central generative organs (clonal deletion), or can replace their self-reactive TCR/BCR (called receptor editing, for T cells or B cells respectively) with nonreactive ones, or finally, they can become anergic (functionally inactivated) in the periphery upon exposure to self-antigens.

With dominant mechanisms, specific T cells control activation and expansion of auto- or over-reactive lymphocytes (in particular T lymphocytes). In this context, Tregs play their pivotal role in immune self- tolerance and homeostasis. Indeed, as previously mentioned, disruption in Treg development or function is a primary cause of autoimmune and inflammatory diseases in humans and animal models. In details, Tregs can modulate immune responses through different strategies (Fig. 10):

1. Tregs can inhibit stimulatory activity of antigen presenting cells (APC), through their engagement of co-stimulatory molecules expressed on the surface of APC, determining a reduction/ absence of activation signals for T naïve/ effector cells. Indeed, Treg cells express CTLA-4 which binds B7 on APC, seizing this molecule from binding with CD28, expressed on T naïve/ effector, or inducing its internalization at APC level (reviewed by Stephens GL.and Sakaguchi S., 2007)

   Tregs produce cytokines like TGFβ and IL10 suppressing APC and T effector activity. In particular, TGF β prevents "classical activation" of macrophages (M1 phenotype), it is actually one of molecule secreted by "alternative" activated macrophages (M2 phenotype). Moreover, this cytokine hampers the activation of other immune cells, like neutrophils and endothelial cells, playing an important role in mitigating and turning off inflammatory responses. Finally, TGF β promotes tissue repair after injury, acting on macrophages and fibroblast for collagen synthesis, for induction of enzymes for extracellular matrix remodelling and for angiogenetic process activation (reviewed by Caridade M. et al., 2013).

   IL10 is involved in controlling innate and cell-mediated immune responses, through inhibition of activated macrophages and dendritic cells (DCs) activity. In particular, IL10 hampers IL12 production from activated macrophages and DC, a cytokine which represents a crucial signal for IFN-γ production. Moreover, IL10 prevents expression of co-stimulatory molecules and MHC class II on these two cell types (Kryczek I. et al., 2006)
2. In some circumstances, Tregs can have direct cytotoxic activity, for instance, through production of perforin/ granzyme and induction of cell death in effector cells (Wing K. et al., 2006; Grossman WJ. et al., 2004; Gondek DC. et al., 2005; Sakaguchi S. et al., 2009; Vignali DA. et al., 2008).
3. In other cases, Tregs can induce a metabolic disruption, inducing for example DC to produce enzyme (i.e. IDO) using amino-acids essential for naïve/ effector cells proliferation (reviewed by Caridade et al., 2013).
4. Since Tregs use huge amount of IL2, they can deprive T effector cells of their growth factor, inducing their apoptosis (Scheffold A. et al., 2005 and 2007).

Treg cells could adopt these and other suppressive mechanisms to switch off immune responses, depending on microenvironment, type of immune responses and biological context they act.

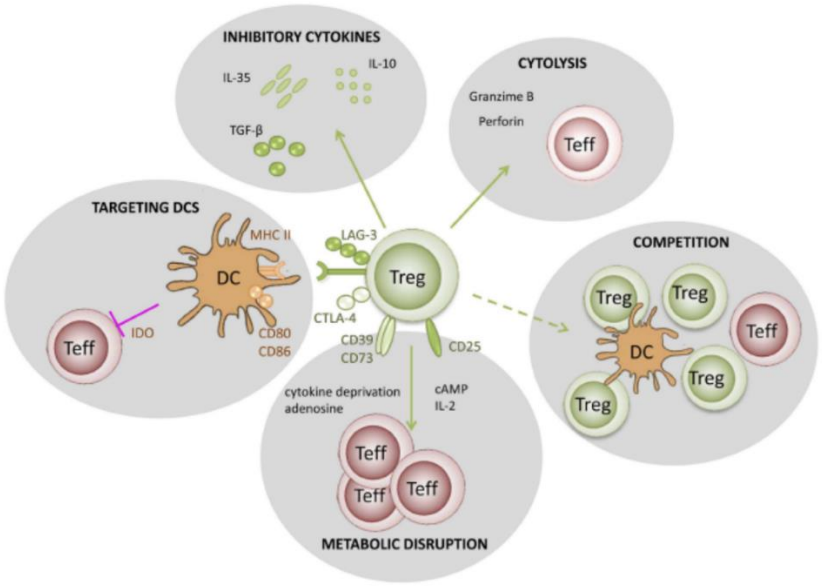


Fig. 10: Schematic representation of possible mechanism used by Tregs to prevent immune responses. (1) DC function inhibition through engagement of co-stimulatory molecules; (2) direct cytotoxic activity on T effector cells; (3) metabolic disruption; (3) completion for critical cytokines essential for T effector proliferation; (5) production of inhibitory cytokines (Caridade et al., 2013).

3.3 Tregs in non-immunological contexts

In last decades several studies underline how Tregs represents a very plastic cell subtype, able to shape their gene expression profile depending on environmental stimuli they receive, both in immunological and non- immunological contexts. In particular, recent works describe the presence of a distinct Treg cell population in several non- lymphoid tissue both in mice and humans, involved in controlling tissue homeostasis and its repair after injury (reviewed by Panduro M. et al., 2016). These "tissue-resident" Tregs are distinguishable from classical lymphoid-organ Tregs both in phenotype and function: they express specific transcription factors, chemokine receptors or TCR repertoire and use mechanisms of action different from other Tregs. These peculiar Tregs subset is found in many peripheral districts, like skin, adipose tissue, intestine, injured skeletal muscle and many hypotheses have been formulated to explain their accumulation in these sites. Since they express patterns of chemokine receptors and adhesion molecules different from their lymphoid counterpart, this subpopulation can "sense" and respond to specific chemotactic molecules produced by parenchymal or stromal cells of a specific tissue, inducing their migration from the circulation in this site. Another possibility involves the recognition of specific tissue antigens which may cause the expansion of specific Treg clones, or the conversion of $CD4^{+}Foxp3^{-}$ T conventional

(a heterogeneous cell population involved in the activation of defences against pathogens, T conv) into peripheral Tregs upon antigen recognition. Finally, another feasible mechanism could imply Treg tissue adaptation and specialization in the new microenvironment (Fig. 11) (Cipolletta D, et al., 2012).

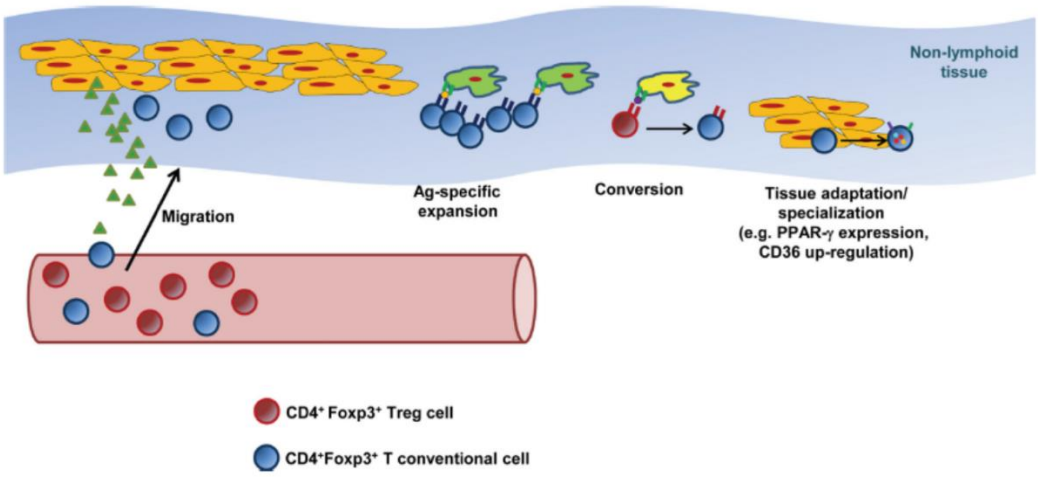


Fig. 11: Possible mechanisms involved in Treg accumulation in non- lymphoid tissues (adapted from Burzyn D. et al., 2013).

3.3.1 Tregs in visceral adipose tissue (VAT)

Feuerer and colleagues (2009) were the first to identify a unique fat-residing Tregs both in murine and human adipose tissue, finding that about 50% of $CD4^+$ T cells in the VAT were $Foxp3^+$ Tregs. In particular, TCRα sequencing revealed that TCR repertoire of this Treg subtype were different from fat T conv and that it represented just a restricted subset of lymph node (LN) Treg TCR repertoire. This evidence suggested two possible mechanisms involved in Treg accumulation in VAT: a continuous recruitment of Treg cells from peripheral LN or an initial migration of tissue-Tregs and subsequent selected clonal expansion in VAT. In line with this, VAT Treg transcriptional profile suggests their thymic origin rather than naïve T cell conversion into this Treg subpopulation (Kolodin D. et al., 2015).

Vasanthkumar et al. (2015) observed that fat Tregs expressed high levels of transcription factor PPARγ, which could be responsible for activation of specific VAT-Treg transcriptional program through interaction with FOXP3. In particular, VAT-Treg phenotype seemed to be due to TCR crosslinking, which induced PPARγ and ST2 (coding for alarmin IL33R, specifically expressed by VAT Tregs) expression through BATF and IRF4 activity, and MyD88 induction of IL33. In line with the hypothesis of a possible Treg adaptation to environmental insults, PPARγ upregulates genes involved in lipid metabolism, like *Dgat1* and *Pcyt1a* on VAT-Tregs, maybe in order to allow

them to survive in lipotoxic environment and/or exploit fatty acids as a potential source of energy supporting their metabolism (Cipolletta D. et al., 2012).

3.3.2 Tregs in intestine

Tregs represent one of the most important cell subtype in preventing immune responses against both food and microbial commensals and in maintenance of intestinal homeostasis. In particular, Tregs constitute about 25-30% and 10-15% of total $CD4^+$ T cells residing in large and small intestine respectively. Transcriptional and functional analysis identified three subsets of intestinal Tregs: $GATA3^+Helios^+$ ($Nrp1^+$), $ROR\gamma t^-Helios^+$ and $ROR\gamma t^-Helios^-$ subtypes (Fig. 12). The first group represent about one third of total intestinal Tregs and most of them express also $Helios^+$ regardless of microbial presence, suggesting a potential thymic origin of these cells. Moreover, they also express ST2 receptor, which binds to IL33, an alarmin induced upon tissue damage. This subtype appears to be involved in suppression of inflammatory responses and promoting tissue repair through amphiregulin (AREG, a member of EGF family) secretion after injury (Schiering C., et al., 2014; Bertheloot D. et al., 2017).

The second subset of intestinal Tregs constitutes half of total colonic Treg cells. The lack of Helios expression suggests a possible extra-thymic origin for these cells. Since their TCRs have been showed to recognize colonic bacteria *in vitro* (Lathrop SK. et al., 2011), they seem to play an important role in the establishment of tolerance to commensals and the expression of cMAF transcription factor appears to be relevant for their protective function.

The identification of $ROR\gamma t^-Helios^-$ intestinal Tregs is recent. They represent about 10-15% of total colonic Treg cells and about 50% of Tregs residing in small intestinal lamina propria. Although their function in colon is not fully understood yet, this subset is probably involved in establishment of tolerance to dietary antigens (Kim KS. et al., 2016).

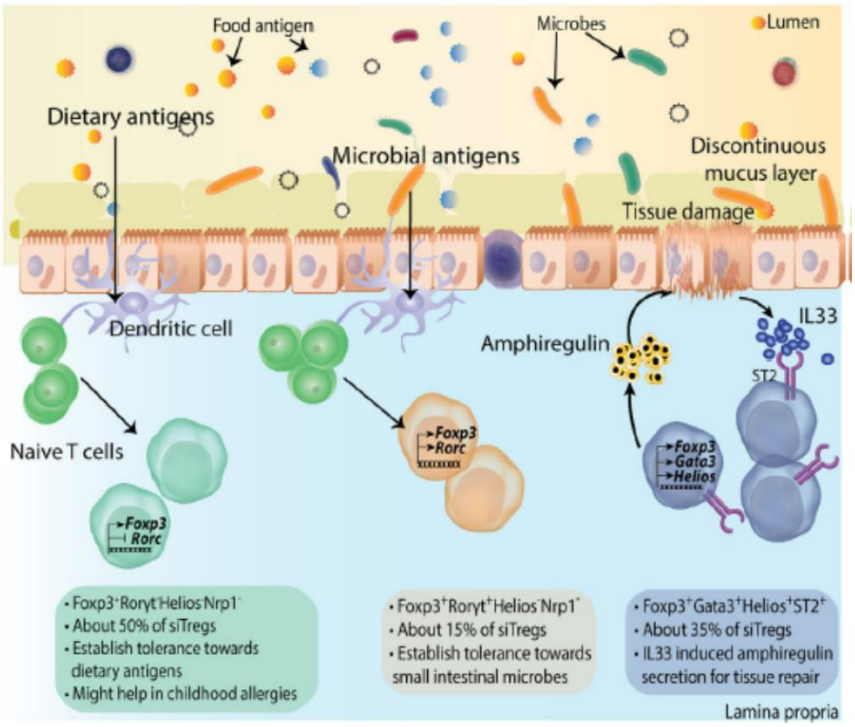


Fig. 12: Schematic representation of identified three subsets constituting of intestinal Tregs: GATA3^{+}Helios^{+} (Nrp1^{+}), RORγt^{-}Helios^{+} and RORγt^{-}Helios^{-} subtypes. Cartoon also shows mechanisms and processes potentially controlled by each of these subpopulation (adapted from Sharma and Rudra, 2018).

3.3.3 Tregs in skin

Tregs play a decisive role in maintenance of self- tolerance and tissue homeostasis also in the skin. Indeed, both in scurfy mice and patients affected by IPEX develop immune responses at skin level, in particular with onset of symptoms like dermatitis and eczema in the latter. In general, Tregs constitute about 20-30% of total CD4^{+} T cell population in human skin, but their origin has not been defined yet. However, some studies showed that cutaneous Treg TCR repertoire is very different from T conv cell one and, moreover, they have demethylated FOXP3 CNS2, suggesting a possible thymic derivation (Sanchez Rodriguez et al. (2014); Povoleri et al. (2013); Huehn et al. (2009)).
Intriguingly, besides the regulation of tolerance towards commensal organisms, in the last years, it was also described a novel role of cutaneous Tregs in modulating hair follicle stem cells (HFSCs) (Ali et al., 2017). In particular, this Treg population co-localizes with HFSCs in skin and expresses *jagged1* gene, coding for a ligand of Notch, whose signaling is fundamental for HFSC proliferation (Fig. 13a)
Moreover, Tregs play a crucial role in cutaneous tissue repair, since their significant accumulation was observed after skin injury, expressing high levels of CD25, CTLA4 and ICOS, typical markers of active state dependent on EGFR pathway. In parallel with limited presence of IFN-γ producing T cells and M1 macrophages in wounds (Nosbaum et al., 2016) (Fig. 13b).

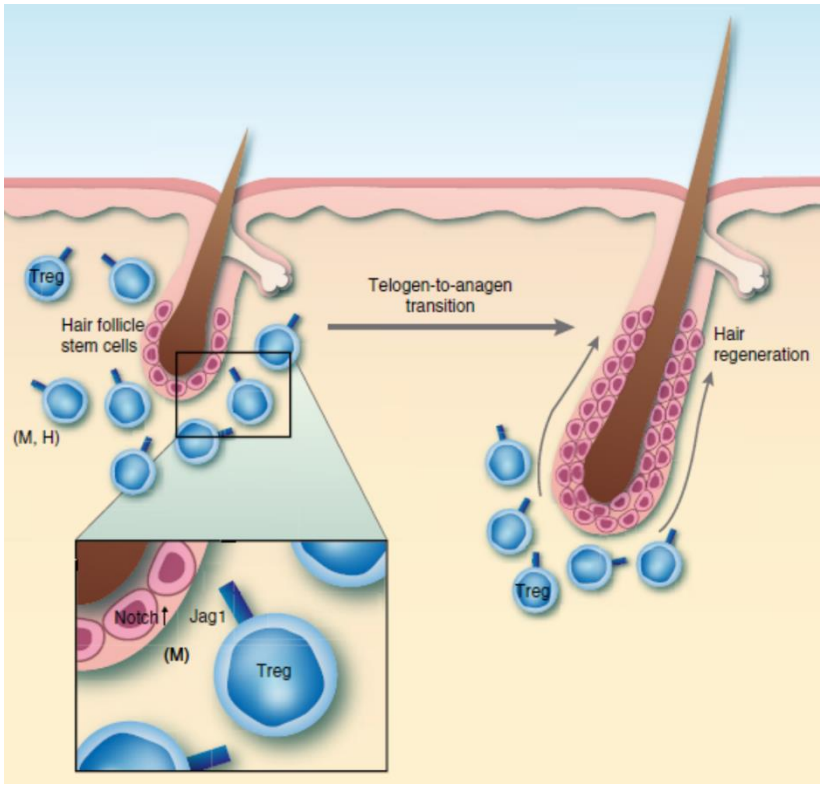


Fig. 13a: Schematic representation of cutaneous Tregs action in skin. Tregs communicate with HFSCs through Notch-Jag1 interaction, promoting hair regeneration (adapted from Ali et al., 2017).

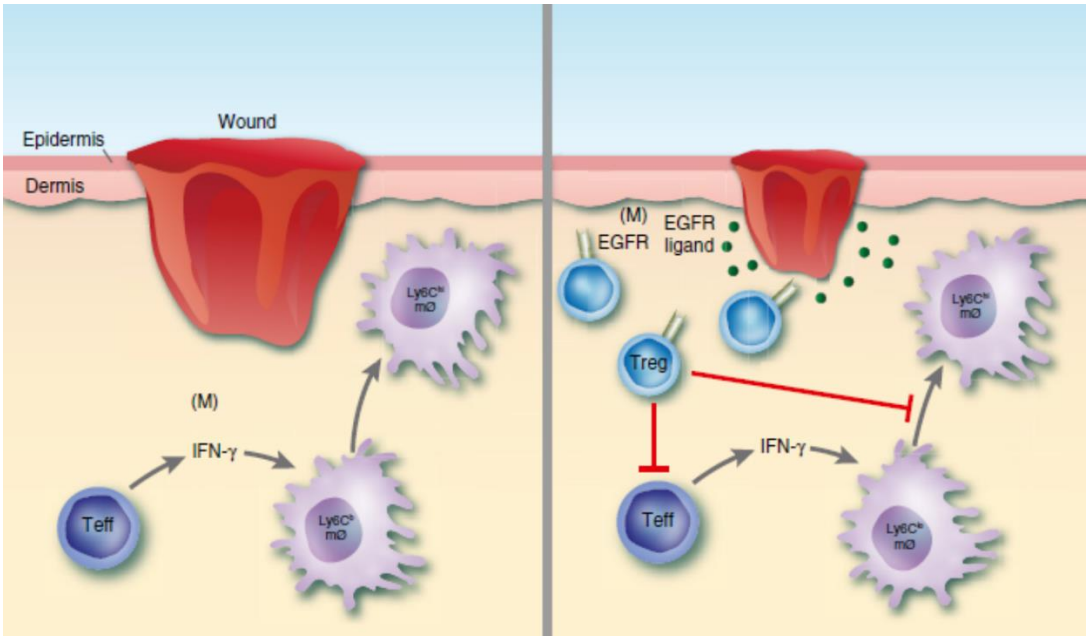


Fig. 13b: Schematic representation of cutaneous Tregs action in skin upon tissue damage. Tregs stimulate wound healing upon stimulation of EGFR pathway (adapted from Ali et al., 2017).

3.3.4 Tregs in skeletal muscle and lung

In line with what observed in skin, recent works demonstrated the involvement of Treg cells also in skeletal muscle regeneration after injury. In particular, Burzyn and colleagues (2013) identified a Treg subpopulation with unique TCR repertoire and function, which accumulated in damaged tissue, constituting up to 50-60% of total $CD4^+$ T cell population recruited in this site. Although they largely overlapped with canonical Treg gene signature, "muscle-infiltrating" Tregs differentially expressed some genes coding for anti-inflammatory cytokines, chemokine receptors, PDGF and AREG, in parallel with the expression of genes related to Treg suppressive function (i.e. IL10, CTLA4, TIM3). This supported the idea that this particular subtype may also have a strong suppressive activity. Tregs can promote muscle regeneration in different ways (Tidball and Villalta, 2010). First, they can regulate damaged tissue- infiltrating myeloid cell populations, by promoting their transition from pro- to anti-inflammatory phenotype. Secondly, they can directly act on satellite cells, which represent an endogenous source of muscle progenitors, promoting their differentiation through AREG stimulation.

In line with this, another Treg cell population was found to be involved in lung repair after injury (Arpaia et al., 2015). This subtype had a peculiar gene expression pattern, suggesting their proficiency in ECM remodelling and tissue repair. In particular, they expressed high levels of IL18R and ST2 and promoted tissue regeneration through secretion of AREG, produced independently on TCR engagement. Moreover, mouse deficient for *Areg* expression showed reduction in lung functioning upon infection with influenza virus, whereas the antiviral response was not affected.

3.3.5 Tregs in feto-maternal tolerance

One of the most significant biological examples of tolerance is that occurring during pregnancy between fetus and mother, and the right establishment of this tolerance is fundamental for pregnancy outset. Both in humans and mice, seminal plasma is rich in TGFβ and prostaglandin E2 (PGE2), two important Treg inducers. This aspect is very important considering that it was found that women with recurrent spontaneous abortions were characterized by reduced number of Tregs (Arruvito et al., 2007). Mold et al (2008) described that maternal antigens could promote fetal tolerogenic Treg development in utero. A recent work demonstrated that human fetal DCs were primarily tolerogenic in their response, including Treg generation, and they were distributed in several fetal tissues, like spleen, skin, gut and lung (McGovern et al., 2017). However, the authors didn't mention also the presence of Treg cells in these anatomic sites.

Although mechanisms underlain feto-maternal tolerance are mostly unknown, Treg function could be fundamental in the establishment of transient tolerance to paternal alloantigens and, subsequently, to fetal ones.

3.3.6 Tumour-infiltrating T regulatory cells (TI-Tregs)

One of the main features of tumour cells is their ability to escape from immunosurveillance. On one hand inflammation can represent an interesting source of growth factors for tumour, but, on the other hand, resolution of inflammation helps cancer to escape from control of immune system. In this context, cancer cells exploit different strategies, like reducing the expression of antigens that can be potentially recognized by effector T cells (in particular from cytotoxic T lymphocytes CTLs), or recruiting immunosuppressive cell populations to switch off antitumor immune responses. Considering their immunosuppressive role, it is not surprising that a significant percentage of $CD4^+$ T cell compartment infiltrating several solid tumours (i.e. hepatocellular, gastric, ovarian, lung, melanoma, breast cancers) is constituted by Tregs (often more than 50%). Moreover, for most of these neoplasms the presence of high number of Tregs correlates with poor prognosis (Wolf D. et al., 2005; Liotta F. et al., 2011; Salama P. et al., 2009).

