Università degli Studi di Palermo

International PhD Program In Immunopharmacology

Dipartimento di Discipline Chirurgiche
Oncologiche e Stomatologiche

BMP7v induces cancer stem cells differentiation and enhances chemotherapy response in colorectal cancer

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Cycle XXIV-SSD MED/04- A.A. 2010-2013

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ABSTRACT

Cancer stem cells (CSCs), characterized by high levels of ATP-binding cassette, anti-apoptotic molecules, active DNA-repair and slow replication capacities, surviving to conventional anti-cancer therapies, able to eradicate only the highly proliferating tumor cells, represent the elective target for new therapies. Colorectal CSCs (CR-CSCs) represent a powerful tool for preclinical validation of target therapies. In particular the elucidation of the mechanisms that govern stem cell survival and differentiation appears very essential for the identification of new molecular targets in cancer therapy. Among the molecules that govern these processes there are the Bone Morphogenetic Proteins (BMPs), members of the TGF-b superfamily. Here we propose that a BMP7 variant (BMP7v) have an important antitumoral and anti angiogenetic effect on CR-CSCs inducing a differentiation program and making these cells more sensitive to conventional chemotherapy drugs. BMP7v *in vitro* administration, activates the BMP signaling pathway in CR-CSCs, reducing the percentage of stem cell marker expression and enhancing epithelial colonic differentiation marker expression. BMP7v reduces self-renewal of CR-CSCs inducing their exit from quiescence and, reducing their typical mesenchymal trait, decreases their invasive and endothelial cord formation capacity. *In vivo*, BMP7v decreases tumor growth and stem cell marker expression, enhancing differentiation compared with control mice and in combination with CRC standard chemotherapy reduces tumor growth, inducing a differentiative and antiproliferative effect, associated with a strong anti-angiogenic role. In addition, BMP7v as second-line of treatment also showed a significant anti-tumor activity in xenografts refractory to chemotherapy. Our data support the use of BMP7v as differentiative agent in combination with cytotoxic drugs for the treatment of CRC, and the use of BMP7v provides a potentially powerful and novel approach for the treatment of tumor disease.

INTRODUCTION

Colorectal cancer and stem cell theory

The colorectal cancer (CRC) is one of the most common cancers in Western countries. It represent the third form of cancer for frequency and the second leading cause of cancer death due to the resistance to current clinical therapies in the world. (1).

Nowadays, the most important approaches, for the managment of this complex pathology remain the prevention and the early diagnosis, although a large numbers of patients after surgery and adjuvant therapy still develop recurrences and metastasis, due to the acquisition of resistance to conventional therapy, such as chemo- and radio-therapy (2).

The colorectal cancer represent a classic example of a multistep pathogenesis, characterized by the acquisition of aberrant function of a proto-oncogene or loss of function of a tumor suppressor gene (3). Many studies showed that at least 4–5 mutations are necessary to generate an invasive carcinoma (4). Some of these mutations seem to follow a constant trend, within the same sequences, and they are shared by many patients affected by colon-rectal cancer, unlike, other different mutations are individuals and therefore necessary to determine the final phenotype of disease (5). Many evidences on colon cancer mutations derived from studies on hereditary forms, representing 5% to 10% of all colon cancer cases. In particular, Familial adenomatous polyposis (FAP) is an autosomal dominant CRC syndrome caused by a mutation in the APC (adenomatous polyposis coli) gene which characterizes multiple CRC (6-8).

In 1990, Fearon and Volgestein proposed a genetic model of colorectal carcinogenesis based on the accumulation of genetic mutations that occur in sequence, defining a particular staging of tumor development (9).

The main event which characterizes the onset of CRC is represented by mutations-inactivating the gene APC that lead to hyperproliferation of the normal intestinal epithelium with the formation of adenomas class I (early adenoma). In fact, the APC mutations are reported as the initiating gatekeeper that regulate positively the Wnt pathway in patients with FAP (10). The key role of APC

protein is represented by the modulation the cytoplasmic levels of beta-catenin, a protein that migrating into the nucleus activates the transcription of genes involved in the regulation of proliferation, differentiation, migration and apoptosis (11).

The progression from early adenoma towards the stage of intermediate adenoma is related to the acquisition of B-RAF and K-RAS mutations. These mutations, mutually exclusive, determine the constitutive activation of the Ras-Raf-MAPK protein signaling pathway.

The loss of heterozygosis involving the chromosome 18q, the mutations in SMAD4 (*Small Mother against DPP homolog 4*), CDC4 (C*ell Division Cycle 4*) and DCC (*Delected in Colorectal Cancer*) or alternatively mismatch repair deficiency, P53, Bax and IGFR2 (insulin-like growth factor receptor 2) are involved in the transition to advanced adenoma (adenoma late) (4, 12, 13).

Finally, a key event in the transition from advanced adenoma to carcinoma is represented by acquisition of mutations in one of the most important tumor suppressor genes, TP53. It is a powerful transcription factor, able to maintain the integrity of the genome through the regulation of the expression of more than 300 genes involved in various cellular processes such as apoptosis, cell cycle arrest, senescence and DNA repair.

The tumor suppressor gene TP53 is mutated in about 95 % of human cancers of various origins. Cancer cells that are non-functional TP53 have a substantial advantage in growth, since they can proliferate actively, even under conditions of stress or damage to the genome, developing resistance to apoptosis.

Finally, the accumulation of additional mutations, many of which are still not known, induce the transformation in metastatic carcinoma (14). Fig.1.

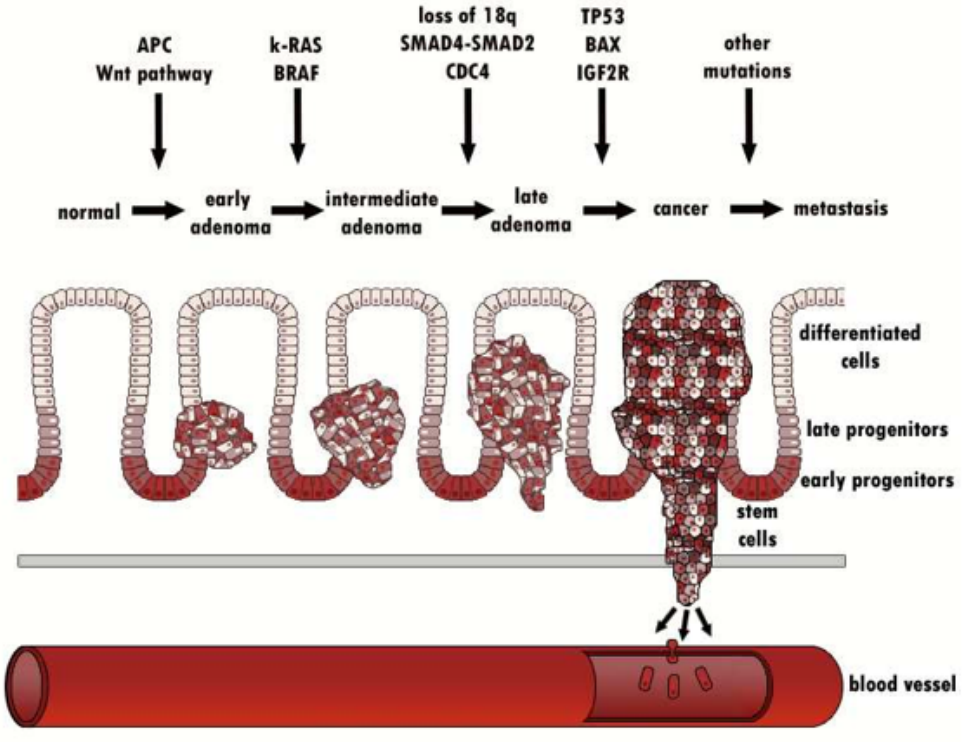


Fig.1. Schematic representation of colorectal cancer multistep progression. (Di Franco S. et al., Encyclopedia of Molecular Cell Biology and Molecular Medicine, Wiley.)

In the last decades the tumor biology has revolutionized the old view of tumourigenesis. CRC, as the other tumors, have long been consider as an exclusively genetic disorder. Nowadays, several studies showed that tumors are constitute by a highly heterogeneous population of tumor cells which differ in morphology, marker expression, proliferation capacity and tumorigenicity. To better describe the role of the different malignant cells within the same tumor, and to explain this morphological, proliferative and functional heterogeneity, two models have been proposed: the stochastic and hierarchical models.

The first model, described by Nowell in 1976 (15) proposed that all cells within a tumor are biologically homogenous and able to regenerate the tumor (16). This model of tumorigenesis, in fact, describes the tumor formation as a process multistep due to the sequential accumulation of mutations in oncogenes and tumor suppressor genes. Accordingly, all cells within the same tumor are able to initiate a new tumors, but this theory does not consider the high cellular heterogeneity, the chemoresistance, the minimal residual disease and the tumor recurrence. In sum, tumors consist of a heterogeneous cell population that, acquiring new mutations, undergoes uncontrolled proliferation and invasivity.

Otherwise, the hierarchical model, considering all the factors intrinsic and extrinsic involved in defining cell behavior, such as, genomic instability, levels of transcription factors, signalling pathways, microenvironment and immune response, is based on the analysis of the high cell heterogeneity within the tumor in terms of features, surface markers expression, proliferation kinetics and tumor initiation capacity (17).

This model suggest that only a subset of tumor cells within the tumor mass, called Cancer Initiating Cells or Cancer Stem Cells (CSCs), can initiate and sustain tumor growth (18). These cells possess the tumorigenic and self-renewal capacity, and the ability to differentiate in non-self renewing cells, that acquiring proliferative capacity, constitute the tumor bulk (19). Fig.2.

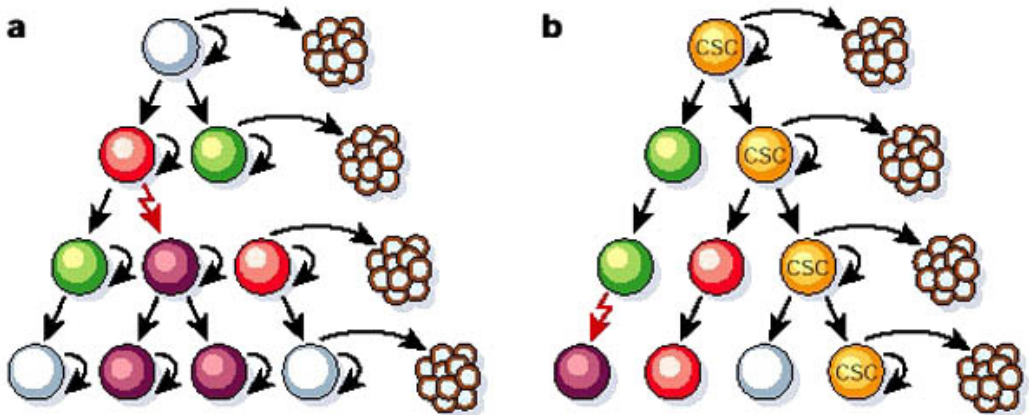


Fig.2. Two general models of heterogeneity in solid cancer cells: a) stochastic and b) hierarchical models. (Reya T. et al. Stem cells, cancer, and cancer stem cells, Nature 2001)

In the last years, novel insights in cancer research have suggested that the capacity to initiate and sustain tumor growth is a unique characteristic of this small subset of cancer cells with stemness properties within the tumor mass, called "cancer stem cells" (CSCs) or "tumor-initiating cells", that have the capacity to propagate the tumor upon transplantation into immuno-compromised mice (19).

CSCs are defined by their stem cell-like features that share with the normal stem cells that are characterized by self-renewal and pluripotent differentiation capacity. These cells are responsible to generate, through several cycles of division, progenitor cells which give rise to non-tumorigenic differentiated population that represent most part of the tumour mass. CSCs could derive either from self-renewing normal stem cells (SCs) that acquire epigenetic and genetic

changes required for tumorigenicity or from proliferative progenitor cells (PCs) that reprogramming themself acquire the self-renewal potential capacity (20).

Emerging evidences suggest that CSCs isolated from a variety of tumors types retaining the tumorigenic capacity are responsible for the propagation, relapse and metastatic dissemination. CSCs can explain the phenomenon of the tumour chemoresistance in which several mutations confer to these cells drug-resistance, altered cell cycle checkpoints and impaired apoptosis machinery. For all these reasons, CSCs survive to conventional treatments giving often rise to minimal residual disease (MRD). Therefore to better understand the mechanisms that maintains stemness features and the subsequent characterization of CSCs could be crucial to develop new most appropriate anti tumor strategy approaches.

Common signaling pathways, including Wnt, Notch and Sonic Hedgehog are involved in the regulation of normal and cancer stem cell. Many evidences underlining the importance of these cellular signalling showed as their deregulation plays an key role in the tumor development (21). Accordingly, several studies suggest the importance of self renewal pathways activation for CSCs maintainance (22).

The CSCs theory has changed the conventional therapeutic approches, suggesting an alternative strategy targeted to these cellular subset. The CSCs are characterized by high resistance to conventional chemotherapeutic drugs that kill the rapidly proliferating cells sparing the slow dividing cells, through a particular upregulation of ATP-binding cassette transporters, active DNA-repair capaciy and overespression of antiapoptotic molecules (23, 24).

Dick and collegues were the first to isolate and characterize CSCs from acute myeloid leukemia (AML) in blood and bone marrow. In particular they isolated a sub-population of $CD34^{+}CD38^{-}$ from patients affected by AML, and they demonstrated that only this cellular subset was able to form colonies *in vitro* experiments. They also analized that only this subset was able to reproduce the parental tumor phenotype, when inoculated into immunodeficient mice (25, 26).

Using a similar procedure many research groups identified a large number of tumor stem cell from a different solid tumor type. In particular, the first CSCs obtained from a solid tumor were a cellular subset $CD44^{+}CD24^{-}$ isolated from breast cancer by Al Hajj et coll. (16). Subsequently, were isolate several different

CSCs tumoral type: brain (27), colon (28-30), head and neck (31), pancreas (32, 33), melanoma (34), mesenchymal (35), hepatic (36), lung (37), prostate (38), and ovarian (39) tumors.

Colon crypts and stem cells

Although the SCs and CSCs characterization has been long studied, several molecules have been identified as a putative stemness markers, up to now, none of the markers studied seems to be exhaustive. Scientific evidences underline the importance to use different combinations of these markers in order to obtain a cell population enriched in stem cells.

The adult intestinal epithelium presents a particular structure ordered into crypts and villi, organized with a hierarchical organization, composed by three different cell types: the colonocytes or columnar cell, the mucin-secreting goblet cells and the endocrine cells, originated from a colonic stem cell. These stem cell, located at the base of the crypt are surrounded by mesenchymal cells that form the stem niche, in which the stem cell, displaying stemness features, can generate through asymmetric division, a cell identical to itself , and a transit cell. The transit cell (rapidly dividing cells) proliferating and differentiating, migrates along the crypt, representing all the intestinal lineage (40-45). According to this theory these stem cells are responsible for the high turn over rate of the colonic epithelial cells (46-48).

This particular and complex structure of the colon crypts has made particular difficult the studies about the mechanisms of crypt formation from a single stem cell. The first study regarding the stem cell position in the colon was conducted by Chang et al. using ^{3}H-thymidine injection, and recently it was confirmed using bromodeoxyuridine DNA-labeling dye (49, 50).

Two different models have been proposed regarding the positioning of the stem cells: the “stem cell zone” model and the “ +4 position” model. According to the first model, the colon stem cell reside at the very bottom of the crypts. Unlike, the second model describes that the stem cells are located at the +4 position above the Paneth cells at the base of the crypts (51).

Although the absence of a specific colonic stem cell markers makes their

identification and positioning very difficult, the colonic stem cell can be characterized by two main features: self-renewal and differentiation capacity. Stem cells may undergo asymmetric division, but they can also generate two identical stem cells via symmetric division that acquiring a diffentiated phenotype regenerate the colon tissue. Then, in the first case, it is possible to obtain a lineage expansion, but in the other case, a lineage extinction, because the differentiated progeny undergo to senescence.

Accordingly with the CSCs theory it is widely accepted that the stem cells are responsible for the origine of the cancer. Their slow division cycle, allow them to accumulate several mutations over time up to define them CSCs (52-54).

Different molecules have been proposed as a stemness markers: Musashi 1(Msi1), EphB receptors, Bmi 1, Lgr5.

Msi1, an RNA binding protein, widely studied in Drosophila Melanogaster, seems to be involved in the asymmetric division that regulate the neural development, also in mammals. In human and murine small intestine it is located at the base of the crypts. Its silencing determines tumor growth arrest by Notch inhibition and p21 upregulation, proteins involved in stemness mainteinance (55-57).

EphB2 receptor is tyrosine kinase receptor, belonging to the family of Wnt target gene, it is expressed in a decresing gradient from the crypt base toward the differentiated cell compartment (58). This expression along the crypts seems regulate the migration and proliferation of intestinal epithelium; mutants in their ligands, or mutant forms of these receptors involve in intestinal compartimentalization defects. Underlining their important role in the intestinal positioning of the different cell types along the crypts. (59).

Bmi1, a repressor of Polycomb group, is involved in hematopoietic, breast and neural self renewal. In the small intestine it is expressed near to the bottom of the crypts (60, 61).

Lgr5 is a G protein coupled receptor, belonging to the family of Wnt target gene. Its expression in a single cells is able to regenerate a crypt-like structure *in vitro* constituted by all cell type of colonic epithelium (62). This and other markers as have been associated with CSCs phenotype.

CR-CSCs identification, isolation and expansion

The existence of colorectal CSCs (CR-CSCs), have been showed, for the first time, thanks to the detection of a new stemness markers, the transmembrane glycoprotein, CD133. This surface expressed polypeptide is associated with self renewal and tumor initiating cells, this protein was first associated as a marker for hematopoietic stem cells and progenitor cells, and successively used in other tumor type: (63): brain (27), prostate, hepatocellular and colon tumors (28-30, 64, 65).

Recently two indipendent groups have revealed that only $CD133^+$ subset of tumor cells within a colon carcinoma, is able to initiate a tumor outgrowth (28-30).

Accordingly, it has been showed that a small group of $CD133^+$ cells is able to serially reproduce the original human tumor phenotype, rather than an high number of $CD133^-$ cells that fail to generate xenograft tumors in immuno-compromised mice. In line with these data, only the tumorigenic $CD133^+$ cells population generated crypt-like structures *in vitro* under differentiation condition on matrigel (65). In addiction, these cells during differentiation *in vitro* and *in vivo* acquire a typical epithelial colonic marker, CK20, reducing at the same time the CD133 stem cell marker.

Accordingly, many clinical reports suggest CD133 as an indipendent prognostic marker and its combination with a nuclear localization of beta catenin is associated with a reduced patients survival (66-69).

In sum, several research groups demonstrated that only the $CD133^+$ cellular sub-population, within a colon carcinoma, is able to initiate and sustain tumor growth (68-70).

Moreover, mantaining the $CD133^+$ cells with the same conditions of neurospheres, these cells were expansed for long term without loss their ability to reproduce human original tumor phenotype, underlining the self renewal and tumor initiation capacity of these sub population (28, 29).

O'Brien and collegues, in order to evaluate whether all the $CD133^+$ cells are CSCs or whether these subpopulation contains also more differentiated progenitors, through serial dilution assays, showed that the $CD133^+$ subpopulation not only contains cancer initiating cells.

Accordingly, recent studies analyzed a new sub-group, contained in $CD133^+$ cells, the $CD44^{+/}$Epcam High/$CD166^+$ stem like cells. Dalerba et al., (30) demonstrated that the $CD44^+$/Epcam high cell subpopulation injected in NOD-SCID mice, is able to reproduce a tumor xenograft phenotypically similar to parental one.

In a similar way, Du's et al., (70) showed that also CD44 could be considered a putative marker, able to discriminate a subpopulation capable to growth *in vitro* as spheres and *in vivo* producing xenografts, resembling the parental human tumor.

The role of CD133 as a marker of stem cell has long been debated, in particular, after the Shmelkov et al. publication, in which it was shown that CD133 is expressed ubiquitously in both undifferentiated and mature colonic cells (71). While the lack of CD133 expression is only found in the stromal and inflammatory cell compartment.

Differently, Zhu et al. analizing the role of Prominin1, the mouse analogue of CD133, in adult colon tissue, showed that the $Prominin1^+$ cells marking the adult colon stem cells, represent the target of tumoral trasformation. (72).

On the bases of these conflicting data, it became clear that the use of a single marker is not adequate for a correct CSCs identification and isolation, to identify the subpopulation of CSCs, it would be more appropriate to use a panel of markers and to standardize protocols that can validate the use of a new marker. For all these reasons, several surface molecules have been proposed to mark colon CSCs, such as CD133, LGR5, CD44 and CD166. (73, 28-30).

Recently, it has been demostrated that undifferentiated tumorigenic CRC cells could be expanded as tumor spheres *in vitro* using a serum-free medium containing EGF and basic FGF (74). The tumor spheres, infact, contain an heterogeneous cell population expressing a variable percentage of CD133, CD166, CD44, CD29, CD24 and nuclear beta-catenin. More recently ALDH1, a detoxifying enzyme, has been proposed as a specific marker able to identify, isolate and track human normal and CSCs during CRC development (75, 76). This marker is expressed by the $CD44^+$ or $CD133^+$ cells, located at the base of normal crypts. After sorting the ALDH1 high cells, injected subcutaneously in NOD SCID mice, generate tumor xenografts (76).

The isolation of CSCs is based essentially on two different approaches: the use of a culture medium serum-free that maintain stemness-selective conditions, originally developed for neural stem cell culture (77) or the direct selection by magnetic sorting or FACS technology for putative markers distinctively expressed by a cell subpopulation. On the bases of these procedures there is a common step in which the surgical excisions of solid tumours are processed by mechanical and/or enzymatic digestion to obtain a single-cell suspension. This freshly digest obtained is constitute by an cells heterogeneous mixture of the original tissue. In order to selectively obtain CSCs, the digest is cultured into ultra-low-adhesion flasks in a specific serum free medium, supplemented with growth factors such as basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF). These conditions allow the undifferentiated tumour cells, stem and transit amplifying cells, to survive and slowly proliferate, while the differentiated cells die through anoikis. Sphere-forming cells are maintained in culture by dissociation and re-plating as single cells, and retain the tumorigenic potential when injected into immunocompromised mice, reproducing the same morphological and antigenic features of the original tumour, data showed by histological examination of xenografts generated from spheres. This tumorigenic capacity is serially maintained, and the xenografts can be digested and the cells obtained can be indefinitely propagate under stemness conditions, maintaining the stem like features as well as the capacity to reform tumors. As previously described, a single clonogenic $CD133^+$ cell, contained in CRC spheres is able to reproduce the original tumor, including the CSC compartment and the differentiated progeny.

Alternatively, the second procedure, involves the selection of putative stem cell marker from the whole digest, by the use of monoclonal antibody directly or indirectly conjugated with magnetic beads. This sorting allow a double positive and negative selection, separating labelled and unlabelled cells, but this procedure permit only a single-marker selection. Another alternative method is represented by FACS sorting, which allows sorting of different populations with multi parameter analysis, testing contemporary several antibodies conjugated to different fluorochromes.

Moreover the sorted cells can be also cultivated in stem cell conditions and also injected into immunocompromised mice to directly test the tumorigenic capacity (74).

Intestinal Niche

Fibroblast, endothelium, inflammatory cells, cytokines and growth factors secreted by these cells, constitute the intestinal niche and are involved in CSCs maintenance at the base of colon crypts. They finely regulate the balance between self-renewal and differentiation (78-80). Indeed the pluripotency of colon SCs is strongly controlled by microenvironment that plays a crucial role ensuring a fine equilibrium between important pathways, such as PTEN-PI3K-Akt (81, 82), Bone Morphogenetic Protein (BMP) (83), Notch (84) and Sonic hedgehog (Shh) (85).

In particular the intestinal sub-epithelial myofibroblast (ISEMFs) surrounding the normal colon stem cells, regulate this balance secreting hepatocyte growth factor (HGF) for maintenance of stem cell in the intestinal niche. The presence of ISEMFs or HGF, as demonstrated by Vermulen et al., restore the stem cell phenotype, inducing a cellular de-differentiation (80).

The colon crypt unit represented in figure 3, shows a particular cellular distribution in which at the basis of the crypt are located the mesenchymal cells (ISEMFs) and their secreted factors responsible for the stem cell niche maintenance, while along the villux apex, where are located several Wnt inhibitors, in order to reduce the stemness features it is possible to identify a progressive cellular differentiation. The pathways mentioned above, are involved in self renewal and are de-regulated both in normal and CSCs (21, 22).

One of the most studied pathway envolved in CSCs progression is represented by the Wnt signalling.

The Wnt signalling in the basis of the crypts promotes nuclear beta catenin accumulation, which activate the transcription of several genes involved in cell cycle regulation and proliferation. Beta catenin also induces the expression of EphB1-2, which regulate stemness, cell migration and differentiation (86-88).

The distribution of Wnt pathway members, results particularly regulated. Wnt ligands are predominantly expressed at the basis of the crypts and are reduced along the crypt where more differentiated cells reside, and where are most express the Wnt inhibitors factors (88-90).

In addiction to Wnt pathway, BMP, Notch and Shh pathways regulate the niche homeostasis.

BMP proteins are TGF-beta superfamily members that through binding to receptors BMPR1A, BMPR1B and BMPR2 can trigger different biological processes in CSCs (91).

The activation of this pathway promotes the phosphorylation of Smad1, 5 and 8 that in association with SMAD 4 translocate into the nucleus and in cooperation with other transcriptor factors regulate the target genes expression (92, 93).

Recent data have demonstrated that the BMPs promote terminal differentiation and apoptosis (94). Kosinsky et al., analyzed the distribution of the different factors along the crypt: the cells at the apex of the crypt express high levels of BMPs, while at the basis of the crypt, their levels are reduced, but the levels of BMPs antagonist, produced by myofibroblasts, contribute to the maintenance of stemness (95, 83).

Notch pathway is envolved in intestinal SCs fate and includes four different trans-membrane receptors (Notch1 to Notch4). The binding of five different ligands (Jagged-1, -2, Delta-like 1, 2 and 4) induces the release of the Notch intracellular domain that translocates into the nucleus where it forms a complex with DNA-binding proteins activating the target genes transcription involved in epithelial cell fate determination (96). Recent reports showed its key role in intestinal homeostasis and neoplastic transformation. Moreover, the expression of Notch intracellular domain blocks cell differentiation, inducing expansion of immature progenitors (97, 84). Defects in Notch pathway were observed in colon cancer stem cells (CCSCs), using an antibody anti DLL4 (an important Notch pathway's component) to directly inhibit human colon cancer xenograft growth.

Finally, Sonic Hedgehog pathway regulate the gut organogenesis, binding to its receptor Patched (PTCH), allowing the release of a G-coupled protein Smoothened (Smo), which with the GLI transcriptors factors migrate into the nucleus inducing target gene activation (85)

In several cancers, such as leukemia, pancreas, stomach, prostate, breast, glioblastoma and colon cancer were found many alterations in the Hedgehog pathway (98-103).

In conclusion, the aberrant alteration of these pathways involved in self-renewal of the intestinal stem cells could be the driving force that promote colon cancer. (104)

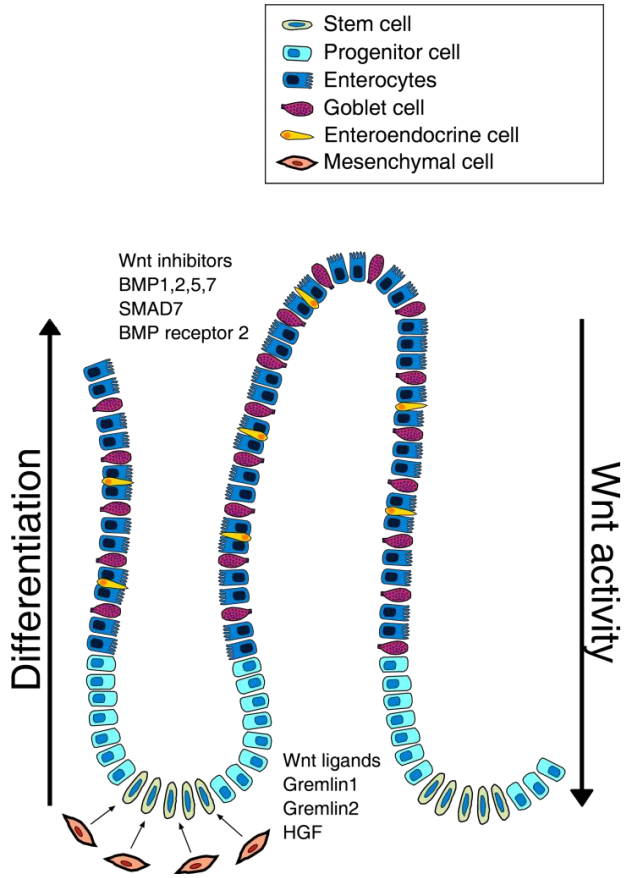


Fig.3. Graphic representation of a colon crypt. (Di Franco S. et al. Colon Cancer Stem Cells: Bench-to-Bedside-New Therapeutical Approaches in Clinical Oncology for Disease Breakdown. Cancers 2011).

Clinical implication of CR-CSCs and BMPs as alternative therapeutic strategy

The CSCs theory, in colorectal cancer, has exciting clinical implications, confirming that the therapy failure and relapses are due to the CSCs resistance. Indeed, CSCs, retaining the stemness features, such as quiescence, self-renewal ability and multidrug resistance, represent the population intrinsically refractoriness to conventional therapies developed to eradicate the rapidly dividing cells that constitute the majority of the non stem cell component within the tumor.

Nowadays, patients with metastatic CRC are treated with two useful protocols FOLFOX (Folinic acid/Fluorouracil and Oxaliplatin) and FOLFIRI (FOLFOX plus vitamin B and irinotecan). Neoadjuvant chemotherapy for these patients, is represented by anti-angiogenic drugs as the Bevacizumab, a humanized monoclonal antibody that targets the vascular endothelial growth factor (VEGF), which plays an inportant role as angiogenic factor in primary and metastatic human CRC (105, 106). Another neo-adjuvant drug is Cetuximab, also known as Erbitux, a monoclonal antibody that inhibits the epidermal growth factor receptor (EGFR), indicated for the treatment of EGFR expressing patients affected by KRAS wild-type metastatic colorectal cancer, alone or in combination with FOLFIRI (107).

The future of diagnostics and treatments of tumor disease should aim to eliminate, not only the terminally differentiated component of the tumor bulk, but, it should be focused, in particular, to the subpopulation of cancer stem cells that represent the driving force for the tumor expansion (19). In order to evaluate the CR-CSCs role in the therapy response, recurrence and metastasis, a recent work showed that the subpopulation of $CD133^{+}$ CR-CSCs results more resistant to both conventional chemotherapeutic drugs, and innovative therapies, compared to epithelial cells that constitute the majority cell population within the tumor (65, 74).