A relevant issue concerns the possible TI-Treg origin. Since many solid tumour microenviroment expressed high level of TGFβ, it is likely that this cell population are generated at the tumour site starting from peripheral Treg cells. However, in tumour mouse models TI-Tregs expressed high level of Nrp1 and Helios, conversely supporting a thymic origin of these infiltrating cells (Bilate et al., 2012). Moreover, TCR sequencing revealed that there was little overlap between TI-Tregs and T conv cells, excluding a possible T conv cell conversion into TI-Tregs (Zheng et al., 2017; Plitas et al., 2016). A possible hypothesis is related to the expression of tumoral antigens in thymus in an *Aire*-dependent manner, responsible for the selection of tumour-specific Treg subpopulations (Malchow S. et al., 2013).

Recent works analysed TI-Tregs transcriptome in different solid tumours and identified some peculiar molecular patterns which can be potentially used as a target for selective therapy (Plitas et al., 2016; De Simone et al., 2016; Zheng et al., 2017). In particular, one of these studies published by my laboratory, revealed that tumor-infiltrating Tregs, isolated from CRC (colorectal cancer) and NSCLC (non-small cell lung cancer) human biopsies, expressed a unique and specific gene signature, including the expression of BATF, CCR8, IL1R2, IL21R, CD30, OX40, TIGIT, MEGEH1, LAYN, PDL1 and 2, together with high level of FOXP3 and CD25 (De Simone M. et al., 2016). Notably, TI- Tregs showed a higher immunosuppressive activity compared to Tregs isolated from adjacent normal tissue or peripheral blood of the same patients. Moreover, the

expression of LAYN, MAGEH1 and CCR8 in TI-Tregs correlated with a poor prognosis both in CRC and NSCLC patients.

Mechanisms exploited by TI-Tregs to suppress immune response are not well understood yet. One hypothesis could be associated to the production of immunosuppressive cytokines, like IL10 or TGFβ, or IL2 sequestration to T effector cells. Other mechanisms can involve direct cytolysis of target lymphocytes through granzyme B and perforin secretion (Cao X. et al., 2007), or contact-based suppression through the interaction of immune checkpoint inhibitors CTLA4 (Wing K. et al., 2008) and PD1 (Sharma MD. et al., 2015; Jacobs JF. et al., 2009), through LAG3 (Liang B. et al., 2008) or TIM3 CD39/CD73 via adenosine receptor 2A (Gautron AS. et al., 2014; Stagg J. et al., 2011; Borsellino G. et al., 2007) (Fig. 14).

In addition, TI-Tregs appears to be more stable in the presence of PTEN and Nrp1 (a VEGF receptor) (Overacre- Delgoffe et al. 2017; Sharma et al., 2015: Delgoff et al., 2013). Indeed, the binding Nrp1 to Semaphorin4a increases nuclear localization of Foxo1 and 3, with the consequent Akt-mediated stabilization of Treg signature and antiapoptotic genes. Moreover, Nrp1 seems to be essential for TI-Tregs, since its depletion makes these cells "fragile", a situation which can occur in presence of IFN γ and HIF1α in hypoxia conditions (Overacre- Delgoffe et al. 2017).

Furthermore, other TI-Tregs were found to express high level of RANKL, which binds its receptor RANK on breast cancer cells, increasing the possibility of lung metastasis (Tan et al., 2011). In particular, this pathway was found to be involved in renewal of breast cancer progenitors and prostate cancer metastasis, through IKKα regulation (Cao et al., 2007; Luo et al., 2007).

All these evidences suggest that TI-Tregs represent a peculiar cell population with specific phenotype and functions, opening a promising possibility to use them as a potential target for antitumor therapeutic strategies.

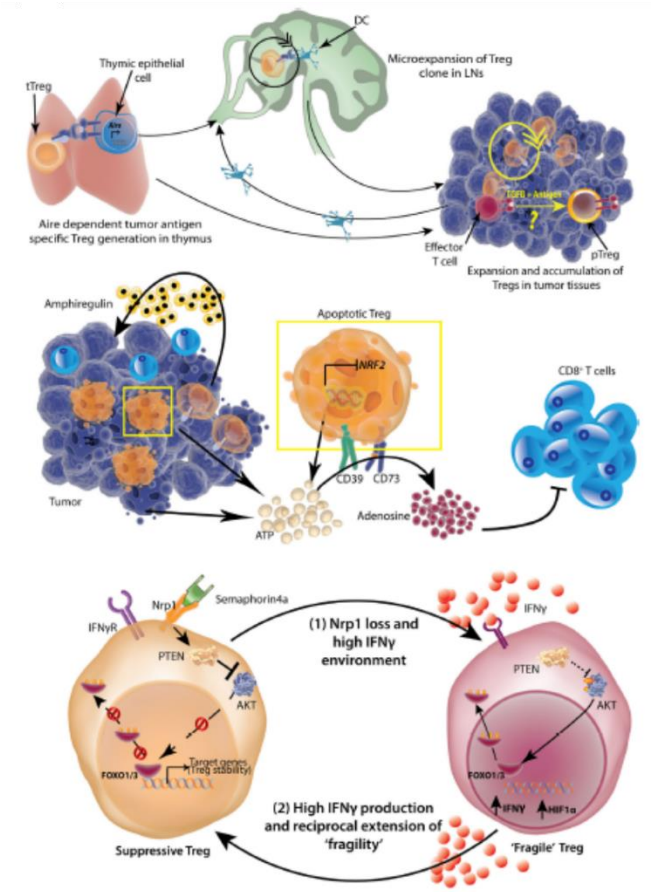


Fig. 14: Schematic representation of TI- Tregs origin, accumulation and function. (A) Possible thymic origin of TI-Tregs due to Aire-mediated expression of specific tumoral antigens in this site, determining selection of tumour-specific antigens. (B) A possible mechanism of action of TI-Tregs: dying TI-Tregs (and also tumoral cells) released high levels of ATP, which can be converted into adenosine by CD39/CD73 expressed on Treg cells. Adenosine represent a powerful inhibitors od cytotoxic T lymphocytes (CTL), promoting tumour progression. (C) Possible regulation of TI-Treg survival and signature in inflammatory condition, mediated by Nrp1 signalling pathway. (Adapted from Sharma and Rudra, 2018).

AIM OF THESIS

Recent advances in 3D culture technology allow embryonic and adult mammalian stem cells to generate organoids *in vitro*, which reflect key structural and functional properties of organs they originate (Clevers H., 2016).

3D organoids represent a powerful tool to study important human physiological and pathological processes, to predict drug response in a personalized manner and to be used for regenerative medicine and gene therapy. In this context, organoids become a fascinating approach to investigate complex processes like tumorigenesis and tumor growth, resembling the *in vivo* mechanisms.

Immune system plays a crucial role in tumor elimination, a process known as immune-surveillance. Indeed, the vast majority of solid tumors is immunogenic and it recruits different leukocytes able to kill cancer cells at their site. However, in many cases, tumor cells can escape the immune surveillance using different strategies: loss of tumor antigens expression and/or recruitment of immune cells with immunosuppressive functions. In this regard, $CD4^+$ T regulatory cells (Tregs), physiologically engaged in the maintenance of immunological self-tolerance and immune homeostasis, are potent suppressors of effector cells and are found at high frequencies in various types of cancer. A recent transcriptome analysis performed in my laboratory (M. De Simone et al., 2016) revealed that tumor-infiltrating Tregs, isolated from CRC (colorectal cancer) and NSCLC (non-small cell lung cancer) patients, expressed a unique and specific gene signature, correlated with patients' survival. In line with our findings, non-lymphoid tissues infiltrating Tregs can exhibit specific phenotypes and transcriptional profile involved in glucose metabolism, tissue repair and muscle regeneration, far from their well-established suppressive roles (Cipolletta D. et al., 2012; Arpaia N. et al., 2015).

Thus, the aim of my project is to evaluate the immune dependent and independent functions of tissues infiltrating Tregs, exploiting a co-culture model with normal and colon cancer-derived organoids. This approach could be suitable to recapitulate primary tumorigenesis, cancer microenvironment effects on Tregs recruitment and phenotype and, vice versa, infiltrating Tregs influence on tumor onset, growth and tissue homeostasis.

MATERIALS AND METHODS

1. GENERATION AND MAINTENANCE OF NORMAL AND TUMORAL COLON DERIVED-ORGANOIDS

Primary human colorectal tumours and non-neoplastic counterparts were obtained from 48 patients with colorectal cancer (CRC), who underwent surgery for therapeutic purposes at Fondazione IRCCS Ca' Granda, Policlinico, Niguarda or San Gerardo Hospitals (Italy). Records were available for all cases and included patients' age at diagnosis, gender, clinic- pathological staging, tumour histotype and grade. No patient received palliative surgery or neoadjuvant chemo- and/or radiotherapy. Informed consent was obtained from all patients, and the study was approved by the Institutional Review Board of the Fondazione IRCCS Ca' Granda (approval n. 30/2014).

1.1 Crypt isolation from normal and tumoral colon biopsies

For crypt isolation we based on Fujii Nature protocol (2016). In particular, we generally used biopsy of 5–10 mm in size and cut them in small pieces of about 5mm for processing. Dissected pieces were placed in 50ml centrifuge tubes and wash at least ten times with cold PBS+ gentamicin (diluted 1:500), until supernatant was free of debris. Then, pieces were incubated in cold PBS+ gentamicin (1:500) + 2.5mM EDTA for 45' on wheel at 4°C. After this treatment, we removed the supernatant and added PBS/1% FBS and pipette up and down (or shake) for at least ten times, in order to release crypts in the supernatant. The former was recovered and centrifuged at 600rpm x 5' at 4°C. The pellet was washed with PBS/1% FBS and centrifuge at 800rpm x 5' at 4°C. Pellet was resuspended in Matrigel (Corning®, 356237), using a ratio crypt: Matrigel that allows to have 50–200 crypts in 25 µl of Matrigel (if you use a 24 well plate, you can plate up to 1000 crypts /60 µl of Matrigel). Finally, 50µl crypts/Matrigel suspension was dispensed in the centre of each well of the prewarmed 24 well/plate and put in a 37°C incubator for at least 30' to solidify the Matrigel. After Matrigel solidification medium is added.

1.2 Organoid media conditions

Organoids have been cultivated in two main culture conditions: WENRAS and ENAS media for normal and tumoral organoids respectively. In particular, each of them is composed by basal medium, which constituted by:

Components	Final concentration
Advance DMEM F12 (Life technologies, 12634)	100%
Penicillin/streptomicin (Euroclone, ECB3001DP)	1%
Gentamicin (Sigma aldrich)	1:500
Hepes (Life technologies, 15630)	10mM
GlutaMAX (Life technologies, 35050)	2mM
N-acetylcystein (Wako, 015-05132, stock of 500mM)	1mM
B27 (Life technologies, 17504 stock 50X)	1X
N2 (Life technologies, 17502 stock 100X)	1X

Thus, organoid media are constituted by:

Components	Final concentration	
	WENRAS	ENAS
Basal medium	50%	100%
Wnt 3A conditioned medium	50%	-
R-spondin 1 (R&D 4645-RS-025, stock 1mg/ml)	1µg/ml	-
Gastrin 1 (Sigma-Aldrich G9145, stock 500µM)	10nM	10nM
EGF(PeproTech, AF100-15, stock 0.5mg/ml)	50ng/ml	50ng/ml
Noggin (PeproTech, 120-10C, stock 200µg/ml)	100ng/ml	100ng/ml
A8301 (Tocris 2939, stock 2.5mM)	0.5µM	0.5µM
SB202190 (Sigma-Aldrich S7067, stock 30mM)	10µM	10µM

Medium was generally changed every 2-3 days, in order to provide organoids always fresh cytokines.

1.3 Split and change matrix organoids

After medium removal, each well containing a Marigel drop was washed three times with cold PBS/gentamicin (10mg/ml). In order to dissolve Matrigel 1ml cold Cell recovery solution (Corning 354253) was added in each well and transfer in 1.5ml tube, incubating for 30' in ice. Organoids were spun at 200g x 3' at 4°C. Organoid pellet was washed with 1ml cold PBS/gentamicin+ 1% FBS and centrifuged at 200g x 3' at 4°C. Pellet was resuspended in Matrigel and plated 50µl Matrigel drop per well in prewarmed 24 well plate and incubate for at least 15' at 37°C before adding organoid medium.

1.4 Wnt-3A conditioned media production

Wnt necessary for organoid culture is obtained starting from L-Wnt3A cell line culture (ATCC®CRL2647™), according to ATCC instructions. These cells are able to produce and release Wnt 3A in the medium thanks to the stable integration of a construct coding for this ligand in their genome. In particular, these cells were plated in petri dish 10cm Ø and cultivated in complete DMEM (10% FBS, 1X Penicillin/streptomycin (Euroclone, ECB3001D), 2mM L-Glutamine (Euroclone, ECB3000D) non-essential amino acids (Euroclone, ECB3054D), 1mM Na/ pyruvate (Euroclone, ECM0542D)). G418 (also known as geneticin, at concentration of 0.4mg/ml) is added to medium at first two passages in order to select cells which stably integrated the construct for Wnt expression in their genome. These cells were generally split every 2-3 days and after the second split they are able to produce Wnt 3A and release it in the medium. Since organoids weren't resistant to geneticin, Wnt 3A collection was performed after cultivating L-Wnt 3A in absence of these antibiotic. The medium containing Wnt 3A was filtered using 0.22µm filters and stored at -80°C.

2 RNA EXTRACTION FOR RNA-seq ANALYSIS

2.1 RNA extraction from organoids

For RNA extraction we directly resuspended Matrigel drop containing organoids in Trizol (Life technologies, 15596018) and stored samples at -80°C until processing. For RNA extraction we used Purelink RNA mini kit (Life technologies, 12183018A). RNA quantification was performed using nanodrop, quantifluor and bioanalyzer. For RNA-seq analysis we used only samples with RIN>7.

2.2 RNA-seq bulk bioinformatic analysis

The quality control (QC) of the reads was performed with FastQC v0.11.7 and MultiQCv1.5. The reads were trimmed using BBDuk and then aligned to the human hg38 reference (GENCODE Release 25 basic gene annotation) using STARv2.5.3a.

The raw count matrix containing all the samples were created by a custom bash script. The mitochondrial genes were removed from the downstream analyses. Normalization and differential analysis were carried out using DESeq2 package (version 1.22.2) and R version 3.5.1. Principal component analysis was performed using the R function *prcomp* considering the 500 most variable transcripts with the parameters center=TRUE, scale=TRUE.

Heatmap and hierarchical clustering were performed using *pheatmap* function with default parameters and Gene set enrichment analysis was performed using the GSEApy package using pre-ranked module with default parameters.

The classification of colorectal cancer primary tissue was performed following the Consensus Molecular Subtype classification, CRC intrinsic subtypes classification and Sadanandam classification. The classification of the primary tumour samples was obtained using default parameters (FDR=0.5, seed=1) of CMScaller R package (https://github.com/peterawe/CMScaller).

3. ChIP-seq ON ORGANOIDS

3.1 Organoid fixation for ChIP

After removing organoid medium each well was washed with cold PBS/Gentamicin for three times. Organoids were collected adding 1ml cold Cell recovery solution in 1.5ml tube and incubated in ice for 30’ to dissolve Matrigel. Organoids were spun at 200g x3’ at 4°C and pellet was washed at least twice with cold PBS/Gentamicin at 200g x 3’ at 4°C, in order to remove any traces of Matrigel. Pellet was fixed in PBS+1% Formaldheyde and incubate samples on wheel for 10’ at RT. Afterwards, we quenched with Glycine 0.125mM and rocked on wheel for 5’ at RT. Samples were centrifuged 200g x 3’ at 4°C. Pellet was washed at least twice with cold PBS spinning at 200g x 3’ at 4°C. supernatant was removed as much as possible, leaving a dry pellet. Fixed organoid pellet was stored at -80°C until further ChIP processing.

3.2 ChIP protocol on organoids

Fixed organoid pellet was lysed in a proper volume of 1X Sonication Buffer (10mM Tris pH 8.0, 0.25% SDS, 2mM EDTA+ 1X protease inhibitors), incubating in ice for at least 30'. Organoids were sonicated at COVARIS M220 in 130 µl cuvettes. In particular, parameters used were: 200 cycles/burst, Peak Incidence 75, duty factor 20%, time: 8' (but these conditions could be changed depending on the amount of starting material). Sonicated fragments should be in the range 200-700bp. After that, lysate was diluted 1:1,5 with Equilibration buffer (10mM Tris, 233mM NaCl, 1.66% TritonX-100, 0.166% DOC, 1mM EDTA, inhibitors) and spun at 14000g 10' 4°C. Supernatant containing chromatin was used for immunoprecipitation (IP); in particular, for this reaction we used 1µg antibody/1µg of chromatin DNA /IP reaction resuspended in RIPA-LS (10mM Tris-HCl pH 8.0, 140mM NaCl, 1mM EDTA pH 8.0, 0.1% SDS, 0.1% Na-Deoxycholate, 1% Triton x-100) and incubated on wheel o/n at 4°C. In the meantime, beads were prepared for IP taking 10µl of protein G Dynabeads (Life technologies), using the concentration 10ul beads/1ug Ab. Afterwards, beads were washed twice with 0.1 % BSA/RIPA L-S and incubated o/n 4°C on wheel, to block beads. Transfer beads to tubes with chromatin and incubate at 4°C for 2 h rotating. Magnet was pre-cooled on ice and we prepared wash buffers by adding protease inhibitors. Beads were washed with 150µl (small cell numbers) - 1000µl (high cell numbers) by adding ice-cold buffer and moving the samples on the magnet 5 times so that the beads move through the solution: 2 washes with RIPA-LS, another 2 with RIPA-HS (10mM Tris-HCl, pH 8.0, 1mM EDTA, pH 8.0, 500mM NaCl, 1% Triton x-100, 0.1% SDS, 0.1% DOC), 2 washes with RIPA-LiCl (10mM Tris-HCl, pH 8.0, 1mM EDTA, pH 8.0, 1mM EDTA, pH 8.0, 0.5% NP-40, 0.5% DOC), 1 wash with 10mM Tris pH 8.0 and a last wash with 1x TE (10mM Tris-HCl pH 8.0, 1mM EDTA pH 8.0). After washing, beads were resuspended in 48µl 1X ChIP Elution Buffer (10mM Tris-HCl pH 8.0, 5mM EDTA pH 8.0, 300mM NaCl, 0.4% SDS) + 2µl Proteinase K (20mg/ml). After that, samples were incubated at 55°C 1h and 65°C for 6-10h (de-crosslinking).

For DNA purification, beads were magnetized and supernatant was transferred into new 1.5ml DNA low-bind tube; then we added another 19µl of ChIP elution buffer + 1µl Proteinase K and incubate 1h for 55°C. Afterwards, we purified with Qiagen MinElute kit and eluted in 22µl Elution Buffer or, alternatively, purified by a 1.8:1 SPRI cleanup eluting in 22µl Qiagen Elution Buffer (10mM Tris pH 8.5). Finally, we measured the ChIPed DNA using fluorescence – based methods and used 1µl of 1:10 diluted sample for qPCR tests on specific targets.

3.3 ChIP-seq bioinformatic analysis

The QC of the reads was performed with FastQC v0.11.7 and MultiQCv1.5. The reads were aligned to the human hg38 reference (GENCODE Release 25 basic gene annotation) using bowtiev1.2.2, sorted using SAMtoolsv1.8 and directly converted into binary files (BAM). PCR duplicates reads were marked and removed using SAMtoolsv1.8. The peaks were called with MACS2v2.1.0, using matched input DNA as a control. Peaks overlapping ENCODE blacklisted regions hg38 were removed, analogously to those found in un-placed and un-localized scaffolds. For the visualization of ChIP-seq tracks, Bedgraph tracks were generated using MACS2 *bdgcmp* function, converted into bigwig using UCSC tools *bedClip and bedGraphToBigWig* functions. pyGenomicTrack tool was used for the visualization of the tracks.

For correlation heatmap of all the histone modifications among the 10 PDO, a consensus peakset was generated using DiffBind v2.6.6 and merging together only peaks detected in at least two tracks. Then, a count matrix of 180250 peaks x 48 samples was created by counting the number of reads per peaks using the dba.count with default parameters. The correlation heatmap and the PCA were produced using *dba.plotHeatmap* and *dba.plotPCA* respectively, with default parameters.

A de-novo chromatin stated characterization of all PDO was performed using a multivariate Hidden Markov Model approach (ChromHMM v1.12) considering 5 histone modifications (H3K4me3, H3K27Ac, H3K4me1, H3K36me3, H3K27me3) across 10 patients and public available data, using default parameters. The datasets were down-sampled to a maximum depth of 45 million reads. The reads count for all the considered samples, were computed in non-overlapping 200-bp bins across the entire genome. The binarization was performed comparing ChIP-seq read count to corresponding input DNA as control to reduce the technical noise. The two states with a strong enrichment of H3K4me1 and H3K27Ac were defined as “Flanking Active Enhancers” and “Active Enhancers”. The state characterized by H3K4me1 was defined as “Weak Enhancers”. The “Elongation” and “Repression” were characterized by the presence of H3K36me3 and H3K27me3, respectively. “Quiescence” state marks regions without any significant enrichment of histone marks.

To estimate the probability to find open chromatin regions in each chromatin states the cancer type-specific ATAC-seq peak set for Colon adenocarcinoma (COAD) was downloaded from the TCGA site (https://gdc.cancer.gov/about-data/publications/ATACseq-AWG/). The number of ATAC-seq peaks inside each chromHMM state was defined by overlapping each chromHMM state with the ATAC-seq peak summit. Then, a conditional probability was calculated to estimate the probability to find open chromatin regions in each chromatin states across the 10 PDO. The probability $p(A|B)$

is the probability that the event A will occur given the knowledge that an event B has already occurred. The conditional probability of A given B is defined as the quotient of the probability of the joint event A and B (both events A and B occur together) and the probability of B.

$$p(A|B) = \frac{p(A \cap B)}{p(B)}$$

With this background in mind, p(A), p(B) and p(A and B) were defined as follow:

$p(A)$= total length of ChromHMM$_i$ state / total length of the genome;

$p(B)$ = total length of ATAC-seq peaks / total length of the genome;

$p(A \cap B)$ = total length of ATAC-seq peaks overlapping ChromHMM$_i$ state / total length of the genome.

Since ATAC-seq peaks were shrinked to the summit of each peak, the length of each peak correspond to 1 bp. From which it was derived the final equation: $p(A|B)$ = total length of ATAC-seq peaks overlapping ChromHMM$_i$ state/ total length of ChromHMM$_i$ state $p(A|B)$ is the probability to find ATAC-seq peaks in each specific ChromHMM state.

4. IMAGING ON ORGANOIDS

4.1 Organoid 3D Immunofluorescence whole mount staining

Importantly, organoids that were destined to whole mount staining were plated in 8-well chamber slide and cultivated in this support until processing. After medium removal, every well, containing 1-2 organoids each, was washed thoroughly with PBS to remove any residues of medium and then fixed with PFA 4% for 30' at 4°C on shaker. Afterwards samples were washed three times with PBS and incubated in NH_4Cl 50mM solution for 30' at 4°C on shaker. Organoids were then wash three times with PBS and permeabilized with PBS+ 0.5% Triton X-100 for 30' on shaker at RT. Samples were washed three times with PBS and we proceeded adding blocking solution constituted by PBS+ 0.2% Triton X-100+ 10% of total sera from animals in which secondary antibodies (which we will use later) were produced and incubated O/N at 4°C on shaker. Samples were then washed three times with PBS and blocking solution (but lacking of Triton X-100) diluted 1:2 and primary antibodies were added and incubated for 33-35 hours at 4°C on shaker. We proceeded with ten washes with PBS on shaker and another one with PBS+ 0.2% Triton X-100 to remove primary

antibody residues and added secondary antibodies incubating O/N at 4°C on shaker. We proceeded with ten washes with PBS on shaker in dark and added with solution of PBS+ 0.2% Triton X-100+ 2μg/ml DAPI, incubating for 1h at RT on shaker (dark). Afterwards, samples were washed five times with PBS on shaker. Stained organoids were preserved in solution with 0.02% NaN_3 until confocal acquisition.

Primary antibodies used: mouse monoclonal anti-human Mucin2 (Santa Cruz Biotechnology- sc515032) 1:50; mouse monoclonal anti- Chromogranin A (Santa Cruz Biotechnology- sc393941) 1:50; rabbit monoclonal anti- human Ki67 (Abcam- 92742) 1:250; rabbit polyclonal anti- human cytokeratin 20 (Abcam- 97511) 1:50; rabbit polyclonal anti- human Fabp1 (SIGMA- HPA028275) 1:300; goat monoclonal anti- human EpCAM (R&D- AF960) 1:40.

Secondary antibodies used: Goat Anti- mouse Alexa 488 (Life Technologies- A11029) 1:400; donkey Anti- goat Alexa 488 (Life Technologies- A11055) 1:400; donkey Anti- rabbit Alexa 568 (Life Technologies- A10042) 1:400; donkey Anti- mouse 647 (Life Technologies- A31571) 1:400.

Other antibody used: Alexa Fluor 647 Phalloidin Life Technologies- A22287, 1:40; mouse anti-human FOXP3 (eBioscience 17-4776-42), 1:50.

4.2 Organoid 3D Immunofluorescence whole mount staining acquisition

Images were acquired using Nikon Eclipse TiE, coupled to sCMOS camera (Andor Zyla VSC-01540). Optical/ spectral configurations: λ1: FITC-exc. 470nm (laser 488), with a laser power of 34% and emission filter: Cy5 (camera: binning 1; global shutter; 520MHz/16bit/1-3dynamic; exposure 700 msec). λ2: DAPI-exc. 395nm, with laser power of 26% and emission filter: DAPI (camera: binning 1; global shutter; 520MHz/16bit/1-3dynamic; exposure 2s). λ3: exc. 568nm (camera: binning 1; global shutter; 520MHz/16bit/1-3dynamic; exposure 1s). Images were then analysed using Fiji software.

4.3 Live imaging on co-culture through IncuCyte instrument

For migration experiments, Tregs- PDO co-cultures were established using ratio organoids: Tregs 1:1 and 1:2. In particular, Tregs were added both to Matrigel drop and on ENAS medium + IL2 (200U/ml). Time- lapse acquisitions of Tregs migration into organoids were performed every 3 hours for a minimum of 4 days up to 10 days of co-culture. Images were then analysed using software provided from Sartorius.

5. TREG ISOLATION FROM BIOLOGICAL SAMPLES

5.1 Tregs isolation from colon primary tissue

For Tregs isolation from human colon biopsies we used the same protocol set up in our laboratory (De Simone M. et al., 2016). Briefly, colorectal cancer (CRC) specimens were cut into small pieces and incubated in DTT 0.16mM (Sigma-Aldrich) for 10 min, then extensively washed in HBSS (Thermo Scientific) and incubated twice in 1mM EDTA (Sigma-Aldrich) for 50' at 37 °C in the presence of 5% CO_2. They were then washed and incubated in type D collagenase solution 0.5mg/ml (Roche Diagnostic) for at least 4h at 37°C. Supernatants containing tumor infiltrating lymphocytes were filtered through 100μm cell strainer, washed with PBS and fractionated 652g for 30 min at RT without brake on a four-step gradient consisting of 100%, 60%, and 40% and 30% Percoll solutions (Pharmacia). The T-cell fraction was recovered from the interface between the 60% and 40% Percoll layers. Tregs were then purified by FACS sorting using the following fluorochrome conjugated antibodies: anti-CD4 APC/Cy7 (Biolegend clone OKT4), anti-IL7R PE (Miltenyi, clone MB15-18C9) and anti-CD25 FITC (eBioscience, clone), using FACSAria II (BD biosciences).

5.2 Tregs isolation from healthy donor peripheral blood

The content of a blood bag was divided into four 50ml tubes and diluted 1:2 in PBS. Blood was fractionated 805g for 20' at RT without brake on Ficoll (Ficoll-Paque™ Plus, GE Healthcare 17-1440-02). PBMC ring was recovered and washed twice with PBS. Tregs were then purified by FACS sorting using the following fluorochrome conjugated antibodies: anti-CD4 APC/Cy7 (Biolegend clone OKT4), anti-IL7R PE (Miltenyi, clone MB15-18C9) and anti-CD25 FITC (eBioscience, clone), using FACSAria II (BD biosciences).

5.3 Tregs expansion *in vitro*

Ex vivo Tregs were expanded *in vitro* in presence of $1x10^6$ Feeders cells/ml, in presence of IL2 (200U/ml) (Miltenyi Biotec) and Anti- CD3 clone OKT3 (30ng/ml) (BioLegend, 317326) in complete RPMI (RPMI 1640 (EuroClone ECM2001L), 10% FBS, 1% Pen/Strep, 10 mM

Na/Pyruvate (Euroclone ECM0542D), non- essential amino acids 1X, (EuroClone ECB3054D)). After three days OKT3 was removed and replaced with fresh medium + IL2 (200U/ml).

6. TREG-PDO CO-CULTURE

For co-culture experiments, tumoroids were dissociated to single cells with TrypLE Express (Gibco, 12605036) at 37°C and wash with PBS. We plated organoids and expanded Tregs in a ratio 1:3 together into Matrigel droplet or Tregs were added to organoid medium after Matrigel drop solidification. Co-cultures were cultivated in ENAS medium with the addition of IL2 (200U/ml), changing medium every 2-3 days.

6.1 GFP$^+$ Tregs production

For GFP lentivirus production, we firstly transfected HEK293T cells, seeding 1x10^6 cells/ well in a 6- well plate in a final volume 3ml of complete DMEM/ well. We prepared transfection solution, constituted by: 4µg psPAX2 plasmid, 2µg of pMD2.G plasmid and 8µg of pGFP plasmid for 100µl of OptiMEM (Gibco, 31985047). The solution was mixed and incubated at RT for 5'. Afterwards, 3µl of PEI/ µg DNA were added and the solution was incubated for 20' at RT. 100µl of transfection solution were added to each well and cells were incubated O/N at 37°C. After that, 2ml of culture medium were replaced with fresh complete DMEM. After 48h post transfection, we collected supernatant containing lentiviral particles which were filtered with 0.45µm filters. For Tregs infection, we added 1ml of lentivirus solution with the addition of polybrene (8 µg/ml) for 1x10^6 Tregs and spinoculated cells for 25' at 805g at 32°C. After spinoculation, 1x10^6 cells were seeded in 24wp in final volume of 2ml complete RPMI+IL2 (200U/ml). After 48h cells were sorted for the expression of GFP protein, using FACSAria II (BD biosciences).

6.2 Treg-organoid co-culture staining for FACS analysis

Co-cultures were dissociated at single cell level, incubating in TryPLE Express at 37°C. Samples were transferred in a "U-bottom" 96wp and centrifuged 5' at 453g RT. Cells were then washed with MACS buffer, centrifuging 5' at 1500rpm RT. Cells were stained with Fixable Viability Stain FVS780 (1:1000) (BD Biosciences, 565388), incubating for 10' at RT (dark). Cells were then washed once with MACS buffer and once with FBS, spinning at 453g for 5' at RT. Afterwards, we proceeded with extracellular staining using the following antibodies: anti- human CD45 BV421 (BD Horizon- Biosciences™ 563879); anti- human CD326 BV605 (BD Horizon- Biosciences™

563182) and anti- human PDL1 PE- Cy7 (Biolegend 329718). Cells were incubated for 20’ at 37°C (dark) and then washed with MACS buffer and spun at 453g for 5’ at RT. For intracellular staining, cells were firstly fixed used solution constituted by Fixation/ Permeabilization concentrate (Invitrogen 00-5123-43) diluted 1:4 in Fixation/ Permeabilization Diluent (Invitrogen 00-5223-56) and incubated for 30’ at 4°C (dark). After washing with MACS buffer at 453g for 5’ at RT, cells were permeabilized using Permeabilization Buffer, diluted 1:10 (Invitrogen 00-8333-56) and we proceeded with intracellular staining, adding 0.5µl of intracellular antibodies for $1x10^6$ cells. In particular, we used anti- human FOXP3 APC (eBioscience 17-4776-42) for Tregs identification. Cells were incubated for 30’ at RT (dark) and then washed once with MACS buffer, spinning at 453g for 5’ at RT. Finally, cells were transferred in FACS tubes and samples were analysed using FACS CANTO II (BD biosciences).

RESULTS

1. PRELIMINARY DATA

As previously mentioned, a recent transcriptome analysis performed in my laboratory (De Simone et al., 2016) evidenced that TI- Tregs, isolated from CRC and NSCLC patients, expressed a specific gene signature, not shared by Tregs isolated from normal counterpart or peripheral blood of healthy donors. Due to their role in promoting tumour growth, it is essential to have the possibility to maintain and expand these cells *in vitro*, to better elucidate mechanisms underlain their immunosuppressive activity in neoplastic context.

However, the protocols used for the *in vitro* expansion of tumor isolated Tregs, can preserve their classical phenotype and proliferation rate, but does not maintain their tumoral signature (Fig. 1).

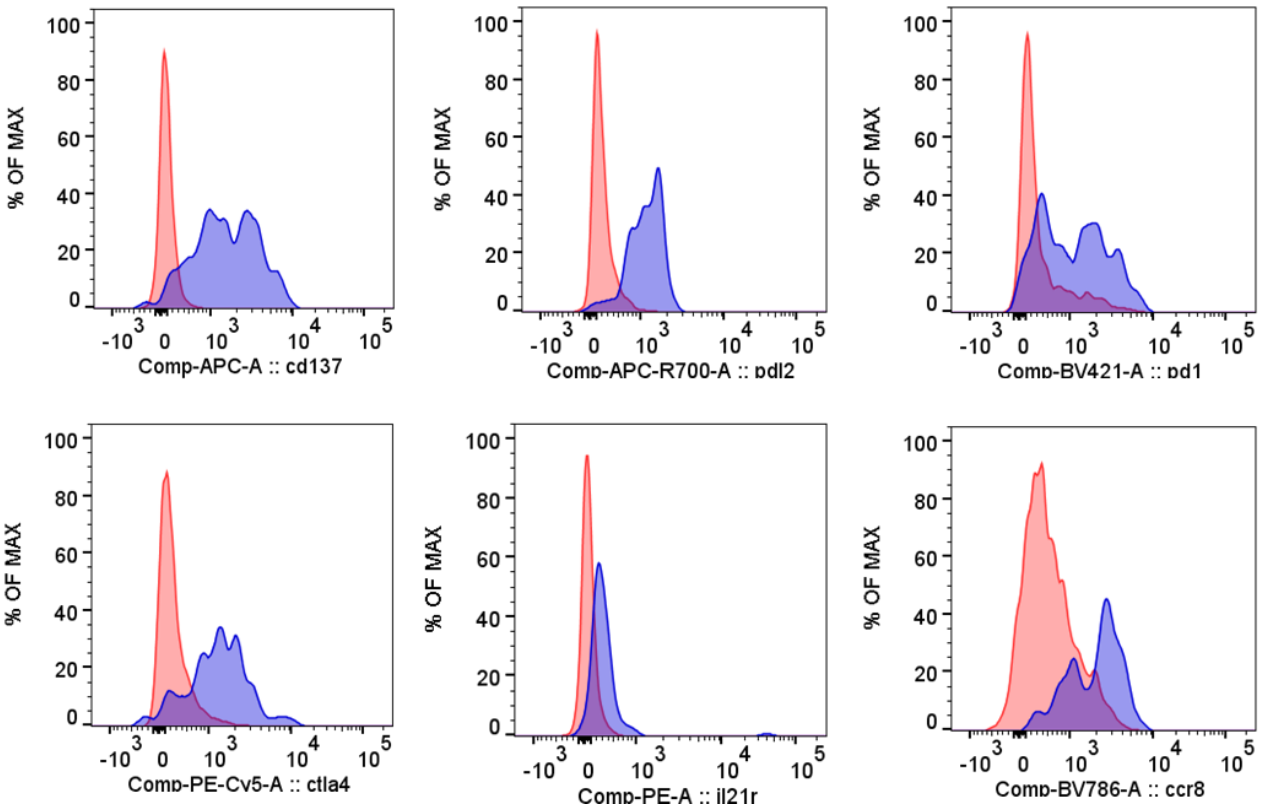


Fig. 1: TI-Treg signature after in *vitro* expansion. Histograms reported the downregulated expression of most of TI-Treg signature genes in TI-Tregs at day 7 post in vitro expansion (red), compared to ex vivo tumor isolated Tregs (blue).

This evidence suggested that the normal lymphocyte culture conditions are not sufficient to sustain their tumoral phenotype, that is likely the result of more complex mechanisms established in the tumour. Therefore, we decided to use the organoid models to set up a co-culture system between

tumoroids and Tregs. Indeed, this experimental strategy, taking advantage from the peculiar capability of the organoids to recapitulate both normal and tumoral primary tissues, would likely provide the proper intrinsic signals to preserve tumor- infilitrating Tregs signature.

The establishment of Tregs and organoids co-culture will allow us to study the effects of organoids on Tregs phenotype and, vice versa, as these T cells could affect tumoroids outcome (Fig. 2).

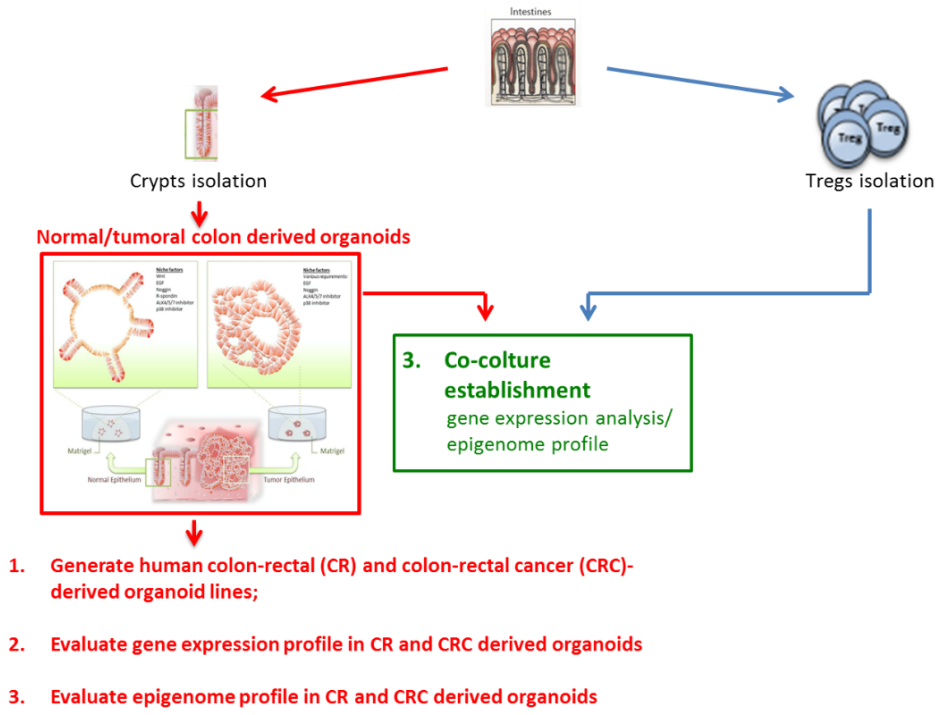


Fig. 2: Experimental strategy for organoids-Tregs co-culture establishment. Our approach consists in isolation both Tregs and crypts from the same colon primary tissue (normal and tumoral), in order to create an organoid biobank which can be co-cultivated with Tregs from the same patient. In parallel, we will perform gene expression and epigenome analysis to gain a molecular characterization of our organoid lines.

2. GENERATION AND CHARACTERIZATION OF CRC- PATIENT DERIVED ORGANOID BIOBANK

2.1 Generation of CRC patient-derived organoids (PDOs)

Surgically resected tissues were obtained from CRC patients. Organoids were generated from crypts isolated from both tumoral biopsies and their normal counterparts of the same patients, according to Fujii's published protocol (2015).

So far, we generated a biobank constituted by 20 and 28 normal colorectal and CRC PDO lines, respectively (Fig. 3), that can be propagated for several passages *in vitro* and frozen and defrosted like any other cell line.

In agreement with Hayflick's limit, supporting that untransformed primary cells can divide only 40-60 times (almost 2-3 months), normal organoids were kept in culture for about 3 months while tumoral organoids showed the possibility of being expanded for an indefined period of time. (Figure 4, A, B panels, respectively). Normal colon organoids showed the formation of numerous buds, from which small symmetrical cysts developed and proliferated into crypt-villus like structures, with a regular morphology, resembling the intestinal crypts (Fig. 18, A panel). On the other hand, tumoral organoids presented a more dysregulated architecture of crypts-villus like structure, that proliferate irregularly trying to reproduce the original tissue.

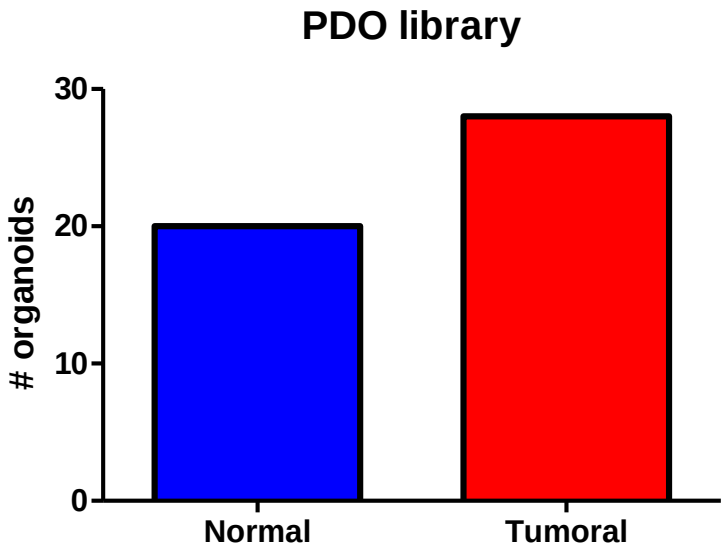
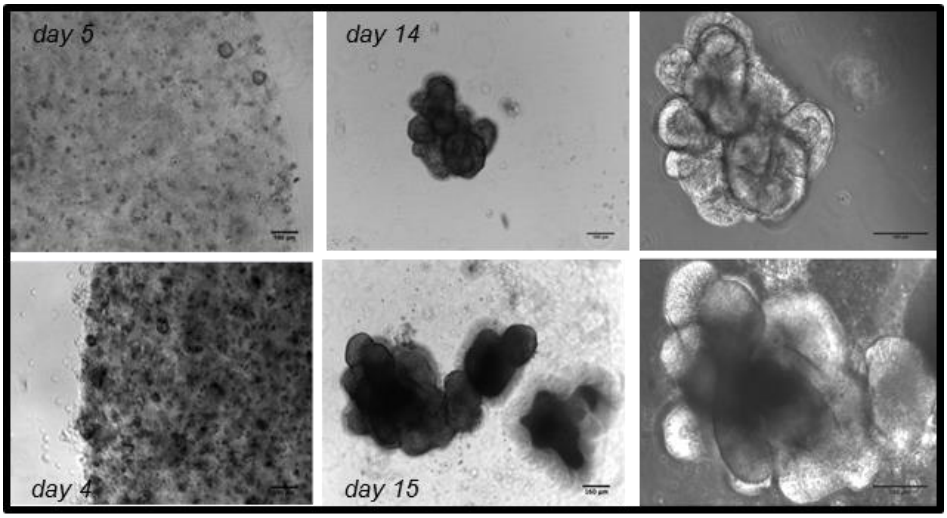


Fig. 3: Schematic representation of total PDO lines generated in our lab so far. In blue organoids generated by normal colon tissues (CR-organoids) are reported, in red those derived from tumoral counterparts (CRC-organoids).

A



B

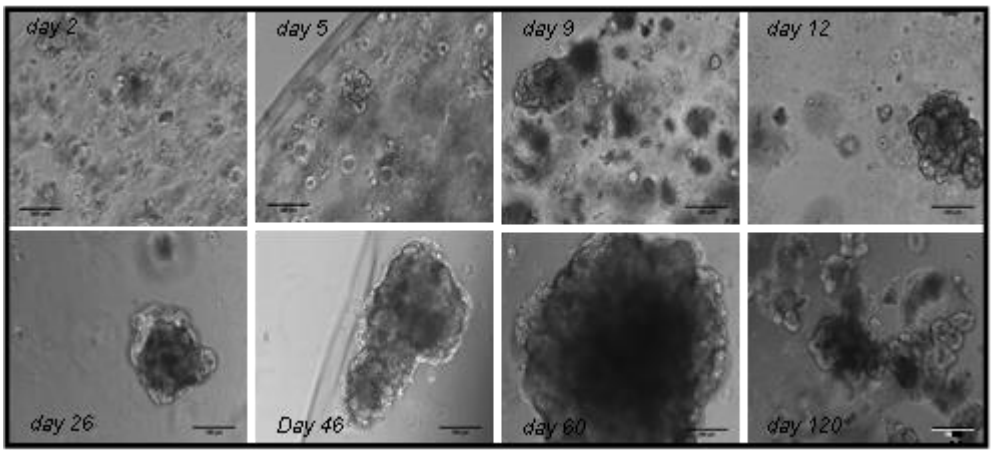


Fig. 4: Normal and tumoral PDO propagation in *vitro*. Panel A shows an example of two normal PDO lines expanded in *vitro* for more than 2 weeks; panel B shows an example of a tumoral PDO line.