These recent findings support the idea that tumor-initiating cells are highly resistant to cytotoxic cancer therapies, underlining the importance of their role in colorectal tumors refractoriness and recurrence also many years after the "successful" treatment of primary tumour.

The CSCs contribute to the poor therapeutic sensitivity through many mechanisms, such as preferential activation of DNA damage checkpoint, high levels expression of ABC transporters and anti-apoptotic molecules, slow replication capacity and other aberrant molecular mechanisms that destroy the normal balance between proliferation and survival or cell death (23).

In addition the CSCs refractoriness is finely regulated by microenvironmental soluble molecules that are involved in many different sectors of tumor development, regulating the growth, migration, and differentiation of all cellular components into both the tumor mass and in the microenvironment.

The possibility to isolate and study CSCs represents a revolutionary approach in cancer research and these cells represent the elective target for new therapies, endowed to high and selective toxicity towards the specific tumor with reduced toxicity for normal cells.

An alternative therapeutic strategy seems to be represented by selectively target of CSCs pathways .

Recent reports have demonstrated that the autocrine production of IL-4 by cancer cells from breast, thyroid, colon, and lung acts as negative regulator of apoptosis, conferring resistance to death receptors and chemotherapy-induced cell death (108). Moreover IL-4 seem to be involved in stimulation of activated B-cell, in T-cell proliferation and the differentiation of $CD4^+$ T-cells into Th2 cells (109). The use of anti–IL-4 neutralizing antibody or IL-4 receptor α antagonist, on CR-CSCs, inhibiting IL-4 signaling transduction pathway, sensitizes these cells to chemotherapeutic agents through down-regulation of anti-apoptotic proteins, such as cFLIP, Bcl-xL, and PED. Moreover, the combined use of IL-4 antibodies plus 5-fluorouracil or oxaliplatin also reduces xenografts tumor growth (65). Concluding, recent studies have evaluated that the CR-CSCs $CD133^+$, are protected by apoptosis phenomenon through the up-regulation of IL-4 (110).

Another important approach could be represented by cancer immunotherapy. Although the cancer cells are less immunogenic than their normal counterpart, the immune system could plays a crucial role in order to effectively and safely increase antitumor responses recognizing and eliminating them (111). Although, these cells do not express MHC molecules, making tumor cells resistant to αβ T cell-mediated cytotoxicity, recently, it has been demonstrated that a subpopulation

of T cells, the γδ T cells, show potent MHC-unrestricted lytic activity versus different tumor cells *in vitro*, suggesting their potential employment in anticancer therapy. γδ T cells have been isolated and identified from tumor infiltrating lymphocytes in different cancer types.

The development of targeted therapies for colorectal cancer requires new therapeutic regiments that aims to eliminate the self-renewal compartment of tumor mass by targeting stemness features owned by CR-CSCs, making this population more sensitive to conventional drugs. This is the main goal that the differentiation therapy aims to achieve.

The therapy based on the induction of differentiation is aimed to affects the self renewal ability of CSCs, and could represent an alternative way to inhibit tumour growth.

Among the molecules that govern stem cell survival and differentiation, the Bone Morphogenetic Proteins (BMPs), a subgroup of TGF-b superfamily members, play an important role in the regulation of colon stem cell features and contribute to the network of the signals that define the intestinal stem cell niche modulating the equilibrium between proliferation and differentiation signalling pathways (112-114)

These proteins and their intracellular signaling components have been conserved in Drosophila and Caenorhabditis elegans and they were originally isolated from bone. The major contribution to their isolation and characterization was made by Sampath and Reddi (115).

BMPs are synthesized as large precursor proteins in the cytoplasm where they are proteolytically processed in a mature proteins (116). The mature BMP molecules are characterized by a cysteine knot with the seven conserved cysteine domains. The dimeric form is active as a homodimer or heterodimer with a molecular weight of about 30-38 kDa.

BMPs bind to two different transmembrane serine/threonine kinase receptors, type I (BMPRI) and type II (BMPRII). Type I receptors include activin receptor type IA (ActRIA or ALK2) and BMP receptors type IA and IB (BMPRIA or ALK3; BMPRIB or ALK6), while type II receptors are represent by BMP receptor type II (BMPRII), activin receptor type IIA and IIB (ActRIIA and ActRIIB) (117, 118). After the formation of heteromeric complexes, type II

receptor phosphorylates the type I receptor (119) and the signalling pathway is then activated through Smad and non-Smad mechanism (117, 119). In addition to the Smad pathway, indeed, the BMPs activate an alternative pathway, which includes p38 and ERK MAP kinases (120). When Smad 1,5,8 protein (R-Smad) are phyosphorylated by the type I receptors, they can interact with co-Smad (Smad4) and after translocate into the nucleus in order to initiate the transcription of BMPs response genes (121).

The Inhibitory Smads, Smad 6/7, inhibits BMP signaling (122), indeed, BMPs activation is tightly regulated by the presence or the absence of antagonists and inhibitors such as Gremlin, Chordin and Noggin (83). BMP antagonists are soluble factors that control BMPs signaling with various degrees of affinity and specifity, binding the BMPs, preventing the functional receptor/ligand interaction: twisted gastrulation (TSG), chordin and noggin and the DAN-family of inhibitors.

BMPs are involved in the earliest stage of development, and play a key role in the normal intestinal development, growth and morphogenesis. Their signalling act to mediator between epithelial and mesenchymal stroma interaction necessary for the correct intestinal homeostasis (123).

During the human and mouse intestine development, BMP7 and BMP6 were found, respectively, on the intestinal epithelium and smooth muscle cells (124, 125). Moreover, the high expression of BMP2 and BMP4 in the mesenchymal site drive the villus formation (126). In particular many reports showed that the BMPs pathway is functional in all three tissue layers of the gastro-intestinal tract, to allow the reciprocal correct interaction. Mice overexpressing the BMP-antagonist noggin, undergo to abnormal villus morphogenesis, associated with stromal and epithelial hyperplasia, ectopic crypt formation, due to low levels of BMPRIA and pSmad1/5/8 (123).

All these results suggest that human gastro-intestinal and chronic intestinal diseases, could be associated with defects in the BMPs signaling pathway (113).

Hardwick et al., have demonstrated that the mature colonocytes at the epithelial surface of normal human and mouse colon, express BMP2, the BMP receptors (Ia, Ib, II), phosphorylated Smad1 and Smad4 (112). Stroma and crypt epithelium of the adult mouse intestine show an high expression of BMPRIA and BMP2 (123,

127). While, Haramis et al showed that BMP4 is expressed in stromal cells and mesenchimal cells surrounding the crypt and glands of the intestine (127, 113).

The BMPs pathway components result highly expressed in colon tops while the BMP antagonists gremlin 1, gremlin 2 and chordin-like 1, produced by myofibroblasts and smooth muscle cells, are located in basal colon crypts.

Juvenile polyposis (JP), is an autosomal dominant hamartomatous polyposis syndrome in which germline mutations of two members of the BMPs pathway are involved. The patients, having mutations or deletions in SMAD4 and BMPR1A genes (128-130) are predisposed to upper gastrointestinal and colorectal cancer, in almost half of the cases. JP patients, with SMAD4 mutations, showed a significant prevalence of gastric polyposis (131). SMAD4, known as a tumor suppressor gene in pancreatic and colon cancer, in JP acts as a susceptibility gene, a "gatekeeper", its loss of function results in polyp formation in which an important role is played by stromal inflammatory response regulating epithelial tumorigenesis (132). Homozygous SMAD4 knockout mice, develop polyps, with thickened intestinal mucosa and loss of villus architecture showing plasma cell infiltrates into the stroma, while mice with conditional SMAD4 deletion in the intestinal epithelial cells did not develop intestinal tumors (133). Although, JP patients with a germline SMAD4 mutation, showed biallelic inactivation of SMAD4 in both the epithelium and stroma, suggesting a common clonal origin (134).

Another gastro-intestinal disease, Familial adenomatous polyposis (FAP), is an autosomal dominant syndrome, characterized by hundreads of adenomatous colorectal polyps due to a deletion in the adenomatous polyposis coli (APC) gene, localized on chromosome 5q21. APC gene, as above mentioned, is involved in ubiquitine-mediated degradation of beta catenin, regulating Wnt signalling and its functional defects leads to uncontrolled cell proliferation (135). In FAP patients, an higher expression of Wnt signaling molecules, results in BMPs signaling downregulation, differently, in intestinal homeostasis, the BMPs inhibit the effects of Wnt-pathway to regulate the intestinal stem cell proliferation and repression of polyps formation (113, 127). Many results showed that heterozygous APC mutant mice, with higher expression of the BMPs antagonist Gremlin 1 and lower BMP2 and BMP4 expression develop severe polyposis and faster rate of tumor growth (136). Accordingly, human FAP tissue specimens in

dysplatic mycroadenomas epithelium do not maintain BMP2 expression (112).

The association of these genetic disorders, with the BMPs signaling pathway, was confirmed by experiments on transgenic mice, overexpressing the BMP antagonist, noggin or mice with conditional inactivation of BMPRIA. These mice showed an increased formation of intestinal polyps (113, 127). Moreover, conditional inactivation of BMPRII in stroma, led to multiple hamartomatous polyp appearance. (137, 138).

These findings indicate that altered BMPs expression plays an important role in aberrant cellular proliferation and tumorigenesis in the human intestine. During the last decade, extensive researches were conducted to explain the factors involved in the initiation and progression of colorectal cancers in particular, BMPs and their signaling pathway have been specially studied. (139, 140).

Many different groups have demonstrated that several BMPs are suppressive for colorectal cancer cells growth (112, 141, 142). Back et al. showed that BMP2 exerts growth suppression by increasing $p21^{WAF1}$ protein levels, and not its transcription, modulating RAS-ERK pathway (143).

Moreover, the loss of BMP signaling seems to be correlated to tumor progression, indeed, the BMPs expression, is lost from late adenoma to early carcinoma.

Abnormal CSCs proliferation in CRCs, is due to an abnormal activation of Wnt signalling pathway and BMPs signalling inhibition, that promote nuclear beta-catenin accumulation through PTEN downregulation and iperactivation of PI3K-Akt pathway (113). Confirming that the BMPs pathway alteration leads to pre-cancerous lesions (138).

The TGF-β signalling inactivation plays a key role in CRC development (144), indeed, it has been reported that the loss of BMPRII and SMAD4 is frequently deleted also in sporadic CRC and that BMPs pathway is inactivated in the majority of colorectal tumors (145, 146). This is strongly supported by a transgenic mice model in which the inactivation of the BMPs pathway leads to polyps formation and up-regulation of Wnt signalling (127).

Considering BMPs' role in regulating SCs differentiation and inducing apoptosis and differentiation program, it is possible to suppose that CSCs treatment with

these molecules could inducing differentiation, make these cells more sensitive to conventional chemotherapies.

Some preliminary studies have been performed on both CSCs of glioblastoma (GBM) and CRCs.

Interestingly, it has been recently demonstrated that in human glioblastoma BMP-BMPR signalling, controls the activity of normal brain stem cells, and inhibits glioblastoma stem like cells (GSLCs) (147). It has been also demonstrated that BMP2, BMP4 and BMP7 treatment inhibits sphere forming capacity and induces differentiation of $CD133^+$ cells; reducing *in vivo* glioblastoma tumor growth (147-149).

Recently, it has been demonstrated that the treatment of CR-CSCs with BMP4 induces *in vitro* differentiation and reduces their tumorigenic potential, sensitizing these cells to conventional chemotherapic drugs reducing the tumor size and inducing complete long-term regression of colon CSC-derived xenograft tumors (94).

Given the regulatory effect of BMPs on neural stem cells, their progenitors and GSLCs, recently, Tate et al. have demonstrated that BMP7 variant (BMP7v) acts on proliferation, differentiation, angiogenesis, and *in vivo* tumorigenicity of GSLCs isolated from surgical specimens of primary GBM. In particular, BMP7v, decreases proliferation of GSLCs, inducing their differentiation into neuronal- and astrocyte cellular phenotypes, and inhibites angiogenic endothelial cord formation. These results were confirmed by *in vivo* analysis of subcutaneous or orthotopic tumor models. Their data suggest that BMP7v therapy, directed against CSCs and angiogenesis, represents a potentially powerful therapeutic option that may improve the poor outcome of conventional treatments (150).

The current therapeutics strategy target and kill differentiated tumor cells that constitute the tumor bulk, failing to affect the rare cancer stem-like cell population.

These data suggest the use BMPs, as alternative therapy, to induce the differentiation of CSCs and to make them more sensitive to conventional chemotherapy. Since the differentiated tumor cells are more sensitive to conventional cancer therapies, therapy differentiative represents another possible

therapeutic strategy to inhibit tumor growth by inducing the differentiation of CSCs, making them more susceptible to the action of chemotherapeutic agents.

The induction of CSCs differentiation provides a potentially powerful and novel approach to the treatment of cancer disease.

AIMS

The high mortality rate of colorectal cancer (CRC) is mainly due to the inefficacy of standard treatments to cure the metastatic disease. The recurrence and relapse characteristic of this kind of cancer suggest that the only one curative therapy could be represented by targeting the subpopulations of tumor cells with tumorigenic potential, the so-called, cancer stem cells (CSCs) (151). Several studies report that radio-chemotherapy, directed against differentiated cells, forming the bulk of tumor cell population, are unable to eradicate the tumorigenic and metastagenic population, without obtaining a long-term clinical remission.

Accordingly, the induction of differentiation, affecting the self-renewal ability of CSCs, represents an alternative way to inhibit tumor growth and to sensitize CSCs to conventional chemotherapy. The treatment of CSCs towards terminally differentiation represent an intriguing concept for future therapy. On the basis of these data my project proposed to investigate the role of a stable BMP7 variant (BMP7v) on CR-CSCs in order to make these cells more sensitive to conventional chemotherapy drugs and to develop a new treatment protocol easily tested in preclinical models to design a future appropriate clinical trials.

MATERIALS AND METHODS

Tissue collection, isolation and culture of cancer cells

Human CRC tissues were obtained from patients undergoing to CR resection, in accordance with the ethical standards of the institutional committee. Normal colon mucosa was obtained from the histologically uninvolved resection. Histological diagnosis was based on the morphological microscopic features of carcinoma cells, determining the histological type and grade.

Surgical specimens were intensively washed in PBS solution containing antibiotics and incubated overnight in DMEM/F12 (GIBCO) containing penicillin (500 U/ml, GIBCO), streptomycin (500 μg/ml, GIBCO) and amphotericin B (1.25 μg/ml, GIBCO) in order to avoid contaminations. Tumor tissues were mechanically and enzymatically digested. Enzymatic dissociation was performed using collagenase and hyaluronidase in DMEM for 1 hour at 37° C. Dissociated CRC cells were then cultured in presence of serum-free medium supplemented with epidermal growth factor (20 ng/mL) and basic fibroblast growth factor (10 ng/mL, both from Sigma-Aldrich, St. Louis, MO 63103, USA) in non-adherent conditions, in ultralow adhesion flasks (Corning, Lowell, MA), to promote the growth of CSCs as spheres. These culture conditions allow the selection and propagation of tumour spheres, containing immature tumour cells, while non malignant or differentiated cells are negatively selected. To achieve *in vitro* differentiation of CSCs, dissociated sphere cells were cultured in DMEM-high glucose supplemented with 10% FBS in adherent conditions. These cells were conventionally indicated as sphere-derived adherent cells (SDACs). All cell cultures were carried out at 37°C in a 5% CO2 humidified incubator. Magnetic cell separation was performed on tumour cell populations obtained from enzymatic dissociation of CRC specimens using microbeads conjugated with CD133/1 (AC133, mouse IgG1, cell isolation kit, Miltenyi). After magnetic sorting, viability was assessed using trypan blue exclusion. Quality of sorting was verified by flow cytometry with an antibody against CD133/2 (293C3-PE, mouse IgG1, Miltenyi) on both $CD133^+$ and $CD133^-$ depleted cell population.

Cell death was evaluated by orange acridine/ethidium bromide staining or by CellTiter Glo Assay Kit (Promega) accordingly to manufacturer's instruction.

BMP7v used for this work was produced by Eli Lilly and Company. The pharmaceutical development of BMP7v represented the major limitation with its suboptimal solubility at neutral pH. Mutations into the N terminus of the BMP7v prodomain were introduced to enhance the cleavage of prodomain and mature domain. Five point mutations were addicted onto the surface of the mature domain, through a random mutagenesis approach, to create a molecule that retained the same signaling properties of wild-type BMP7 but had greater expression and enhanced biophysical properties such as solubility and stability. BMP7v material can be made available to researchers upon request to the Lilly authors (152).

In order to detect the proportion of differentiated and undifferentiated cells, dissociated spheres were cultured in stem cell medium in presence of BMP7v (100ng/ml) up to 18 days. At different time points, the adherent cells were harvested with trypsin and mixed with floating cells. The cell mixture was then cytospun and stained for CK20 and CD133.

To evaluate BMP7v role in differentiation *in vitro*, the spheres were dissociated into single cells and cultured in the presence of BMP7v (100ng/ml) for 48 hours or 90 minutes in order to evaluate the p-Smad 1, 5, 8 nuclear traslocation.

Histochemistry and Immunohistochemistry /Immunofluorescence

Histochemical and immunohistochemical/immunofluorescence analyses were performed on 5 μm paraffin-embedded sections of human normal colon and CRC tissues or subcutaneous tumor xenografts and cytospuns of freshly sorted cells and spheres cells exposed to BMP7v

The following antibodies were used: BMP7 (164311, mouse IgG2B; R&D system), CD133 (AC133, mouse IgGb; Miltenyi), BMPRIA, (87933, mouse IgG2b; R&D Systems), BMPR1B (88614, mouse IgG2a; R&D Systems), BMPR2 (73805, mouse IgG2b; R&D Systems), pSmad 1,5,8 (rabbit polyclonal; CST), cytokeratin 20 (Ks20.8, mouse IgGa; Dako Cytomation), p21(#2947, Rabbit IgG; CST), E-cadherin (rabbit polyclonal; CST), Vimentin (#39325, Rabbit; CST), Beta-catenin (H102, rabbit polyclonal; Santa Cruz Biotechnology), CD166 (MOG/07, Mouse Monoclonal Antibody; Leica), Lgr5 (RB 14211, Rabbit Ig; ABGENT) Ki67 (MIB-1, mouse IgG1; Dako Cytomation), CD31 (clone JC70A,

mouse IgG1Kappa; Dako ytomation), VEGFR2 (goat IgG; R&D System) or isotype-matched controls at appropriate dilutions.

For immunohistochemistry (IHC) the dewaxed slides were heated for 1 min at 450 W and 5 min at 100 W in a microwave oven in 0.1M citrate buffer pH 6.0 or pH 9.0 only for KI67 staining. For cytoplasmatic epitopes detection, samples were permeabilized with 0.1% TritonX-100 in PBS for 10 min sections and after the slides were incubated with Tris-buffered saline (TBS) containing 10% AB human serum to block unspecific binding. After elimination of excess serum the sections were exposed overnight at 4°C to specific Abs against BMP7, CD133, CD166, Beta-catenin, Lgr5, Ki67, CK20, CD31, VEGFR2 or isotype-matched controls at appropriate dilutions. Following exposure to primary Abs, sections were treated with biotinylated anti-mouse- rabbit and anti goat immunoglobulins, washed in PBS and then incubated with streptavidin peroxidase (LSAB 2 Kit; Dako Cytomation or Vectastain kit; Vector). Stainings were detected using 3-amino-9-ethylcarbazole (AEC) chromogen. Counterstain of nuclei was performed using aqueous hematoxylin (Sigma).

For hematoxylin and eosin (H&E) staining, dewaxed sections were stained in Hematoxylin (Sigma) for 1 minutes, washed in water and then exposed for 30 seconds to eosin (Sigma). Stained sections were dehydrated and mounted with syntetic resin.

For Azan Mallory, sections were stained with azocarmine G (Sigma) for 1 hour and with 5% of phosphovolframic acid for an additional hour. Then, sections were stained with aniline blue/orange G (Sigma) and mounted in synthetic resin.

All IHC images were analyzed with Imaging Analyzer Software

Apoptotic events were determined by TUNEL labeling using In Situ Cell Death Detection, AP Kit (Boehringer Mannheim) (roche). DNA strand breaks were detected by 5-bromo-4-chloro-3-indolyl-phosphate (BCIP, Dako Cytomation) substrate.

Immunofluorescence stainings were performed on 5-µm-thick embedded sections of human CRC tissues, on cytospun freshly sorted cells, spheres cells allowed to differentiate in 10% FBS or after exposure to BMP7v. Cells were fixed in 2% PFA for 20 min at 37°C. For cytoplasmatic epitopes detection, samples were permeabilized with 0.1% TritonX-100 in PBS for 10 min, blocked with 3% BSA

for 30 min and exposed overnight at 4°C to antibodies against BMP7, CD133, BMPRIA, BMPRIB and BMPRII, p-SMAD1,5,8, CK20, p21, E-cadherin, Vimentin and beta-catenin or isotype-matched controls at appropriate dilutions. Then, cells were treated with FITC or Rhodamine anti-mouse or anti-rabbit antibodies (Molecular Probes, Inc.) plus RNase (200 ng/ml, Sigma). Nuclei were counterstained using Toto-3 iodide (642/660, Molecular Probes). Confocal analysis was used to acquire fluorescence stainings.

RNA isolation and *Real-time PCR*

Total RNA from cell pellet was obtained using the Rneasy Mini Kit (Qiagen GmbH), the residual amounts of DNA remaining was removed using the RNase-Free DNase according to manufacturer's instructions. The yield of the extracted RNA was determined by measuring the optical density at 260 nm by Nanodrop ND-1000 (Nanodrop, Wilmington, DE).

1 µg of total RNA was retro-transcribed using High-Capacity cDNA Archive Kit (Applied Biosystems) according to manufacturer's instructions. PCR amplification and detection of the PCR amplified gene products were performed with the SYBR Green PCR master mix (SuperArray Bioscience, Frederick, MD) All amplification reactions were done in triplicate, and the relative quantitation of gene expression was calculated using the comparative Ct method (ΔΔCt). Levels of mRNA expression were expressed after normalization with endogenous control, GAPDH. For SYBR green chemistry, the following primers were purchased from MWG: BMPR1A forward primer 5' GTC ATA CGA AGA TAT GCG TGA GGT TGT 3', BMPR1A reverse primer 5' ATG CTG TGA GTC TGG AGG CTG GAT T 3, BMPR1B forward primer 5' AAG GCT CAG ATT TTC AGT GTC GGG A 3', BMPR1B reverse primer 5' GGA GGC AGT GTA GGG TGT AGG TCT TTA TT 3', BMPR2 forward primer 5' GTG ACT GGG TAA GCT CTT GCC GTC T 3', BMPR2 reverse primer 5' GCA GGT TTA TAA TGA TCT CCT CGT GGT 3', GAPDH forward primer 5' GCT TCG CTC TCT GCT CCT CCT GT 3', GAPDH reverse primer 5' TAC GAC CAA ATC CGT TGA CTC CG 3'.

Flow cytometry

Flow cytometry was performed on freshly purified colon cancer cells after magnetic $CD45^+$ cells depletion, SDAC and dissociated sphere cells untreated and treated with BMP7v. Cell preparations were fixed in 2% paraformaldehyde for 10 min at 37°C and permeabilized by 0.1% Triton-X 100 for 10 min at 4°C to detect cytoplasmatic epitopes. Cells were washed twice with 0.5% bovine serum albumin (BSA, Sigma) in PBS and exposed to antibodies against CD133/2 (293C3-PE, mouse IgG2b, Miltenyi), CD133/1 (AC133, mouse IgG2b, Miltenyi), CK20 (Ks20.8, mouse IgG2a, DAKO Cytomation). Samples were then incubated with FITC-conjugated anti-mouse or anti-rabbit antibodies (Molecular Probes). Cells were subjected to flow cytometry analysis using a FACSCalibur cytometer and Cell Quest Software (Becton Dickinson). Only cells with staining intensities above the maximal level of isotype-matched controls were defined as positive cells.

Cell cycle analysis was performed on dissociated sphere cells untreated and treated with BMP7v for 48 hours and SDACs. The cells were washed in PBS and fixed in ice-cold 70% ethanol at 4°C overnight and then incubated with PBS containing propidium iodide (50 µg/ml, Sigma), sodium citrate (3.8 Mm, Sigma) and RNase (10 µg/ml, Sigma) at 37°C for 30 minutes. Samples were analyzed using a flow cytometer (BD Biosciences).

PKH26 (Sigma) staining was performed, according to manufacturer's instructions, on dissociated sphere cells, untreated and treated with BMP7v (100ng/ml) for 48 hours, up to 14 days, at different time points these cells were harvested for the FACS analysis,

Protein isolation and immunoblotting

Cell pellets were re-suspended in ice-cold NP40 lysis buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EGTA, and 1% NP40] containing proteases and phosphatases inhibitors and fractioned on SDS-polyacrylamide gels and blotted on nitrocellulose membranes. Membranes were blocked for 1 hour with nonfat dry milk in TBS containing 0.05% Tween 20 and successively incubated with antibodies specific against PTEN (17A, mouse IgMk, Neomarkers), AKT (rabbit polyclonal, CST), p-AKT (Ser 473, rabbit polyclonal, CST), GSK3ß (rabbit

polyclonal, CST), p-GSK3ß (Ser9, rabbit polyclonal, CST) and beta-actin (JLA20, mouse IgM, Calbiochem) used as loading control. Membranes were then washed, incubated for 1 hour with horseradish peroxidase-conjugated anti-mouse, anti-rabbit or anti-goat immunoglobulins (Amersham Biosciences) and developed with a chemiluminescence detection system (SuperSignal West Pico/Dura Extended duration Substrate, Pierce Biotechnology). Densitometric analysis of protein expression level was performed by Vision Works LS (UVP). Results were expressed as protein/beta-actin OD ratio.

Clonogenic and colony forming assays

Dissociated sphere cells untreated and treated with BMP7v for 48 hours were plated in presence of stem cell medium, on ultra-low-adhesion 96-well plates at a concentration of a single cell per well. Wells containing either none or more than one cell were excluded for this analysis, but these valors was used to calculate the cancer stem cell frequency, with ELDA software.

Colony forming was performed on dissociated sphere cells untreated and treated with BMP7v, 5FU+Oxaliplatin alone or in combination. Cells were plated on Agarose Sea Plague Agar (Invitrogen), and maintained up to 21 days at 37°C in presence of 5% CO_2. The colonies were stained with 10% Cristal Violet.

Invasion assay

Cell migration capacity was assessed using growth factor–depleted matrigel–coated (BD Biosciences) transwell insert. Dissociated sphere cells ($2x10^3$) untreated and treated with BMP7v 100ng/ml for 48 hours were plated into matrigel-coated transwell of 8 μm pore size (Corning). Supernatant of NIH-3T3 cells, cultured in stem cell medium was used as chemoattractant in the transwell lower part. Migration was observed and counted microscopically up to 96 hours.

RT^2 Profiler™ PCR Array.

The RT^2 Profiler PCR array was assessed for genes related to Wnt targets (PAHS-243ZR-12; SuperArray Bioscience). It was used to simultaneously examine the

mRNA levels of 89 genes closely associated with Wnt pathway, including five "housekeeping genes" in 96-well plates following the manufacturer's protocol.

Cycle threshold values were calculated for all the genes present on the array and normalized using the average of 5 housekeeping genes (ACTB, B2M, HPRT1, RPLP0 and GAPDH). RT^2 Profiler PCR Array Data Analysis was represented by clustergrams based on Pearson's Correlation of 2^ (ΔCt).

Animals and tumor model

Mice were obtained from Charles River Laboratories (Milan, Italy) and maintained according to institutional guidelines of the University of Palermo animal care and committee. Dissociated CRC sphere cells ($5x10^5$) were implanted subcutaneously (s.c.) with matrigel GF reduced (BD Biosciences) at a 1:1 ratio in a total volume of 100 µL into the flank of five-to-six week old NOD-SCID mice. Tumor mass size was calculated according to the formula: (π/6) x larger diameter x (smaller diameter)^2. When tumors were palpable the mice were treated three times a week by intraperitoneal injection of BMP7v for three weeks at different concentrations: 50, 5, 0.5 and 0.05µg/kg, and PBS as control, in order to test the efficacy dose.

Then the other set of mice were treated three times a week for three weeks by intraperitoneal injection (IP) of PBS, as control, BMP7v (50µg/kg) alone or in combination with 5FU and Oxaliplatin. (5-FU: 15 mg/kg/day for 2 days a week for 3 weeks; and Oxa: 0.25 mg/kg once a week for 3 weeks).

Histological examination, analysis of differentiation, incidence of cell death and mitotic index were determined on tumor xenografts using AC133, CK20, TUNEL and Ki67 staining.

To test the BMP7v activity as second line treatment, when the tumor xenografts obtained as previous described, were palpable, the standard adjuvant treatment, Oxaliplatin (0.25 mg/kg once a week for 3 weeks) and 5-FU (15 mg/kg/day for 2 days a week for 3 weeks) was performed intraperitoneally on eight mice for group. After this first line of treatment with 5FU plus Oxaliplatin alone for two weeks when tumor re-growth, the follow IP treatments were performed: PBS, 5FU+Oxaliplatin, BMP7v and BMP7v+5FU+Oxaliplatin, in a standard protocol

for three weeks. The tumor xenografts obtained after 13 weeks from injection were used for histological examination.

Endothelial tube formation assay

The effects of BMP7v on *in vitro* endothelial tube formation were evaluate using HUVEC, obtained from Lonza (Clonetics, Verviers, Belgium) and grown in endothelial growth medium (EGM) according to supplier's information. HUVEC, pretreated with BMP4 (2nM R&D System) and BMP7v (100ng/ml) for 24, 48, 72 hours, were plated (70,000 cells/well) in Matrigel-coated 24 well plate (BD Bioscience), and incubated up to 5 hrs at 37°C. Endothelial tube formation, evaluated by phase-contrast microscopy, was photographed at different time points and the cables length was measured manually with the IMAGE-J software.

Statistical analysis

Data were expressed as mean ± standard deviation of the mean. Immunohistochemical scores were calculated from the positivity observed on paraffin-embedded engrafted tumor tissues counted by two independent observers.

Statistical significance was determined by Analysis of Variance (one-way or two-way) with Bonferroni post-test. Results were considered significant when p values were less than 0.05. * indicates $P<0.05$, ** indicate $P<0.01$, *** indicate $P<0.001$.