2.2 Molecular classification of PDOs

As previously mentioned in the introduction, colorectal cancer represents a highly heterogeneous disease from both molecular and clinical point of view, and accordingly, this heterogeneity is retained in *vitro* by the model of 3D organoids. In the first step of this study, we obtained a pool of PDOs recapitulating the molecular heterogeneity of human CRC. For this, we performed RNA-sequencing (RNA-seq) on the primary tumours and screened them for markers of microsatellite instability (MSI) and three recently published gene expression classifiers (Guinney et al., 2015; Sadanandam et al., 2013; Isella et al., 2017), contextually generating PDOs from the same donors.

Analyses on primary tumours allowed to select a collection of 10 PDOs representing a balanced library that recapitulates the molecular diversity of the cancer-cell intrinsic features of human primary CRCs (Fig. 5).

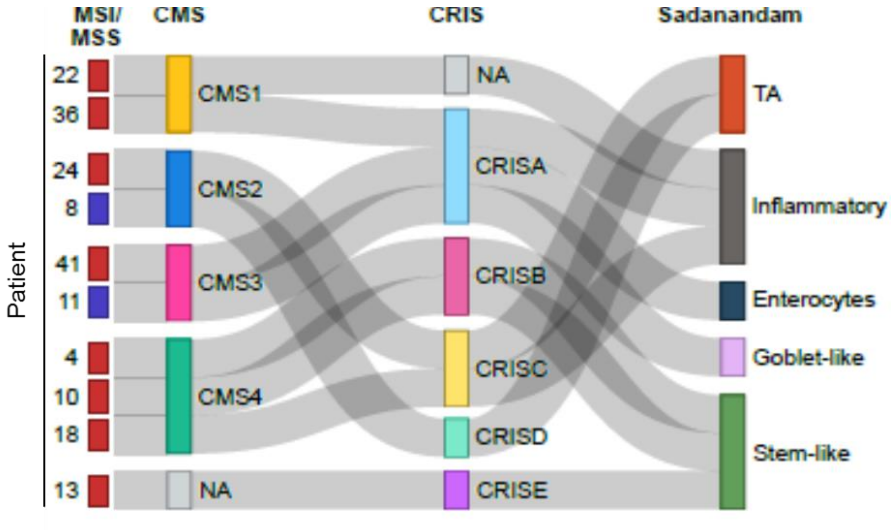


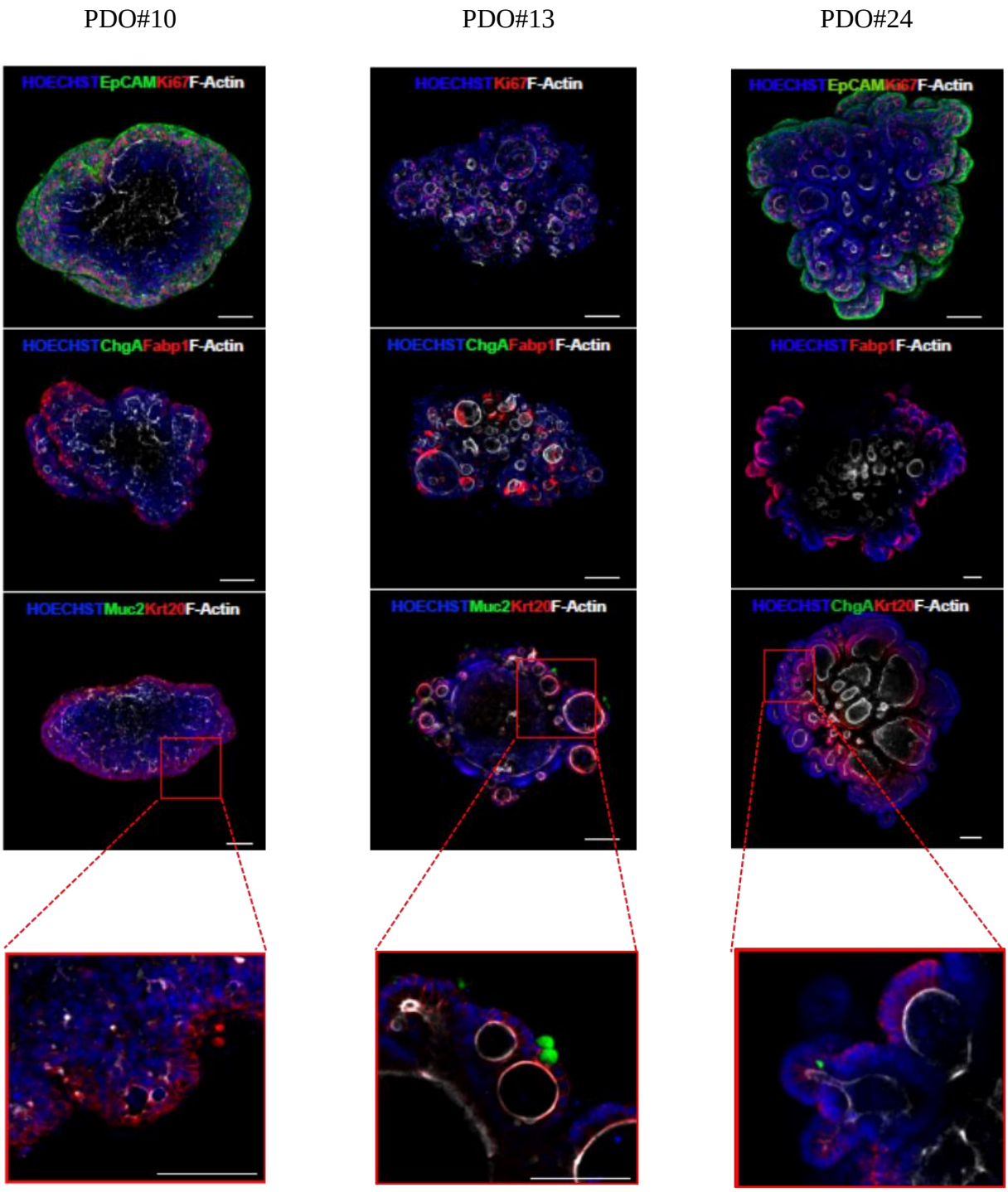
Fig. 5: Schematic representation of molecular classification of our primary tumours. From left to right: classification based on presence/absence microsatellite instability (blue presence, red absence); consensus molecular subtype (CMS) based classification (Guinney et al., 2015); CRIS classification according to Isella et al. (2017) and Sadanandam classification (2013).

2.3 Morphological characterization of PDOs

Next, we used 3D immunofluorescence (IF) whole mount approach to validate whether PDOs retain the same dysregulated architecture and morphology of their primary CRC, from which they were derived.

As shown in Fig. 6 (first row), PDOs presented a disorganized epithelial polarity (as highlighted by Epcam and F-actin staining) and a random distribution of proliferating cells (indicated as $Ki67^{+}$ cells in Fig. 6). Moreover, we observed a displaced localization of enterocytes ($FABP1^{+}$ cells, Fig. 6, second row) and cytokeratin 20 positive cells (represented as $KRT20^{+}$, third and fourth rows in the figure), recapitulating the common dysplastic features of human CRC. Interestingly, goblet-specific mucin 2 was absent in most PDOs but massively expressed in the organoid derived from the mucinous adenocarcinoma of patient 13 (Fig. 6, third and fourth rows), consistent with the histopathological features of the clinical specimen. Analogously, the detection of chromogranin A, a marker of enteroendocrine cells, specifically expressed in PDO#24 but not in others, indicated the

presence of various levels of de-/differentiation among our organoid lines, representing a further evidence of the heterogeneity of our library reflecting CRC inter-individual variability.



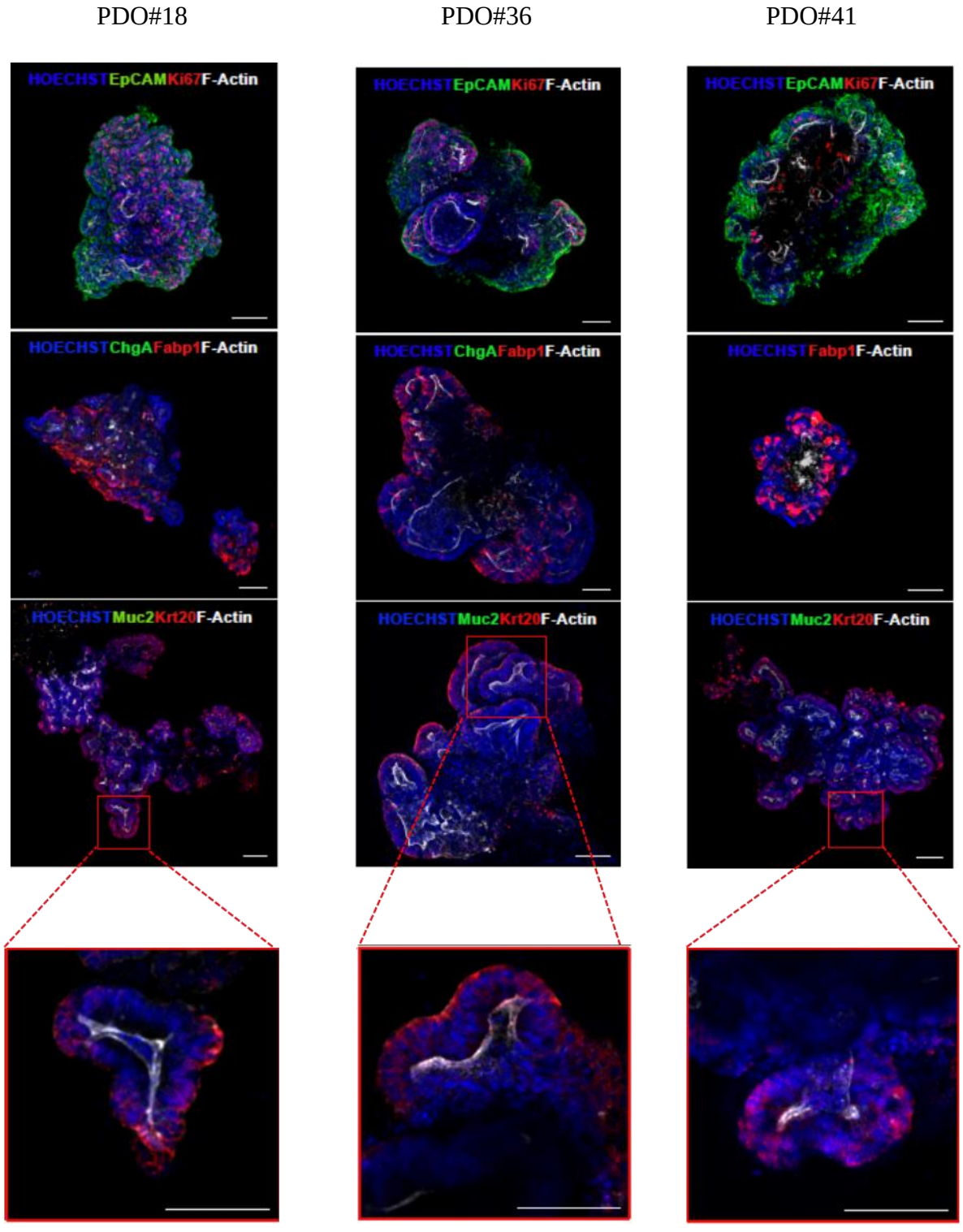
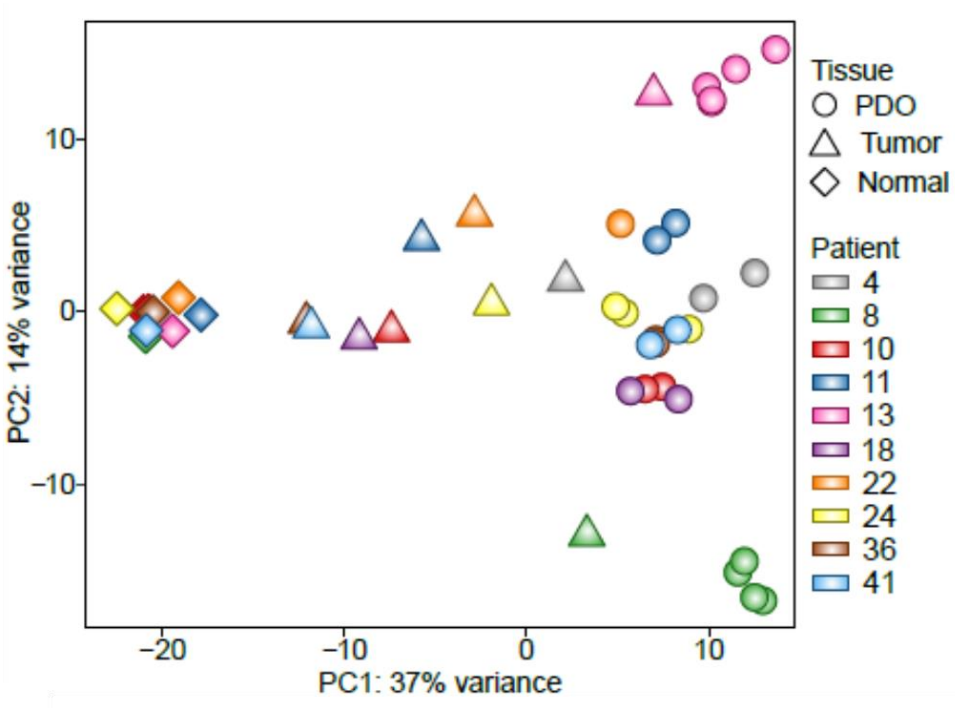


Fig. 6: 3D immunofluorescence analysis performed on three of our PDO library. We used: Hoechst for nuclei, F-actin staining cytoskeleton; Ki67 as a marker of proliferation; FABP1 for enterocytes; Krt20 and Epcam as a marker for respectively top crypts epithelial cells and general colon epithelial cells; Muc2 staining for goblet cells staining; ChgA for enteroendocine cells. Images showed altered architecture in our tumoral PDOs as highlighted by distribution of proliferating cells ($Ki67^+$ cells) and enterocytes localization ($FABP1^+$ cells).

2.4 Transcriptional characterization of PDOs

Afterwards, we assessed gene expression profile of our PDOs by RNA-sequencing, in order to characterized them from the transcriptional point of view. Principal component analysis (PCA) performed on our library revealed that normal colon primary tissues derived from the same CRC patients were transcriptionally stable and grouped together far from adjacent neoplastic tissues (Fig. 7A, represented by rhombuses). Conversely, tumoral primary tissues distributed along all second principal component (PC2), representing a clear evidence of patient-driven heterogeneity (Fig. 7A, indicated with triangles), and the same distribution is preserved in our PDOs, since our organoid lines shared the same spread spatial localization of their tumoral parental tissues (Fig. 7A, indicated with spheres). The same distribution of both PDOs and tumoral tissues along PC2 was maintained also when we excluded normal primary tissues from our PCA analysis, confirming once again the conserved transcriptional inter-individual variability among these two groups (Fig. 7B).
Moreover, organoids derived from the same patients cluster together along both PC1 and 2 (Fig. 7, reported in graphs as spheres with the same colour), suggesting a transcriptional stability of our lines in culture, consistent with the lack of changes in culture morphology and proliferation rate.

A



B

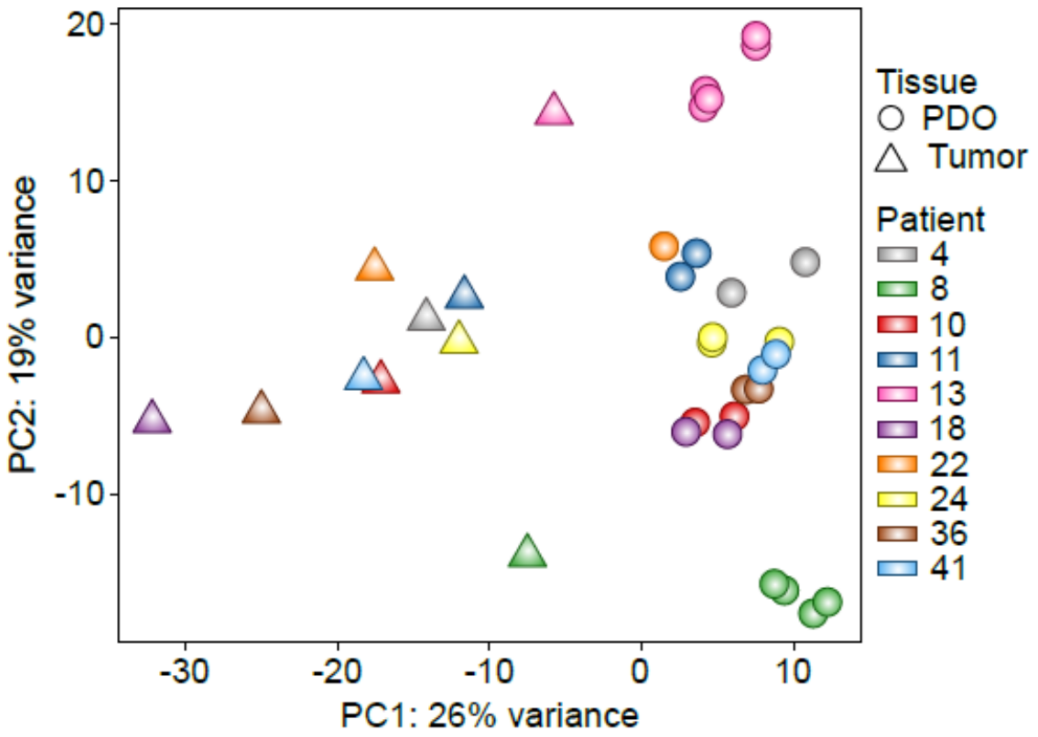


Fig. 7: PCA analysis performed on RNA-seq data from normal and tumoral colon primary tissues and PDOs. Samples collected from the same patients are indicated with the same colour.

Panel A: Normal tissues (rhombuses) clustered together along PC1, far from tumoral counterparts (triangles) as expected, but also along PC2, indicating transcriptional homogeneity. Tumoral tissues distributed along PC2, indicating the intrinsic heterogeneity among different patients and the same was maintained in PDOs derived from the same patients (spheres).

Panel B: the same distribution of tumoral tissue and PDOs was preserved also after normal tissue removal from analysis.

Afterwards, we deepened the transcriptional comparison between PDOs and parental tumours. We found that 85% of expressed genes were concordant between PDOs and tumours (Fig. 9, Venn and correlation diagrams). In particular, when we focused on transcriptional differences between this two groups we observed that discordant genes (expressed in primary tumours but not in PDOs) in this comparison was enriched for gene signatures of stromal cells (Fig. 8, enrichment plot). Consistent with previous works (Isella et al., 2015; Calon et al., 2015; Fujii et al., 2016) tumoral tissues presented a prominent stromal component compared with PDOs. Our data indicated that PDOs retained the CRC gene signature but they were deprived of stromal contamination, representing a purified system to decipher tumour-related molecular profile of colorectal cancer.

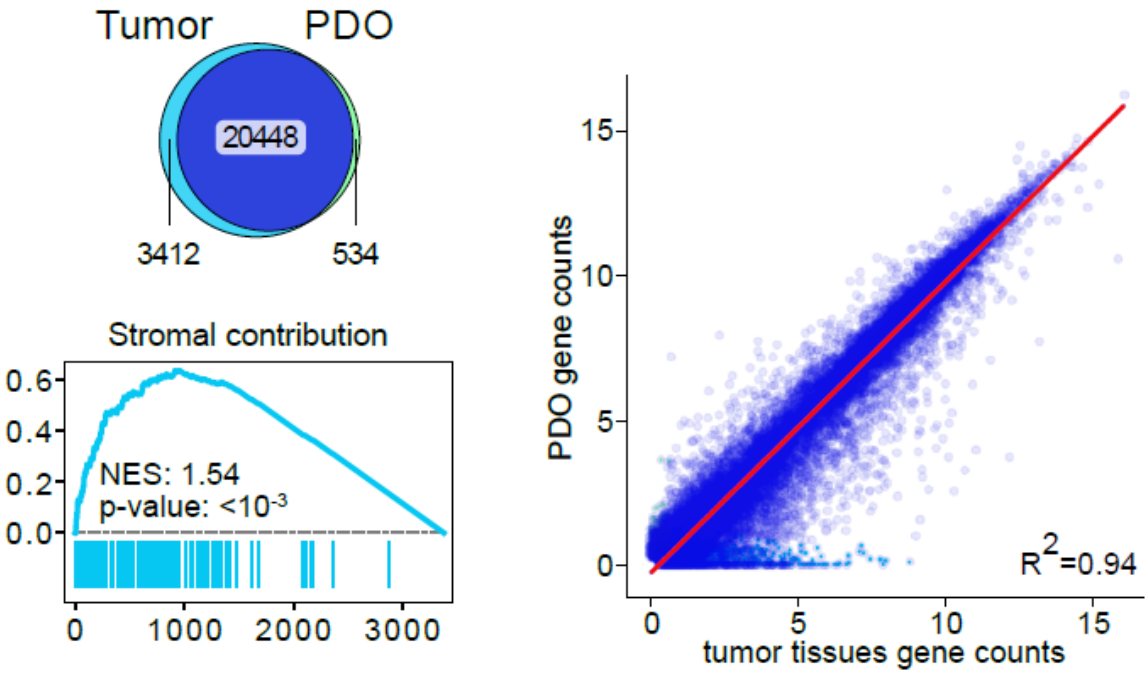


Fig. 8: Transcriptional concordance between PDOs and tumoral primary tissues. Venn diagram showed that 20448 genes overlapping between the two groups (about 85% of total genes). On the right, graph reported that expression levels across genes were well correlated between the two groups. Below enrichment graph underlain that non-overlapping genes expressed in primary tumours are associated to stromal component.

Moreover, we performed a differential expression (DE) analysis between tumoral and normal tissues for the detection of transcriptional changes associated to cancer development. In order to assess whether these are preserved in our organoids, we included PDOs in the analysis and hierarchical clustering revealed that the tumour-related signature groups PDOs together with primary tumours and separately from normal colon mucosa (Fig. 9).