RESULTS

BMP7 is widely expressed in colo-rectal cancer tissue but not in colo-rectal cancer stem cells

Human colo-rectal cancer (CRC) specimens were provided by the Surgical Department of Policlinico "Paolo Giaccone", Palermo.

These tumors and their normal counterpart (obtained from the edge of the resected specimen) were analysed for BMP7 expression with immunohistochemistry: CRC specimens widely expressed BMP7, compared to their normal counterpart (Fig.4A).

Expression of this cytokine was also analysed with immunofluorescence (IF) on paraffin embedded cancer tissue: BMP7 was localized along the upper part of the crypt, with the exception of the base of the crypt where stem cells reside (Fig.4B).

These findings prompted us to investigate whether there is differential expression of BMP7 between cancer stem cells (CSCs) and their differentiated counterpart using our *in vitro* models, i.e. sphere cells and sphere-derived adherent cells (SDACs), respectively. While SDACs displayed high levels of BMP7 expression, sphere cells showed very low positivity.

We also analyzed BMP7 expression in CR-CSCs sorted for CD133 positivity, a known CSC marker: IF analysis showed that BMP7 is exclusively expressed by $CD133^-$ cells (Fig.4C-D).

All *in vitro* experiments shown in this report were conducted in three different cell lines. The images are from one cell line, but are representative of results obtained for all three cell lines.

These data suggest a correlation between BMP7 expression and differentiation of CRC cells, and therefore a possible role in CSCs differentiation.

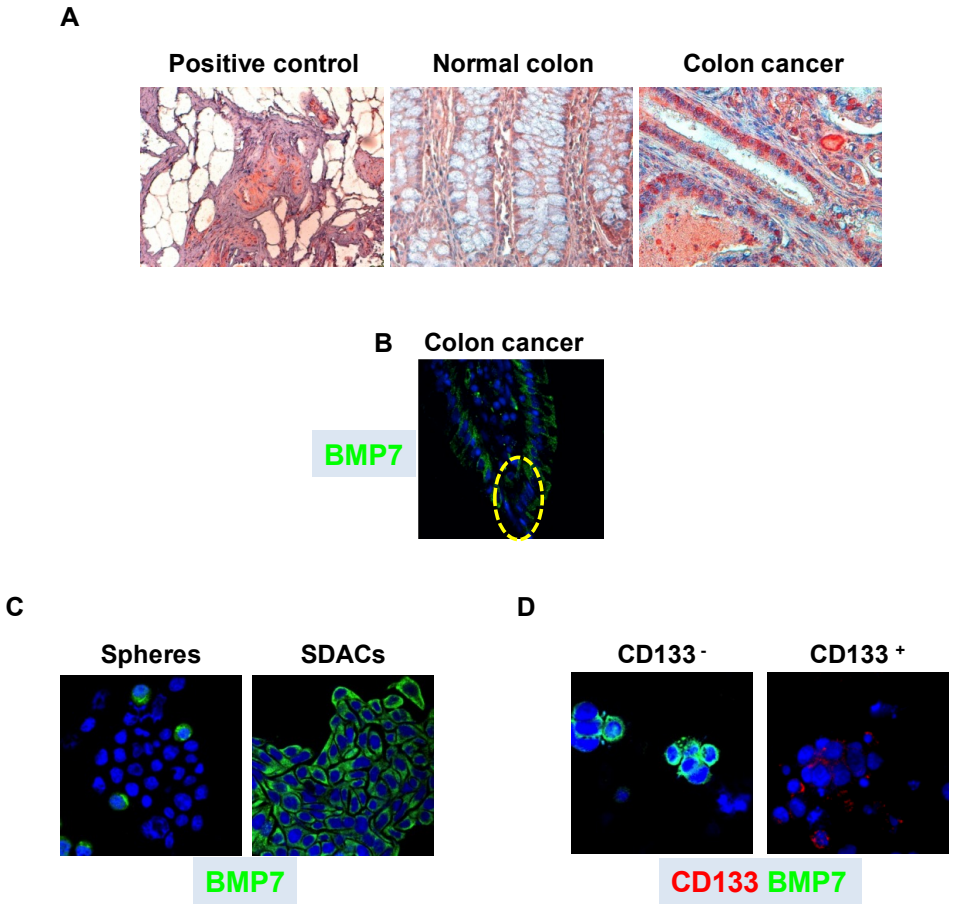


Fig.4. CD133$^+$ CR-CSCs do not express BMP7. A) Immunohistochemical analysis for BMP7 on normal and tumoral colon paraffin-embedded sections. Nuclei are revealed by hematoxylin staining (*blue*). **B)** Confocal microscopy analysis of BMP7 (*green*) on CRC paraffin-embedded tissue. Nuclei were counterstained with Toto-3 (*blue*). **C)** Representative images of immunofluorescence analysis of BMP7 (*green*) in sphere cells (*Spheres*) and SDACs. Nuclei were counterstained with Toto-3 (*blue*). **D)** Representative images of immunofluorescence analysis of BMP7 (*green*) on CD133$^-$ and CD133$^+$ CRC cells sorted from the sample as in C. Nuclei were counterstained by Toto-3 (*blue*).

BMP7v *in vitro* administration activates the BMP signaling pathway in CR-CSCs.

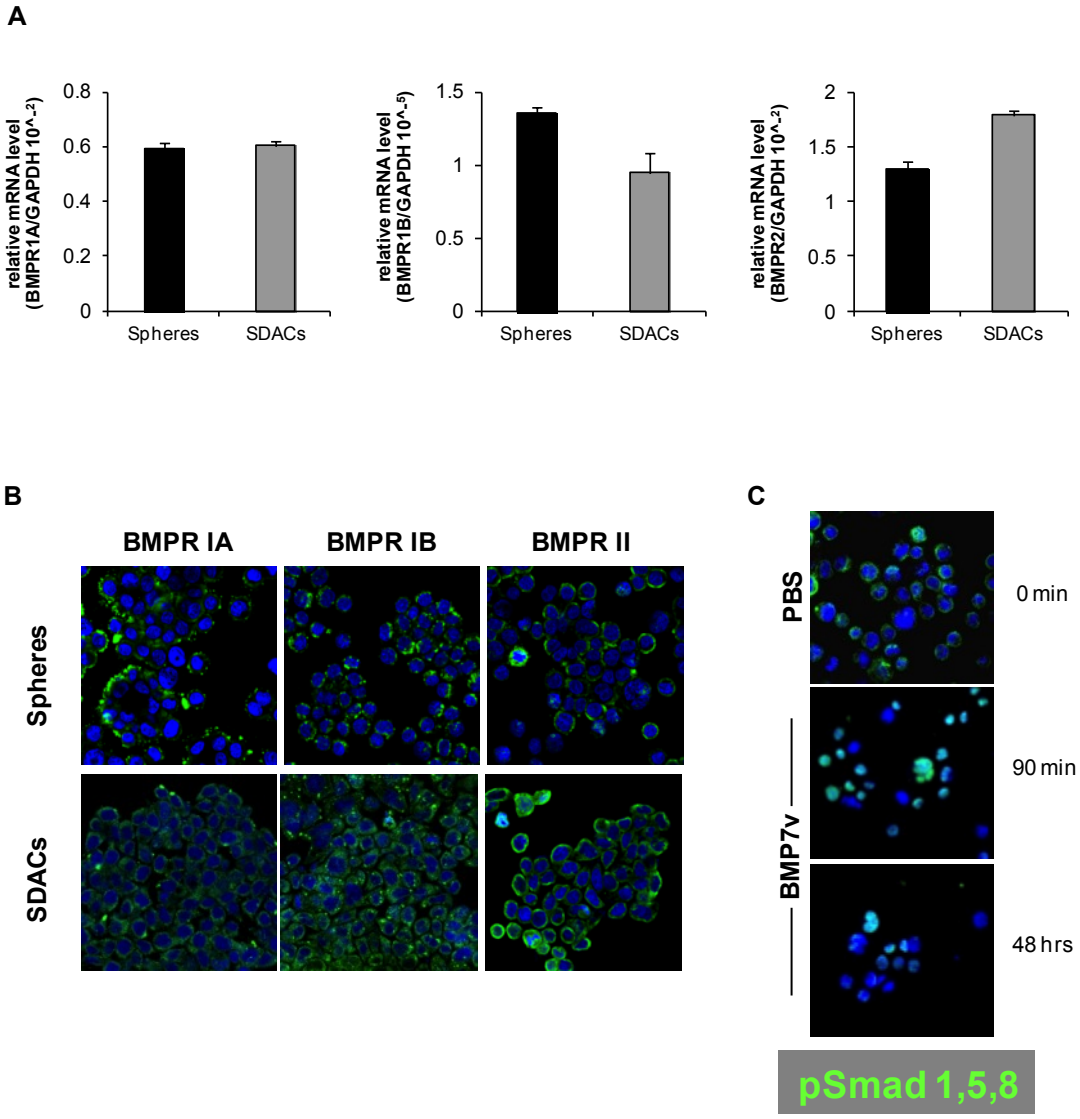
To evaluate the possible use of BMP7v as a differentiative agent of CSCs, we analysed the expression of BMP receptors through real-time PCR and IF analysis.

Both CRC sphere cells and SDACs expressed BMPR1A, BMPR1B and BMPR2 (Fig.5A).

In order to verify the BMPRs functionality in CR-CSCs, we evaluated p-SMAD1-5-8 localization with IF after treatment with BMP7v (100ng/ml): activation of the BMP7 pathway was confirmed by the prevalent p-Smad1,5,8 nuclear translocation following treatment (Fig.5B-C).

Western blot analysis of downstream targets to the BMP7 pathway are consistent with these findings (Fig.5D).

These results indicate that exogenous addition of BMP7v can activate the canonical BMP signaling pathway in CR-CSCs.



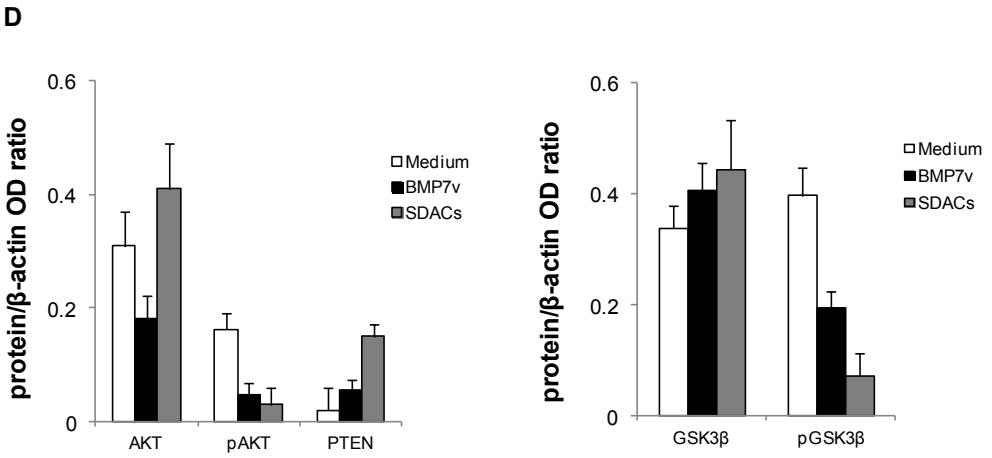


Fig.5. BMP7v administration activates the canonical signaling pathway in CR-CSCs. A) Relative quantification of BMPR1A, BMPR1B, and BMPR2 mRNA expression levels in sphere cells (*Spheres*) and SDACs. **B)** Representative images of immunofluorescence analysis of BMPR1A, BMPR1B, and BMPR2 (*green*) in sphere cells and SDACs. Nuclei were counterstained with Toto-3 (*blue*). **C)** Representative images of confocal microscopy analysis of pSmad1,5,8 (*green*) in sphere cells, untreated or treated with BMP7v for 90 minutes or 48 hours. Nuclei were stained with Toto-3 (*blue*). **D)** Densitometric analyses of protein expression levels of AKT, p-AKT, PTEN , GSK3β and p- GSK3β in sphere cells, untreated or exposed to BMP7v for 48 hours, and SDACs. Loading control was assessed by β-actin.

BMP7v induces *in vitro* differentiation in CR-CSCs and reduces the percentage of CD133$^+$ cells

To evaluate the role of BMP7v in inducing differentiation, we tested whether sphere cells could be forced to differentiate upon exposure to the morphogenetic factor. CRC sphere cells were cultured in the presence of BMP7v or 10% FBS. BMP7v alone induced a rapid differentiation of CR-CSCs, evaluated by plastic adherence and acquisition of the typical differentiated appearance, i.e. large size and polygonal shape (Fig.6A).

Accordingly, BMP7v treatment reduced the percentage of CD133$^+$ and increased CK20 expression in CR-CSCs (Fig.6B-C).

Reduction of the CD133$^+$ cells following 48 hours of BMP7v treatment was also confirmed by flow cytometry (Fig.6D).

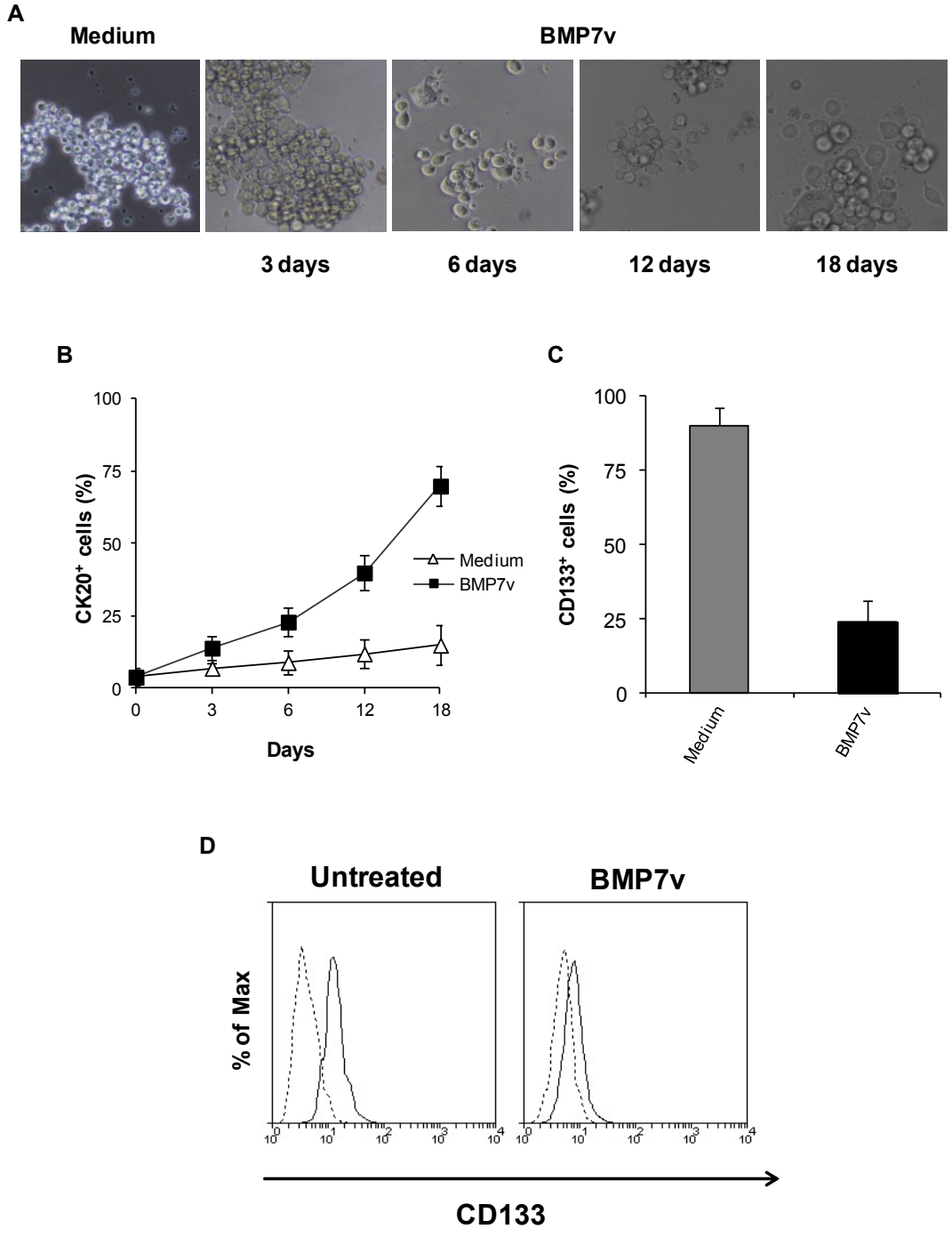


Fig.6. BMP7v promotes *in vitro* differentiation of CR-CSCs. A) Representative images of phase-contrast microscopy of dissociated sphere cells, untreated or treated with BMP7v up to 18 days. **B)** Percentage of $CK20^+$ sphere cells, untreated or treated with BMP7v up to 18 days. **C)** Percentage of $CD133^+$ sphere cells, untreated or treated with BMP7v. **D)** Representative flow cytometry profile of CD133 expression in sphere cells, untreated or treated with BMP7v for 48 hours.

BMP7v reduces self-renewal of CR-CSCs

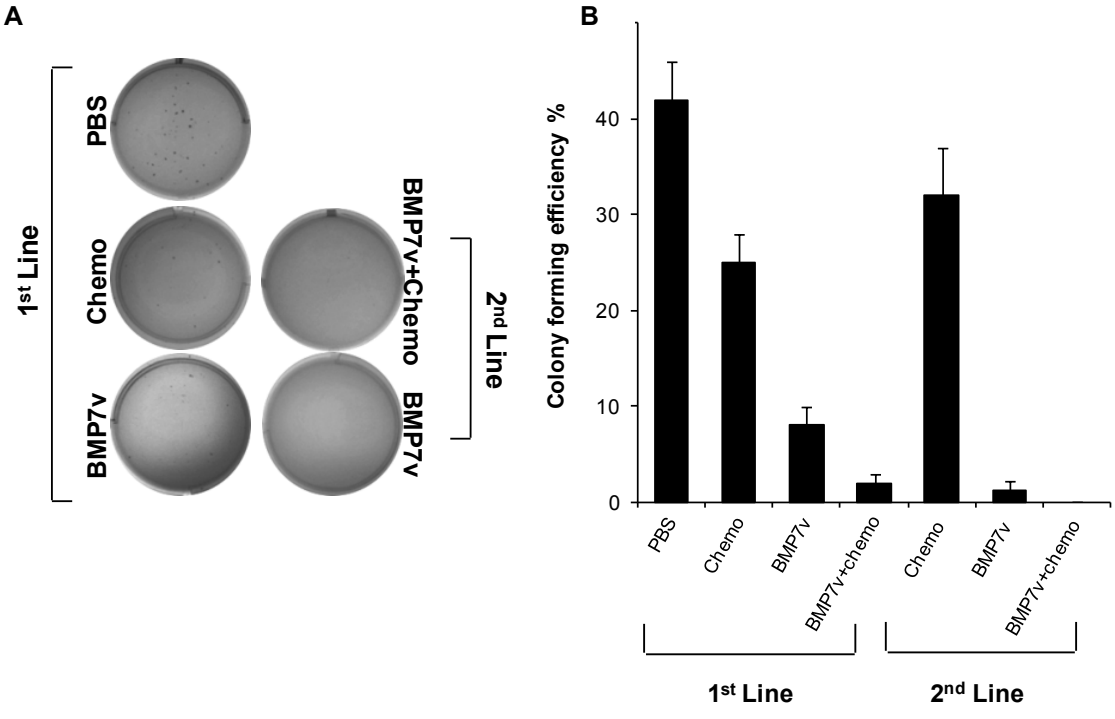
To evaluate the effect of BMP7v treatment on CR-CSCs self renewal capacity, we performed the *in vitro* colony forming assay. BMP7v, given for 48 hours, reduced the CR-CSCs sphere forming capacity.

We also tested the effect of combining BMP7v to standard chemotherapy (5FU+Oxa): this treatment resulted in a more significant reduction of clonogenic capacity compared to chemotherapy alone in CR-CSCs.

To further test the CR-CSC sensibility to BMP7v, one week after the first step of treatment, the cells were re-treated: this treatment reduced the colony forming efficiency even more drastically (Fig.7A-B).

As p21 seems to be a key regulator of CR-CSC self renewal, we also evaluated its cellular localization with IF: BMP7v treatment induces depletion of nuclear p21, confirming the induction of cell cycle progression, with consequent cancer stem cell clone exhaustion (Fig.7C).

We showed that BMP7v treatment not only reduces CR-CSCs self-renewal but also potentiates the effect of standard chemotherapy.



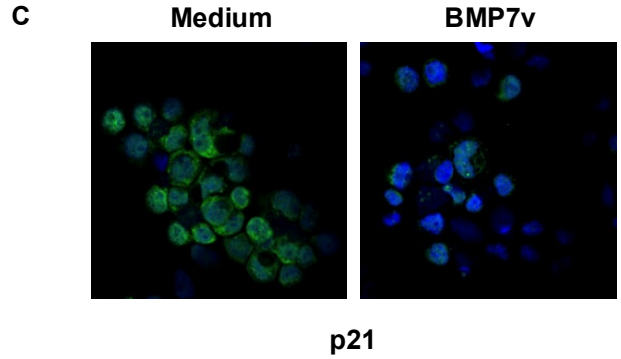


Fig.7. BMP7v reduces self renewal of CR-CSCs. A) Representative colony forming assay of sphere cells, untreated or treated with 5FU+Oxaliplatin (chemo), BMP7v alone (BMP7v), BMP7v+5FU+Oxaliplatin (BMP7v+chemo), for 48 hours; these treatments were performed once as a 1st line or repeated after one week as a 2nd line of treatment. **B)** Percentage of colony forming efficiency in sphere cells, untreated or treated as in A. **C)** Representative images of confocal microscopy analysis of p21 (*green*) in sphere cells, untreated and treated with BMP7v for 48 hrs. Nuclei were stained with Toto-3 (*blue*).

BMP7v induces CR-CSCs to exit from quiescence

We then evaluated the effect of BMP7v on cell cycle distribution. FACS analysis revealed that 48 hours of BMP7v treatment, unlike to BMP4, induced a significant increase in the percentage of cells in the G2/M phase, with a reduction of cells in G0/G1.

Interestingly, the BMP7v induced cell cycle distribution in sphere cells was similar to the baseline SDACs profile (Fig.8A-B).

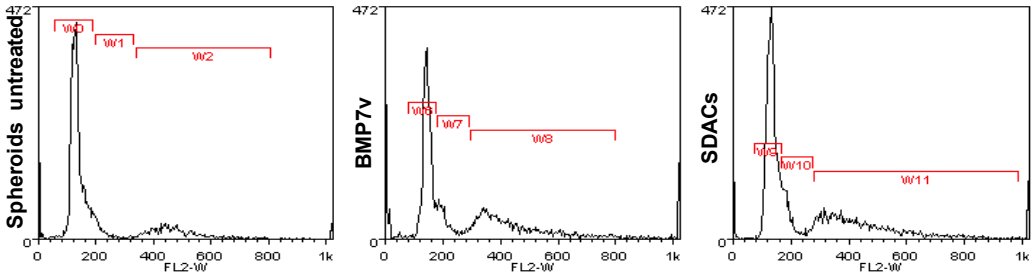
To evaluate the effect of BMP7v on proliferation of CR-CSCs, we performed PKH26 staining: treatment induced a reduction of PKH26 high cells, confirming the induction of proliferation in a time dependent manner (Fig.8C-D).

These data suggest that, in addition to differentiation induction, BMP7v treatment displays an unexpected proliferative effect.

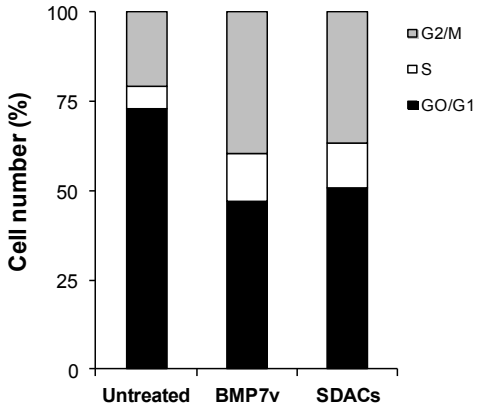
This cytokine drives different cell subpopulations present in sphere cultures to exit from the quiescent state, characteristic of stem cells, thus making them more sensitive to standard chemotherapies.

Taken together, these *in vitro* results indicate that BMP7v treatment is able to reduce stemness of CR-CSCs.

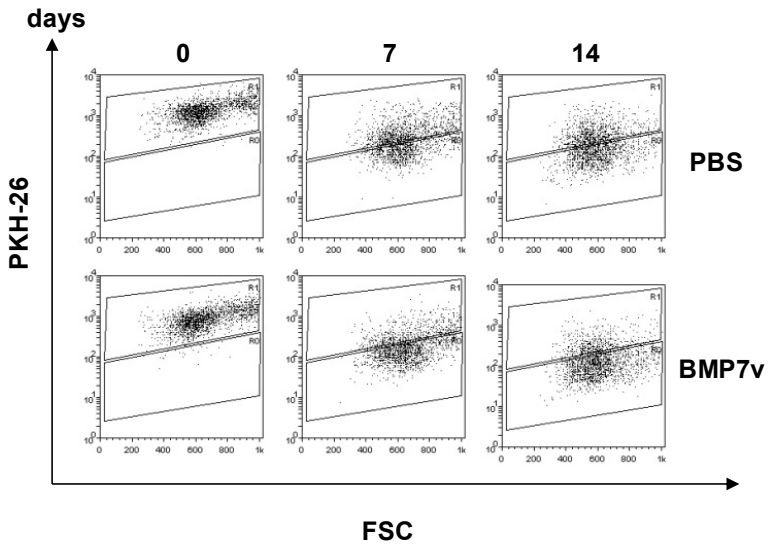
A


Spheroids untreated
BMP7v
SDACs
FL2-W

B


Cell number (%)
100
75
50
25
0
G2/M
S
GO/G1
Untreated
BMP7v
SDACs

C


days
0
7
14
PBS
BMP7v
PKH-26
FSC

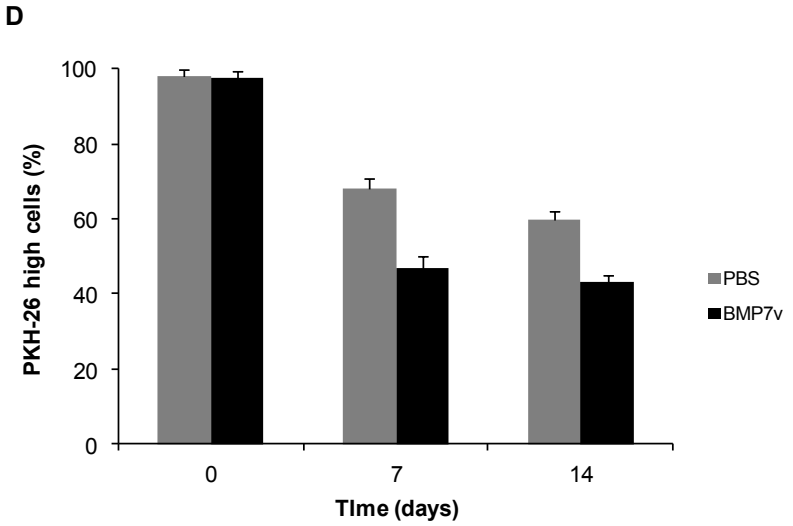
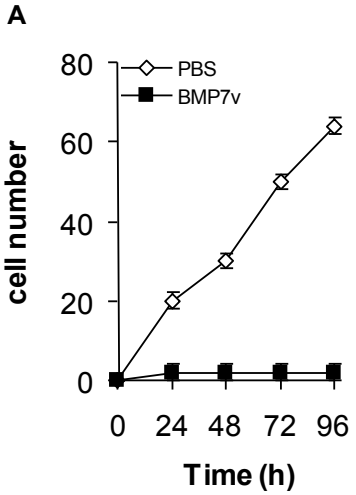


Fig.8. BMP7v induces CR-CSCs to exit from quiescence. A) Representative cell-cycle profile of sphere cells, untreated or treated with BMP7v for 48 hours, and SDACs. **B)** Cell-cycle distribution of sphere cells treated as in A. **C)** Representative PKH-26 profile of sphere cells, untreated or treated with BMP7v up to 14 days. **D)** Percentage of PKH-26 high cells treated as in C.

BMP7v reduces the mesenchymal CR-CSCs traits

Given the link between CSCs, epithelial-mesenchymal transition (EMT) and metastasis, we tested the effect of BMP7v treatment on CR-CSCs invasive capacity, using a transwell migration assay: BMP7v drastically reduces CR-CSC motility and invasiveness (Fig.9A).



We then analysed expression of EMT markers, such as E-caderin, Vimentin and beta-catenin: BMP7v suppressed the mesenchymal trait, inducing E-cadherin expression and cytoplasmic localization of beta-catenin, while silencing Vimentin (Fig.9B).

Given the role of the Wnt pathway in EMT, a Wnt signaling trascriptional profile was performed on CR-CSCs after 48 hours of BMP7v treatment to verify its action on regulation of Wnt targets: results revealed a strong regulation of Wnt targets involved in stemness and migration.

BMP7v induced upregulation of Wnt inhibitors such as SFRP2 and Axin2, associated with down regulation of stem cell markers, such as SOX2 e Nanog. In addition, the treatment reduced expression of migration regulator genes, such as Met and MMP2 and 7 (Fig.9C).

These results confirm the induction of differentiation *via* the Wnt pathway.

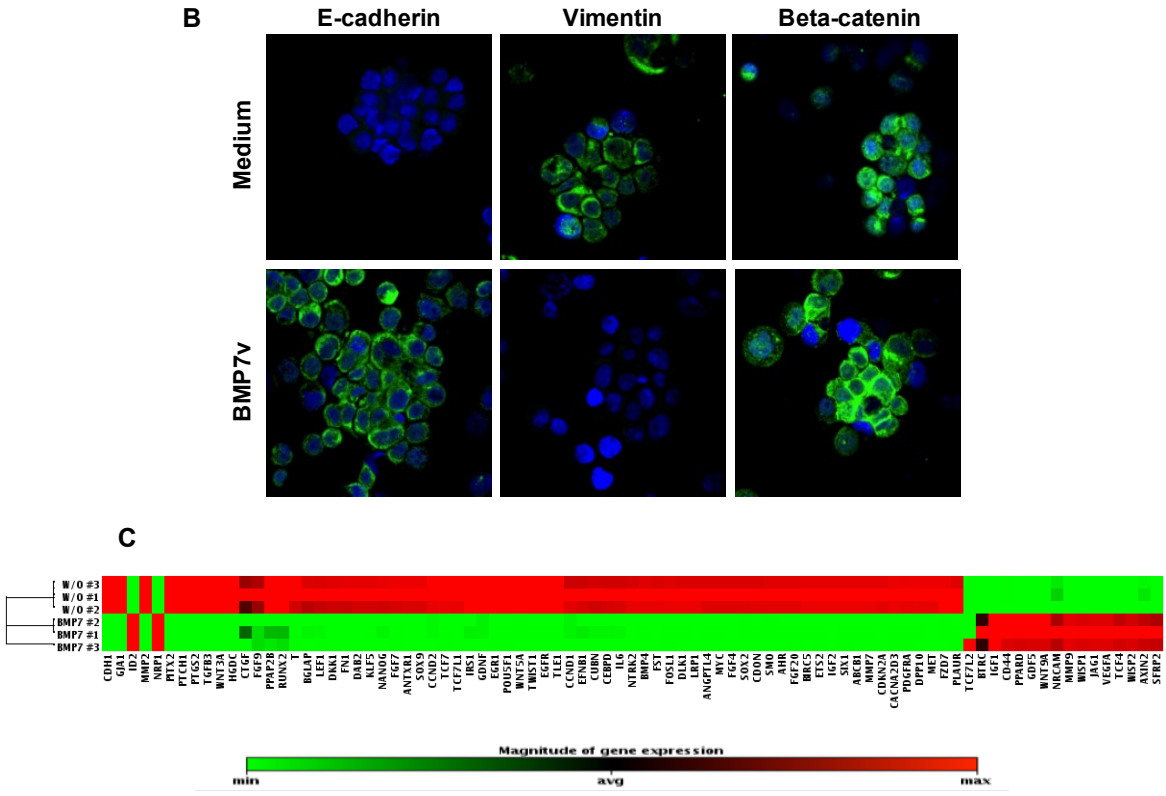


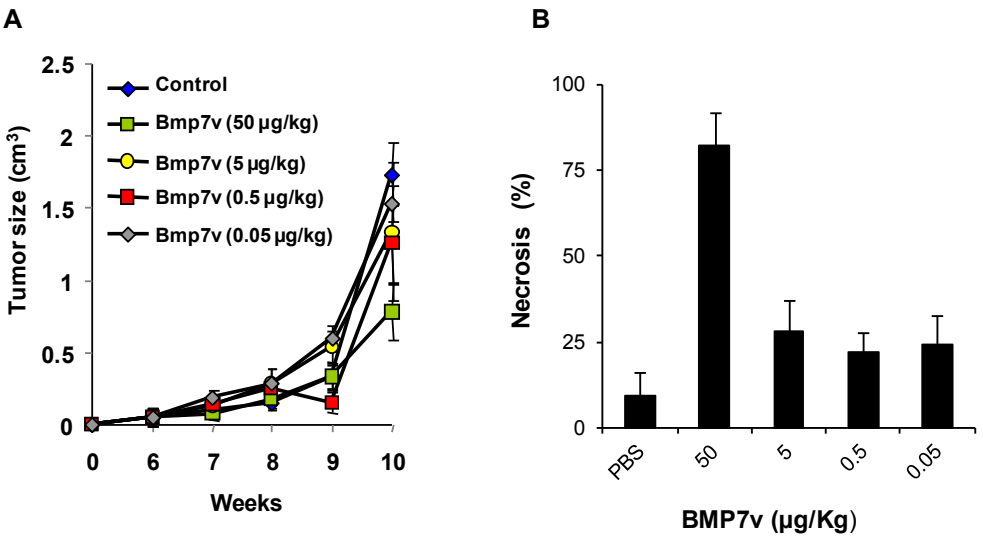
Fig.9. BMP7v reduces the mesenchymal CR-CSCs traits. A) Invasion assay of sphere cells, untreated or treated with BMP7v for 48 hours at different time points up to 96 hrs. **B)** Representative images of confocal microscopy analysis of E-cadherin, Vimentin and Beta-catenin (*green*) in sphere cells, untreated or treated as in A. Nuclei were stained with Toto-3 (*blue*). **C)** Wnt target card clustergram on sphere cells untreated or treated as in A.

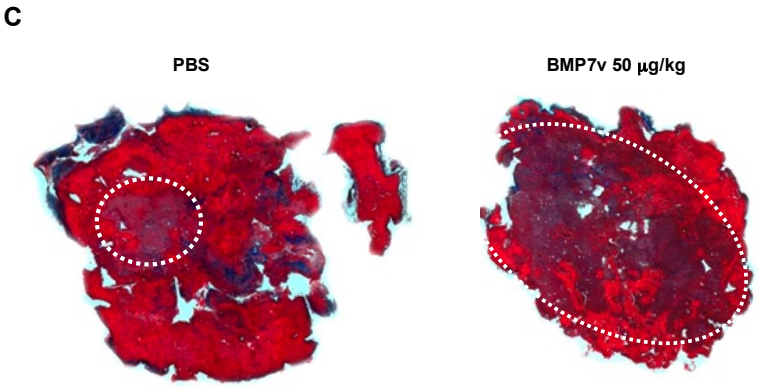
BMP7v in combination with standard chemotherapy reduces CR-CSCs tumor growth *in vivo*

To evaluate the effects of BMP7v on CR-CSCs tumor growth *in vivo,* different concentrations were administered intraperitoneally (IP) three times a week.

Three to four weeks after subcutaneous injection of sphere cells, palpable tumors were observed in immuno-compromised mice. BMP7v treatment was started at different doses listed below: 50, 5, 0.5 and 0.05µg/kg.

The results showed a reduction in tumor size and a notable necrotic effect confirmed by Azan Mallory staining on paraffin embedded xenografts sections: these effects were more pronounced in the 50µg/kg group (Fig.10A-C).





Based on these findings, we decided to use 50μg/kg in combination with standard chemotherapy, to evaluate whether BMP7v could enhance its cytotoxic effect *in vivo*.

Tumors were obtained injecting Smad4-wt, Smad4-null and Smad4-null PI3K/CA sphere cells. They were then exposed to Oxaliplatin plus 5-FU alone or in combination with intraperitoneal injection of PBS or BMP7v.

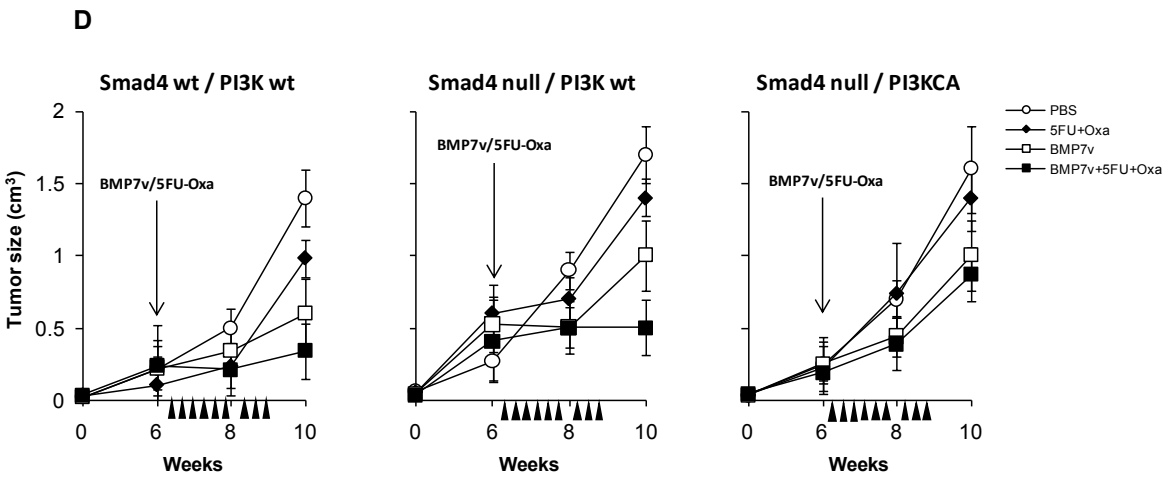
Combined treatment resulted in a considerable reduction of tumor growth in Smad4-wt and Smad4-null, while it was less effective in Smad4 null harboring PI3KCA mutation.

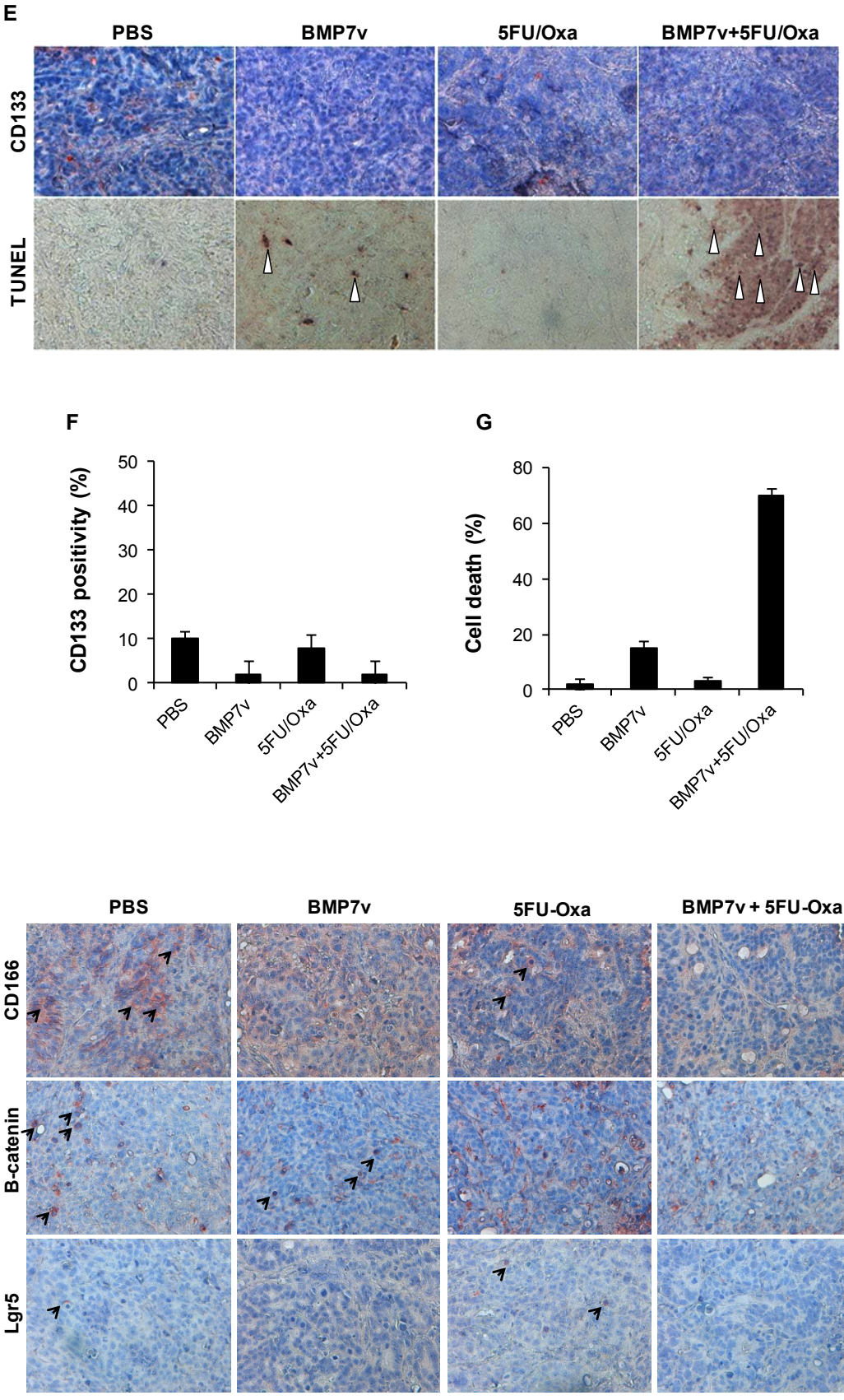
BMP7v alone gave a more pronounced growth delay than standard chemotherapy (Fig.10D).

Histological analysis of xenografts treated with BMP7v plus chemotherapy showed an high percentage of dying cells and large areas of fibrosis, highlighted by TUNEL staining (Fig.10E).

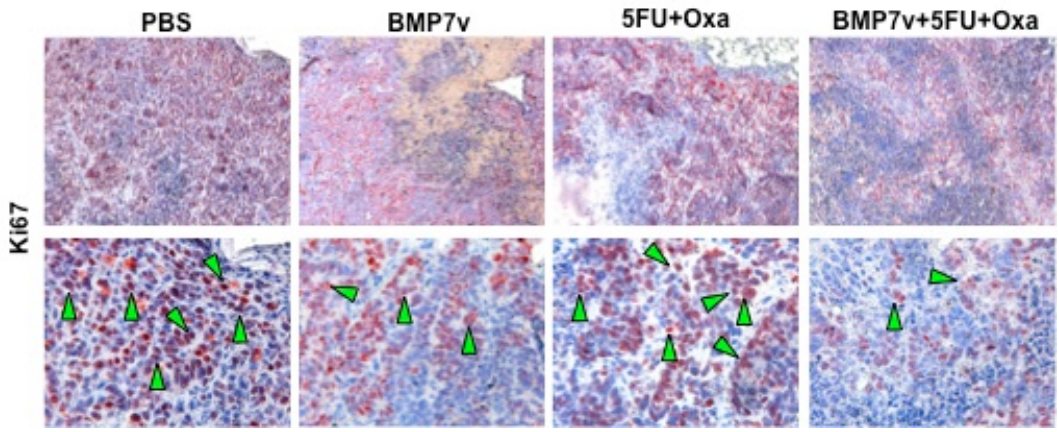
BMP7v plus chemotherapy treated tumors showed lower expression of CD133, CD166, β-catenin, Lgr5 and Ki67, with a significant increase of CK20 compared to control, suggesting anti-proliferative and pro-differentiative effects *in vivo* (Fig.10E-L).

Our findings confirm the rationale for combining a pro-differentiation agent with chemotherapy drugs, supporting the use of BMP7v in colorectal cancer patients.

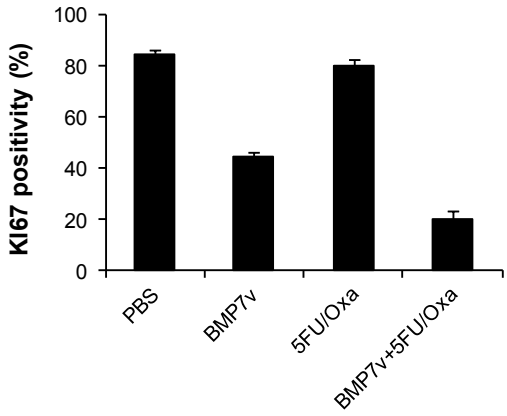



E
PBS
BMP7v
5FU/Oxa
BMP7v+5FU/Oxa
CD133
TUNEL
F
CD133 positivity (%)
50
40
30
20
10
0
PBS
BMP7v
5FU/Oxa
BMP7v+5FU/Oxa
G
Cell death (%)
80
60
40
20
0
PBS
BMP7v
5FU/Oxa
BMP7v+5FU/Oxa
H
PBS
BMP7v
5FU-Oxa
BMP7v + 5FU-Oxa
CD166
B-catenin
Lgr5

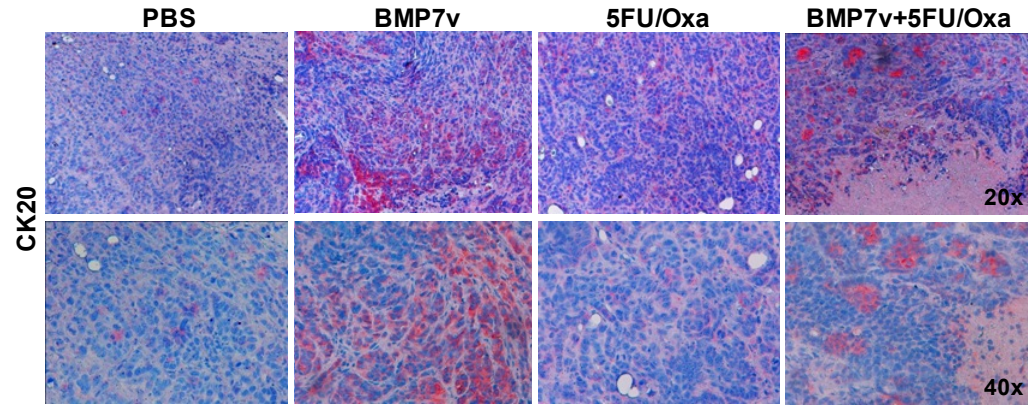
I


PBS
BMP7v
5FU+Oxa
BMP7v+5FU+Oxa
Ki67

J


KI67 positivity (%)
100
80
60
40
20
0
PBS
BMP7v
5FU/Oxa
BMP7v+5FU/Oxa

K


PBS
BMP7v
5FU/Oxa
BMP7v+5FU/Oxa
CK20
20x
40x

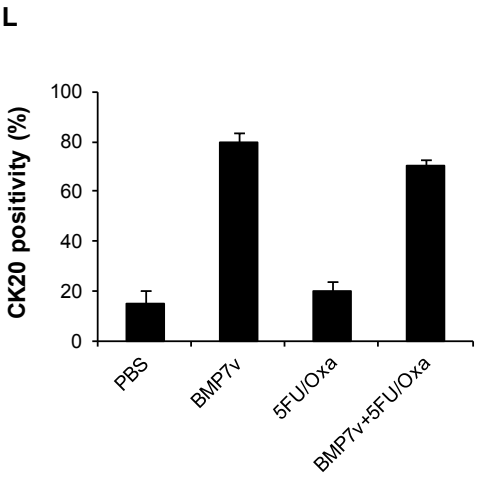


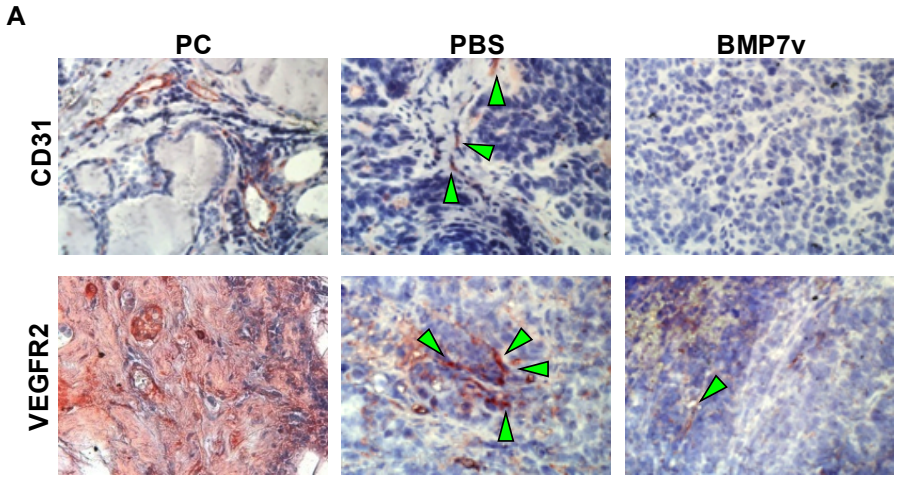
Fig.10. BMP7v in combination with standard chemotherapy reduces CR-CSCs tumor growth *in vivo*. A) Size of subcutaneous tumors following injection of sphere cells. When tumor xenografts were palpable immuno-compromised mice were treated intraperitoneally three times a week with BMP7v (50, 5, 0.5 or 0.05 ug/Kg) or with PBS as control. **B)** Percentage of necrosis evaluated on paraffin-embedded sections of xenografts, untreated or treated as in A. **C)** Representative Azan-Mallory staining on paraffin embebbed xenografts untreated (PBS) or treated as in A. **D)** Size of subcutaneous CRC xenografts derived from injection of sphere cells. Mice were untreated (PBS) or treated intraperitoneally with BMP7v (50ug/Kg), 5-FU plus Oxaliplatin alone or in combination with BMP7v. The arrows indicate the starting point of treatment. Data were obtained on three different cell lines .**E)** Immunohistochemical analysis of CD133 and TUNEL (dark blue) revealed by AEC (red) on paraffin-embedded sections of xenografts obtained as in D. Nuclei are revealed by hematoxylin staining (blue). Arrowheads indicate TUNEL positive cells. **F)** Percentage of $CD133^+$ cells evaluated on paraffin-embedded sections of tumors, untreated (PBS) or treated as in D. **G)** Percentage of TUNEL positive cells evaluated on paraffin-embedded sections of tumors untreated (PBS) or treated as in D. **H)** Immunohistochemical analysis of CD166, beta-catenin and Lgr5 revealed by AEC (red) on paraffin-embedded sections of xenografts obtained as in D. Nuclei are revealed by hematoxylin staining (blue). Arrowheads indicate CD166, beta-catenin and Lgr5 positive cells. **I)** Immunohistochemical analysis of Ki67 revealed by AEC (red) on paraffin-embedded sections of xenografts obtained as in D. Nuclei are revealed by hematoxylin staining (blue). Arrowheads indicate Ki67 positive cells. (upper: low magnification; lower: high magnification) **J)** Percentage of Ki67 positive cells evaluated on paraffin-embedded sections of tumors untreated (PBS) or treated as in D. **K)** Immunohistochemical analysis of CK20 revealed by AEC (red) on paraffin-embedded sections of xenografts obtained as in D. Nuclei are revealed by hematoxylin staining (blue). (upper: low magnification; lower: high magnification) **L)** Percentage of CK20 positive cells evaluated on paraffin-embedded sections of tumors, untreated (PBS) or treated as in D.

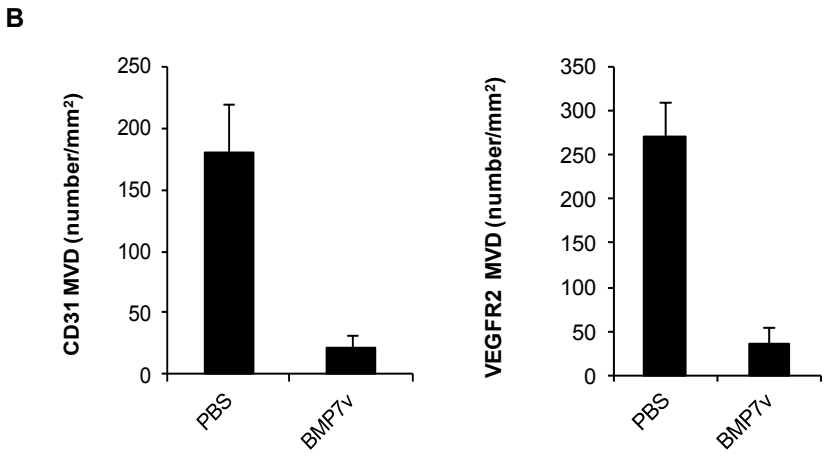
BMP7v has a strong anti-angiogenic effect *in vivo*

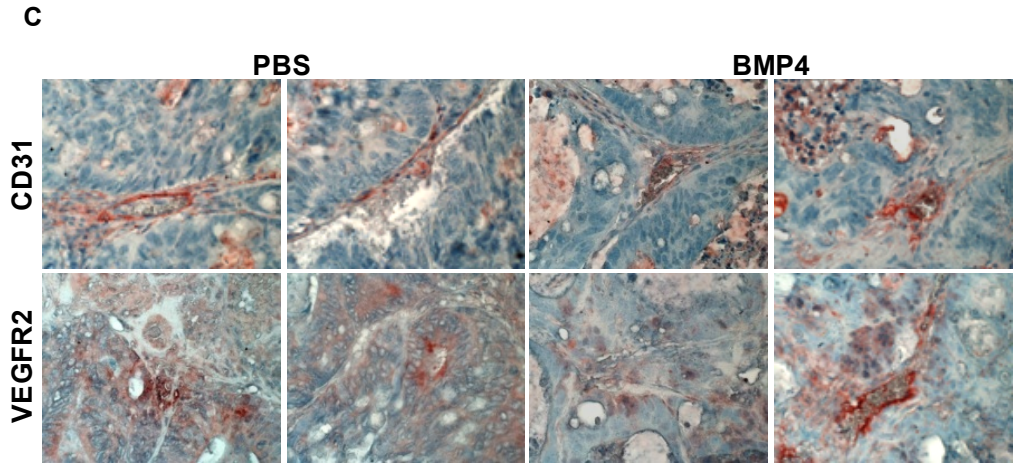
Since BMP7v treatment resulted in a considerable growth delay associated with a potent necrotic effect, we investigated further its anti-angiogenic potential.

Xenografts were exposed to BMP7v or BMP4, another member of the BMP family with an anti-tumour activity.

Histological examination showed a significant reduction of human CD31 and VEGFR2 expression following BMP7v, but not BMP4 treatment (Fig.11A-C).







We also performed an endothelial tube formation assay using endothelial cells from the umbilical vein (HUVEC): BMP7v affected the ability to form vessels after 48 hrs of treatment, while this was not observed for BMP4 (Fig.11D-F).

These findings confirm a specific anti-angiogenic effect of BMP7v in colorectal cancer xenografts.

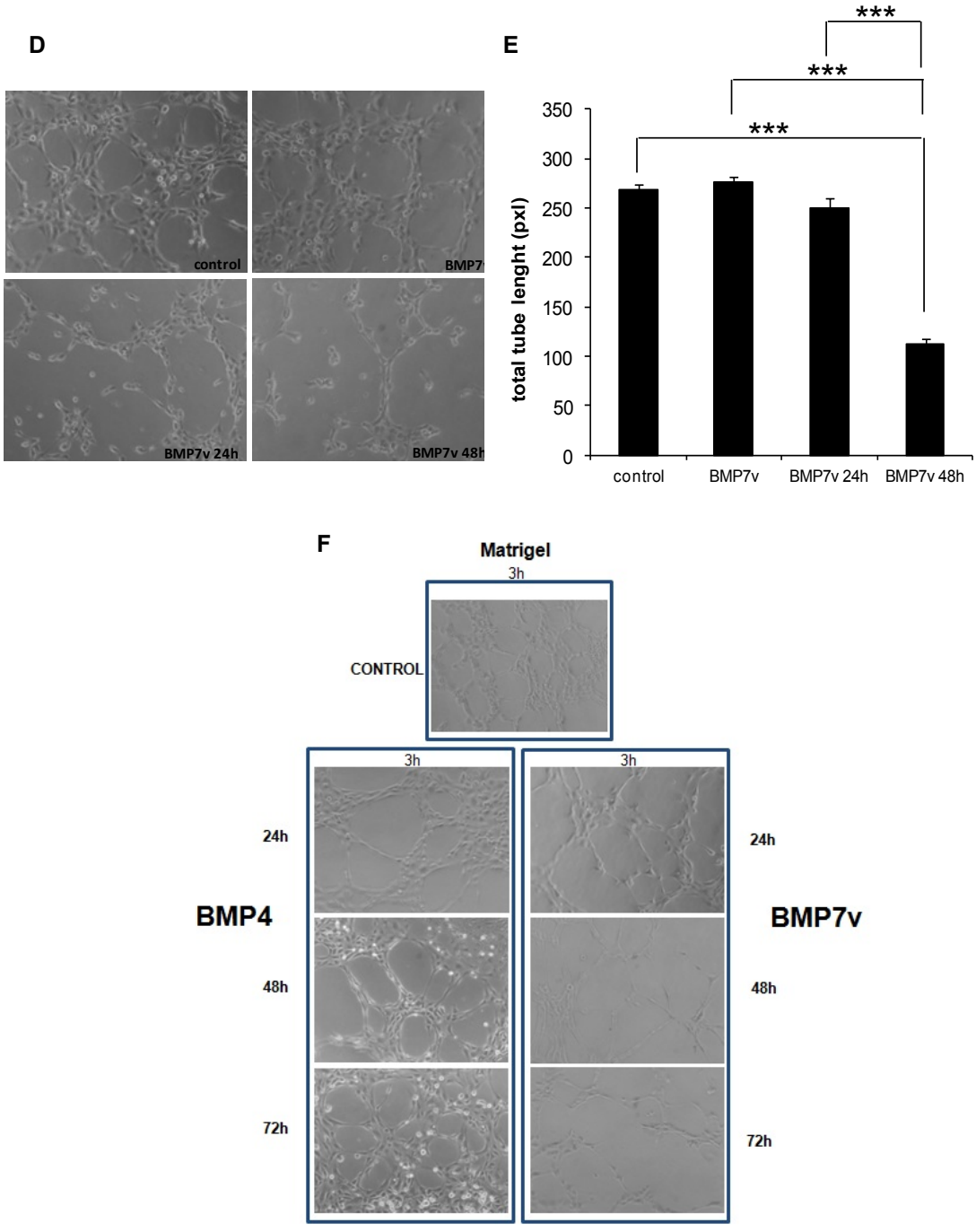


Fig.11. BMP7v has a strong anti-angiogenic effect. A) Immunohistochemical analysis of CD31 and VEGFR2 revealed by AEC (red) on paraffin-embedded section of xenografts obtained after PBS or BMP7v treatment (50ug/Kg). Nuclei are revealed by hematoxylin staining (blue). **B)** Percentages of CD31 and VEGFR2 expression evaluated on paraffin embedded sections of tumors

treated as in A. **C)** Immunohistochemical analysis of CD31 and VEGFR2 revealed by AEC (red) on paraffin-embedded section of xenografts obtained after intra-tumoral injection of PBS or BMP4 loaded beads. Nuclei are revealed by hematoxylin staining (blue). **D)** Representative images of phase-contrast microscopy of endothelial tube formation assay. Huvec cells, untreated (EGM medium as control) or pretreated with BMP7v (100ng/ml) at different time points. **E)** Measure (pxl) of total tube length obtained with Huvec treated as in D. **F)** Representative images of phase-contrast microscopy of endothelial tube formation assay. Huvec cells untreated (EGM medium as control) or pretreated with BMP4 (2nM) and BMP7v (100ng/ml) at different time points.

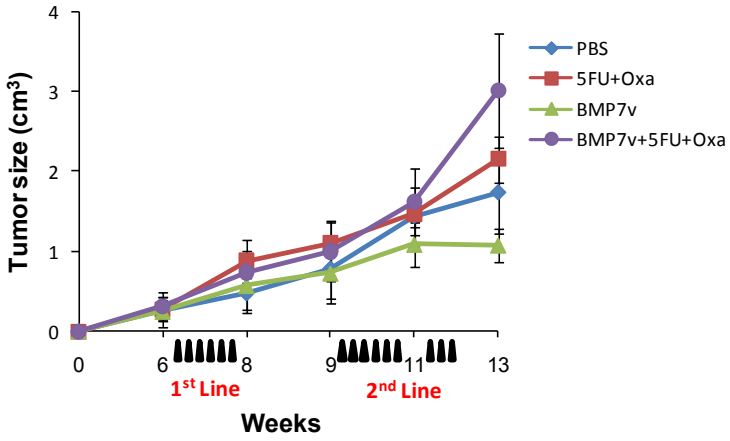
BMP7v as second-line treatment shows significant anti-tumor activity in xenografts refractory to chemotherapy

To test the activity of BMP7v as second line treatment, first line treatment with 5FU plus Oxaliplatin was administrated for two weeks. After tumour re-growth, mice were treated with: PBS, 5FU+Oxaliplatin, BMP7v or BMP7v+5FU+Oxaliplatin, for three weeks. BMP7v alone showed greater efficacy compared to combined treatment in tumor xenografts previously treated with standard chemotherapy (Fig.12A).