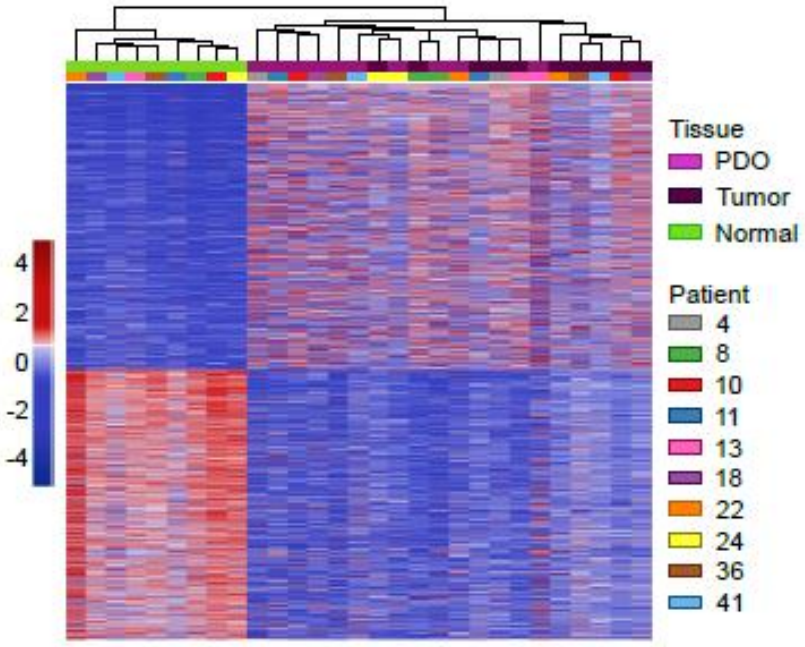


Fig. 9: Differential expression analysis on normal and tumoral primary tissues and PDO lines. The heatmap showed that tumoral primary tissues and PDOs clustered together, whereas normal tissues grouped separately.

Moreover, to better validate that PDOs recapitulate parental tumours from the transcriptional point of view, we performed gene set enrichment analysis (GSEA), using published tumour-associated gene signature. As shown in Fig. 10, genes up-regulated in our PDOs were enriched for the same genes found to be up modulated in public colon carcinoma gene set and the same was observed for genes down- regulated in our organoid lines.

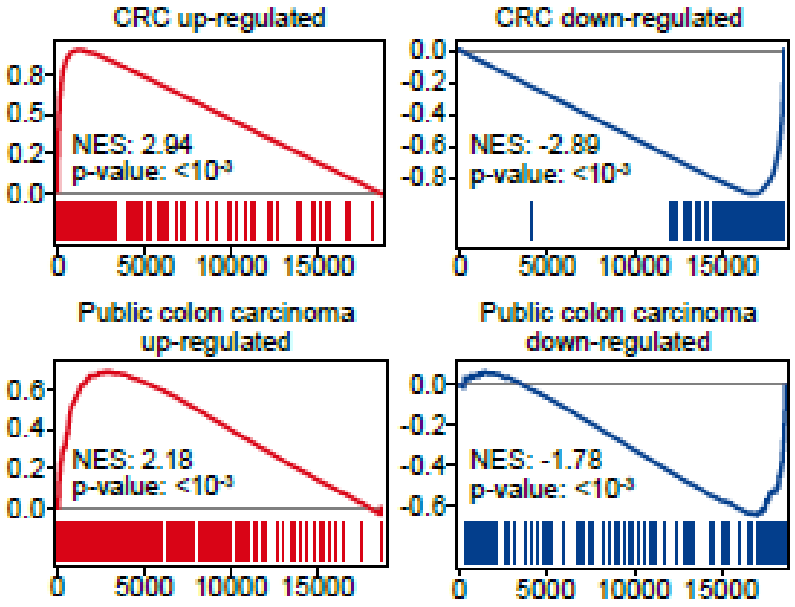


Fig. 10: Gene set enrichment analysis on PDO library. Analysis shows that genes up- and down- regulated (in red and in blue, respectively) in our organoid lines correlated with the same observed in public colon carcinoma datasets.

All these findings demonstrated that we generated a balanced PDO library, able to recapitulate the morphological, molecular and transcriptional heterogeneity, typical of colorectal cancer, in *vitro*. In addition, our PDOs were transcriptionally stable during passages and preserved high percentage of transcriptional concordance with their parental tissues, hence representing a suitable system for a comprehensive knowledge of CRC gene expression profile.

2.5 Epigenetic characterization of PDOs

Cancer epigenome represents one fascinating aspect to be investigated due to its potential role in the establishment and maintenance of specific cancer transcriptional programs (Easwaran H., 2014). In this context, our balanced and heterogeneous CRC PDOs biobank, faithfully reproducing transcriptional and histopathological features of primary tumours, can be exploited to investigate the most relevant epigenetic events and blueprints of cancer development and growth.
In line with this, we first performed a multi-factorial integrative analysis of genome-wide chromatin immunoprecipitation sequencing (ChIP-seq) for a core set of five histone modifications (H3K4me3, H3K27ac, H3K4me1, H3K36me3, and H3K27me3) on all PDOs (Fig. 11). Consistent with a good enrichment signal, our analysis revealed that the distribution of histone modifications across genes reflected their expected localization in relation to the gene body as well as the transcription start (TSS) and end (TES) sites (Fig. 11).

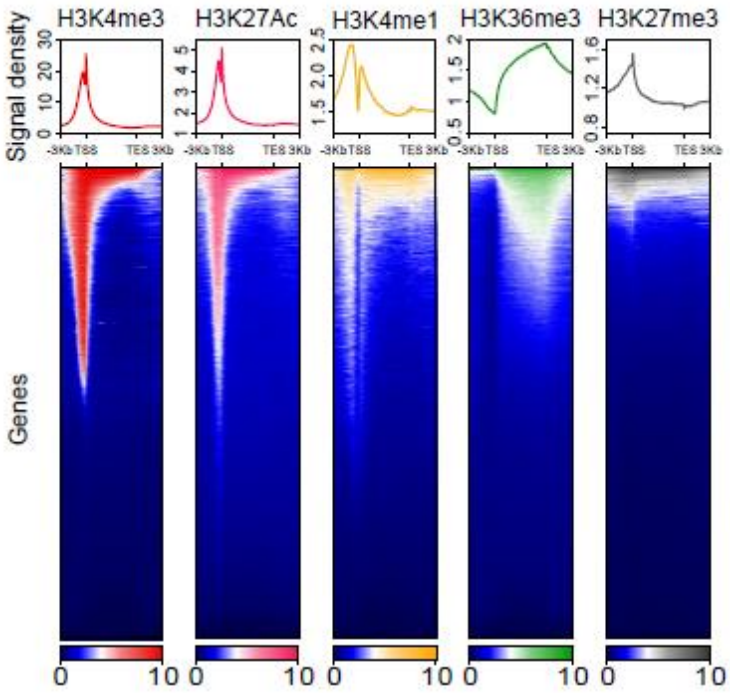


Fig. 11: Distribution of histone modifications across genes. The graph shows that each modification was detected in expected localization in relation to the gene body as well as the transcription start (TSS) and end (TES) sites.

Interestingly, unsupervised hierarchical clustering (Fig. 12) revealed that PDO derived from different patients shared a similar epigenomic landscape: the chromatin regions marked by the repressive marker H3K27me3, the elongation marker H3K36me3, and the block of histone marks defining active regulatory regions (H3K4me3, H3K27ac, and H3K4me1) were largely conserved between PDOs.

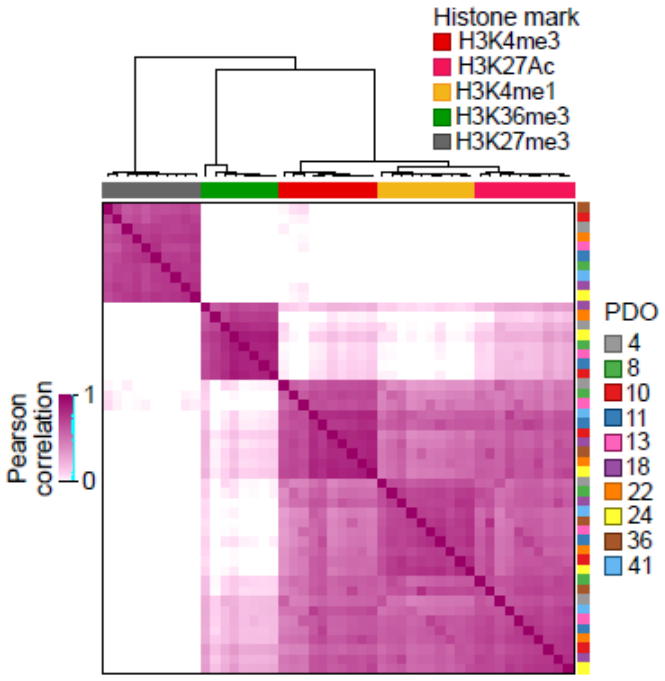


Fig. 12: Correlation analysis on histone modifications of our PDOs. Heatmap shows that markers associated to transcriptional activation clustered together (H3K4me3, H3K27ac, and H3K4me1), far from histone modifications related to transcriptional repression (H3K27me3) or from elongation marker (H3K36me3).

In order to have a more comprehensive view of CRC epigenomic complexity, we improved machine learning approaches to perform *de novo* chromatin state characterization on the whole ChIP-seq data for the PDOs. To this end, we exploit ChromHMM (Ernst and Kellis, 2012), a software generally used for learning and characterizing chromatin states. Thanks to this tool, we explored the combinatorial patterns of the five histone marks in an 8-state model and predicted specific genomic features with high resolution across samples (Fig. 13). With this approach we identified two promoter states ("Active TSS" and "Flanking Active TSS"), two active and one weak enhancer states ("Flanking Active Enhancers", "Active Enhancers", and "Weak Enhancers"), an elongation, a repressed, and finally, a quiescent state (Fig. 13).

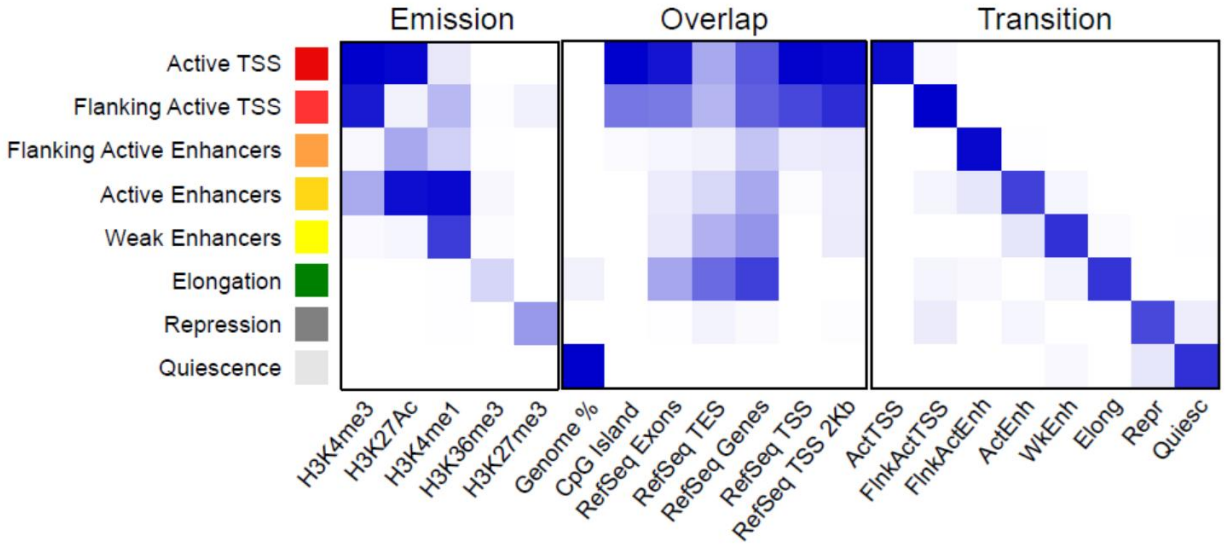


Fig. 13: ChromHMM on PDO library. We identified 8 total chromatin states: 2 promoter states (defined as active TSS and flanking active TSS); 2 active enhancer states (defined as flanking active enhancers and active enhancers); 1 weak enhancer state; 1 corresponding to elongation state, 1 as repression state and, finally, 1 quiescent state.

In order to support our results obtained with ChromHMM, we compared these data with chromatin accessibility for colon adenocarcinoma using ATAC-seq (Assay for Transposase Accessible Chromatin with high-throughput sequencing) datasets obtained from The Cancer Genome Atlas (TCGA) (Corces et al, 2018). The chromatin states identified in our PDOs remarkably concurred with chromatin accessibility, with active states displaying the highest and more inactive regions the lowest ATAC-seq signals, respectively (Fig. 14). Therefore, our PDOs represent a faithful resource to investigate the epigenetic landscape of CRC, and our ChromHMM states can provide a valuable atlas to decipher the functional role of ATAC-seq-defined open chromatin regions.

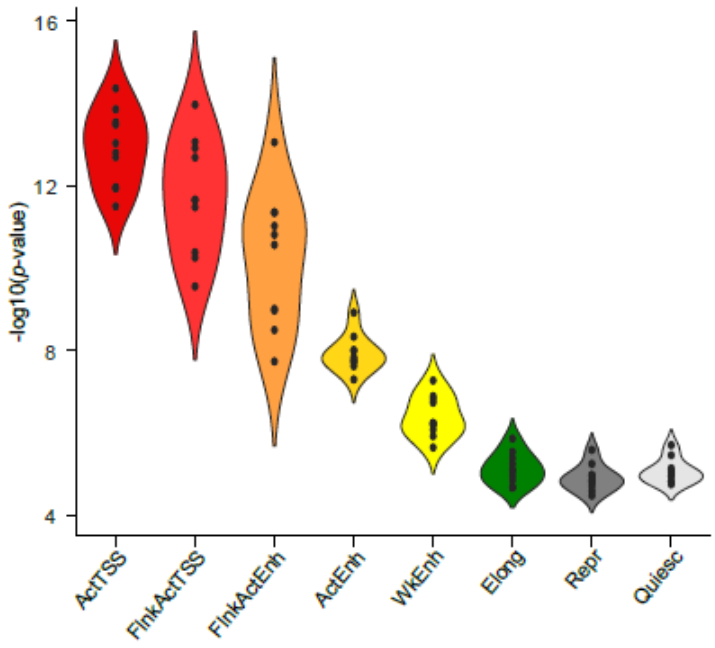


Fig.14: Comparative analysis between ChromHMM data of PDOs and ATAC-seq public data from TCGA. The graph showed the probability of each ChromHMM state overlapping ATAC-seq regions for TCGA colon adenocarcinoma across PDO library.

Human CRC PDO were characterized by extensive cellular and molecular heterogeneity. Yet, they shared a large fraction of their transcriptional and epigenetic programs evident by Fig. 9 and the conservation of histone marks profiles across PDOs (Fig. 12), respectively. As an example of epigenetic conservation, we showed the chromatin states pattern of FABP1, a marker of enterocytic differentiation expressed by all PDOs (Fig. 6); all PDOs display an active chromatin state at this locus as visualized by the peaks associated to active histone marks (H3K4me3, H3K27ac, and H3K4me1) at the promoter and flanking region (Fig. 15).

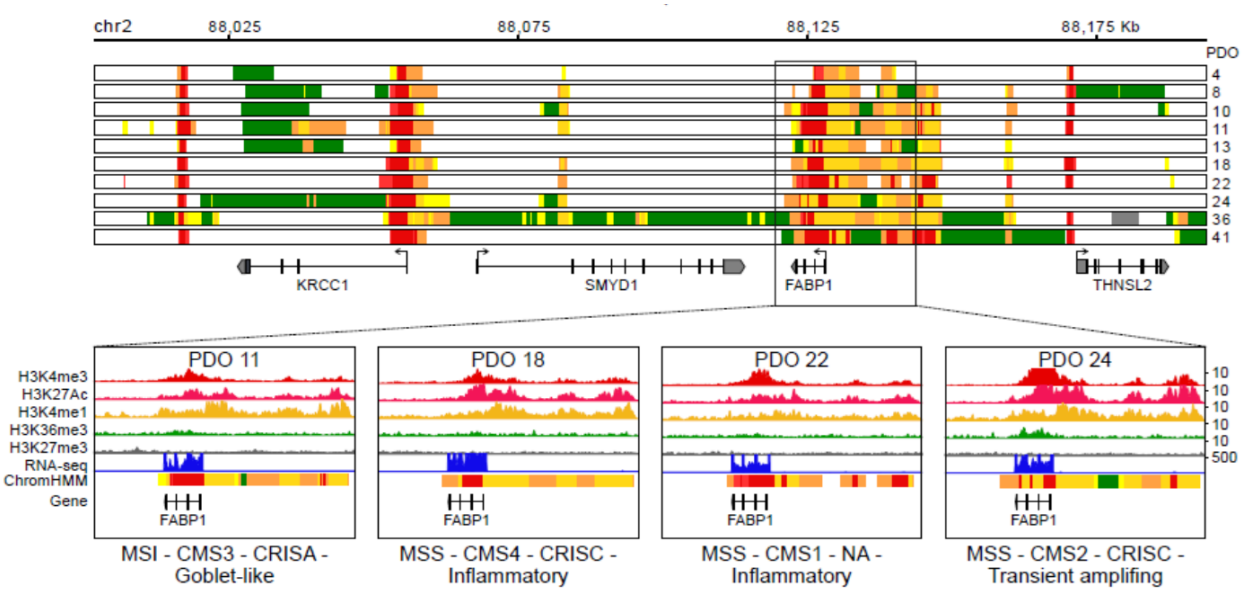


Fig. 15: Comparative analysis between RNA-seq data (on the top) and ChIP-seq data (on the bottom) on FABP1 locus. Epigenome profiles of 4 PDOs belonging to different molecular subtypes shows the same pattern of transcriptional activation.

Our epigenetic analyses also allowed to identify tumour-specific epigenetic features. Regulatory variability across PDOs was observed in the gene encoding for laminin subunit α-5 (LAMA5) (Fig. 16), a marker of cell adhesion and migration reported to be involved in metastasis (Bartolini et al., 2016). Active transcription in PDO#11 was indicated by a ChromHMM profile that associated with active states around the TSS and with an elongation state along the gene body. On the contrary, LAMA5 was actively silenced in PDO#18 evident by the loss of H3K36me3 and the accumulation of the H3K27me3-repressive mark at the promoter and throughout the gene body.

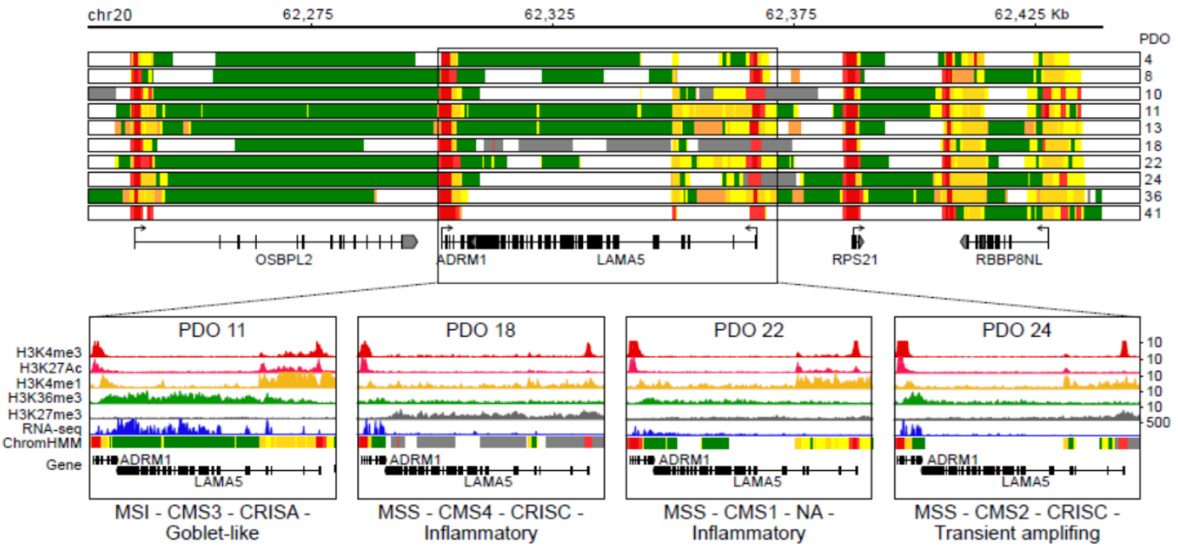


Fig. 16: Comparative analysis between RNA-seq data (on the top) and ChIP-seq data (on the bottom) on FABP1 locus. Epigenome profiles of 4 PDOs belonging to different molecular subtypes shows the same pattern of transcriptional activation.

In conclusion, our molecular data allowed us to shed light on epigenome and transcriptional landscape of our CRC PDOs, and consequently of primary human colorectal cancer, giving the opportunity to identify both common and specific active regions across different CRC molecular subtypes. Moreover, a deepest interrogation of PDOs ChromHMM data will help us to better identify all the active and repressed regulatory elements that control the epigenetic mechanisms and transcriptional profiles at the base of human colorectal cancer.

3. PDO-TREGS CO-CULTURE ESTABLISHMENT

3.1 Generation of human PDOs and TILs isolation from the same CRC biopsy

In the previous paragraphs we showed that human CRC PDOs represent a powerful model to study human colorectal cancer, mimicking its molecular, transcriptional and histopathological profiles. In the introduction section, we mentioned that one limit of the organoid model is determined by the absence of stromal compartment, with particular attention to the role of tumour infiltrating lymphocytes (TILs). Therefore, with this background in mind, we decided to set up a co-culture between tumoral organoids and TILs. In particular, considering the role of Tregs in colorectal cancer (De Simone et al., 2016), we added this type of immune cells to the tumoral organoids. To this extent, we set up a protocol which allowed us to isolate TI-Tregs from normal and tumoral colon biopsies (De Simone et al., 2016). Briefly, each primary tissue was simultaneously processed using two different protocols (Figure 17): one for crypt isolation (according to Sato's protocol, 2015), and a second for TIL extraction (De Simone et al., 2016).

For co-culture experiments we used freshly isolated (ex vivo) Tregs or *in vitro* expanded Tregs isolated from the same tumoral biopsies (Fig. 18, 17 respectively). The last ones offer the possibility to work with higher number of cells, though representing a biological challenge due to loss of their tumoral phenotype during the expansion in culture (Figure 1).

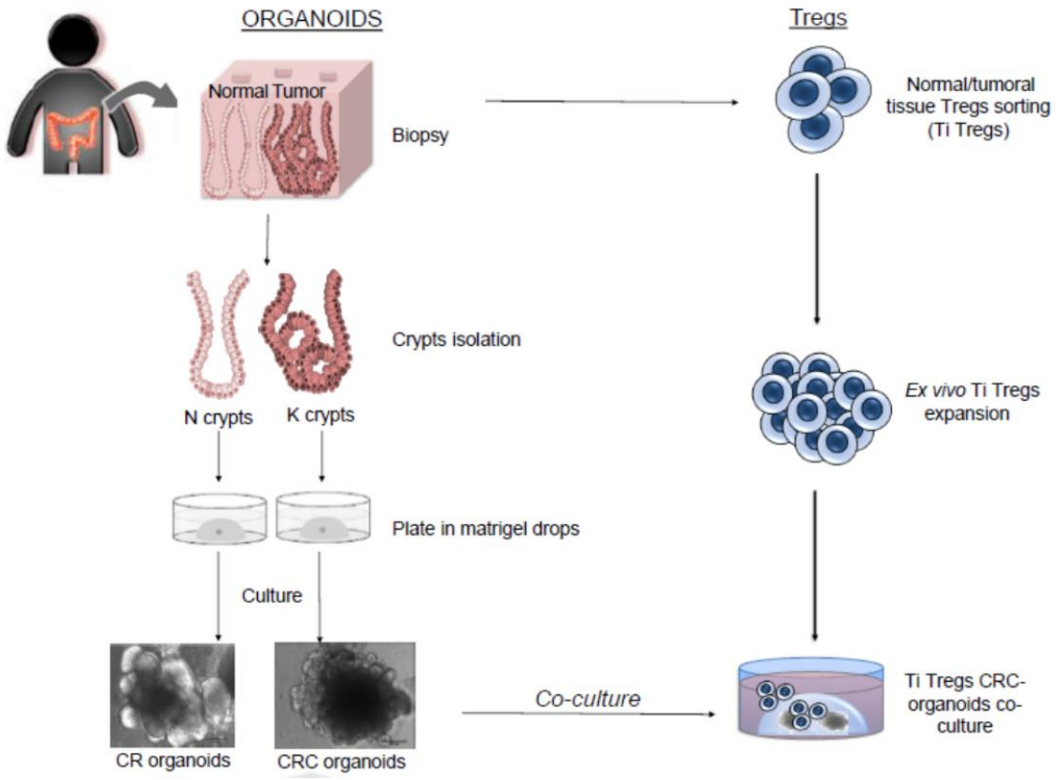


Fig. 17: Co-culture experimental strategy using expanded TI-Tregs. Our procedure involved both crypts and Treg isolation from the same colon primary tissue. Once we managed to generate organoids we could use them to be co-cultivated with TI-Tregs, previously expanded *in vitro.*

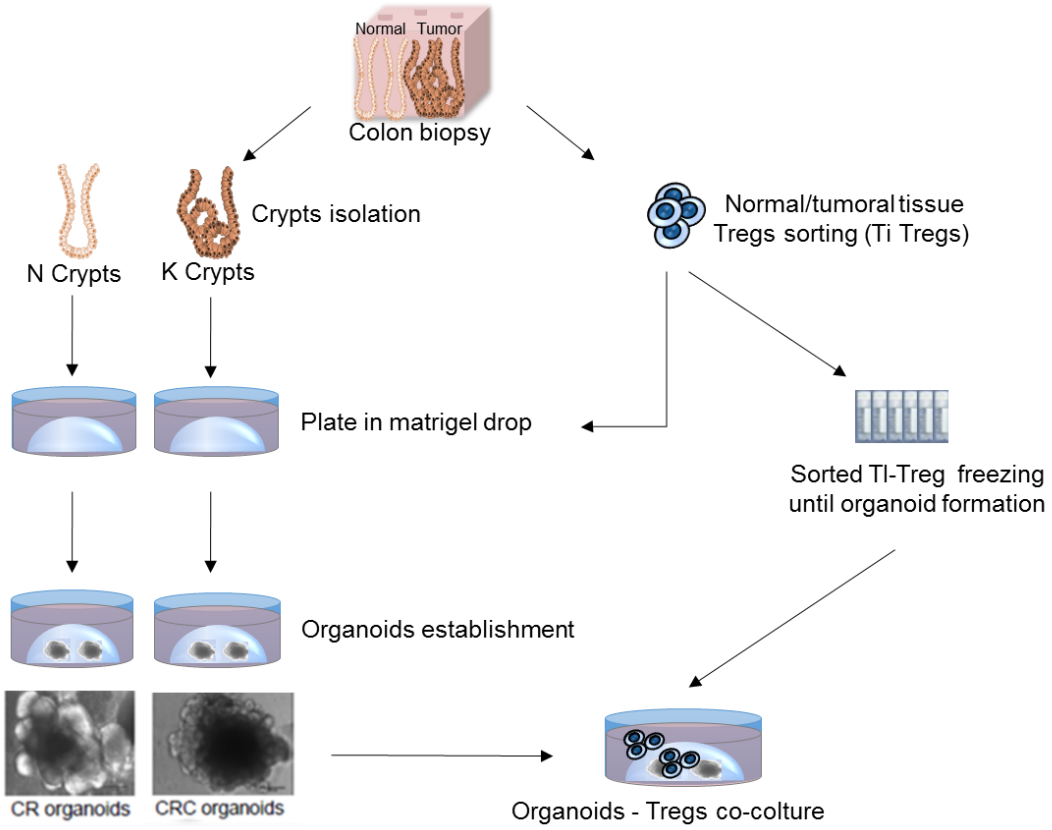
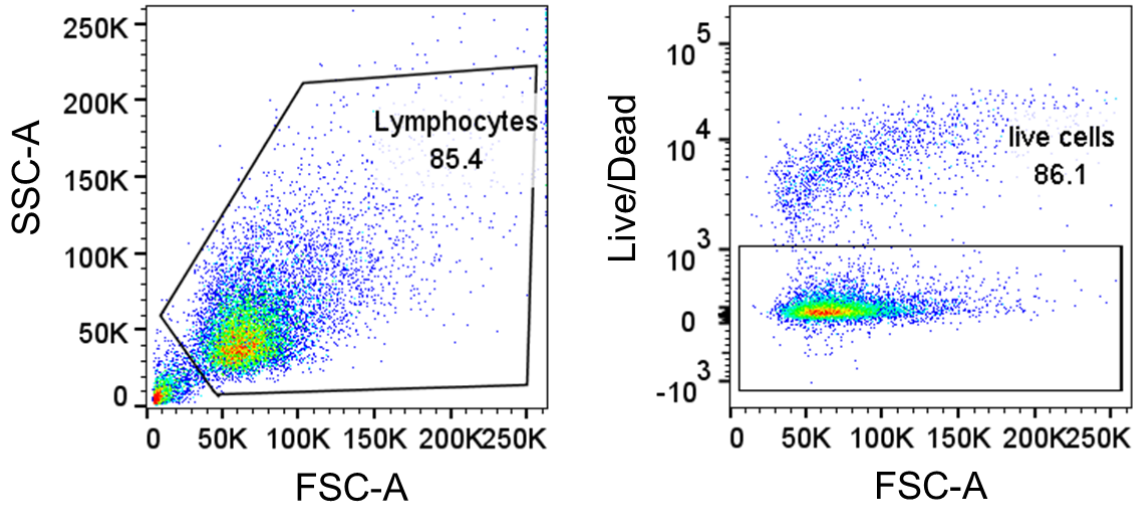


Fig. 18: Co-culture experimental procedure using TI-Tregs ex *vivo*. Two possibilities were available: one involved the co-culture between crypt and Tregs ex *vivo*, to evaluate a feasible advantage conferred by these cells to organoid formation; the second involved instead the co-culture between organoid and TI-Tregs ex *vivo*, to assess if these cells were able to preserve their "tumoral" phenotype *in vitro*, exploiting organoid 3D culture.

3.2 Tregs viability in organoid culture conditions

First of all, our co-culture system required the possibility to grow both PDO and Treg in the same culture conditions. Indeed, we need to test the effect of all the components of organoid medium, essential for *in vitro* PDO maintenance, on Tregs viability and phenotype. In particular, as previously mentioned in the introduction, tumoroids were generally cultivated in ENAS medium, constituted by basal medium (for its components see experimental procedure) supplemented by: Gastrin, EGF, BMP-inhibitor Noggin, MAPK inhibitor SB202190, TGFβ inhibitor A8301. On the other hand, Tregs usually grow in complete RPMI medium with the addition of IL2 (200U/ml). In line with this, we decided to check first Tregs viability in ENAS organoid media with the addition of IL2 for Tregs survival (after three, five and seven days using flow cytometry analysis (FC). We started these experiments using expanded Tregs isolated from peripheral blood of healthy donors (HD PB Tregs) instead of TI-Tregs, for technical reasons (high number recovery, easily available). To check Tregs viability were stained with Live/Dead FVS780, a dye which, diffusing into cells with impaired membrane integrity, cannot colour live cells. As shown in Fig. 19, HD PB viability was not affected by organoid culture conditions.

A



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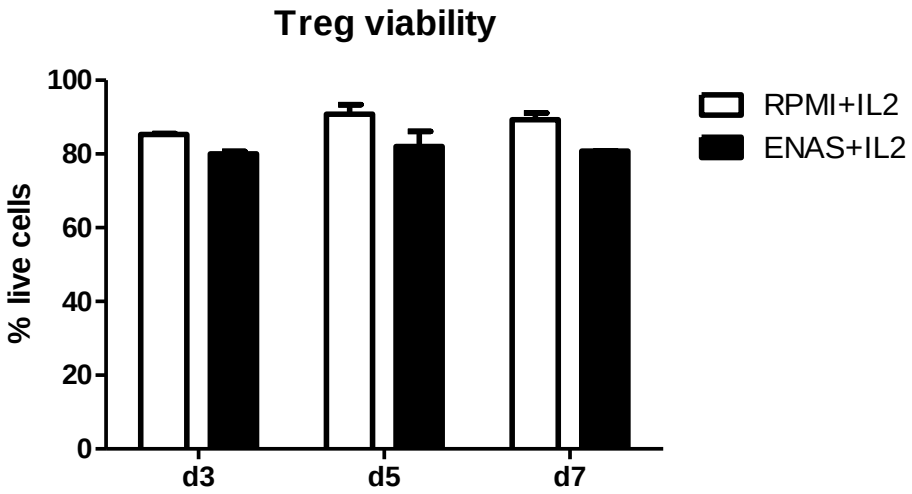
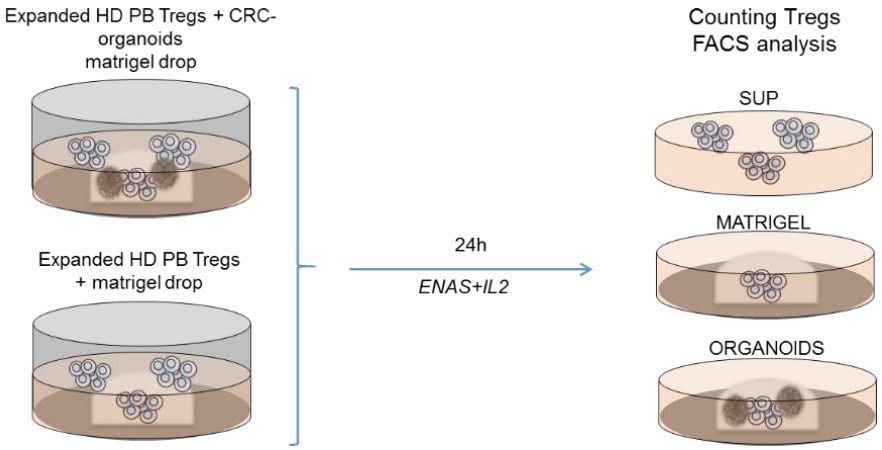


Fig. 19: Treg viability on organoid culture conditions. In panel A gating strategy used for FC analysis is reported: after selection based on physical parameters, we evaluated % negative cells stained with Live/Dead. Panel B shows that Treg viability were not affected by organoid culture conditions (N=2 independent experiments).

3.3 Treg migration into Matrigel drop

Matrigel is another essential component for organoid culture, mimicking the fundamental support of extracellular matrix (ECM). To this end, we tested the ability of expanded HD PB Tregs to migrate inside Matrigel drop independently from the presence of the organoids, when added to culture medium after 24h by FC analysis. We collected Tregs from each fraction: cells remained in the medium, those able to cross Matrigel drop and, when present, those entered within the organoids (Fig. 20A). Based on previous reports (Dijkstra K.K. et al., 2018) and to push the system, we decided to use a ratio Treg: organoid 5:1 in favour of T cells. For each fraction we counted live cells and we indentified Tregs as $CD4^{+}CD25^{+}CD127^{-}$ population (Fig 20B).

A



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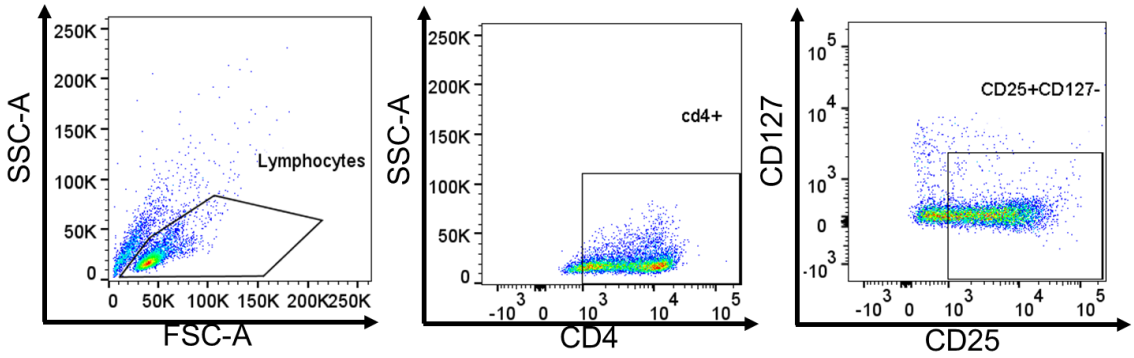


Fig. 20: Tregs migration assay. Panel A: Experimental strategy showed expanded HD PB Tregs were added to medium in presence of Matrigel drop alone or in presence of organoids. After 24h cell fraction in the medium, in Matrigel and inside organoids were collected and counted live cells for FC analysis. Panel B: Gating strategy for Tregs identification in each fraction: cells were selected as $CD4^{+}CD25^{+}CD127^{-}$ (N=2 independent experiments).

As showed in Fig. 21 no significant differences in Tregs viability were detected when Tregs were plated in presence of Matrigel drop alone or in presence of organoids after 24h. Moreover, we didn't observe any variation in the expression of markers CD4, CD25 and CD127 in each fraction. When we analysed CD4+CD25+$CD127^{-}$ population in each fraction (see gating strategy shown in fig. 20B), about 47% of total live cells remained in the medium, about 35% migrated into Matrigel drop but outside organoids, whereas 18% migrated inside organoids. These percentages differed in absence of organoids: we observed that right after 24h more than 40% of Tregs could cross Matrigel drop when alone, and that this percentage reached 53% in presence of organoids (18%+ 35%). This means that probably some factors or cytokines produced by tumoroids were able to attract more Treg cells at the tumour site (Fig. 21).

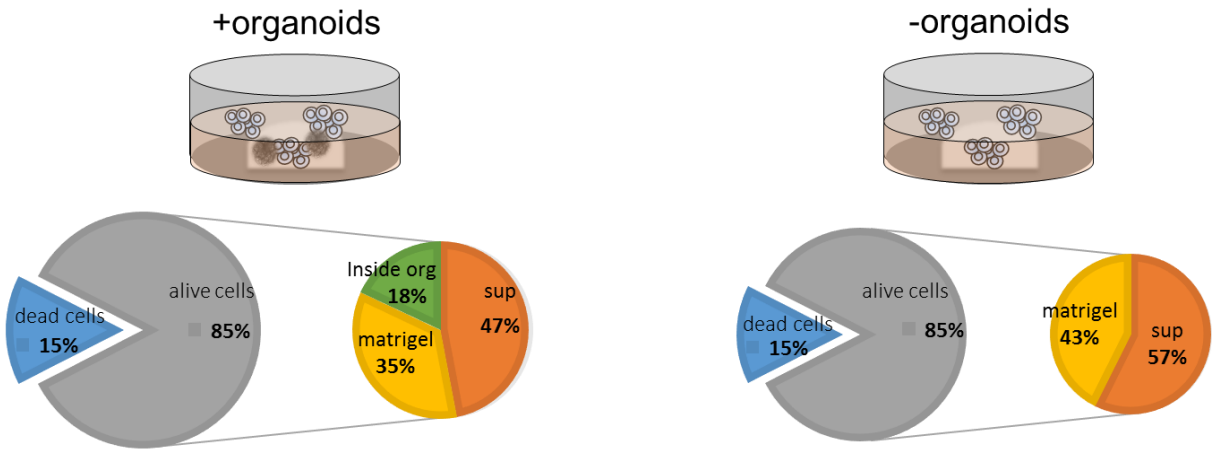


Fig. 21: Tregs migration into Matrigel drop. Pie chart showed percentages of Tregs detected in each fraction in presence (on the left) or absence (on the right) of organoids. In presence of organoids, about 53% of Tregs migrate into matrigel drop (35% remained around organoids and 18% moved into organoids) against 43% detected in absence of organoids.

In conclusion, our preliminary experiments supported the feasibility to set up a co-culture model to assess the interplay between organoids and TI-Tregs.

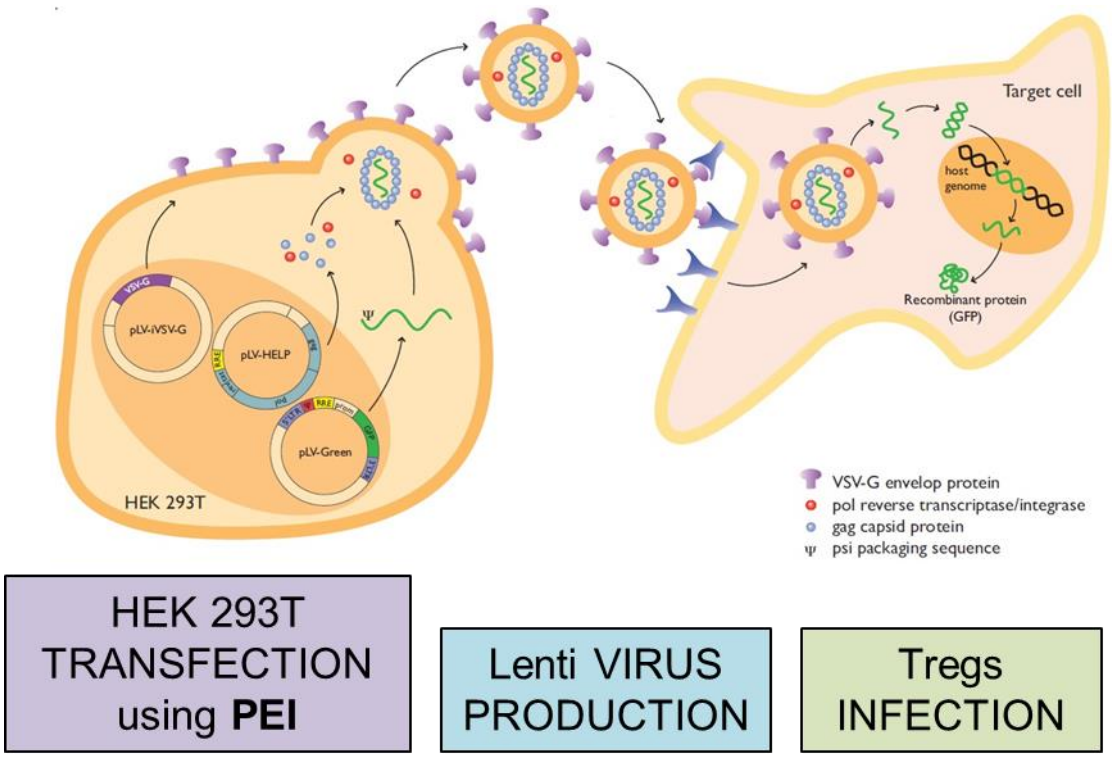
3.4 PDO-Tregs preliminary co-culture experiments

3.4.1 Tregs lentiviral transduction for GFP expression

Having analysed some basic aspects of co-culture conditions, we performed some co-culture experiments to follow live Treg migration in/out organoids, through time lapse acquisitions at microscope.

For a better visualization of Tregs, we stably infected Tregs with GFP- expressing lentiviral vector (Fig.22A). We tested the efficiency of transduction both on expanded HD PB Tregs and TI-Tregs by FC analysis. As showed in Fig. 22B, we reached an efficiency of about 24% for HD PB Tregs (in blue in Fig. 22B) and almost 40% for TI-Tregs (in red in Fig. 22B), an important result, especially for TI-Tregs.

A



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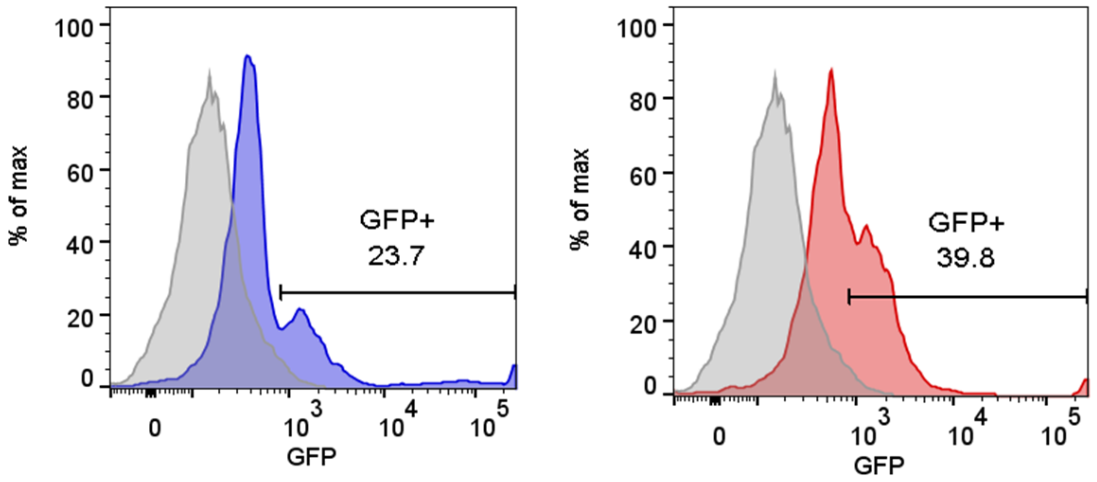

Fig. 22: Tregs lentiviral infection. Panel A: experimental strategy used for setting up Tregs lentiviral transduction. Panel B: Tregs transduction efficiency. Histograms showed transduction efficiency in expanded HD PB Tregs (in blue) and in expanded TI-Tregs (in red) compared to the control (in grey).

3.4.2 Live imaging co-culture experiments

Once obtained stable GFP-expressing Tregs, we performed co-culture experiments and followed Tregs migration through time lapse acquisition at microscope. We exploited IncuCyte S3 live-cell analysis system, an instrument that allowed a real-time quantitative live-cell imaging and represents a suitable platform for kinetic studies, drug responses and other different applications. Another great advantage provided by this instrument is the possibility to follow real-time our co-culture experiment directly in cell incubator, since the camera for acquisition was installed inside, without external perturbations. In the experiment we both used GFP^+ HD PB and GFP^+ TI-Tregs which were added to Matrigel drop or to culture medium and co-cultivated for 10 days in presence of PDOs. Fig. 23 shows a middle time-point of GFP^+ Tregs co-cultured with the organoids. This picture, taken by the software at a central focus of the well and corresponding to the first half of Matrigel drop, clearly demonstrated that Tregs were inside the organoids, with an elongated and likely activated shape and capable to survive in these conditions, migrating in and out the organoids. The video provided as media supplemented material further confirmed these observations. The movie showed a fast organoids growth rate, starting from the very early time points (before 24h), displaying new buds of active proliferation, likely indicating that in the first phases Tregs could not affect their proliferation, and further quantitative analysis will better define this point.