Hystological examination of xenografts, obtained 13 weeks after injection, revealed a strong necrotic effect. This was also confirmed with Azan Mallory staining (Fig.12B-C).

These results further support the possible use of BMP7v in colorectal cancer, providing evidence for its efficacy in pre-treated patients.

A



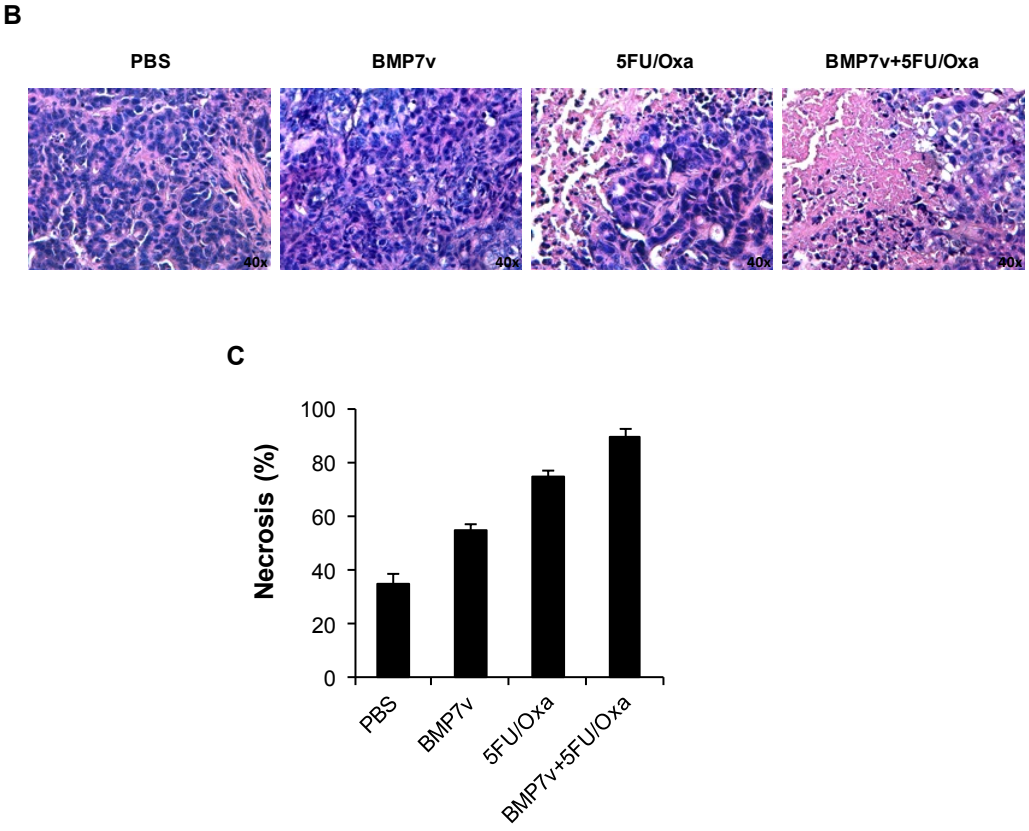


Fig.12. BMP7v shows an anti tumor activity as second line treatment in xenografts refractory to chemotherapy. A) Size of subcutaneous CRC xenografts derived from injection of sphere cells. Mice were untreated (PBS) or treated intraperitoneally with 5-FU+Oxaliplatin for two weeks. After tumor re-growth mice were treated with PBS, BMP7v (50ug/Kg), 5-FU+Oxaliplatin alone or combination. **B)** H&E staining of paraffin-embedded sections of xenografts obtained as in A. **C)** Percentage of necrosis evaluated on paraffin-embedded sections of xenografts untreated (PBS) or treated as in A.

DISCUSSION AND CONCLUSIONS

Current therapies mostly hit the differentiated cells, sparing the tumorigenic population which is responsible for the tumor relapse. Although clinical protocols are traditionally directed against the bulk of tumor cell population, increasing evidence suggests that curative therapies can be established only by targeting the subpopulations of tumor cells with tumorigenic potential. Several reports indicate that chemotherapy is more active on differentiated cells and generally ineffective against the tumorigenic population. In fact, traditional debulking agents often fail to produce long-term clinical remission and tumor relapses as a result of the inability to target cancer initiating cells (151).

The induction of differentiation affecting the self-renewal ability of CSCs represents an alternative way to inhibit tumor growth and to sensitize CSCs to conventional chemotherapy drugs.

According to recent publications, the tumor-initiating cells have stem like characteristics such as abilities of self-renewal, differentiation and invasion. Several research groups have identified tumorigenic populations with stem-like features in CRC (28-30, 73, 76, 80). These cellular subpopulation is able to promote tumor growth and is considered as an optimal cellular target to obtain effective therapies.

Many reports suggest that the balance between self-renewal and differentiation in normal stem cells results deregulated in CR-CSCs.

Indeed, the activation of Wnt signaling plays a key role in maintaining the normal stem cell population in the gut and promoting self-renewal of CR-CSCs (153-156) but also the cytokines, released in the microenvironment, contribute significantly to maintain the undifferentiated status and clonogenic activity of the tumorigenic cells (157).

Thus, the differentiation therapy could represent a considerable therapeutic option for the treatment of colon cancer, inhibiting CSCs self-renewal ability and eradicating the tumor-driving cell population. Although this approach does not directly kill the cancer cells, it could make the conventional therapies more effective in the eradication of the tumor bulk.

For years retinoic acid has been used in the treatment and differentiation of promyelocytic leukemia, thus validating this concept (158). The pro-differentiative effect of Bone Morphogenetic Protein 4 (BMP4) has been already proposed as a therapeutic option for human glioblastoma and colorectal cancer (147, 158-160)

Moreover these treatments enhanced the antitumor activity of chemotherapeutic drugs, whose concomitant administration is able to induce a complete therapeutic response, also after treatment interruption. Thus supporting the combined use of differentiative and cytotoxic agents for cancer therapy. Accordingly, the induction of differentiation, affecting the self-renewal ability of CSCs, represents an alternative way to inhibit tumor growth and to sensitize CSCs to conventional chemotherapy.

On the basis of these data, this work proposed to investigate the role of a stable BMP7 variant (BMP7v) on CSCs purified from CRC sample, in order to make these cells more sensitive to conventional chemotherapy drugs and to develop a new treatment protocol easily tested in preclinical models to design a future appropriate clinical trials.

This study identifies, the use of BMP7v, as new potential therapeutic approach that activates CRC differentiation program. As previously described, the pro-differentiation activity of BMPs, is perfectly detectable in normal gut in which these molecules are expressed following a decreasing gradient from the intestine lumen up to the crypt, thus limiting the stem cell expansion at the bottom of the crypts promoting the intestinal epithelial cell differentiation along the upper part of the crypts (113, 114).

The present data show that all CRC specimens analysed, widely express BMP7, compared to their normal counterpart and its expression is limited to the differentiated progeny of CRC epithelial cells, which constitute the major population of the tumor mass. Here we demonstrated that BMP7 is localized along the upper part of the crypt, with the exception of the base of the crypt where stem cells reside. On the contrary, its expression is undetectable in the $CD133^{+}$ CR-CSCs fraction. BMP7 results exclusively expressed by $CD133^{-}$ cells or in their differentiated counterpart, the sphere-derived adherent cells (SDACs). Although both CRC sphere cells and SDACs express BMPR1A, BMPR1B and

BMPR2. These results suggest a correlation between BMP7 expression and differentiation of CRC cells, and therefore a possible role in CSCs differentiation.

The activation of the BMP signalling pathway was confirmed by the prevalent p-Smad1,5,8 nuclear translocation following BMP7v treatment, as confirmed by analysis of downstream protein targets. Accordingly, BMP7v treatment increased PTEN levels inhibiting PI3K/AKT survival pathway confirming its differentiative role on CR-CSCs (161).

BMP7v treatment induced a rapid differentiation of CR-CSCs, morphologically evaluated by plastic adherence and acquisition of the typical differentiated phenotype, into large and polygonal colonic cells. Consequently, this treatment on CR-CSCs reduced the percentage of $CD133^{+}$ subpopulation increasing the CK20 expression, a typical markers of epithelial colonic differentiation.

BMP7v, also reduce the sphere forming capacity, and maintain this capacity also after a first step of treatment, showing that this second treatment reduces the colony forming efficiency even more drastically. Moreover BMP7v not only decreases CR-CSCs self-renewal but also potentiates the effect of standard chemotherapy.

Taken together, these *in vitro* results indicate that this cytokine drives the different cell subpopulations present in sphere cultures to exit from the quiescent state, characteristic of stem cells, thus making them more sensitive to standard chemotherapies, as showed by cell cycle analysis. The BMP7v treatment in fact increased the percentage of cells in the G2/M phase, reducing the cells in G0/G1, confirming that BMP7v treatment is able to reduce CR-CSCs stemness trait.

According to these data the treatment reduces CR-CSC motility and invasiveness, underlining the link between CSCs, epithelial-mesenchymal transition (EMT) and metastasis, as showed by the analysis of putative EMT markers: BMP7v suppressed the mesenchymal trait, inducing E-cadherin expression and cytoplasmic localization of beta-catenin, reducing Vimentin expression.

To confirm the correlation between BMP and Wnt pathways suggested by western blotting analysis a Wnt signaling trascriptional profile was performed on CR-CSCs after 48 hours of BMP7v treatment: BMP7v induced up-regulation of Wnt inhibitors such as SFRP2 and Axin2, associated with down regulation of stem cell markers, such as SOX2 e Nanog. In addition, the treatment reduced expression of

migration regulator genes, such as Met and MMP2 and 7. These results confirm the induction of differentiation *via* the Wnt pathway.

The clinical results obtained in leukemia, sustain the combination of a pro-differentiation agent with chemotherapy, suggesting the synergic action of BMP7v in combination with conventional drugs used in the management of CRC disease. BMP7v in combination with standard chemotherapy reduced CR-CSCs tumor growth *in vivo* as showed by strong reduction of tumor size, and by lower expression of CD133, CD166, beta-catenin, Lgr5 and Ki67, with a significant increase of CK20 compared to control, suggesting anti-proliferative and pro-differentiative effects *in vivo.*

Our findings confirm the rationale for combining a pro-differentiation agent with chemotherapy drugs, supporting the use of BMP7v in colorectal cancer patients.

Since BMP7v treatment resulted in a notable necrotic effect, paraffin embedded xenografts sections were analyzed for CD31 and VEGFR2 expression.

The histological examination showed a significant reduction of human CD31 and VEGFR2 expression following BMP7v treatment, but not BMP4 treatment. This anti-angiogenetic effect was confirmed through an endothelial tube formation assay. These findings confirm a specific anti-angiogenic effect of BMP7v in colorectal cancer xenografts

BMP7v also showed a significant anti-tumor activity in xenografts refractory to chemotherapy, revealing a strong necrotic effect and a greater efficacy compared to combined treatment in tumor xenografts previously treated with standard chemotherapy.

These results also support the possible use of BMP7v in colorectal cancer, providing evidence for its efficacy also in pre-treated patients.

Concluding, the clinical benefit obtained by the combination of a prodifferentiative agent with chemotherapy led us to propose the combination of BMP7v with current standard chemotherapy regimens for CRC further supporting the usefulness of CSCs differentiation as a CRC therapy.

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ABSTRACT

Cancer stem cells (CSCs), characterized by high levels of ATP-binding cassette, anti-apoptotic molecules, active DNA-repair and slow replication capacities, surviving to conventional anti-cancer therapies, able to eradicate only the highly proliferating tumor cells, represent the elective target for new therapies. Colorectal CSCs (CR-CSCs) represent a powerful tool for preclinical validation of target therapies. In particular the elucidation of the mechanisms that govern stem cell survival and differentiation appears very essential for the identification of new molecular targets in cancer therapy. Among the molecules that govern these processes there are the Bone Morphogenetic Proteins (BMPs), members of the TGF-b superfamily. Here we propose that a BMP7 variant (BMP7v) have an important antitumoral and anti angiogenetic effect on CR-CSCs inducing a differentiation program and making these cells more sensitive to conventional chemotherapy drugs. BMP7v *in vitro* administration, activates the BMP signaling pathway in CR-CSCs, reducing the percentage of stem cell marker expression and enhancing epithelial colonic differentiation marker expression. BMP7v reduces self-renewal of CR-CSCs inducing their exit from quiescence and, reducing their typical mesenchymal trait, decreases their invasive and endothelial cord formation capacity. *In vivo*, BMP7v decreases tumor growth and stem cell marker expression, enhancing differentiation compared with control mice and in combination with CRC standard chemotherapy reduces tumor growth, inducing a differentiative and antiproliferative effect, associated with a strong anti-angiogenic role. In addition, BMP7v as second-line of treatment also showed a significant anti-tumor activity in xenografts refractory to chemotherapy. Our data support the use of BMP7v as differentiative agent in combination with cytotoxic drugs for the treatment of CRC, and the use of BMP7v provides a potentially powerful and novel approach for the treatment of tumor disease.

INTRODUCTION

Colorectal cancer and stem cell theory

The colorectal cancer (CRC) is one of the most common cancers in Western countries. It represent the third form of cancer for frequency and the second leading cause of cancer death due to the resistance to current clinical therapies in the world. (1).

Nowadays, the most important approaches, for the managment of this complex pathology remain the prevention and the early diagnosis, although a large numbers of patients after surgery and adjuvant therapy still develop recurrences and metastasis, due to the acquisition of resistance to conventional therapy, such as chemo- and radio-therapy (2).

The colorectal cancer represent a classic example of a multistep pathogenesis, characterized by the acquisition of aberrant function of a proto-oncogene or loss of function of a tumor suppressor gene (3). Many studies showed that at least 4–5 mutations are necessary to generate an invasive carcinoma (4). Some of these mutations seem to follow a constant trend, within the same sequences, and they are shared by many patients affected by colon-rectal cancer, unlike, other different mutations are individuals and therefore necessary to determine the final phenotype of disease (5). Many evidences on colon cancer mutations derived from studies on hereditary forms, representing 5% to 10% of all colon cancer cases. In particular, Familial adenomatous polyposis (FAP) is an autosomal dominant CRC syndrome caused by a mutation in the APC (adenomatous polyposis coli) gene which characterizes multiple CRC (6-8).

In 1990, Fearon and Volgestein proposed a genetic model of colorectal carcinogenesis based on the accumulation of genetic mutations that occur in sequence, defining a particular staging of tumor development (9).

The main event which characterizes the onset of CRC is represented by mutations-inactivating the gene APC that lead to hyperproliferation of the normal intestinal epithelium with the formation of adenomas class I (early adenoma). In fact, the APC mutations are reported as the initiating gatekeeper that regulate positively the Wnt pathway in patients with FAP (10). The key role of APC

protein is represented by the modulation the cytoplasmic levels of beta-catenin, a protein that migrating into the nucleus activates the transcription of genes involved in the regulation of proliferation, differentiation, migration and apoptosis (11).

The progression from early adenoma towards the stage of intermediate adenoma is related to the acquisition of B-RAF and K-RAS mutations. These mutations, mutually exclusive, determine the constitutive activation of the Ras-Raf-MAPK protein signaling pathway.

The loss of heterozygosis involving the chromosome 18q, the mutations in SMAD4 (*Small Mother against DPP homolog 4*), CDC4 (C*ell Division Cycle 4*) and DCC (*Delected in Colorectal Cancer*) or alternatively mismatch repair deficiency, P53, Bax and IGFR2 (insulin-like growth factor receptor 2) are involved in the transition to advanced adenoma (adenoma late) (4, 12, 13).

Finally, a key event in the transition from advanced adenoma to carcinoma is represented by acquisition of mutations in one of the most important tumor suppressor genes, TP53. It is a powerful transcription factor, able to maintain the integrity of the genome through the regulation of the expression of more than 300 genes involved in various cellular processes such as apoptosis, cell cycle arrest, senescence and DNA repair.

The tumor suppressor gene TP53 is mutated in about 95 % of human cancers of various origins. Cancer cells that are non-functional TP53 have a substantial advantage in growth, since they can proliferate actively, even under conditions of stress or damage to the genome, developing resistance to apoptosis.

Finally, the accumulation of additional mutations, many of which are still not known, induce the transformation in metastatic carcinoma (14). Fig.1.

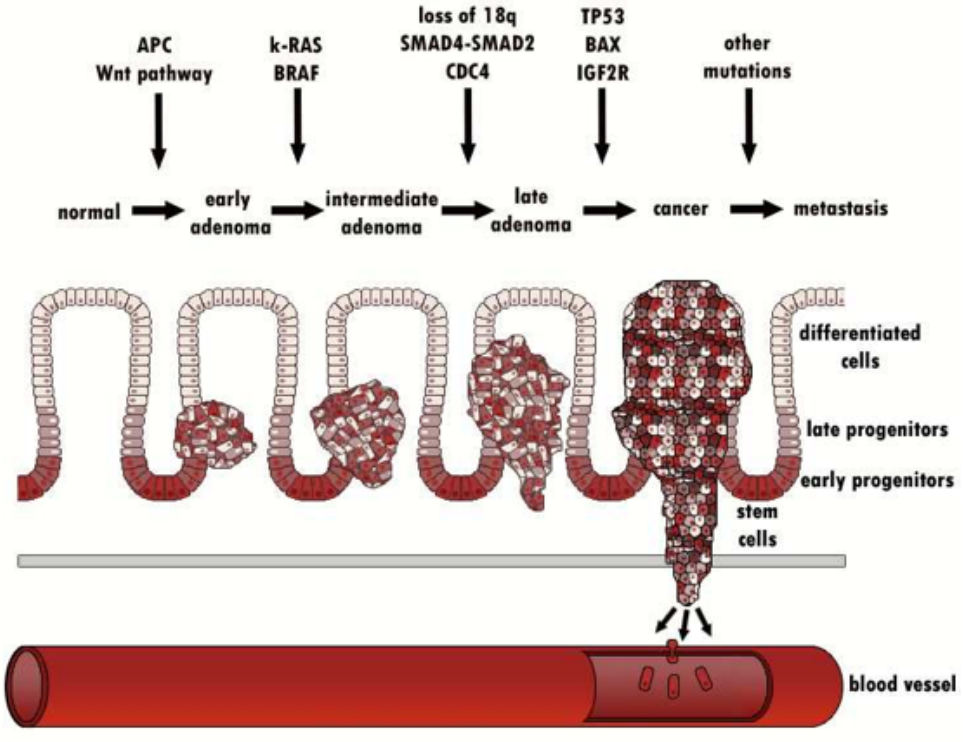


Fig.1. Schematic representation of colorectal cancer multistep progression. (Di Franco S. et al., Encyclopedia of Molecular Cell Biology and Molecular Medicine, Wiley.)

In the last decades the tumor biology has revolutionized the old view of tumourigenesis. CRC, as the other tumors, have long been consider as an exclusively genetic disorder. Nowadays, several studies showed that tumors are constitute by a highly heterogeneous population of tumor cells which differ in morphology, marker expression, proliferation capacity and tumorigenicity. To better describe the role of the different malignant cells within the same tumor, and to explain this morphological, proliferative and functional heterogeneity, two models have been proposed: the stochastic and hierarchical models.

The first model, described by Nowell in 1976 (15) proposed that all cells within a tumor are biologically homogenous and able to regenerate the tumor (16). This model of tumorigenesis, in fact, describes the tumor formation as a process multistep due to the sequential accumulation of mutations in oncogenes and tumor suppressor genes. Accordingly, all cells within the same tumor are able to initiate a new tumors, but this theory does not consider the high cellular heterogeneity, the chemoresistance, the minimal residual disease and the tumor recurrence. In sum, tumors consist of a heterogeneous cell population that, acquiring new mutations, undergoes uncontrolled proliferation and invasivity.

Otherwise, the hierarchical model, considering all the factors intrinsic and extrinsic involved in defining cell behavior, such as, genomic instability, levels of transcription factors, signalling pathways, microenvironment and immune response, is based on the analysis of the high cell heterogeneity within the tumor in terms of features, surface markers expression, proliferation kinetics and tumor initiation capacity (17).

This model suggest that only a subset of tumor cells within the tumor mass, called Cancer Initiating Cells or Cancer Stem Cells (CSCs), can initiate and sustain tumor growth (18). These cells possess the tumorigenic and self-renewal capacity, and the ability to differentiate in non-self renewing cells, that acquiring proliferative capacity, constitute the tumor bulk (19). Fig.2.

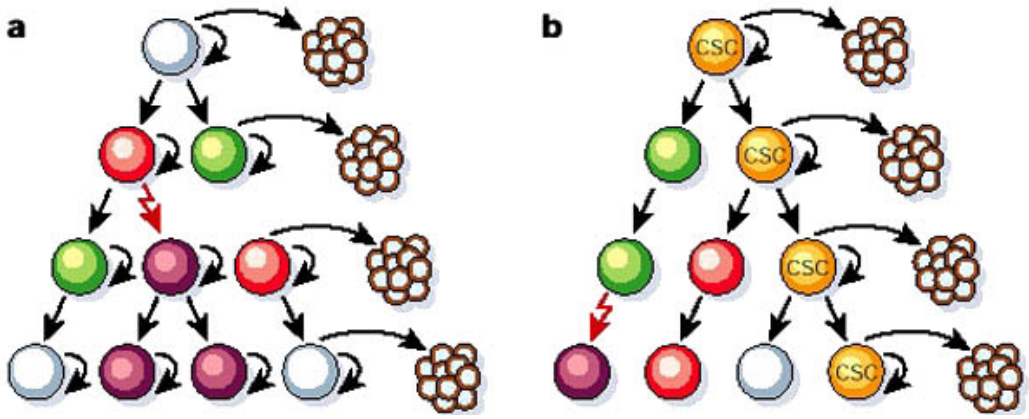


Fig.2. Two general models of heterogeneity in solid cancer cells: a) stochastic and b) hierarchical models. (Reya T. et al. Stem cells, cancer, and cancer stem cells, Nature 2001)

In the last years, novel insights in cancer research have suggested that the capacity to initiate and sustain tumor growth is a unique characteristic of this small subset of cancer cells with stemness properties within the tumor mass, called "cancer stem cells" (CSCs) or "tumor-initiating cells", that have the capacity to propagate the tumor upon transplantation into immuno-compromised mice (19).

CSCs are defined by their stem cell-like features that share with the normal stem cells that are characterized by self-renewal and pluripotent differentiation capacity. These cells are responsible to generate, through several cycles of division, progenitor cells which give rise to non-tumorigenic differentiated population that represent most part of the tumour mass. CSCs could derive either from self-renewing normal stem cells (SCs) that acquire epigenetic and genetic

changes required for tumorigenicity or from proliferative progenitor cells (PCs) that reprogramming themself acquire the self-renewal potential capacity (20).

Emerging evidences suggest that CSCs isolated from a variety of tumors types retaining the tumorigenic capacity are responsible for the propagation, relapse and metastatic dissemination. CSCs can explain the phenomenon of the tumour chemoresistance in which several mutations confer to these cells drug-resistance, altered cell cycle checkpoints and impaired apoptosis machinery. For all these reasons, CSCs survive to conventional treatments giving often rise to minimal residual disease (MRD). Therefore to better understand the mechanisms that maintains stemness features and the subsequent characterization of CSCs could be crucial to develop new most appropriate anti tumor strategy approaches.

Common signaling pathways, including Wnt, Notch and Sonic Hedgehog are involved in the regulation of normal and cancer stem cell. Many evidences underlining the importance of these cellular signalling showed as their deregulation plays an key role in the tumor development (21). Accordingly, several studies suggest the importance of self renewal pathways activation for CSCs maintainance (22).

The CSCs theory has changed the conventional therapeutic approches, suggesting an alternative strategy targeted to these cellular subset. The CSCs are characterized by high resistance to conventional chemotherapeutic drugs that kill the rapidly proliferating cells sparing the slow dividing cells, through a particular upregulation of ATP-binding cassette transporters, active DNA-repair capaciy and overespression of antiapoptotic molecules (23, 24).

Dick and collegues were the first to isolate and characterize CSCs from acute myeloid leukemia (AML) in blood and bone marrow. In particular they isolated a sub-population of $CD34^{+}CD38^{-}$ from patients affected by AML, and they demonstrated that only this cellular subset was able to form colonies *in vitro* experiments. They also analized that only this subset was able to reproduce the parental tumor phenotype, when inoculated into immunodeficient mice (25, 26).

Using a similar procedure many research groups identified a large number of tumor stem cell from a different solid tumor type. In particular, the first CSCs obtained from a solid tumor were a cellular subset $CD44^{+}CD24^{-}$ isolated from breast cancer by Al Hajj et coll. (16). Subsequently, were isolate several different

CSCs tumoral type: brain (27), colon (28-30), head and neck (31), pancreas (32, 33), melanoma (34), mesenchymal (35), hepatic (36), lung (37), prostate (38), and ovarian (39) tumors.

Colon crypts and stem cells

Although the SCs and CSCs characterization has been long studied, several molecules have been identified as a putative stemness markers, up to now, none of the markers studied seems to be exhaustive. Scientific evidences underline the importance to use different combinations of these markers in order to obtain a cell population enriched in stem cells.

The adult intestinal epithelium presents a particular structure ordered into crypts and villi, organized with a hierarchical organization, composed by three different cell types: the colonocytes or columnar cell, the mucin-secreting goblet cells and the endocrine cells, originated from a colonic stem cell. These stem cell, located at the base of the crypt are surrounded by mesenchymal cells that form the stem niche, in which the stem cell, displaying stemness features, can generate through asymmetric division, a cell identical to itself , and a transit cell. The transit cell (rapidly dividing cells) proliferating and differentiating, migrates along the crypt, representing all the intestinal lineage (40-45). According to this theory these stem cells are responsible for the high turn over rate of the colonic epithelial cells (46-48).

This particular and complex structure of the colon crypts has made particular difficult the studies about the mechanisms of crypt formation from a single stem cell. The first study regarding the stem cell position in the colon was conducted by Chang et al. using ^{3}H-thymidine injection, and recently it was confirmed using bromodeoxyuridine DNA-labeling dye (49, 50).

Two different models have been proposed regarding the positioning of the stem cells: the “stem cell zone” model and the “ +4 position” model. According to the first model, the colon stem cell reside at the very bottom of the crypts. Unlike, the second model describes that the stem cells are located at the +4 position above the Paneth cells at the base of the crypts (51).

Although the absence of a specific colonic stem cell markers makes their

identification and positioning very difficult, the colonic stem cell can be characterized by two main features: self-renewal and differentiation capacity. Stem cells may undergo asymmetric division, but they can also generate two identical stem cells via symmetric division that acquiring a diffentiated phenotype regenerate the colon tissue. Then, in the first case, it is possible to obtain a lineage expansion, but in the other case, a lineage extinction, because the differentiated progeny undergo to senescence.

Accordingly with the CSCs theory it is widely accepted that the stem cells are responsible for the origine of the cancer. Their slow division cycle, allow them to accumulate several mutations over time up to define them CSCs (52-54).

Different molecules have been proposed as a stemness markers: Musashi 1(Msi1), EphB receptors, Bmi 1, Lgr5.

Msi1, an RNA binding protein, widely studied in Drosophila Melanogaster, seems to be involved in the asymmetric division that regulate the neural development, also in mammals. In human and murine small intestine it is located at the base of the crypts. Its silencing determines tumor growth arrest by Notch inhibition and p21 upregulation, proteins involved in stemness mainteinance (55-57).

EphB2 receptor is tyrosine kinase receptor, belonging to the family of Wnt target gene, it is expressed in a decresing gradient from the crypt base toward the differentiated cell compartment (58). This expression along the crypts seems regulate the migration and proliferation of intestinal epithelium; mutants in their ligands, or mutant forms of these receptors involve in intestinal compartimentalization defects. Underlining their important role in the intestinal positioning of the different cell types along the crypts. (59).

Bmi1, a repressor of Polycomb group, is involved in hematopoietic, breast and neural self renewal. In the small intestine it is expressed near to the bottom of the crypts (60, 61).

Lgr5 is a G protein coupled receptor, belonging to the family of Wnt target gene. Its expression in a single cells is able to regenerate a crypt-like structure *in vitro* constituted by all cell type of colonic epithelium (62). This and other markers as have been associated with CSCs phenotype.

CR-CSCs identification, isolation and expansion

The existence of colorectal CSCs (CR-CSCs), have been showed, for the first time, thanks to the detection of a new stemness markers, the transmembrane glycoprotein, CD133. This surface expressed polypeptide is associated with self renewal and tumor initiating cells, this protein was first associated as a marker for hematopoietic stem cells and progenitor cells, and successively used in other tumor type: (63): brain (27), prostate, hepatocellular and colon tumors (28-30, 64, 65).

Recently two indipendent groups have revealed that only $CD133^+$ subset of tumor cells within a colon carcinoma, is able to initiate a tumor outgrowth (28-30).

Accordingly, it has been showed that a small group of $CD133^+$ cells is able to serially reproduce the original human tumor phenotype, rather than an high number of $CD133^-$ cells that fail to generate xenograft tumors in immuno-compromised mice. In line with these data, only the tumorigenic $CD133^+$ cells population generated crypt-like structures *in vitro* under differentiation condition on matrigel (65). In addiction, these cells during differentiation *in vitro* and *in vivo* acquire a typical epithelial colonic marker, CK20, reducing at the same time the CD133 stem cell marker.

Accordingly, many clinical reports suggest CD133 as an indipendent prognostic marker and its combination with a nuclear localization of beta catenin is associated with a reduced patients survival (66-69).

In sum, several research groups demonstrated that only the $CD133^+$ cellular sub-population, within a colon carcinoma, is able to initiate and sustain tumor growth (68-70).

Moreover, mantaining the $CD133^+$ cells with the same conditions of neurospheres, these cells were expansed for long term without loss their ability to reproduce human original tumor phenotype, underlining the self renewal and tumor initiation capacity of these sub population (28, 29).

O'Brien and collegues, in order to evaluate whether all the $CD133^+$ cells are CSCs or whether these subpopulation contains also more differentiated progenitors, through serial dilution assays, showed that the $CD133^+$ subpopulation not only contains cancer initiating cells.