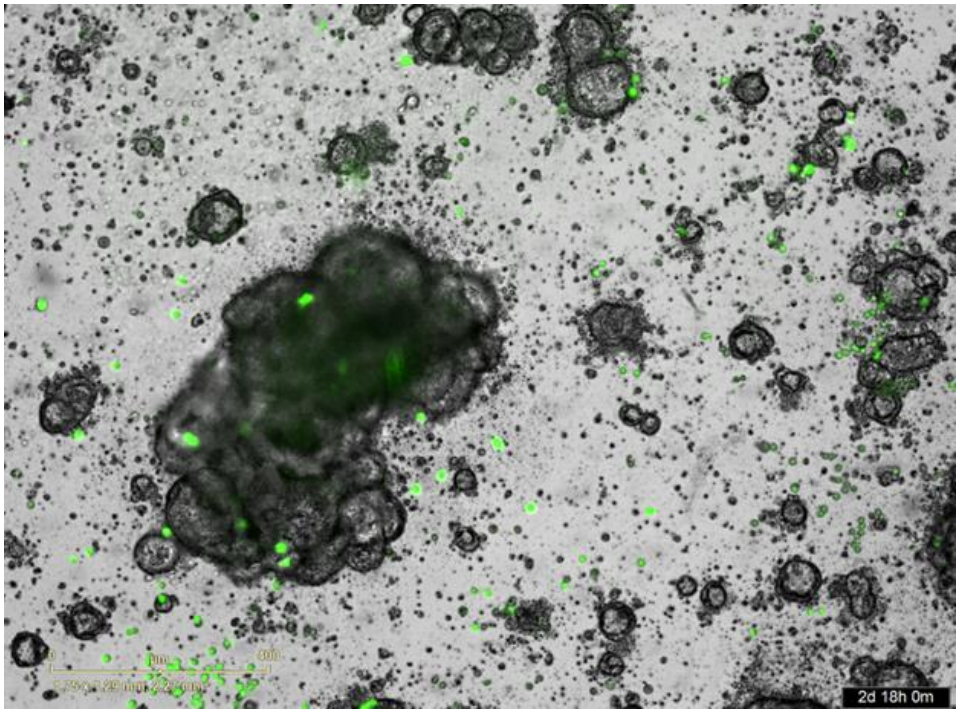

Fig.23: Unmatched expanded GFP^+ HD PB Tregs- tumoral organoids co-culture. In this experiment Tregs were added directly to organoids. Image showed that Tregs were still alive and migrated into organoids for 10 days of co-culture.

Collecting GFP signal and integrating it with the organoids area, the instrument could measure Tregs migration into the tumoroids. This analysis revealed that Tregs started to migrate into the organoids around day1 with an exponential trend that begins at day 3 up to the end of culture, in both big and small organoids (Fig. 24A and B). These results suggest that Tregs require a short time to adapt to co-culture conditions, as we also observed in 24h migration experiments (Fig. 21) and independently of organoids dimensions, are attracted to them. Interestingly, the trend was the same when Tregs were added both to medium (Fig. 24A) and directly to Matrigel drop (Fig. 24B), meaning that in addition to Matrigel, the organoids could attract Tregs with cytokines and chemokines, likely released in the medium with paracrine effect.

Using the same software, we also evaluated the growth rate of our organoids in presence of different ratio Tregs: organoids (1:1 and 2:1), observing the same trend of exponential growth in both cases for 10 days, indicating that Tregs cannot macroscopically affect organoids grow rate (Fig. 24C). Further analysis at a single cell level could maybe decipher if some organoids cell types could be more influenced.

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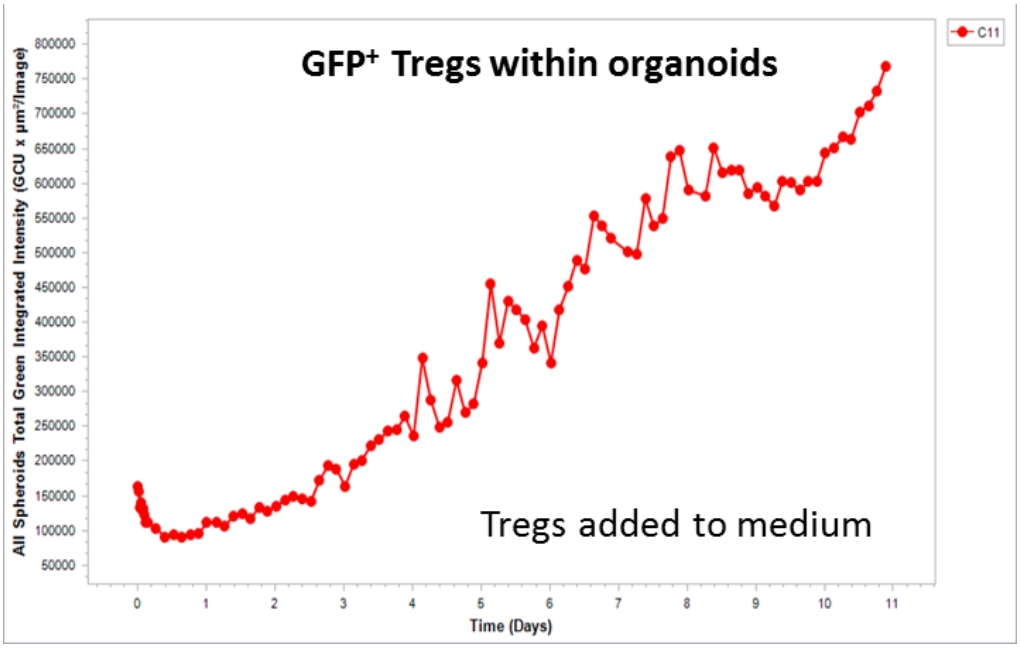

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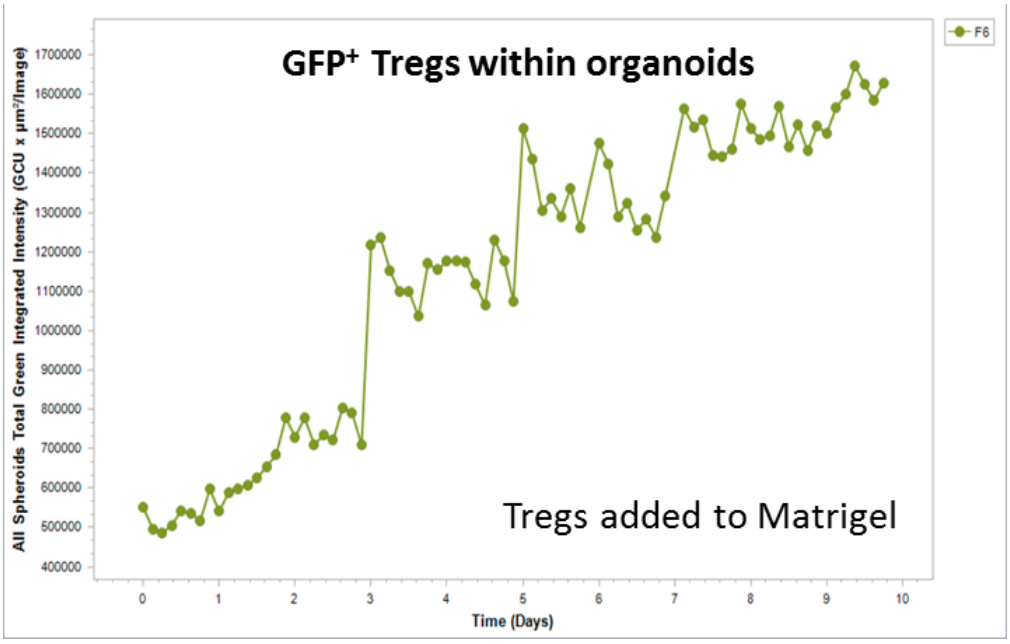

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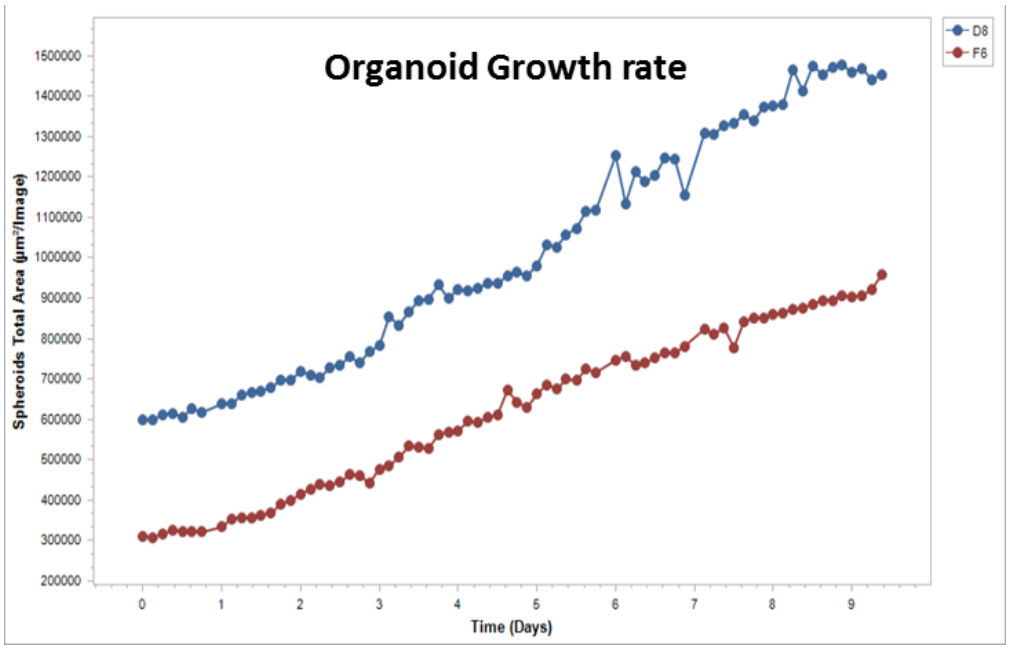

Fig. 24: Panel A and B: Kinetics of GFP+ Treg migration in unmatched co-culture experiment for 10 days. In particular, Tregs migration were reported when added to medium (A) or to Matrigel (B), calculating the GFP signal detected inside spheroids (Y axis). Both graphs showed increasing number of Tregs moved into organoid during passing of time. Panel C: organoid growth rate in presence of a different ratio Tregs: organoids (1:1 (in blue in the graph) and 2:1 (red in the graph)). Growth was evaluated as total area constituted by spheroids (Y axis).

Analogously, we performed the same experiment using matched PDO and expanded GFP$^+$ TI-Tregs isolated from the same tumoral primary tissue and followed this co-culture for 4 days. Interestingly

expanded TI-Tregs showed the same behaviour observed for GFP^+ HD PB Tregs, with a similar kinetics of migration inside organoids (Fig. 25).

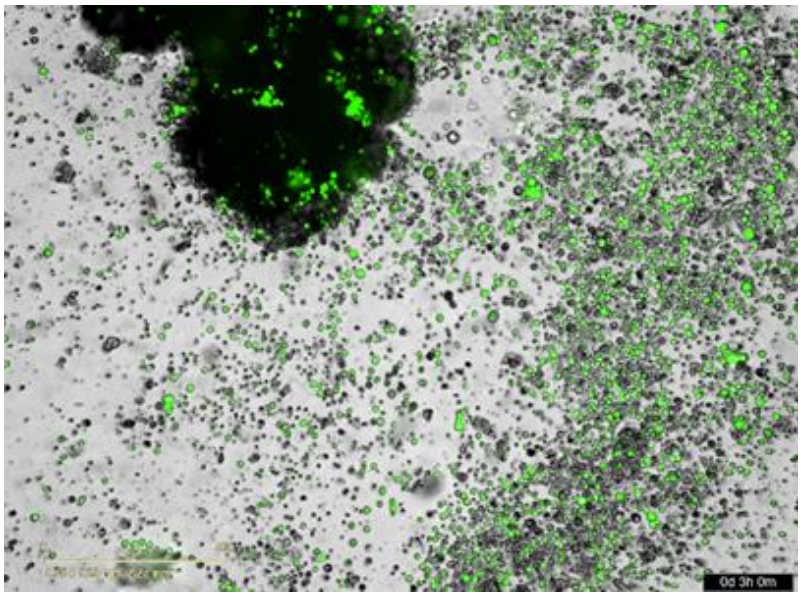

Fig. 25: Matched expanded GFP^+ TI-Tregs and tumoral organoid co-culture. In this case, Tregs survived and migrated in/out organoids for 4 days in co-culture.

To further confirm the presence of Tregs within the PDOs, we also performed on this matched co-culture analysed at the IncuCyte, a 3D immunofluorescence (IF) whole mount staining. In order to detect each signal associated to the presence of Tregs inside the organoids we acquired very thin Z-stacks (of 2,5µm), and, as we showed in Fig. 26, reporting a Z-stack corresponding to a central region of the organoids, we detected GFP signals inside the organoids. These images indicate that Tregs were able to migrate deeply within the organoids and these last ones were highly proliferating in presence of TI-Tregs (given by high amount of Ki67+ cells), confirming what we showed in the time-lapse experiment.

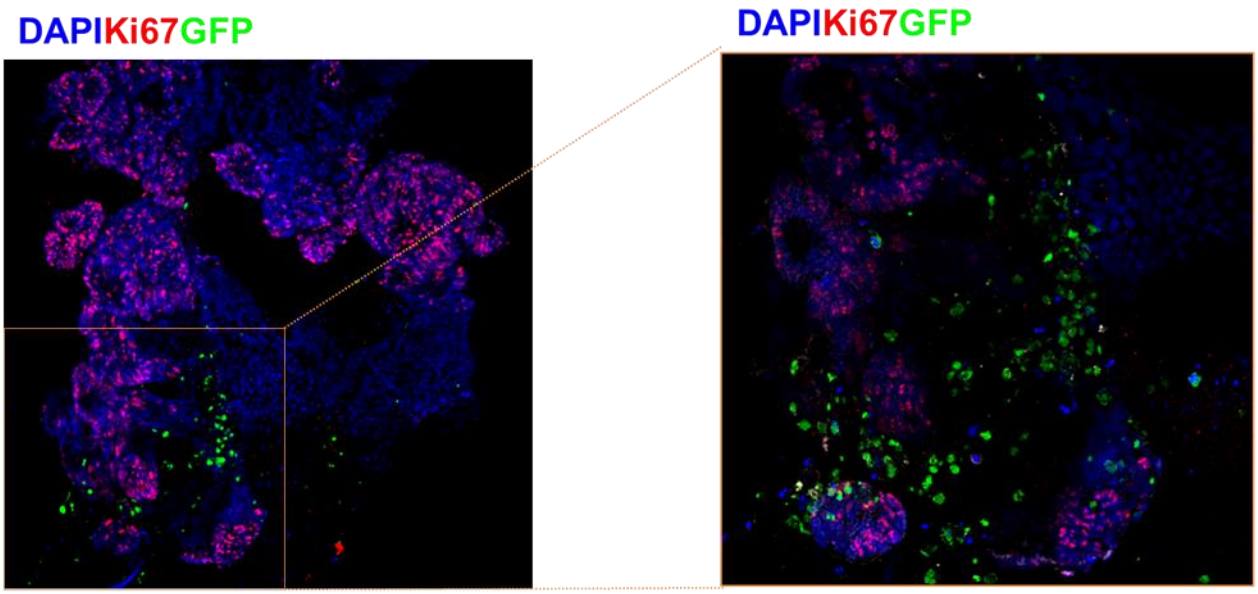

Fig. 26: 3D IF on matched TI-Tregs and tumoral organoids from patient CRC#66 after 4 days in co-culture. Maximum projection at 20X and at 40X are reported on the left and on the right, respectively. We acquired 18 Z stacks of 2.5µm of thickness. The images showed nuclei with DAPI, TI-Tregs as GFP^+ and proliferating cells as $Ki67^+$. Images showed Tregs which migrated into high proliferating organoids.

Furthermore, in order to assess the feasibility of long- term Treg-organoid co-culture model, we put matched TI-Tregs and tumoral organoids together in the Matrigel drop and co-cultured them for 30 days. We subsequently performed a 3D IF whole mount staining to detect the presence of Tregs. To this end, we stained for CD4 and FOXP3 and acquired very thin Z-stacks to detect all signals coming from Tregs across the organoids. Although it represents a qualitative approach, we were able to identify the presence of Tregs inside the organoids, displaying the capability of Tregs to survive and maintain Foxp3 expression even after a long period of time (Fig. 27). However further more quantitative experiments will be necessary to better define their phenotype inside the organoids.

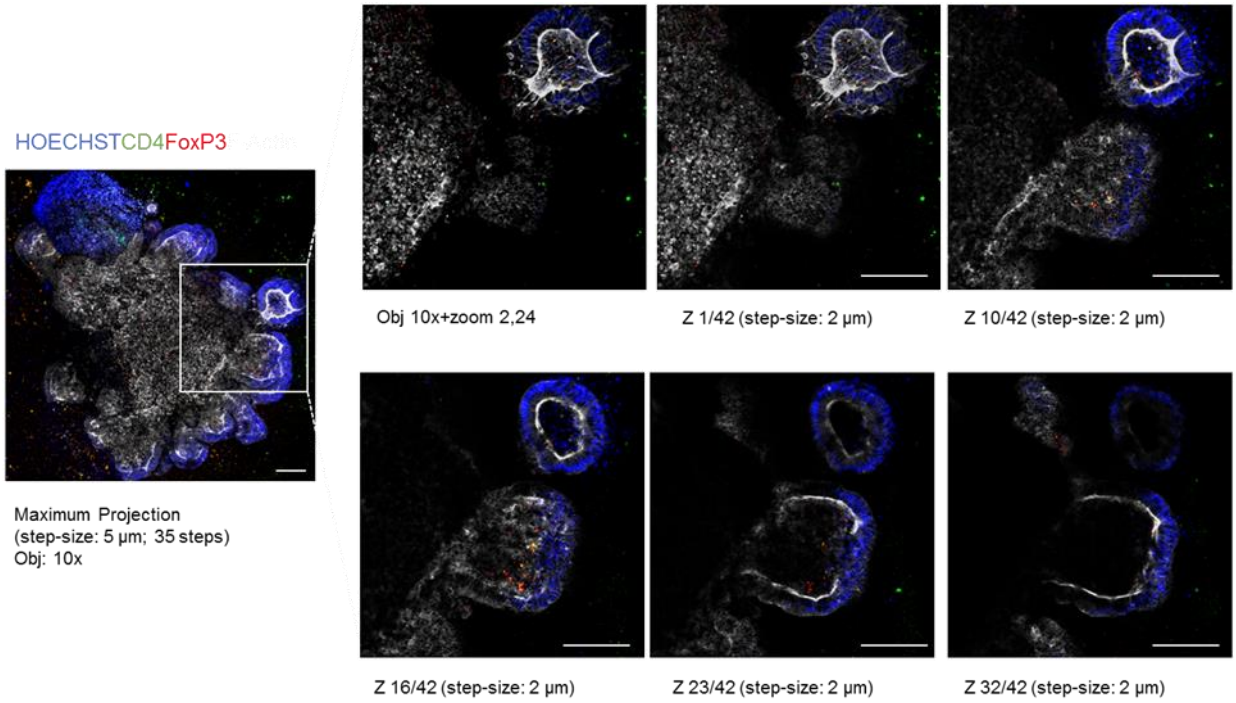

Fig. 27: 3D IF on matched TI-Tregs- tumoral organoids co-culture. Nuclei were stained with Hoechst, Tregs with CD4 (green) and Foxp3 (red). Images showed that Tregs were still present inside organoids after 30 days of co-culture.

In conclusion, our data demonstrated that human Tregs were able to survive in organoid culture conditions, migrate into Matrigel drop and the organoids, mimicking their infiltration capability at the tumour site *in vivo*. We obtained similar results adding both Tregs in medium or directly into Matrigel droplets, further evidencing the organoids ability to attract Tregs to neoplastic cells, with likely paracrine stimuli. These observations suggested the possibility to exploit the organoid model to understand the mechanisms underlain Tregs and colon cancer crosstalk favouring tumour growth and proliferation. Next analysis will evaluate the ability of co-culture models to induce/restore TI-Tregs specific phenotype on *in vitro* expanded tumour Tregs, used for co-culture experiments.

3.5 Organoid- Tregs interplay in co-culture model

Taking into account our setting up co-culture experiments and the viability of Tregs in the presence of organoids media, we decided to use ENAS plus IL2 to culture together these two cell type populations.

To assess co-culture feasibility to induce a tumoral gene signature in tumor expanded Tregs, we put unmatched expanded TI-Tregs and tumoroids (3:1 ratio) co-culture in the same Matrigel drop, and we analysed Tregs phenotype after 7 days by Flow cytometry (Fig. 28). We collected both Tregs migrated inside the organoids and those moved out from Matrigel drop. Interestingly, we observed a significant increase of PDL1 expression (one of the markers reported in TI-Treg gene signature, see De Simone 2016) in Tregs detected inside (61%) compared to those migrated out of the Matrigel (supernatant co-culture -10%) and to the same cells cultured without the tumoroids (Fig. 28 panel B). This meant that our co-culture model could be suitable to recapitulate one in *vivo* situation, where PDL1 induction on TI-Tregs can likely be expressed thanks to the direct interaction between Tregs and cancer cells.