Accordingly, recent studies analyzed a new sub-group, contained in $CD133^+$ cells, the $CD44^{+/}$Epcam High/$CD166^+$ stem like cells. Dalerba et al., (30) demonstrated that the $CD44^+$/Epcam high cell subpopulation injected in NOD-SCID mice, is able to reproduce a tumor xenograft phenotypically similar to parental one.

In a similar way, Du's et al., (70) showed that also CD44 could be considered a putative marker, able to discriminate a subpopulation capable to growth *in vitro* as spheres and *in vivo* producing xenografts, resembling the parental human tumor.

The role of CD133 as a marker of stem cell has long been debated, in particular, after the Shmelkov et al. publication, in which it was shown that CD133 is expressed ubiquitously in both undifferentiated and mature colonic cells (71). While the lack of CD133 expression is only found in the stromal and inflammatory cell compartment.

Differently, Zhu et al. analizing the role of Prominin1, the mouse analogue of CD133, in adult colon tissue, showed that the $Prominin1^+$ cells marking the adult colon stem cells, represent the target of tumoral trasformation. (72).

On the bases of these conflicting data, it became clear that the use of a single marker is not adequate for a correct CSCs identification and isolation, to identify the subpopulation of CSCs, it would be more appropriate to use a panel of markers and to standardize protocols that can validate the use of a new marker. For all these reasons, several surface molecules have been proposed to mark colon CSCs, such as CD133, LGR5, CD44 and CD166. (73, 28-30).

Recently, it has been demostrated that undifferentiated tumorigenic CRC cells could be expanded as tumor spheres *in vitro* using a serum-free medium containing EGF and basic FGF (74). The tumor spheres, infact, contain an heterogeneous cell population expressing a variable percentage of CD133, CD166, CD44, CD29, CD24 and nuclear beta-catenin. More recently ALDH1, a detoxifying enzyme, has been proposed as a specific marker able to identify, isolate and track human normal and CSCs during CRC development (75, 76). This marker is expressed by the $CD44^+$ or $CD133^+$ cells, located at the base of normal crypts. After sorting the ALDH1 high cells, injected subcutaneously in NOD SCID mice, generate tumor xenografts (76).

The isolation of CSCs is based essentially on two different approaches: the use of a culture medium serum-free that maintain stemness-selective conditions, originally developed for neural stem cell culture (77) or the direct selection by magnetic sorting or FACS technology for putative markers distinctively expressed by a cell subpopulation. On the bases of these procedures there is a common step in which the surgical excisions of solid tumours are processed by mechanical and/or enzymatic digestion to obtain a single-cell suspension. This freshly digest obtained is constitute by an cells heterogeneous mixture of the original tissue. In order to selectively obtain CSCs, the digest is cultured into ultra-low-adhesion flasks in a specific serum free medium, supplemented with growth factors such as basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF). These conditions allow the undifferentiated tumour cells, stem and transit amplifying cells, to survive and slowly proliferate, while the differentiated cells die through anoikis. Sphere-forming cells are maintained in culture by dissociation and re-plating as single cells, and retain the tumorigenic potential when injected into immunocompromised mice, reproducing the same morphological and antigenic features of the original tumour, data showed by histological examination of xenografts generated from spheres. This tumorigenic capacity is serially maintained, and the xenografts can be digested and the cells obtained can be indefinitely propagate under stemness conditions, maintaining the stem like features as well as the capacity to reform tumors. As previously described, a single clonogenic $CD133^+$ cell, contained in CRC spheres is able to reproduce the original tumor, including the CSC compartment and the differentiated progeny.

Alternatively, the second procedure, involves the selection of putative stem cell marker from the whole digest, by the use of monoclonal antibody directly or indirectly conjugated with magnetic beads. This sorting allow a double positive and negative selection, separating labelled and unlabelled cells, but this procedure permit only a single-marker selection. Another alternative method is represented by FACS sorting, which allows sorting of different populations with multi parameter analysis, testing contemporary several antibodies conjugated to different fluorochromes.

Moreover the sorted cells can be also cultivated in stem cell conditions and also injected into immunocompromised mice to directly test the tumorigenic capacity (74).

Intestinal Niche

Fibroblast, endothelium, inflammatory cells, cytokines and growth factors secreted by these cells, constitute the intestinal niche and are involved in CSCs maintenance at the base of colon crypts. They finely regulate the balance between self-renewal and differentiation (78-80). Indeed the pluripotency of colon SCs is strongly controlled by microenvironment that plays a crucial role ensuring a fine equilibrium between important pathways, such as PTEN-PI3K-Akt (81, 82), Bone Morphogenetic Protein (BMP) (83), Notch (84) and Sonic hedgehog (Shh) (85).

In particular the intestinal sub-epithelial myofibroblast (ISEMFs) surrounding the normal colon stem cells, regulate this balance secreting hepatocyte growth factor (HGF) for maintenance of stem cell in the intestinal niche. The presence of ISEMFs or HGF, as demonstrated by Vermulen et al., restore the stem cell phenotype, inducing a cellular de-differentiation (80).

The colon crypt unit represented in figure 3, shows a particular cellular distribution in which at the basis of the crypt are located the mesenchymal cells (ISEMFs) and their secreted factors responsible for the stem cell niche maintenance, while along the villux apex, where are located several Wnt inhibitors, in order to reduce the stemness features it is possible to identify a progressive cellular differentiation. The pathways mentioned above, are involved in self renewal and are de-regulated both in normal and CSCs (21, 22).

One of the most studied pathway envolved in CSCs progression is represented by the Wnt signalling.

The Wnt signalling in the basis of the crypts promotes nuclear beta catenin accumulation, which activate the transcription of several genes involved in cell cycle regulation and proliferation. Beta catenin also induces the expression of EphB1-2, which regulate stemness, cell migration and differentiation (86-88).

The distribution of Wnt pathway members, results particularly regulated. Wnt ligands are predominantly expressed at the basis of the crypts and are reduced along the crypt where more differentiated cells reside, and where are most express the Wnt inhibitors factors (88-90).

In addiction to Wnt pathway, BMP, Notch and Shh pathways regulate the niche homeostasis.

BMP proteins are TGF-beta superfamily members that through binding to receptors BMPR1A, BMPR1B and BMPR2 can trigger different biological processes in CSCs (91).

The activation of this pathway promotes the phosphorylation of Smad1, 5 and 8 that in association with SMAD 4 translocate into the nucleus and in cooperation with other transcriptor factors regulate the target genes expression (92, 93).

Recent data have demonstrated that the BMPs promote terminal differentiation and apoptosis (94). Kosinsky et al., analyzed the distribution of the different factors along the crypt: the cells at the apex of the crypt express high levels of BMPs, while at the basis of the crypt, their levels are reduced, but the levels of BMPs antagonist, produced by myofibroblasts, contribute to the maintenance of stemness (95, 83).

Notch pathway is envolved in intestinal SCs fate and includes four different trans-membrane receptors (Notch1 to Notch4). The binding of five different ligands (Jagged-1, -2, Delta-like 1, 2 and 4) induces the release of the Notch intracellular domain that translocates into the nucleus where it forms a complex with DNA-binding proteins activating the target genes transcription involved in epithelial cell fate determination (96). Recent reports showed its key role in intestinal homeostasis and neoplastic transformation. Moreover, the expression of Notch intracellular domain blocks cell differentiation, inducing expansion of immature progenitors (97, 84). Defects in Notch pathway were observed in colon cancer stem cells (CCSCs), using an antibody anti DLL4 (an important Notch pathway's component) to directly inhibit human colon cancer xenograft growth.

Finally, Sonic Hedgehog pathway regulate the gut organogenesis, binding to its receptor Patched (PTCH), allowing the release of a G-coupled protein Smoothened (Smo), which with the GLI transcriptors factors migrate into the nucleus inducing target gene activation (85)

In several cancers, such as leukemia, pancreas, stomach, prostate, breast, glioblastoma and colon cancer were found many alterations in the Hedgehog pathway (98-103).

In conclusion, the aberrant alteration of these pathways involved in self-renewal of the intestinal stem cells could be the driving force that promote colon cancer. (104)

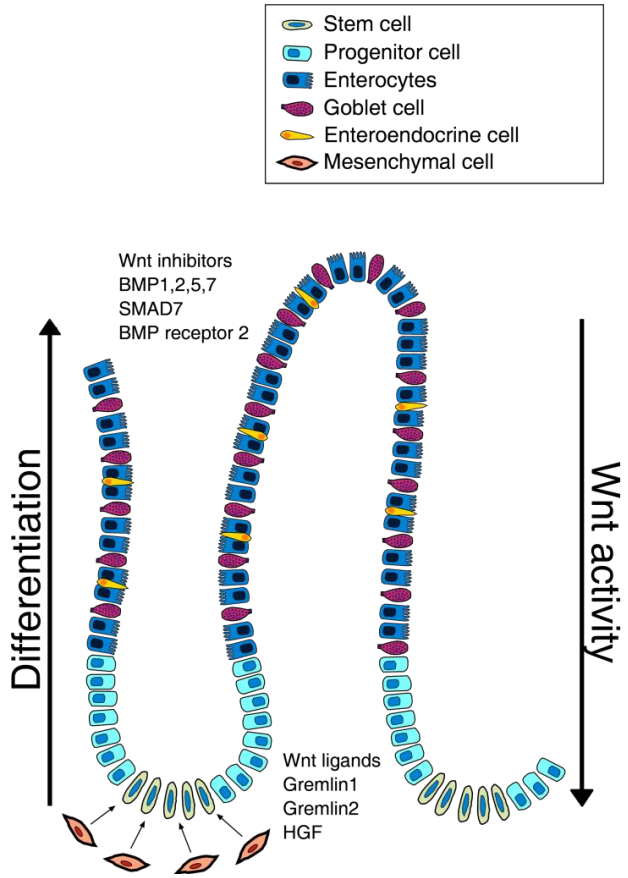


Fig.3. Graphic representation of a colon crypt. (Di Franco S. et al. Colon Cancer Stem Cells: Bench-to-Bedside-New Therapeutical Approaches in Clinical Oncology for Disease Breakdown. Cancers 2011).

Clinical implication of CR-CSCs and BMPs as alternative therapeutic strategy

The CSCs theory, in colorectal cancer, has exciting clinical implications, confirming that the therapy failure and relapses are due to the CSCs resistance. Indeed, CSCs, retaining the stemness features, such as quiescence, self-renewal ability and multidrug resistance, represent the population intrinsically refractoriness to conventional therapies developed to eradicate the rapidly dividing cells that constitute the majority of the non stem cell component within the tumor.

Nowadays, patients with metastatic CRC are treated with two useful protocols FOLFOX (Folinic acid/Fluorouracil and Oxaliplatin) and FOLFIRI (FOLFOX plus vitamin B and irinotecan). Neoadjuvant chemotherapy for these patients, is represented by anti-angiogenic drugs as the Bevacizumab, a humanized monoclonal antibody that targets the vascular endothelial growth factor (VEGF), which plays an inportant role as angiogenic factor in primary and metastatic human CRC (105, 106). Another neo-adjuvant drug is Cetuximab, also known as Erbitux, a monoclonal antibody that inhibits the epidermal growth factor receptor (EGFR), indicated for the treatment of EGFR expressing patients affected by KRAS wild-type metastatic colorectal cancer, alone or in combination with FOLFIRI (107).

The future of diagnostics and treatments of tumor disease should aim to eliminate, not only the terminally differentiated component of the tumor bulk, but, it should be focused, in particular, to the subpopulation of cancer stem cells that represent the driving force for the tumor expansion (19). In order to evaluate the CR-CSCs role in the therapy response, recurrence and metastasis, a recent work showed that the subpopulation of $CD133^{+}$ CR-CSCs results more resistant to both conventional chemotherapeutic drugs, and innovative therapies, compared to epithelial cells that constitute the majority cell population within the tumor (65, 74).

These recent findings support the idea that tumor-initiating cells are highly resistant to cytotoxic cancer therapies, underlining the importance of their role in colorectal tumors refractoriness and recurrence also many years after the "successful" treatment of primary tumour.

The CSCs contribute to the poor therapeutic sensitivity through many mechanisms, such as preferential activation of DNA damage checkpoint, high levels expression of ABC transporters and anti-apoptotic molecules, slow replication capacity and other aberrant molecular mechanisms that destroy the normal balance between proliferation and survival or cell death (23).

In addition the CSCs refractoriness is finely regulated by microenvironmental soluble molecules that are involved in many different sectors of tumor development, regulating the growth, migration, and differentiation of all cellular components into both the tumor mass and in the microenvironment.

The possibility to isolate and study CSCs represents a revolutionary approach in cancer research and these cells represent the elective target for new therapies, endowed to high and selective toxicity towards the specific tumor with reduced toxicity for normal cells.

An alternative therapeutic strategy seems to be represented by selectively target of CSCs pathways .

Recent reports have demonstrated that the autocrine production of IL-4 by cancer cells from breast, thyroid, colon, and lung acts as negative regulator of apoptosis, conferring resistance to death receptors and chemotherapy-induced cell death (108). Moreover IL-4 seem to be involved in stimulation of activated B-cell, in T-cell proliferation and the differentiation of $CD4^+$ T-cells into Th2 cells (109). The use of anti–IL-4 neutralizing antibody or IL-4 receptor α antagonist, on CR-CSCs, inhibiting IL-4 signaling transduction pathway, sensitizes these cells to chemotherapeutic agents through down-regulation of anti-apoptotic proteins, such as cFLIP, Bcl-xL, and PED. Moreover, the combined use of IL-4 antibodies plus 5-fluorouracil or oxaliplatin also reduces xenografts tumor growth (65). Concluding, recent studies have evaluated that the CR-CSCs $CD133^+$, are protected by apoptosis phenomenon through the up-regulation of IL-4 (110).

Another important approach could be represented by cancer immunotherapy. Although the cancer cells are less immunogenic than their normal counterpart, the immune system could plays a crucial role in order to effectively and safely increase antitumor responses recognizing and eliminating them (111). Although, these cells do not express MHC molecules, making tumor cells resistant to αβ T cell-mediated cytotoxicity, recently, it has been demonstrated that a subpopulation

of T cells, the γδ T cells, show potent MHC-unrestricted lytic activity versus different tumor cells *in vitro*, suggesting their potential employment in anticancer therapy. γδ T cells have been isolated and identified from tumor infiltrating lymphocytes in different cancer types.

The development of targeted therapies for colorectal cancer requires new therapeutic regiments that aims to eliminate the self-renewal compartment of tumor mass by targeting stemness features owned by CR-CSCs, making this population more sensitive to conventional drugs. This is the main goal that the differentiation therapy aims to achieve.

The therapy based on the induction of differentiation is aimed to affects the self renewal ability of CSCs, and could represent an alternative way to inhibit tumour growth.

Among the molecules that govern stem cell survival and differentiation, the Bone Morphogenetic Proteins (BMPs), a subgroup of TGF-b superfamily members, play an important role in the regulation of colon stem cell features and contribute to the network of the signals that define the intestinal stem cell niche modulating the equilibrium between proliferation and differentiation signalling pathways (112-114)

These proteins and their intracellular signaling components have been conserved in Drosophila and Caenorhabditis elegans and they were originally isolated from bone. The major contribution to their isolation and characterization was made by Sampath and Reddi (115).

BMPs are synthesized as large precursor proteins in the cytoplasm where they are proteolytically processed in a mature proteins (116). The mature BMP molecules are characterized by a cysteine knot with the seven conserved cysteine domains. The dimeric form is active as a homodimer or heterodimer with a molecular weight of about 30-38 kDa.

BMPs bind to two different transmembrane serine/threonine kinase receptors, type I (BMPRI) and type II (BMPRII). Type I receptors include activin receptor type IA (ActRIA or ALK2) and BMP receptors type IA and IB (BMPRIA or ALK3; BMPRIB or ALK6), while type II receptors are represent by BMP receptor type II (BMPRII), activin receptor type IIA and IIB (ActRIIA and ActRIIB) (117, 118). After the formation of heteromeric complexes, type II

receptor phosphorylates the type I receptor (119) and the signalling pathway is then activated through Smad and non-Smad mechanism (117, 119). In addition to the Smad pathway, indeed, the BMPs activate an alternative pathway, which includes p38 and ERK MAP kinases (120). When Smad 1,5,8 protein (R-Smad) are phyosphorylated by the type I receptors, they can interact with co-Smad (Smad4) and after translocate into the nucleus in order to initiate the transcription of BMPs response genes (121).

The Inhibitory Smads, Smad 6/7, inhibits BMP signaling (122), indeed, BMPs activation is tightly regulated by the presence or the absence of antagonists and inhibitors such as Gremlin, Chordin and Noggin (83). BMP antagonists are soluble factors that control BMPs signaling with various degrees of affinity and specifity, binding the BMPs, preventing the functional receptor/ligand interaction: twisted gastrulation (TSG), chordin and noggin and the DAN-family of inhibitors.

BMPs are involved in the earliest stage of development, and play a key role in the normal intestinal development, growth and morphogenesis. Their signalling act to mediator between epithelial and mesenchymal stroma interaction necessary for the correct intestinal homeostasis (123).

During the human and mouse intestine development, BMP7 and BMP6 were found, respectively, on the intestinal epithelium and smooth muscle cells (124, 125). Moreover, the high expression of BMP2 and BMP4 in the mesenchymal site drive the villus formation (126). In particular many reports showed that the BMPs pathway is functional in all three tissue layers of the gastro-intestinal tract, to allow the reciprocal correct interaction. Mice overexpressing the BMP-antagonist noggin, undergo to abnormal villus morphogenesis, associated with stromal and epithelial hyperplasia, ectopic crypt formation, due to low levels of BMPRIA and pSmad1/5/8 (123).

All these results suggest that human gastro-intestinal and chronic intestinal diseases, could be associated with defects in the BMPs signaling pathway (113).

Hardwick et al., have demonstrated that the mature colonocytes at the epithelial surface of normal human and mouse colon, express BMP2, the BMP receptors (Ia, Ib, II), phosphorylated Smad1 and Smad4 (112). Stroma and crypt epithelium of the adult mouse intestine show an high expression of BMPRIA and BMP2 (123,

127). While, Haramis et al showed that BMP4 is expressed in stromal cells and mesenchimal cells surrounding the crypt and glands of the intestine (127, 113).

The BMPs pathway components result highly expressed in colon tops while the BMP antagonists gremlin 1, gremlin 2 and chordin-like 1, produced by myofibroblasts and smooth muscle cells, are located in basal colon crypts.

Juvenile polyposis (JP), is an autosomal dominant hamartomatous polyposis syndrome in which germline mutations of two members of the BMPs pathway are involved. The patients, having mutations or deletions in SMAD4 and BMPR1A genes (128-130) are predisposed to upper gastrointestinal and colorectal cancer, in almost half of the cases. JP patients, with SMAD4 mutations, showed a significant prevalence of gastric polyposis (131). SMAD4, known as a tumor suppressor gene in pancreatic and colon cancer, in JP acts as a susceptibility gene, a "gatekeeper", its loss of function results in polyp formation in which an important role is played by stromal inflammatory response regulating epithelial tumorigenesis (132). Homozygous SMAD4 knockout mice, develop polyps, with thickened intestinal mucosa and loss of villus architecture showing plasma cell infiltrates into the stroma, while mice with conditional SMAD4 deletion in the intestinal epithelial cells did not develop intestinal tumors (133). Although, JP patients with a germline SMAD4 mutation, showed biallelic inactivation of SMAD4 in both the epithelium and stroma, suggesting a common clonal origin (134).

Another gastro-intestinal disease, Familial adenomatous polyposis (FAP), is an autosomal dominant syndrome, characterized by hundreads of adenomatous colorectal polyps due to a deletion in the adenomatous polyposis coli (APC) gene, localized on chromosome 5q21. APC gene, as above mentioned, is involved in ubiquitine-mediated degradation of beta catenin, regulating Wnt signalling and its functional defects leads to uncontrolled cell proliferation (135). In FAP patients, an higher expression of Wnt signaling molecules, results in BMPs signaling downregulation, differently, in intestinal homeostasis, the BMPs inhibit the effects of Wnt-pathway to regulate the intestinal stem cell proliferation and repression of polyps formation (113, 127). Many results showed that heterozygous APC mutant mice, with higher expression of the BMPs antagonist Gremlin 1 and lower BMP2 and BMP4 expression develop severe polyposis and faster rate of tumor growth (136). Accordingly, human FAP tissue specimens in

dysplatic mycroadenomas epithelium do not maintain BMP2 expression (112).

The association of these genetic disorders, with the BMPs signaling pathway, was confirmed by experiments on transgenic mice, overexpressing the BMP antagonist, noggin or mice with conditional inactivation of BMPRIA. These mice showed an increased formation of intestinal polyps (113, 127). Moreover, conditional inactivation of BMPRII in stroma, led to multiple hamartomatous polyp appearance. (137, 138).

These findings indicate that altered BMPs expression plays an important role in aberrant cellular proliferation and tumorigenesis in the human intestine. During the last decade, extensive researches were conducted to explain the factors involved in the initiation and progression of colorectal cancers in particular, BMPs and their signaling pathway have been specially studied. (139, 140).

Many different groups have demonstrated that several BMPs are suppressive for colorectal cancer cells growth (112, 141, 142). Back et al. showed that BMP2 exerts growth suppression by increasing $p21^{WAF1}$ protein levels, and not its transcription, modulating RAS-ERK pathway (143).

Moreover, the loss of BMP signaling seems to be correlated to tumor progression, indeed, the BMPs expression, is lost from late adenoma to early carcinoma.

Abnormal CSCs proliferation in CRCs, is due to an abnormal activation of Wnt signalling pathway and BMPs signalling inhibition, that promote nuclear beta-catenin accumulation through PTEN downregulation and iperactivation of PI3K-Akt pathway (113). Confirming that the BMPs pathway alteration leads to pre-cancerous lesions (138).

The TGF-β signalling inactivation plays a key role in CRC development (144), indeed, it has been reported that the loss of BMPRII and SMAD4 is frequently deleted also in sporadic CRC and that BMPs pathway is inactivated in the majority of colorectal tumors (145, 146). This is strongly supported by a transgenic mice model in which the inactivation of the BMPs pathway leads to polyps formation and up-regulation of Wnt signalling (127).

Considering BMPs' role in regulating SCs differentiation and inducing apoptosis and differentiation program, it is possible to suppose that CSCs treatment with

these molecules could inducing differentiation, make these cells more sensitive to conventional chemotherapies.

Some preliminary studies have been performed on both CSCs of glioblastoma (GBM) and CRCs.

Interestingly, it has been recently demonstrated that in human glioblastoma BMP-BMPR signalling, controls the activity of normal brain stem cells, and inhibits glioblastoma stem like cells (GSLCs) (147). It has been also demonstrated that BMP2, BMP4 and BMP7 treatment inhibits sphere forming capacity and induces differentiation of $CD133^+$ cells; reducing *in vivo* glioblastoma tumor growth (147-149).

Recently, it has been demonstrated that the treatment of CR-CSCs with BMP4 induces *in vitro* differentiation and reduces their tumorigenic potential, sensitizing these cells to conventional chemotherapic drugs reducing the tumor size and inducing complete long-term regression of colon CSC-derived xenograft tumors (94).

Given the regulatory effect of BMPs on neural stem cells, their progenitors and GSLCs, recently, Tate et al. have demonstrated that BMP7 variant (BMP7v) acts on proliferation, differentiation, angiogenesis, and *in vivo* tumorigenicity of GSLCs isolated from surgical specimens of primary GBM. In particular, BMP7v, decreases proliferation of GSLCs, inducing their differentiation into neuronal- and astrocyte cellular phenotypes, and inhibites angiogenic endothelial cord formation. These results were confirmed by *in vivo* analysis of subcutaneous or orthotopic tumor models. Their data suggest that BMP7v therapy, directed against CSCs and angiogenesis, represents a potentially powerful therapeutic option that may improve the poor outcome of conventional treatments (150).

The current therapeutics strategy target and kill differentiated tumor cells that constitute the tumor bulk, failing to affect the rare cancer stem-like cell population.

These data suggest the use BMPs, as alternative therapy, to induce the differentiation of CSCs and to make them more sensitive to conventional chemotherapy. Since the differentiated tumor cells are more sensitive to conventional cancer therapies, therapy differentiative represents another possible

therapeutic strategy to inhibit tumor growth by inducing the differentiation of CSCs, making them more susceptible to the action of chemotherapeutic agents.

The induction of CSCs differentiation provides a potentially powerful and novel approach to the treatment of cancer disease.

AIMS

The high mortality rate of colorectal cancer (CRC) is mainly due to the inefficacy of standard treatments to cure the metastatic disease. The recurrence and relapse characteristic of this kind of cancer suggest that the only one curative therapy could be represented by targeting the subpopulations of tumor cells with tumorigenic potential, the so-called, cancer stem cells (CSCs) (151). Several studies report that radio-chemotherapy, directed against differentiated cells, forming the bulk of tumor cell population, are unable to eradicate the tumorigenic and metastagenic population, without obtaining a long-term clinical remission.

Accordingly, the induction of differentiation, affecting the self-renewal ability of CSCs, represents an alternative way to inhibit tumor growth and to sensitize CSCs to conventional chemotherapy. The treatment of CSCs towards terminally differentiation represent an intriguing concept for future therapy. On the basis of these data my project proposed to investigate the role of a stable BMP7 variant (BMP7v) on CR-CSCs in order to make these cells more sensitive to conventional chemotherapy drugs and to develop a new treatment protocol easily tested in preclinical models to design a future appropriate clinical trials.

MATERIALS AND METHODS

Tissue collection, isolation and culture of cancer cells

Human CRC tissues were obtained from patients undergoing to CR resection, in accordance with the ethical standards of the institutional committee. Normal colon mucosa was obtained from the histologically uninvolved resection. Histological diagnosis was based on the morphological microscopic features of carcinoma cells, determining the histological type and grade.

Surgical specimens were intensively washed in PBS solution containing antibiotics and incubated overnight in DMEM/F12 (GIBCO) containing penicillin (500 U/ml, GIBCO), streptomycin (500 μg/ml, GIBCO) and amphotericin B (1.25 μg/ml, GIBCO) in order to avoid contaminations. Tumor tissues were mechanically and enzymatically digested. Enzymatic dissociation was performed using collagenase and hyaluronidase in DMEM for 1 hour at 37° C. Dissociated CRC cells were then cultured in presence of serum-free medium supplemented with epidermal growth factor (20 ng/mL) and basic fibroblast growth factor (10 ng/mL, both from Sigma-Aldrich, St. Louis, MO 63103, USA) in non-adherent conditions, in ultralow adhesion flasks (Corning, Lowell, MA), to promote the growth of CSCs as spheres. These culture conditions allow the selection and propagation of tumour spheres, containing immature tumour cells, while non malignant or differentiated cells are negatively selected. To achieve *in vitro* differentiation of CSCs, dissociated sphere cells were cultured in DMEM-high glucose supplemented with 10% FBS in adherent conditions. These cells were conventionally indicated as sphere-derived adherent cells (SDACs). All cell cultures were carried out at 37°C in a 5% CO2 humidified incubator. Magnetic cell separation was performed on tumour cell populations obtained from enzymatic dissociation of CRC specimens using microbeads conjugated with CD133/1 (AC133, mouse IgG1, cell isolation kit, Miltenyi). After magnetic sorting, viability was assessed using trypan blue exclusion. Quality of sorting was verified by flow cytometry with an antibody against CD133/2 (293C3-PE, mouse IgG1, Miltenyi) on both $CD133^+$ and $CD133^-$ depleted cell population.

Cell death was evaluated by orange acridine/ethidium bromide staining or by CellTiter Glo Assay Kit (Promega) accordingly to manufacturer's instruction.

BMP7v used for this work was produced by Eli Lilly and Company. The pharmaceutical development of BMP7v represented the major limitation with its suboptimal solubility at neutral pH. Mutations into the N terminus of the BMP7v prodomain were introduced to enhance the cleavage of prodomain and mature domain. Five point mutations were addicted onto the surface of the mature domain, through a random mutagenesis approach, to create a molecule that retained the same signaling properties of wild-type BMP7 but had greater expression and enhanced biophysical properties such as solubility and stability. BMP7v material can be made available to researchers upon request to the Lilly authors (152).

In order to detect the proportion of differentiated and undifferentiated cells, dissociated spheres were cultured in stem cell medium in presence of BMP7v (100ng/ml) up to 18 days. At different time points, the adherent cells were harvested with trypsin and mixed with floating cells. The cell mixture was then cytospun and stained for CK20 and CD133.

To evaluate BMP7v role in differentiation *in vitro*, the spheres were dissociated into single cells and cultured in the presence of BMP7v (100ng/ml) for 48 hours or 90 minutes in order to evaluate the p-Smad 1, 5, 8 nuclear traslocation.

Histochemistry and Immunohistochemistry /Immunofluorescence

Histochemical and immunohistochemical/immunofluorescence analyses were performed on 5 μm paraffin-embedded sections of human normal colon and CRC tissues or subcutaneous tumor xenografts and cytospuns of freshly sorted cells and spheres cells exposed to BMP7v

The following antibodies were used: BMP7 (164311, mouse IgG2B; R&D system), CD133 (AC133, mouse IgGb; Miltenyi), BMPRIA, (87933, mouse IgG2b; R&D Systems), BMPR1B (88614, mouse IgG2a; R&D Systems), BMPR2 (73805, mouse IgG2b; R&D Systems), pSmad 1,5,8 (rabbit polyclonal; CST), cytokeratin 20 (Ks20.8, mouse IgGa; Dako Cytomation), p21(#2947, Rabbit IgG; CST), E-cadherin (rabbit polyclonal; CST), Vimentin (#39325, Rabbit; CST), Beta-catenin (H102, rabbit polyclonal; Santa Cruz Biotechnology), CD166 (MOG/07, Mouse Monoclonal Antibody; Leica), Lgr5 (RB 14211, Rabbit Ig; ABGENT) Ki67 (MIB-1, mouse IgG1; Dako Cytomation), CD31 (clone JC70A,

mouse IgG1Kappa; Dako ytomation), VEGFR2 (goat IgG; R&D System) or isotype-matched controls at appropriate dilutions.

For immunohistochemistry (IHC) the dewaxed slides were heated for 1 min at 450 W and 5 min at 100 W in a microwave oven in 0.1M citrate buffer pH 6.0 or pH 9.0 only for KI67 staining. For cytoplasmatic epitopes detection, samples were permeabilized with 0.1% TritonX-100 in PBS for 10 min sections and after the slides were incubated with Tris-buffered saline (TBS) containing 10% AB human serum to block unspecific binding. After elimination of excess serum the sections were exposed overnight at 4°C to specific Abs against BMP7, CD133, CD166, Beta-catenin, Lgr5, Ki67, CK20, CD31, VEGFR2 or isotype-matched controls at appropriate dilutions. Following exposure to primary Abs, sections were treated with biotinylated anti-mouse- rabbit and anti goat immunoglobulins, washed in PBS and then incubated with streptavidin peroxidase (LSAB 2 Kit; Dako Cytomation or Vectastain kit; Vector). Stainings were detected using 3-amino-9-ethylcarbazole (AEC) chromogen. Counterstain of nuclei was performed using aqueous hematoxylin (Sigma).