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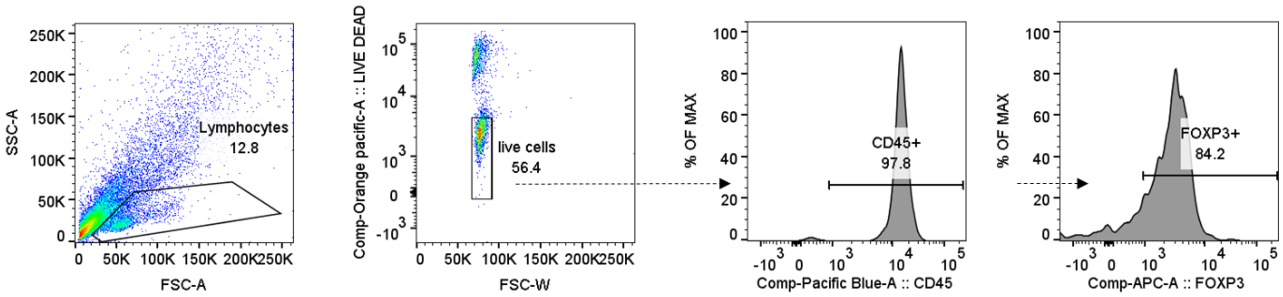

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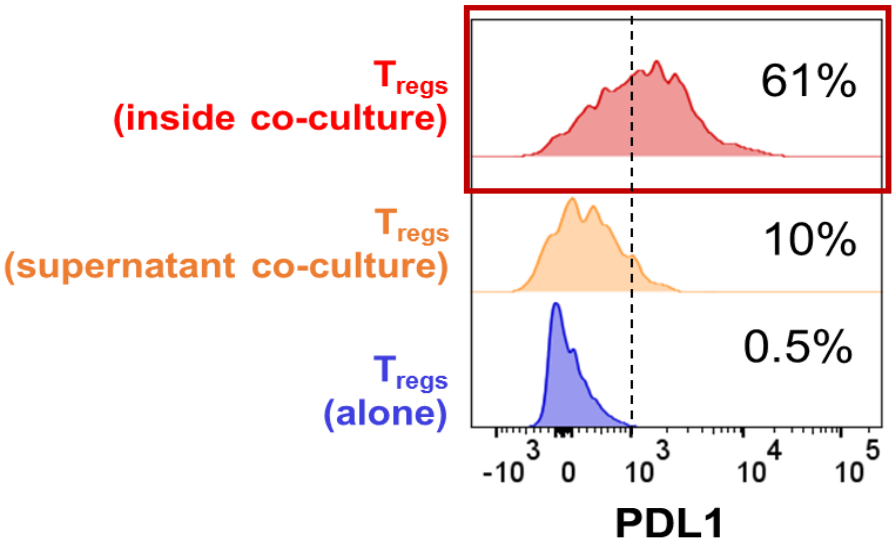

Fig. 28: Expanded TI-Treg- organoid co-culture for 7 days. Panel A: Gating strategy adopted to identify Tregs in co-culture model: cells were selected as live/dead negative, $CD45^{+}FOXP3^{+}$ subset. Panel B: PDL1 expression in Tregs inside co-culture (red), in Tregs collected from co-culture medium (orange) and in Tregs cultivated in absence of organoids.

Intriguingly, when we perform the same co-culture but using expanded Tconv cells in place of Tregs, we did not detect any significant differences in PDL1 expression, independently of the presence of the tumoroids (Fig. 29). This meant that tumoroids were able to upregulate characteristic markers on specific cell population potentially conferring them a peculiar phenotype.

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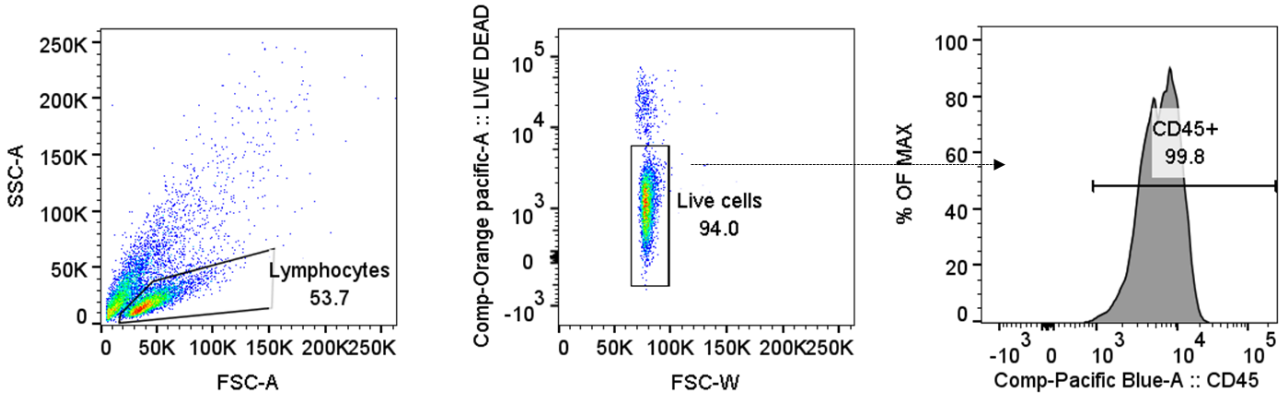

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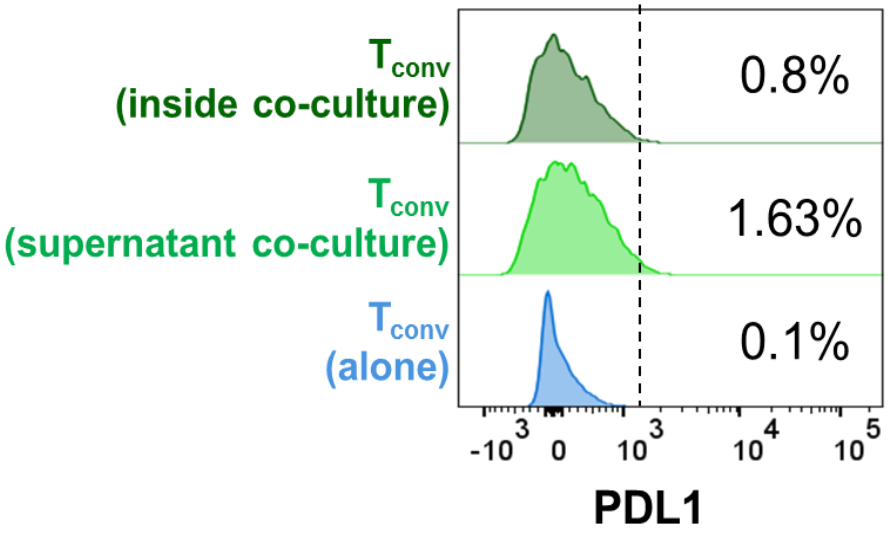

Fig. 29: Expanded TI-Tconv- organoid co-culture for 7 days. Panel A: Gating strategy adopted to indentify Tconv cells. Panel B: PDL1 expression on Tconv cells inside co-culture (dark green), in Tconv collected from co-culture medium (light green) and in Tconv cultivated in absence of organoids (light blue).

Moreover, since PDL1 was also a marker generally upregulated in tumour cells, we assessed if there were any differences in its expression in our tumoroids in our co-culture experiment. Intriguingly, in presence of expanded TI-Tregs we observed an increased expression of PDL1 on organoids compared to those cultivated alone (Fig. 30). Interestingly, its significant down modulation was detected when co-cultivated with expanded Tconv cells. This suggested that TI-Tregs and TI- Tconv cells could potentially have a different effect on expression of specific markers on tumour cells.

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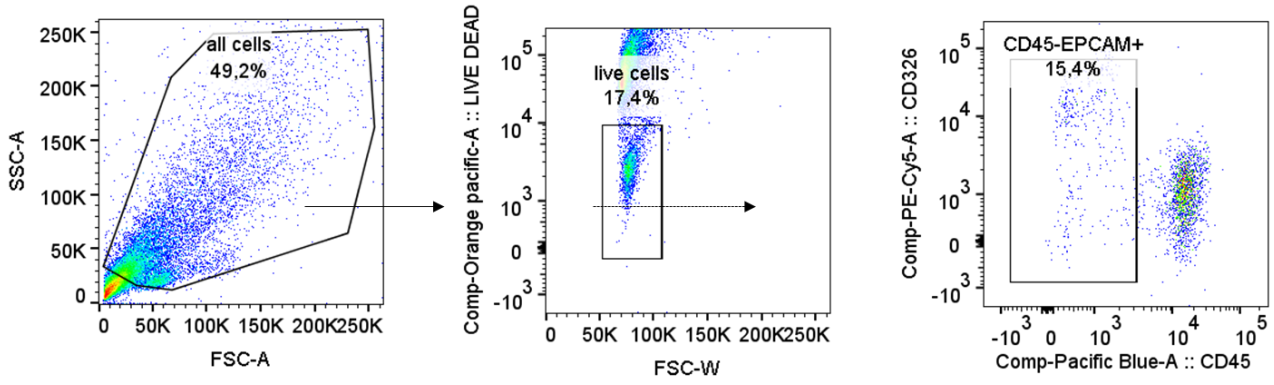

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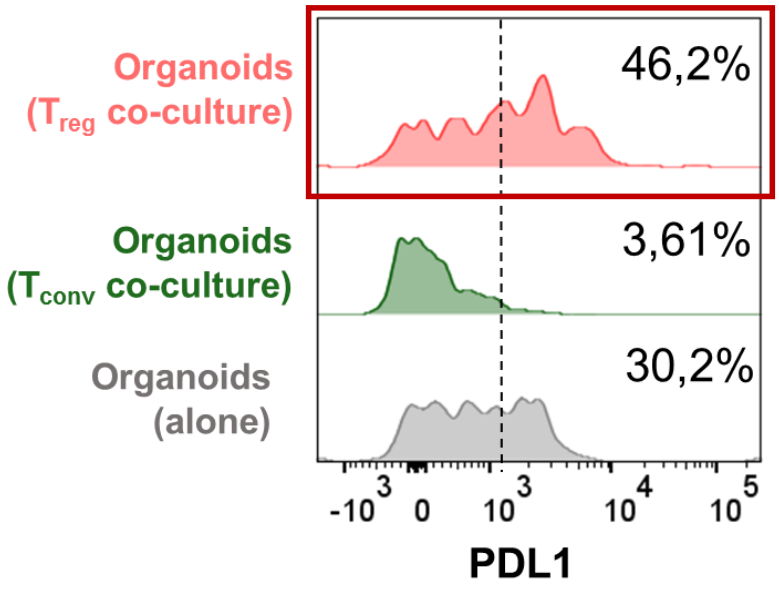
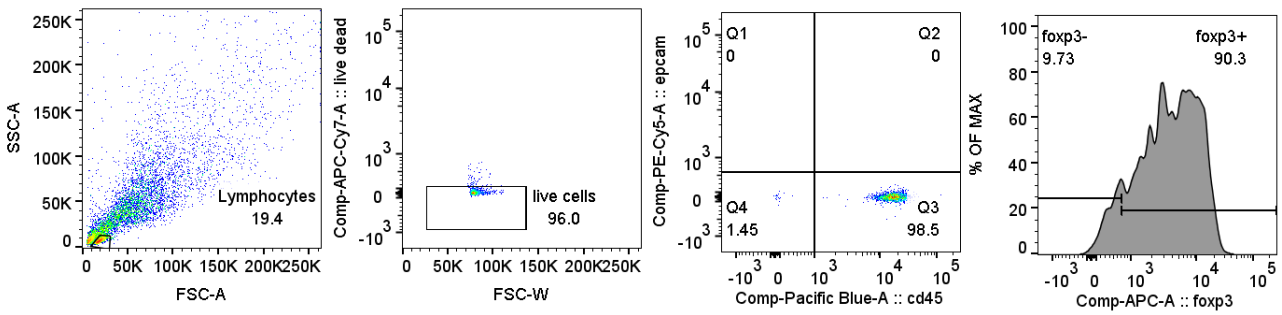


Fig. 30: Expanded TI-Treg- organoid co-culture for 7 days. Panel A: Gating strategy adopted to identify organoid cells (selected as $CD45^{-}EPCAM^{+}$). Panel B: PDL1 expression on organoids in co-culture with Tregs (red), with Tconv (green) and alone (grey).

In order to rule out that our observations were due to a possible "alloreactivity" caused by the use of a heterologous system, we decided to exploit a matched co-culture model, using expanded TI-Tregs and organoids derived from the same patient. After 7 days of co-culture we observed that Tregs upregulated PDL1 expression (64%), compared those migrated out of the Matrigel (supernatant co-culture -0.65%) and to the same cells cultured without the tumoroids (0.46%) (Fig. 31, panel B), confirming that our previous observations, obtained from our experiments with unmatched co-culture systems, were not affected by the use of a heterologous system.

A



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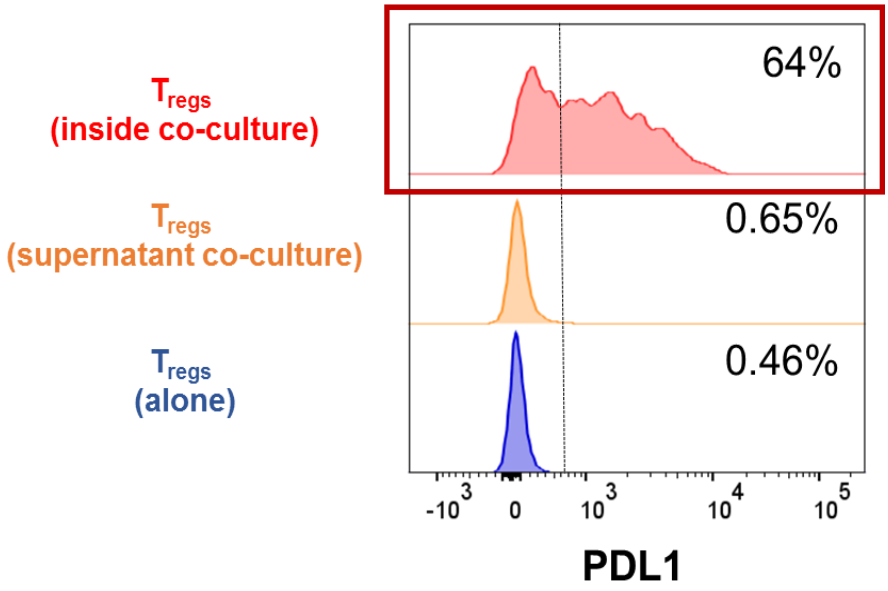

Fig. 31: Matched expanded TI-Treg- organoid co-culture for 7 days. Panel A: Gating strategy adopted to identify Tregs in co-culture model: cells were selected as live/dead negative, $CD45^{+}FOXP3^{+}$ subset. Panel B: PDL1 expression in Tregs inside co-culture (red), in Tregs collected from co-culture medium (orange) and in Tregs cultivated in absence of organoids.

In conclusion, our evidences suggested the possibility to exploit organoid model to understand the mechanisms underlain Tregs and colon cancer crosstalk favouring tumour growth and proliferation. In particular, our preliminary data on co-culture experiments showed how this system could potentially be used to investigate the mutual influence between tumour and Treg cells on determining a specific phenotype. Further analysis (in particular scRNA-seq analysis) on co-culture model could shed light on the mechanisms underlain TI-Treg specific gene signature acquisition. To this end, we are also setting up co-culture experiments at different time points to be analysed through Symphony FACS instrument, which will allow us a multi-parametric analysis of TI-Treg signature gene expression on co-cultivated expanded Tregs, to detect the potential acquisition of most Treg tumoral markers at once.

DISCUSSION

The aim of my PhD thesis consists in proposing organoid model as a powerful system to study the interaction between tumour and Tregs. To this end, we generated a biobank of 20 and 28 human-normal and tumoral colon- derived organoid lines, respectively, from tumoral biopsies and the adjacent normal mucosa of patients affected by colorectal cancer. Remarkably, these organoid lines can be propagated, frozen and defrosted like any tumour cell line and morphologically recapitulate the cellular composition and architecture of colon primary tissue. Importantly, we obtained a balanced PDO library, representative of all CRC molecular subtypes, giving the opportunity to follow divergent molecular aspects and evolutions of these CRC subsets *in vitro*, features that are difficult to track with available 2D culture systems.

Moreover, the transcriptomic analysis on our organoid lines unveiled their transcriptional stability across passages and the maintenance of the inter-individual heterogeneity, offering the opportunity to exploit each PDO as an "avatar *in vitro*" of every patient. This interesting aspect would potentially allow to predict, for example, patient-specific tumour progression or drug responses, using a system able to recapitulate *in vitro* the extreme tumoral complexity, thus providing the possibility to develop new personalized therapies neoplasia dependent.

Considering the importance that epigenetic is acquiring in cancer field and to completely fullfill the molecular characterization of our 3D model, we performed ChIP-seq analysis on the main histones modifications, in our CRC PDOs library. To this extent, we combined more than 80 ChIP-seq to reconstruct the *de novo* chromatin state discovery (ChromHMM, Figure 13, Section - Results) of human colorectal cancer, identifying 8 different chromatin states. This reconstruction allowed us to precisely discriminate genome-wide location of promoter regions, transcription start sites, regulatory elements, elongating and repressed genomic regions, providing a global scenario of the epigenetic landscape of the human CRC. Moreover, this kind of analysis can distinguish functional elements from only open chromatin regions, representing a great advantage comparing to other "omics" epigenetic technologies (ATAC-seq, DNase-seq, etc), and an innovative approach to investigate epigenetic processes leading to CRC development and progression. Indeed, most of the knowledge in this field has been acquired thanks to the use of 2D cell lines or from analysis performed on primary tissue from CRC patients (Akhtar-Zaidi B. et al., 2012; Cohen AJ. et al., 2017; Jäger R. et al., 2015; Liao Q. et al., 2016). Organoids, arising halfway between the 2D system and the in *vivo* models and considering their cellular complexity compared to common cell lines, could offer a powerful deepening of epigenetic processes underlain CRC onset and progression, nowadays not fully understood yet.

Another field in which the organoid model could offer great opportunity to better reflect the "in vivo" situation is that of co-culture systems. Accordingly, in the last year several groups focused their interest in exploiting the organoid model to study the crosstalk between cancer cells and the components of tumour microenvironment, with particular attention to stromal and immune cells (Dijkstra K.K. et al., 2018; Neal JT. et al., 2018; Tsai S. et al., 2018). In this context, considering the pivotal role of TI-Tregs in hampering immune surveillance and the peculiar gene signature correlated to poor patient prognosis that these cells acquire (De Simone et al., 2016; Plitas et al., 2016), we decided to use PDOs as a suitable platform to shed new light on the relationship between Tregs and CRC.

Several important biological questions in this topic are still unanswered: how Tregs acquire tumoral gene signature? How these T cells affect tumor growth and maintenance? Can tumoroid Tregs co-culture system recapitulate tumor Tregs gene expression profile?

Considering that tumor isolated and "in vitro" expanded Tregs lose their tumoral phenotype (Figure 1, Section Results) and trying to reply to these important questions, we set up a co-culture system between PDO and human Tregs. In doing that, we firstly demonstrated the feasibility of the system and Tregs viability, survival and maintenance of their classical phenotype, in organoid culture conditions. We also evaluated Tregs ability to migrate into 3D organoid structure, overcoming matrigel droplet (which mimics extracellular matrix) and then creeping into the 3D architecture of organoids, recapitulating the same behaviour they have when they are recruited at the tumour site.

Regarding the *in vitro* restoration of tumor Tregs signature in co-culture system, we obtained an interesting result in our preliminary experiments. Indeed, we observed the upregulation of PDL1 (one of TI-Tregs signature gene - De Simone et al., 2016), in Tregs but not in Tconv co-cultured with PDOs. Notably, only the fraction of Tregs in contact with the organoids (those recovered from matrigel drop) showed this upregulation, further supporting the requirement of direct crosstalk between these cells and the tumor ones. Moreover, a similar induction of PDL1 expression was also detected on the tumoroids co-cultured with Tregs but not Tconv, still reinforcing the cooperation between these specific immune cells and the tumor. In addition, our results acquire a greater relevance if we consider that the co-culture experiments were performed with *in vitro* expanded human TI-Tregs, which, as we showed (Figure 1, Results) lose their peculiar phenotype in culture. These preliminary experiments demonstrate for the first time the feasibility of this system to revert *in vitro* cultured Ti-Tregs to an *in vivo* phenotype, giving the opportunity to study the behaviour of this peculiar Treg subset with an innovative approach, previously unsuccessful with common 2D culture systems.

Applying this co-culture system to Tregs coming from normal adjacent counterpart or peripheral blood, could be helpful to understand the source of TI-Tregs. Although some published works based on TCR studies indicate a possible derivation from peripheral blood (Ahmadzadeh M. et al., 2019; Hindley JP. et al., 2011; Zhou G. et al., 2007), there is still an open debate about this topic. In addition, due to the lack of stromal cellular components (as shown in Figure 8, Section-Results) Tregs-PDO co-culture model could potentially suggest any processes or molecules involved in the lymphocyte recall at the tumour site, intrinsically promoted by neoplastic cells. Moreover, the generation of our balanced PDO biobank, representative of all CRC molecular subtypes, could offer the opportunity to potentially detect different mechanisms for Tregs recruitment, depending on the CRC molecular subgroup they infiltrate. Accordingly, these experiments could partially explain the conflicting evidences associated to good or poor prognosis of patients with high numbers of Tregs recruited at tumour site, likely playing different roles in promoting or preventing neoplastic growth.

Another interesting aspect of PDO-Tregs co-culture system is represented by the influence of TI-Tregs on tumour outcome. In line with this, our preliminary co-culture experiments showed that expanded TI-Tregs enhanced PDL1 expression on tumoroid cells, suggesting that Tregs can affect tumoroid cells phenotype, likely providing a selective advantage during tumour progression and an escape from immune surveillance.

Accordingly, recent studies demonstrated that Tregs can communicate with specific cell types population, in particular with stem cell compartment of different anatomic districts (Cipolletta D. et al., 2012; Burzyn D. et al., 2013; Arpaia N. et al., 2015). Intriguingly, these works highlighted the existence of non-lymphoid tissue Tregs exhibiting a peculiar phenotype and transcriptional profile, mainly involved in glucose metabolism, tissue repair and muscle regeneration, far from their well-established suppressive functions (Cipolletta D. et al., 2012; Burzyn D. et al., 2013; Arpaia N. et al., 2015). In particular, an interesting crosstalk between Tregs and stem cell niches was observed in all of these contexts, where Tregs were found to play a relevant role in wound healing and tissue homeostasis. In line with this, Biton M. and colleagues (2018) recently exploited murine intestinal organoids to study the interaction between different T-cell subsets and intestinal stem cell niche. Importantly, they observed that pro-inflammatory signals and organoid co-culture with Th cells promoted differentiation, while Tregs and their released cytokines reduced it. This work demonstrated the great potentiality of using T cell-organoid co-culture system to investigate complex cellular interactions, *in vitro* recapitulating the cellular composition, architecture and molecular features of the primary tissue.

Intriguingly, similar to what happens in physiological conditions, TI-Tregs could communicate with stem cells of tumoral mass (cancer stem cells - CSCs), sustaining their survival and exploiting the

same mechanisms used to trigger tissue repair. Thus, it is likely that, in tumoral context, Tregs can play a dual pro-tumoral role: hampering anti-tumoral immune response on one hand and supporting CSCs compartment on the other. The availability of technologies like single-cell RNA-seq (scRNA-seq) will provide us more information and identification of possible neoplastic subpopulations which can benefit from Tregs presence in tumoral milieu, giving them a selective advantage in cancer progression.

In conclusion, the exploitation of TI-Treg-PDO co-culture could really represent a powerful tool to shed light on all these significant aspects and, the deep understanding of the interplay between these two components will give us the opportunity to interfere with this crosstalk and design a peculiar anticancer therapy targeting TI-Tregs in a personalized manner.

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