For hematoxylin and eosin (H&E) staining, dewaxed sections were stained in Hematoxylin (Sigma) for 1 minutes, washed in water and then exposed for 30 seconds to eosin (Sigma). Stained sections were dehydrated and mounted with syntetic resin.

For Azan Mallory, sections were stained with azocarmine G (Sigma) for 1 hour and with 5% of phosphovolframic acid for an additional hour. Then, sections were stained with aniline blue/orange G (Sigma) and mounted in synthetic resin.

All IHC images were analyzed with Imaging Analyzer Software

Apoptotic events were determined by TUNEL labeling using In Situ Cell Death Detection, AP Kit (Boehringer Mannheim) (roche). DNA strand breaks were detected by 5-bromo-4-chloro-3-indolyl-phosphate (BCIP, Dako Cytomation) substrate.

Immunofluorescence stainings were performed on 5-µm-thick embedded sections of human CRC tissues, on cytospun freshly sorted cells, spheres cells allowed to differentiate in 10% FBS or after exposure to BMP7v. Cells were fixed in 2% PFA for 20 min at 37°C. For cytoplasmatic epitopes detection, samples were permeabilized with 0.1% TritonX-100 in PBS for 10 min, blocked with 3% BSA

for 30 min and exposed overnight at 4°C to antibodies against BMP7, CD133, BMPRIA, BMPRIB and BMPRII, p-SMAD1,5,8, CK20, p21, E-cadherin, Vimentin and beta-catenin or isotype-matched controls at appropriate dilutions. Then, cells were treated with FITC or Rhodamine anti-mouse or anti-rabbit antibodies (Molecular Probes, Inc.) plus RNase (200 ng/ml, Sigma). Nuclei were counterstained using Toto-3 iodide (642/660, Molecular Probes). Confocal analysis was used to acquire fluorescence stainings.

RNA isolation and *Real-time PCR*

Total RNA from cell pellet was obtained using the Rneasy Mini Kit (Qiagen GmbH), the residual amounts of DNA remaining was removed using the RNase-Free DNase according to manufacturer's instructions. The yield of the extracted RNA was determined by measuring the optical density at 260 nm by Nanodrop ND-1000 (Nanodrop, Wilmington, DE).

1 µg of total RNA was retro-transcribed using High-Capacity cDNA Archive Kit (Applied Biosystems) according to manufacturer's instructions. PCR amplification and detection of the PCR amplified gene products were performed with the SYBR Green PCR master mix (SuperArray Bioscience, Frederick, MD) All amplification reactions were done in triplicate, and the relative quantitation of gene expression was calculated using the comparative Ct method (ΔΔCt). Levels of mRNA expression were expressed after normalization with endogenous control, GAPDH. For SYBR green chemistry, the following primers were purchased from MWG: BMPR1A forward primer 5' GTC ATA CGA AGA TAT GCG TGA GGT TGT 3', BMPR1A reverse primer 5' ATG CTG TGA GTC TGG AGG CTG GAT T 3, BMPR1B forward primer 5' AAG GCT CAG ATT TTC AGT GTC GGG A 3', BMPR1B reverse primer 5' GGA GGC AGT GTA GGG TGT AGG TCT TTA TT 3', BMPR2 forward primer 5' GTG ACT GGG TAA GCT CTT GCC GTC T 3', BMPR2 reverse primer 5' GCA GGT TTA TAA TGA TCT CCT CGT GGT 3', GAPDH forward primer 5' GCT TCG CTC TCT GCT CCT CCT GT 3', GAPDH reverse primer 5' TAC GAC CAA ATC CGT TGA CTC CG 3'.

Flow cytometry

Flow cytometry was performed on freshly purified colon cancer cells after magnetic $CD45^+$ cells depletion, SDAC and dissociated sphere cells untreated and treated with BMP7v. Cell preparations were fixed in 2% paraformaldehyde for 10 min at 37°C and permeabilized by 0.1% Triton-X 100 for 10 min at 4°C to detect cytoplasmatic epitopes. Cells were washed twice with 0.5% bovine serum albumin (BSA, Sigma) in PBS and exposed to antibodies against CD133/2 (293C3-PE, mouse IgG2b, Miltenyi), CD133/1 (AC133, mouse IgG2b, Miltenyi), CK20 (Ks20.8, mouse IgG2a, DAKO Cytomation). Samples were then incubated with FITC-conjugated anti-mouse or anti-rabbit antibodies (Molecular Probes). Cells were subjected to flow cytometry analysis using a FACSCalibur cytometer and Cell Quest Software (Becton Dickinson). Only cells with staining intensities above the maximal level of isotype-matched controls were defined as positive cells.

Cell cycle analysis was performed on dissociated sphere cells untreated and treated with BMP7v for 48 hours and SDACs. The cells were washed in PBS and fixed in ice-cold 70% ethanol at 4°C overnight and then incubated with PBS containing propidium iodide (50 µg/ml, Sigma), sodium citrate (3.8 Mm, Sigma) and RNase (10 µg/ml, Sigma) at 37°C for 30 minutes. Samples were analyzed using a flow cytometer (BD Biosciences).

PKH26 (Sigma) staining was performed, according to manufacturer's instructions, on dissociated sphere cells, untreated and treated with BMP7v (100ng/ml) for 48 hours, up to 14 days, at different time points these cells were harvested for the FACS analysis,

Protein isolation and immunoblotting

Cell pellets were re-suspended in ice-cold NP40 lysis buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EGTA, and 1% NP40] containing proteases and phosphatases inhibitors and fractioned on SDS-polyacrylamide gels and blotted on nitrocellulose membranes. Membranes were blocked for 1 hour with nonfat dry milk in TBS containing 0.05% Tween 20 and successively incubated with antibodies specific against PTEN (17A, mouse IgMk, Neomarkers), AKT (rabbit polyclonal, CST), p-AKT (Ser 473, rabbit polyclonal, CST), GSK3ß (rabbit

polyclonal, CST), p-GSK3ß (Ser9, rabbit polyclonal, CST) and beta-actin (JLA20, mouse IgM, Calbiochem) used as loading control. Membranes were then washed, incubated for 1 hour with horseradish peroxidase-conjugated anti-mouse, anti-rabbit or anti-goat immunoglobulins (Amersham Biosciences) and developed with a chemiluminescence detection system (SuperSignal West Pico/Dura Extended duration Substrate, Pierce Biotechnology). Densitometric analysis of protein expression level was performed by Vision Works LS (UVP). Results were expressed as protein/beta-actin OD ratio.

Clonogenic and colony forming assays

Dissociated sphere cells untreated and treated with BMP7v for 48 hours were plated in presence of stem cell medium, on ultra-low-adhesion 96-well plates at a concentration of a single cell per well. Wells containing either none or more than one cell were excluded for this analysis, but these valors was used to calculate the cancer stem cell frequency, with ELDA software.

Colony forming was performed on dissociated sphere cells untreated and treated with BMP7v, 5FU+Oxaliplatin alone or in combination. Cells were plated on Agarose Sea Plague Agar (Invitrogen), and maintained up to 21 days at 37°C in presence of 5% CO_2. The colonies were stained with 10% Cristal Violet.

Invasion assay

Cell migration capacity was assessed using growth factor–depleted matrigel–coated (BD Biosciences) transwell insert. Dissociated sphere cells ($2x10^3$) untreated and treated with BMP7v 100ng/ml for 48 hours were plated into matrigel-coated transwell of 8 μm pore size (Corning). Supernatant of NIH-3T3 cells, cultured in stem cell medium was used as chemoattractant in the transwell lower part. Migration was observed and counted microscopically up to 96 hours.

RT^2 Profiler™ PCR Array.

The RT^2 Profiler PCR array was assessed for genes related to Wnt targets (PAHS-243ZR-12; SuperArray Bioscience). It was used to simultaneously examine the

mRNA levels of 89 genes closely associated with Wnt pathway, including five "housekeeping genes" in 96-well plates following the manufacturer's protocol.

Cycle threshold values were calculated for all the genes present on the array and normalized using the average of 5 housekeeping genes (ACTB, B2M, HPRT1, RPLP0 and GAPDH). RT^2 Profiler PCR Array Data Analysis was represented by clustergrams based on Pearson's Correlation of 2^ (ΔCt).

Animals and tumor model

Mice were obtained from Charles River Laboratories (Milan, Italy) and maintained according to institutional guidelines of the University of Palermo animal care and committee. Dissociated CRC sphere cells ($5x10^5$) were implanted subcutaneously (s.c.) with matrigel GF reduced (BD Biosciences) at a 1:1 ratio in a total volume of 100 µL into the flank of five-to-six week old NOD-SCID mice. Tumor mass size was calculated according to the formula: (π/6) x larger diameter x (smaller diameter)^2. When tumors were palpable the mice were treated three times a week by intraperitoneal injection of BMP7v for three weeks at different concentrations: 50, 5, 0.5 and 0.05µg/kg, and PBS as control, in order to test the efficacy dose.

Then the other set of mice were treated three times a week for three weeks by intraperitoneal injection (IP) of PBS, as control, BMP7v (50µg/kg) alone or in combination with 5FU and Oxaliplatin. (5-FU: 15 mg/kg/day for 2 days a week for 3 weeks; and Oxa: 0.25 mg/kg once a week for 3 weeks).

Histological examination, analysis of differentiation, incidence of cell death and mitotic index were determined on tumor xenografts using AC133, CK20, TUNEL and Ki67 staining.

To test the BMP7v activity as second line treatment, when the tumor xenografts obtained as previous described, were palpable, the standard adjuvant treatment, Oxaliplatin (0.25 mg/kg once a week for 3 weeks) and 5-FU (15 mg/kg/day for 2 days a week for 3 weeks) was performed intraperitoneally on eight mice for group. After this first line of treatment with 5FU plus Oxaliplatin alone for two weeks when tumor re-growth, the follow IP treatments were performed: PBS, 5FU+Oxaliplatin, BMP7v and BMP7v+5FU+Oxaliplatin, in a standard protocol

for three weeks. The tumor xenografts obtained after 13 weeks from injection were used for histological examination.

Endothelial tube formation assay

The effects of BMP7v on *in vitro* endothelial tube formation were evaluate using HUVEC, obtained from Lonza (Clonetics, Verviers, Belgium) and grown in endothelial growth medium (EGM) according to supplier's information. HUVEC, pretreated with BMP4 (2nM R&D System) and BMP7v (100ng/ml) for 24, 48, 72 hours, were plated (70,000 cells/well) in Matrigel-coated 24 well plate (BD Bioscience), and incubated up to 5 hrs at 37°C. Endothelial tube formation, evaluated by phase-contrast microscopy, was photographed at different time points and the cables length was measured manually with the IMAGE-J software.

Statistical analysis

Data were expressed as mean ± standard deviation of the mean. Immunohistochemical scores were calculated from the positivity observed on paraffin-embedded engrafted tumor tissues counted by two independent observers.

Statistical significance was determined by Analysis of Variance (one-way or two-way) with Bonferroni post-test. Results were considered significant when p values were less than 0.05. * indicates $P<0.05$, ** indicate $P<0.01$, *** indicate $P<0.001$.

RESULTS

BMP7 is widely expressed in colo-rectal cancer tissue but not in colo-rectal cancer stem cells

Human colo-rectal cancer (CRC) specimens were provided by the Surgical Department of Policlinico "Paolo Giaccone", Palermo.

These tumors and their normal counterpart (obtained from the edge of the resected specimen) were analysed for BMP7 expression with immunohistochemistry: CRC specimens widely expressed BMP7, compared to their normal counterpart (Fig.4A).

Expression of this cytokine was also analysed with immunofluorescence (IF) on paraffin embedded cancer tissue: BMP7 was localized along the upper part of the crypt, with the exception of the base of the crypt where stem cells reside (Fig.4B).

These findings prompted us to investigate whether there is differential expression of BMP7 between cancer stem cells (CSCs) and their differentiated counterpart using our *in vitro* models, i.e. sphere cells and sphere-derived adherent cells (SDACs), respectively. While SDACs displayed high levels of BMP7 expression, sphere cells showed very low positivity.

We also analyzed BMP7 expression in CR-CSCs sorted for CD133 positivity, a known CSC marker: IF analysis showed that BMP7 is exclusively expressed by $CD133^-$ cells (Fig.4C-D).

All *in vitro* experiments shown in this report were conducted in three different cell lines. The images are from one cell line, but are representative of results obtained for all three cell lines.

These data suggest a correlation between BMP7 expression and differentiation of CRC cells, and therefore a possible role in CSCs differentiation.

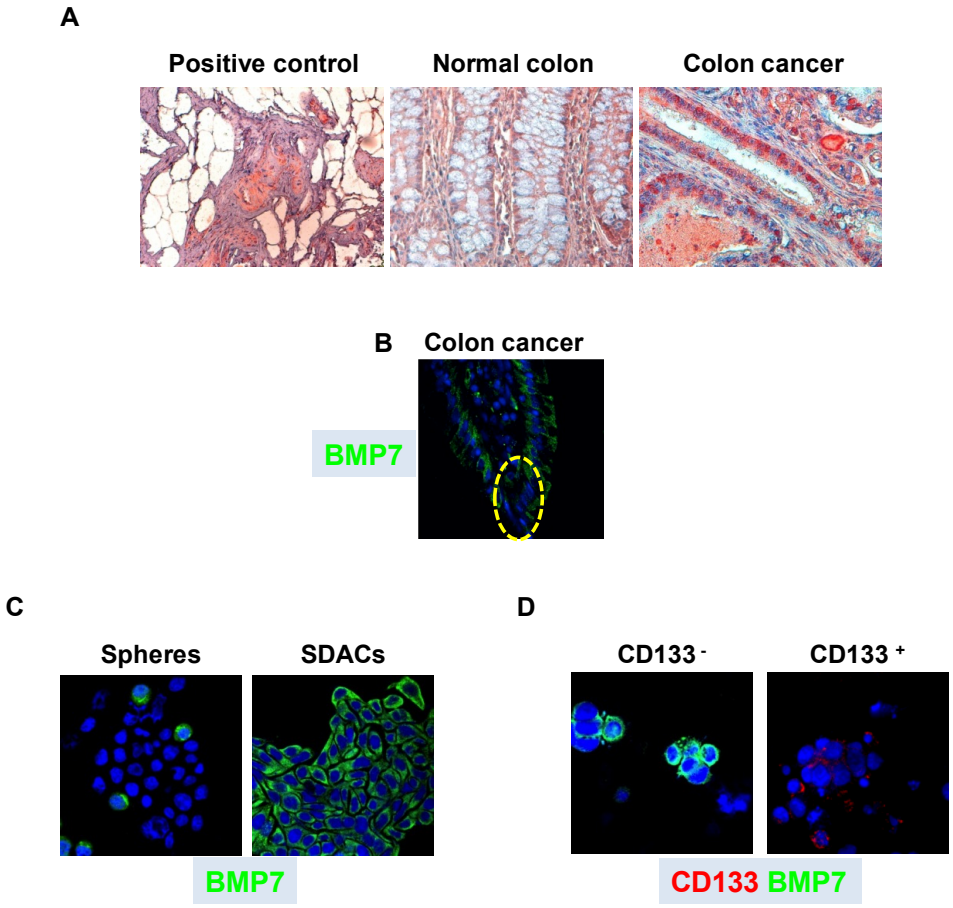


Fig.4. CD133$^+$ CR-CSCs do not express BMP7. A) Immunohistochemical analysis for BMP7 on normal and tumoral colon paraffin-embedded sections. Nuclei are revealed by hematoxylin staining (*blue*). **B)** Confocal microscopy analysis of BMP7 (*green*) on CRC paraffin-embedded tissue. Nuclei were counterstained with Toto-3 (*blue*). **C)** Representative images of immunofluorescence analysis of BMP7 (*green*) in sphere cells (*Spheres*) and SDACs. Nuclei were counterstained with Toto-3 (*blue*). **D)** Representative images of immunofluorescence analysis of BMP7 (*green*) on CD133$^-$ and CD133$^+$ CRC cells sorted from the sample as in C. Nuclei were counterstained by Toto-3 (*blue*).

BMP7v *in vitro* administration activates the BMP signaling pathway in CR-CSCs.

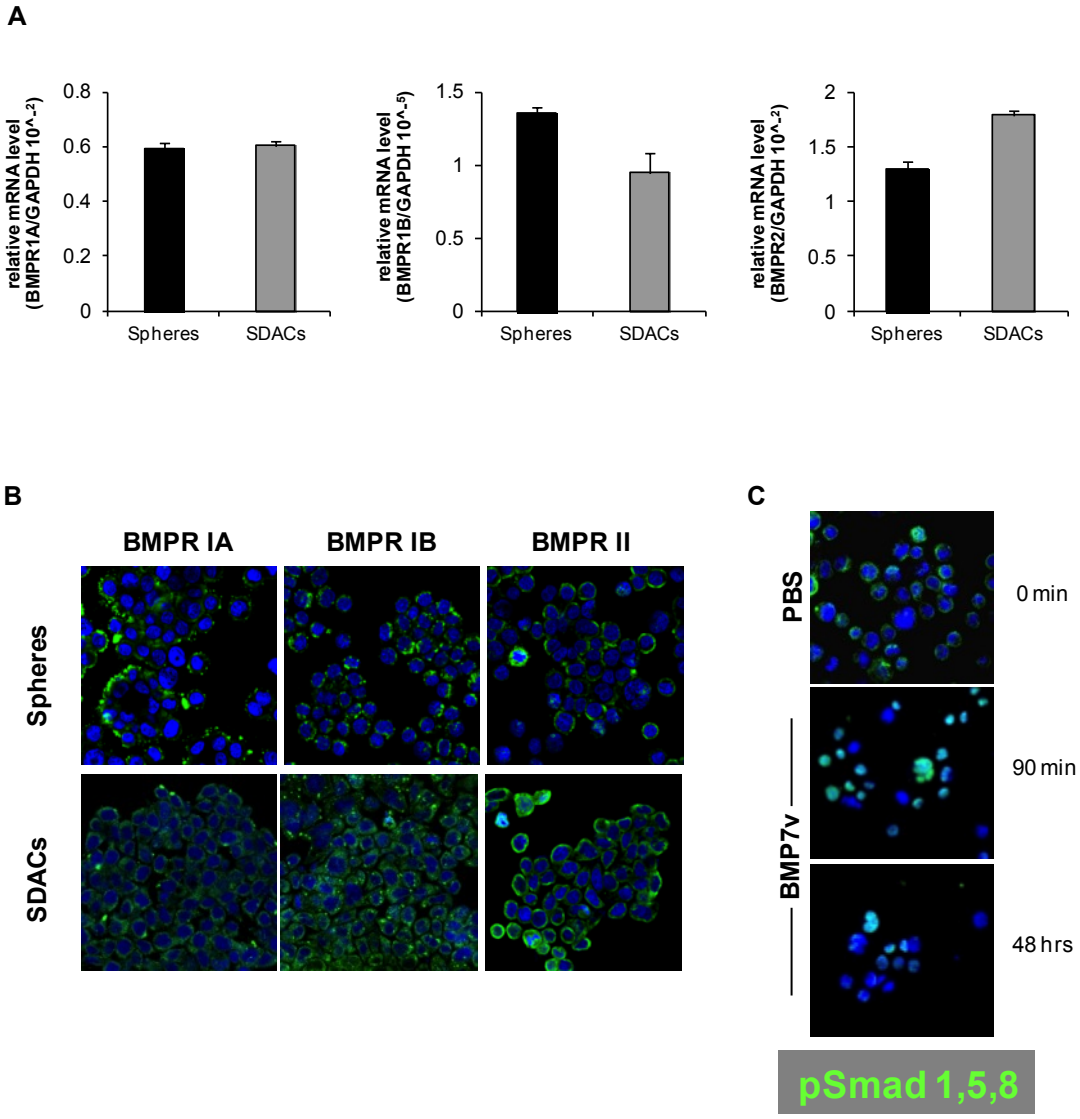
To evaluate the possible use of BMP7v as a differentiative agent of CSCs, we analysed the expression of BMP receptors through real-time PCR and IF analysis.

Both CRC sphere cells and SDACs expressed BMPR1A, BMPR1B and BMPR2 (Fig.5A).

In order to verify the BMPRs functionality in CR-CSCs, we evaluated p-SMAD1-5-8 localization with IF after treatment with BMP7v (100ng/ml): activation of the BMP7 pathway was confirmed by the prevalent p-Smad1,5,8 nuclear translocation following treatment (Fig.5B-C).

Western blot analysis of downstream targets to the BMP7 pathway are consistent with these findings (Fig.5D).

These results indicate that exogenous addition of BMP7v can activate the canonical BMP signaling pathway in CR-CSCs.



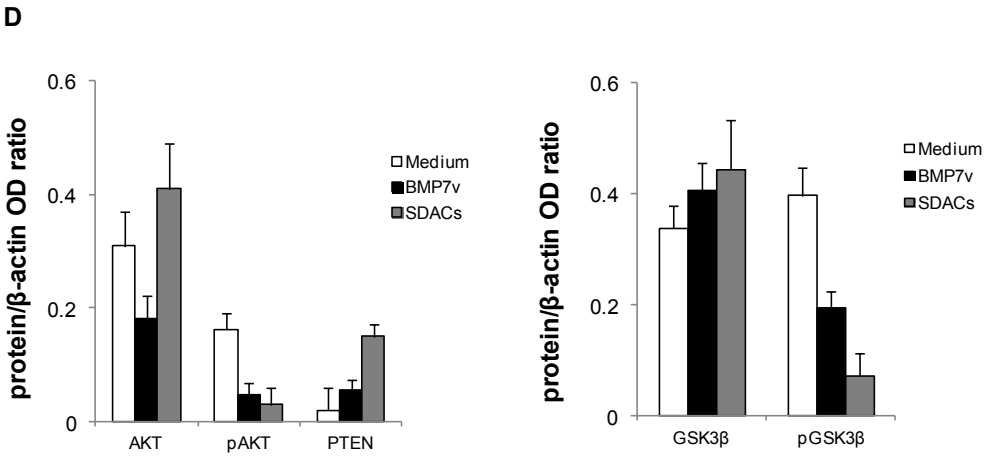


Fig.5. BMP7v administration activates the canonical signaling pathway in CR-CSCs. A) Relative quantification of BMPR1A, BMPR1B, and BMPR2 mRNA expression levels in sphere cells (*Spheres*) and SDACs. **B)** Representative images of immunofluorescence analysis of BMPR1A, BMPR1B, and BMPR2 (*green*) in sphere cells and SDACs. Nuclei were counterstained with Toto-3 (*blue*). **C)** Representative images of confocal microscopy analysis of pSmad1,5,8 (*green*) in sphere cells, untreated or treated with BMP7v for 90 minutes or 48 hours. Nuclei were stained with Toto-3 (*blue*). **D)** Densitometric analyses of protein expression levels of AKT, p-AKT, PTEN , GSK3β and p- GSK3β in sphere cells, untreated or exposed to BMP7v for 48 hours, and SDACs. Loading control was assessed by β-actin.

BMP7v induces *in vitro* differentiation in CR-CSCs and reduces the percentage of CD133$^+$ cells

To evaluate the role of BMP7v in inducing differentiation, we tested whether sphere cells could be forced to differentiate upon exposure to the morphogenetic factor. CRC sphere cells were cultured in the presence of BMP7v or 10% FBS. BMP7v alone induced a rapid differentiation of CR-CSCs, evaluated by plastic adherence and acquisition of the typical differentiated appearance, i.e. large size and polygonal shape (Fig.6A).

Accordingly, BMP7v treatment reduced the percentage of CD133$^+$ and increased CK20 expression in CR-CSCs (Fig.6B-C).

Reduction of the CD133$^+$ cells following 48 hours of BMP7v treatment was also confirmed by flow cytometry (Fig.6D).

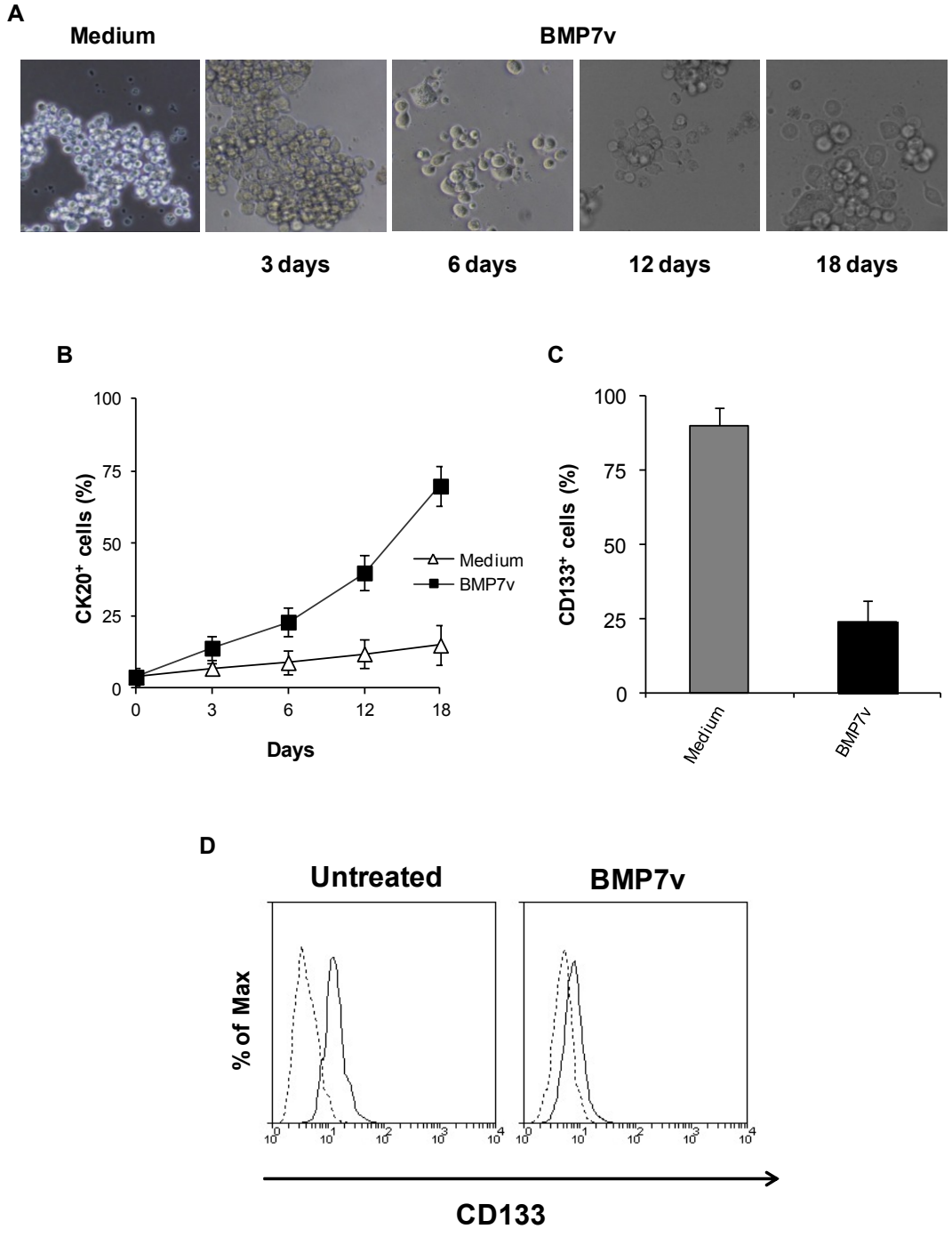


Fig.6. BMP7v promotes *in vitro* differentiation of CR-CSCs. A) Representative images of phase-contrast microscopy of dissociated sphere cells, untreated or treated with BMP7v up to 18 days. **B)** Percentage of $CK20^+$ sphere cells, untreated or treated with BMP7v up to 18 days. **C)** Percentage of $CD133^+$ sphere cells, untreated or treated with BMP7v. **D)** Representative flow cytometry profile of CD133 expression in sphere cells, untreated or treated with BMP7v for 48 hours.

BMP7v reduces self-renewal of CR-CSCs

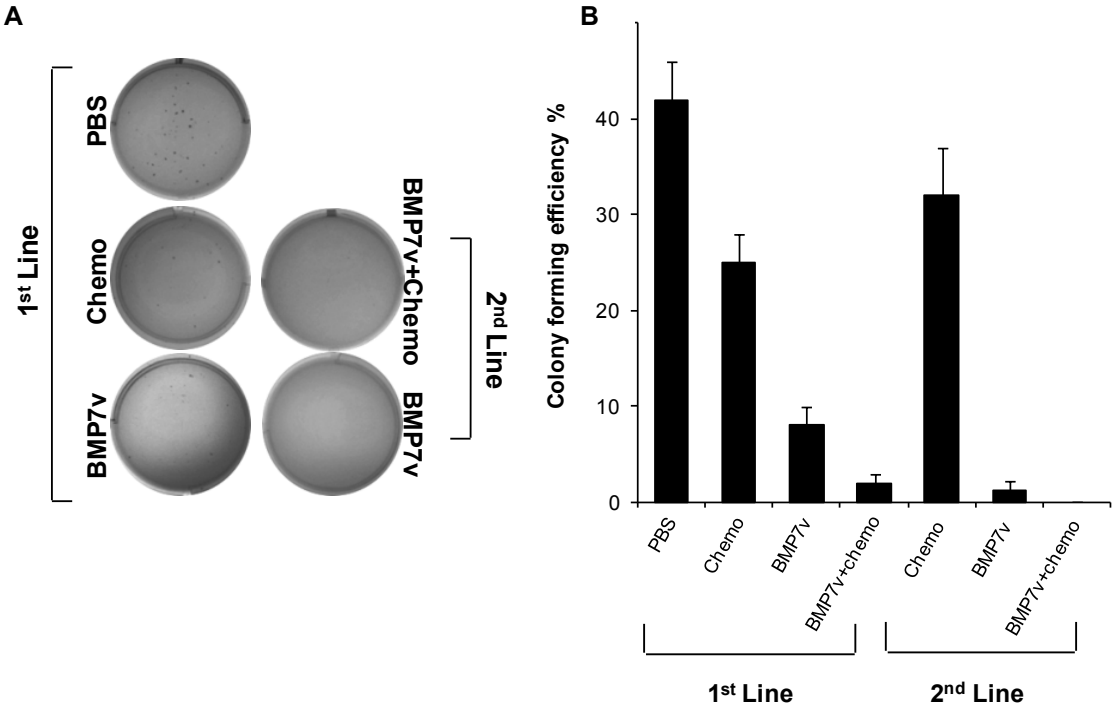
To evaluate the effect of BMP7v treatment on CR-CSCs self renewal capacity, we performed the *in vitro* colony forming assay. BMP7v, given for 48 hours, reduced the CR-CSCs sphere forming capacity.

We also tested the effect of combining BMP7v to standard chemotherapy (5FU+Oxa): this treatment resulted in a more significant reduction of clonogenic capacity compared to chemotherapy alone in CR-CSCs.

To further test the CR-CSC sensibility to BMP7v, one week after the first step of treatment, the cells were re-treated: this treatment reduced the colony forming efficiency even more drastically (Fig.7A-B).

As p21 seems to be a key regulator of CR-CSC self renewal, we also evaluated its cellular localization with IF: BMP7v treatment induces depletion of nuclear p21, confirming the induction of cell cycle progression, with consequent cancer stem cell clone exhaustion (Fig.7C).

We showed that BMP7v treatment not only reduces CR-CSCs self-renewal but also potentiates the effect of standard chemotherapy.



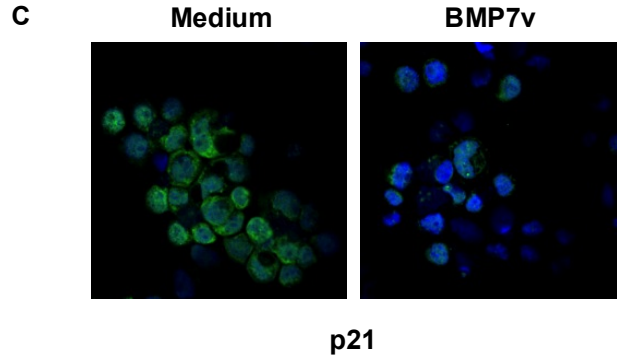


Fig.7. BMP7v reduces self renewal of CR-CSCs. A) Representative colony forming assay of sphere cells, untreated or treated with 5FU+Oxaliplatin (chemo), BMP7v alone (BMP7v), BMP7v+5FU+Oxaliplatin (BMP7v+chemo), for 48 hours; these treatments were performed once as a 1st line or repeated after one week as a 2nd line of treatment. **B)** Percentage of colony forming efficiency in sphere cells, untreated or treated as in A. **C)** Representative images of confocal microscopy analysis of p21 (*green*) in sphere cells, untreated and treated with BMP7v for 48 hrs. Nuclei were stained with Toto-3 (*blue*).

BMP7v induces CR-CSCs to exit from quiescence

We then evaluated the effect of BMP7v on cell cycle distribution. FACS analysis revealed that 48 hours of BMP7v treatment, unlike to BMP4, induced a significant increase in the percentage of cells in the G2/M phase, with a reduction of cells in G0/G1.

Interestingly, the BMP7v induced cell cycle distribution in sphere cells was similar to the baseline SDACs profile (Fig.8A-B).

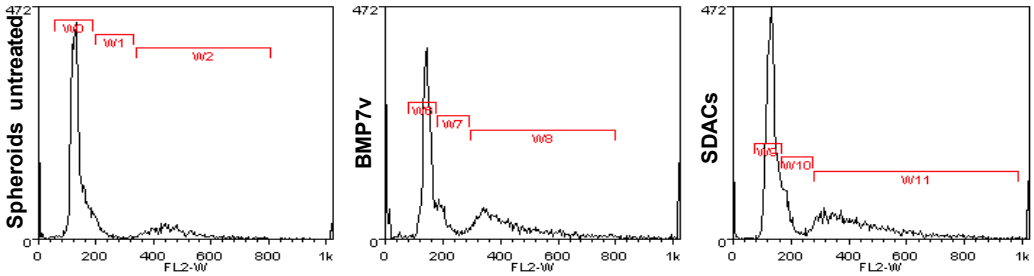
To evaluate the effect of BMP7v on proliferation of CR-CSCs, we performed PKH26 staining: treatment induced a reduction of PKH26 high cells, confirming the induction of proliferation in a time dependent manner (Fig.8C-D).

These data suggest that, in addition to differentiation induction, BMP7v treatment displays an unexpected proliferative effect.

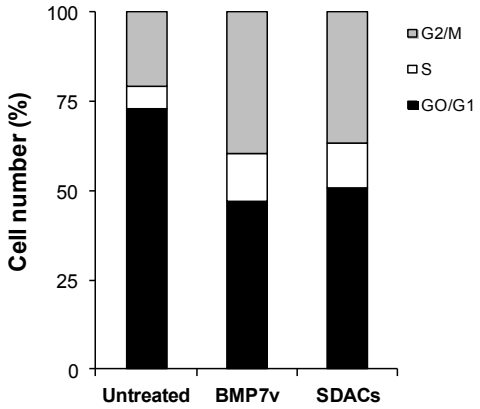
This cytokine drives different cell subpopulations present in sphere cultures to exit from the quiescent state, characteristic of stem cells, thus making them more sensitive to standard chemotherapies.

Taken together, these *in vitro* results indicate that BMP7v treatment is able to reduce stemness of CR-CSCs.

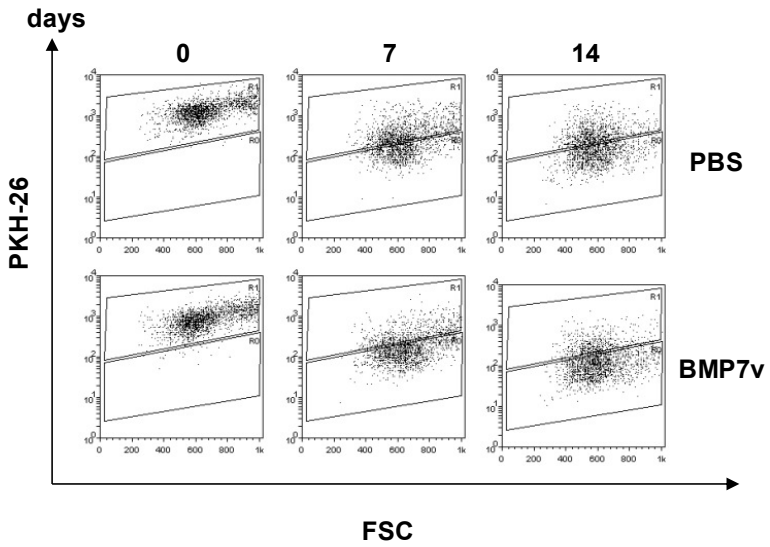
A


Spheroids untreated
BMP7v
SDACs
FL2-W

B


Cell number (%)
100
75
50
25
0
G2/M
S
GO/G1
Untreated
BMP7v
SDACs

C


days
0
7
14
PBS
BMP7v
PKH-26
FSC

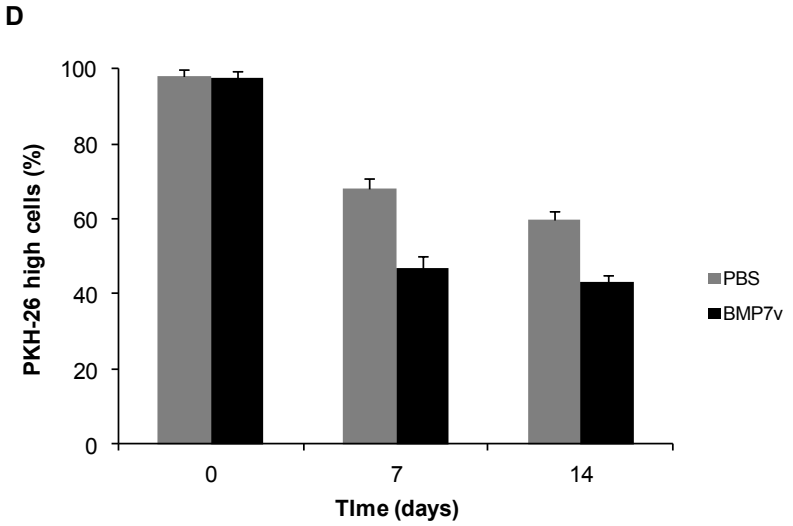
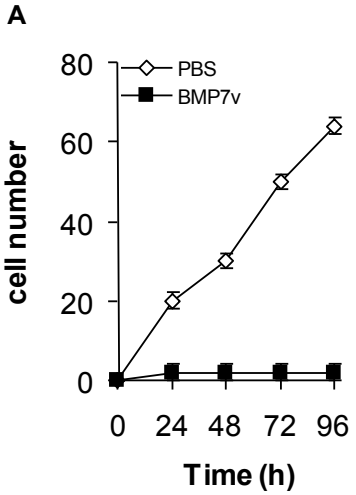


Fig.8. BMP7v induces CR-CSCs to exit from quiescence. A) Representative cell-cycle profile of sphere cells, untreated or treated with BMP7v for 48 hours, and SDACs. **B)** Cell-cycle distribution of sphere cells treated as in A. **C)** Representative PKH-26 profile of sphere cells, untreated or treated with BMP7v up to 14 days. **D)** Percentage of PKH-26 high cells treated as in C.

BMP7v reduces the mesenchymal CR-CSCs traits

Given the link between CSCs, epithelial-mesenchymal transition (EMT) and metastasis, we tested the effect of BMP7v treatment on CR-CSCs invasive capacity, using a transwell migration assay: BMP7v drastically reduces CR-CSC motility and invasiveness (Fig.9A).



We then analysed expression of EMT markers, such as E-caderin, Vimentin and beta-catenin: BMP7v suppressed the mesenchymal trait, inducing E-cadherin expression and cytoplasmic localization of beta-catenin, while silencing Vimentin (Fig.9B).

Given the role of the Wnt pathway in EMT, a Wnt signaling trascriptional profile was performed on CR-CSCs after 48 hours of BMP7v treatment to verify its action on regulation of Wnt targets: results revealed a strong regulation of Wnt targets involved in stemness and migration.

BMP7v induced upregulation of Wnt inhibitors such as SFRP2 and Axin2, associated with down regulation of stem cell markers, such as SOX2 e Nanog. In addition, the treatment reduced expression of migration regulator genes, such as Met and MMP2 and 7 (Fig.9C).

These results confirm the induction of differentiation *via* the Wnt pathway.

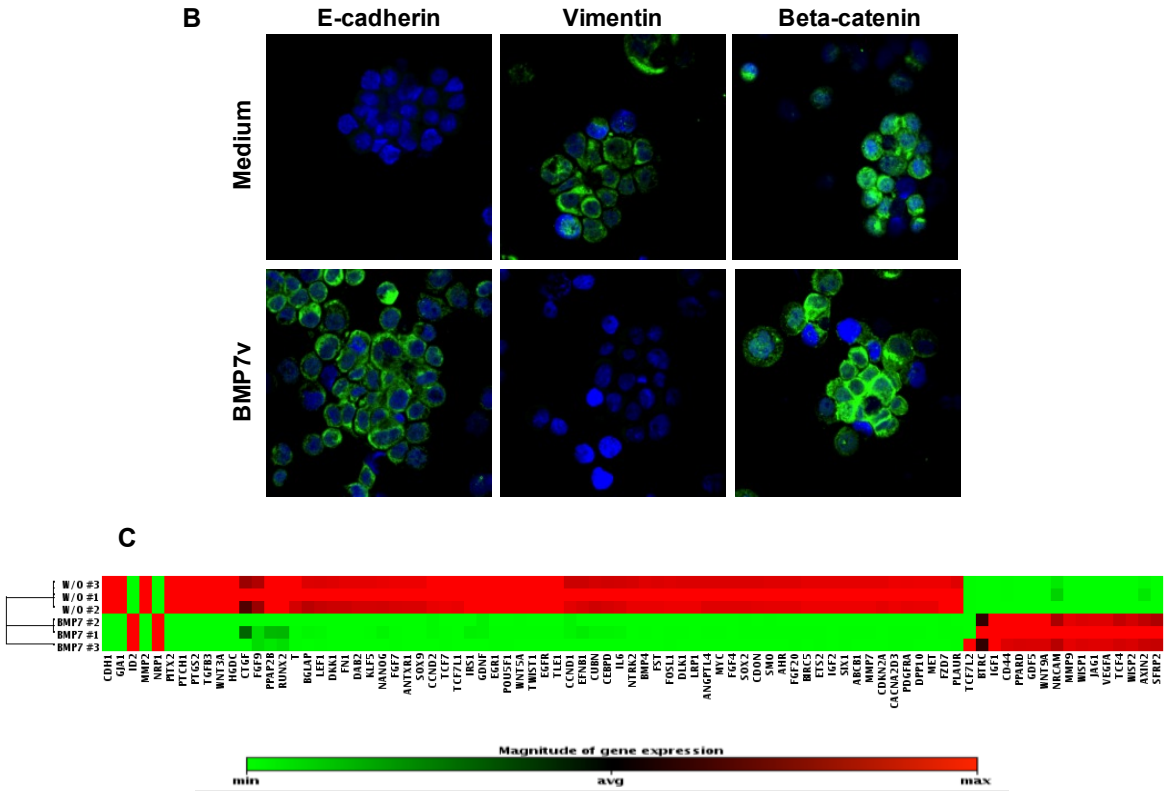


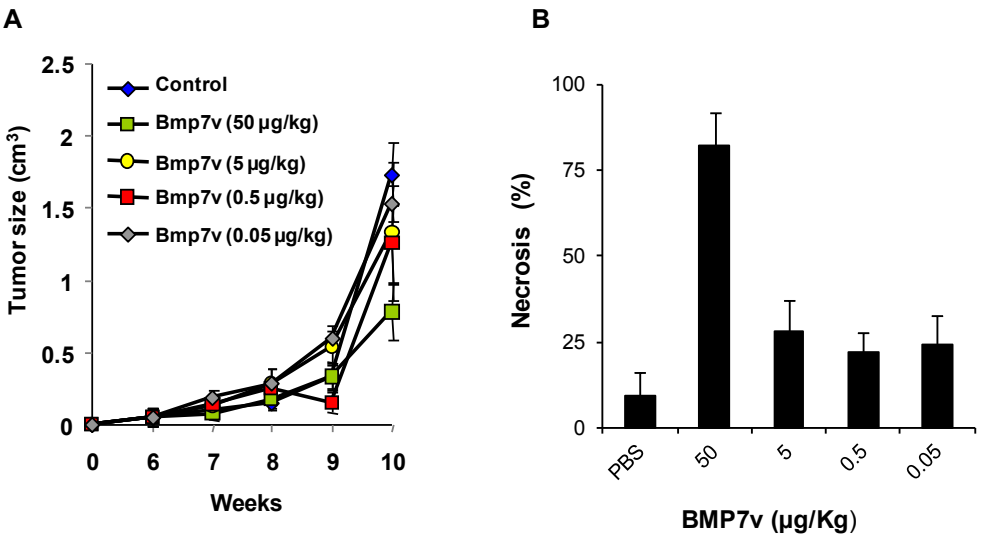
Fig.9. BMP7v reduces the mesenchymal CR-CSCs traits. A) Invasion assay of sphere cells, untreated or treated with BMP7v for 48 hours at different time points up to 96 hrs. **B)** Representative images of confocal microscopy analysis of E-cadherin, Vimentin and Beta-catenin (*green*) in sphere cells, untreated or treated as in A. Nuclei were stained with Toto-3 (*blue*). **C)** Wnt target card clustergram on sphere cells untreated or treated as in A.

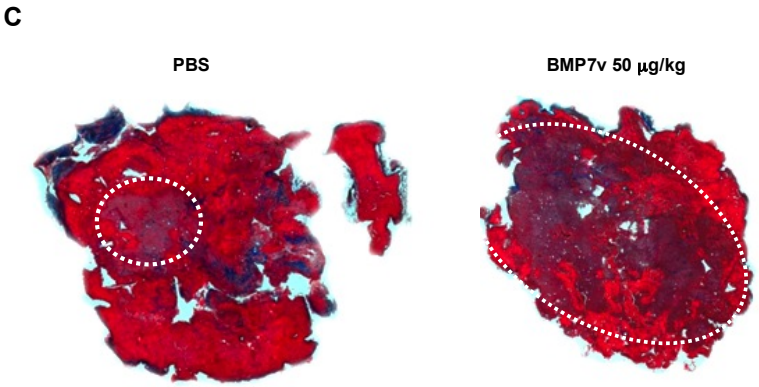
BMP7v in combination with standard chemotherapy reduces CR-CSCs tumor growth *in vivo*

To evaluate the effects of BMP7v on CR-CSCs tumor growth *in vivo,* different concentrations were administered intraperitoneally (IP) three times a week.

Three to four weeks after subcutaneous injection of sphere cells, palpable tumors were observed in immuno-compromised mice. BMP7v treatment was started at different doses listed below: 50, 5, 0.5 and 0.05µg/kg.

The results showed a reduction in tumor size and a notable necrotic effect confirmed by Azan Mallory staining on paraffin embedded xenografts sections: these effects were more pronounced in the 50µg/kg group (Fig.10A-C).





Based on these findings, we decided to use 50μg/kg in combination with standard chemotherapy, to evaluate whether BMP7v could enhance its cytotoxic effect *in vivo*.

Tumors were obtained injecting Smad4-wt, Smad4-null and Smad4-null PI3K/CA sphere cells. They were then exposed to Oxaliplatin plus 5-FU alone or in combination with intraperitoneal injection of PBS or BMP7v.

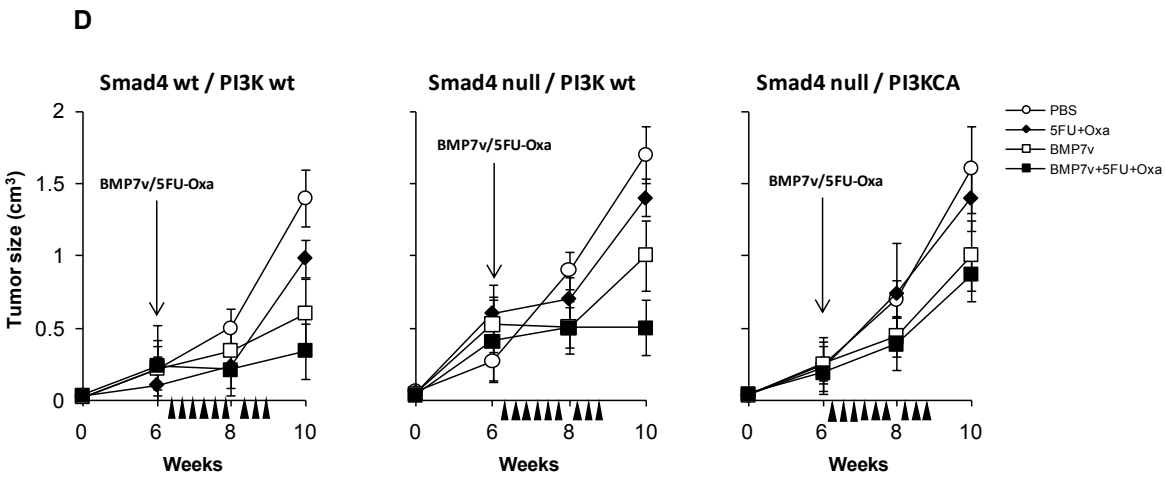
Combined treatment resulted in a considerable reduction of tumor growth in Smad4-wt and Smad4-null, while it was less effective in Smad4 null harboring PI3KCA mutation.

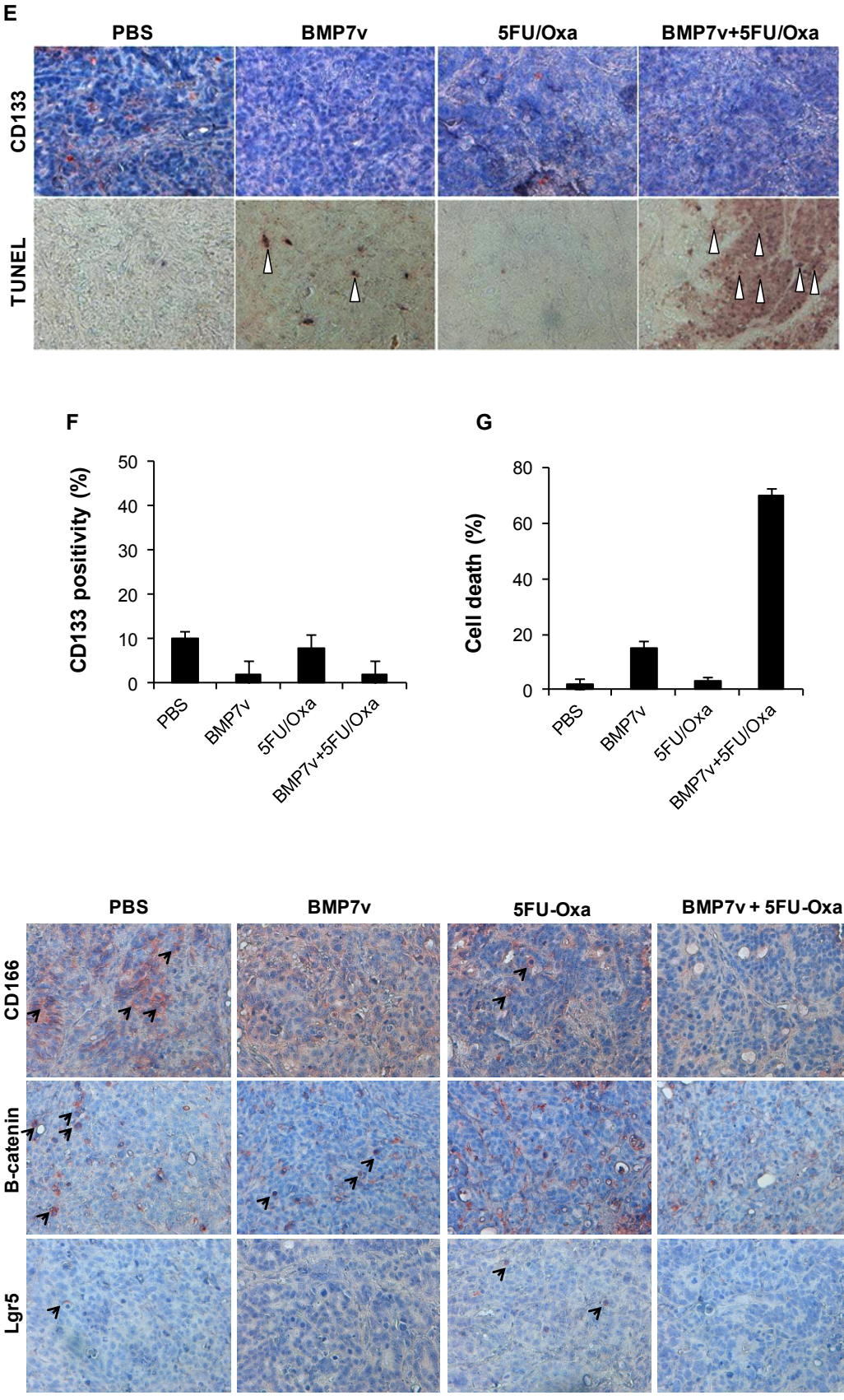
BMP7v alone gave a more pronounced growth delay than standard chemotherapy (Fig.10D).

Histological analysis of xenografts treated with BMP7v plus chemotherapy showed an high percentage of dying cells and large areas of fibrosis, highlighted by TUNEL staining (Fig.10E).

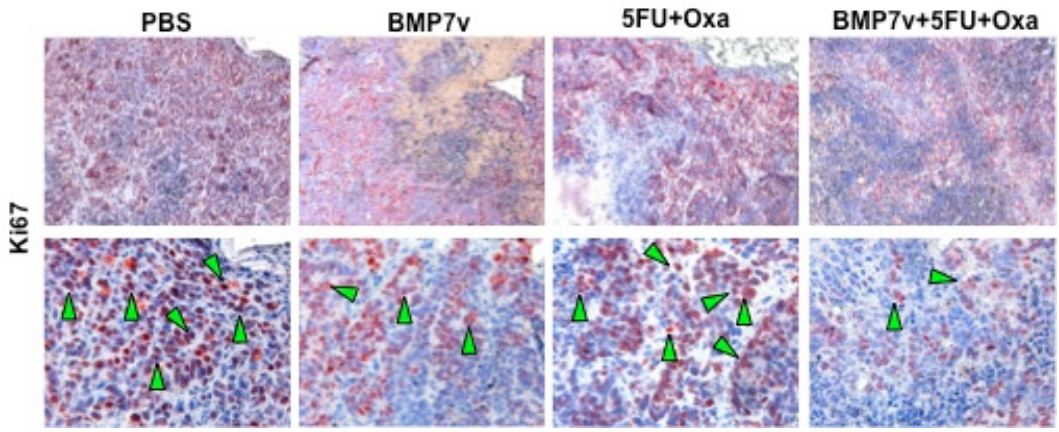
BMP7v plus chemotherapy treated tumors showed lower expression of CD133, CD166, β-catenin, Lgr5 and Ki67, with a significant increase of CK20 compared to control, suggesting anti-proliferative and pro-differentiative effects *in vivo* (Fig.10E-L).

Our findings confirm the rationale for combining a pro-differentiation agent with chemotherapy drugs, supporting the use of BMP7v in colorectal cancer patients.

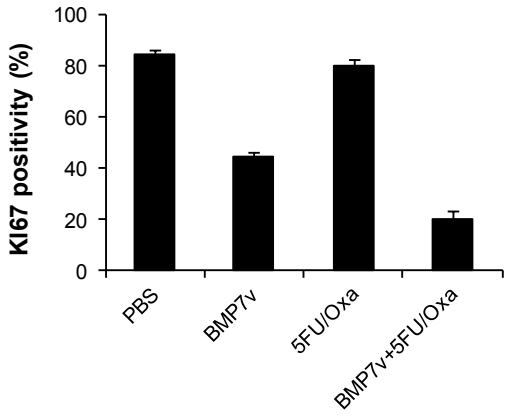



E
PBS
BMP7v
5FU/Oxa
BMP7v+5FU/Oxa
CD133
TUNEL
F
CD133 positivity (%)
50
40
30
20
10
0
PBS
BMP7v
5FU/Oxa
BMP7v+5FU/Oxa
G
Cell death (%)
80
60
40
20
0
PBS
BMP7v
5FU/Oxa
BMP7v+5FU/Oxa
H
PBS
BMP7v
5FU-Oxa
BMP7v + 5FU-Oxa
CD166
B-catenin
Lgr5

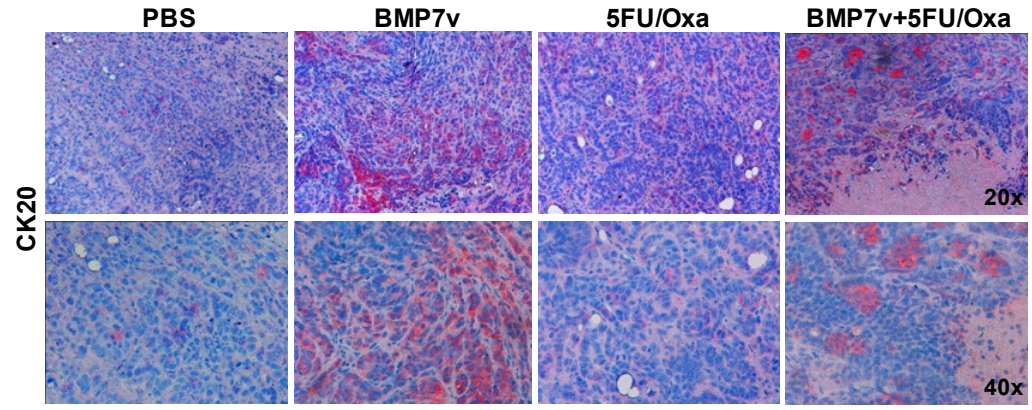
I


PBS
BMP7v
5FU+Oxa
BMP7v+5FU+Oxa
Ki67

J


KI67 positivity (%)
100
80
60
40
20
0
PBS
BMP7v
5FU/Oxa
BMP7v+5FU/Oxa

K


PBS
BMP7v
5FU/Oxa
BMP7v+5FU/Oxa
CK20
20x
40x

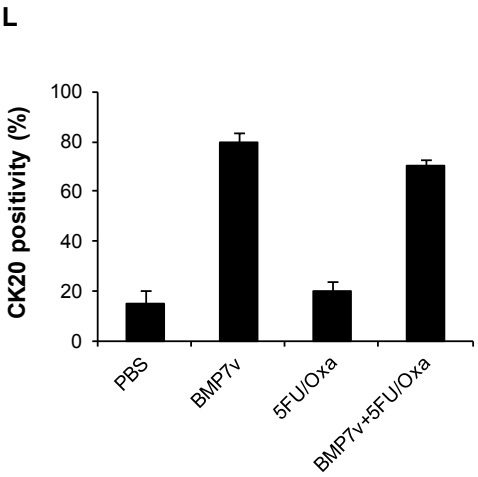


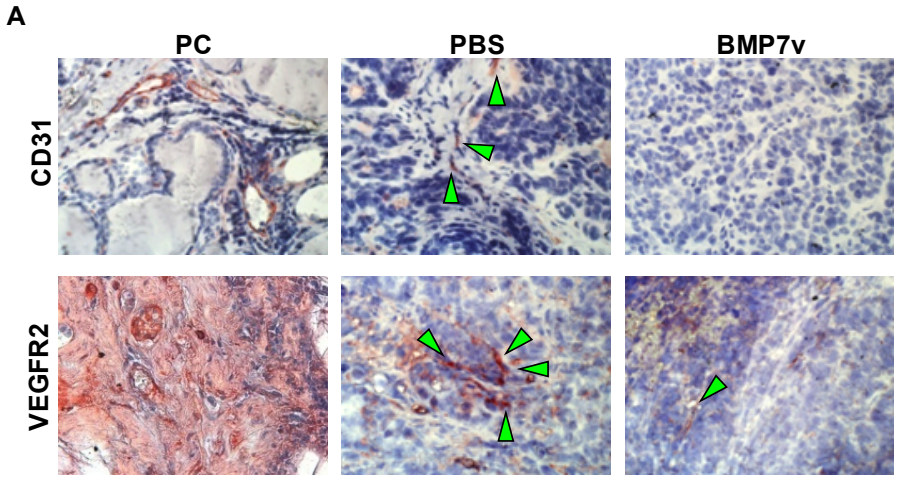
Fig.10. BMP7v in combination with standard chemotherapy reduces CR-CSCs tumor growth *in vivo*. A) Size of subcutaneous tumors following injection of sphere cells. When tumor xenografts were palpable immuno-compromised mice were treated intraperitoneally three times a week with BMP7v (50, 5, 0.5 or 0.05 ug/Kg) or with PBS as control. **B)** Percentage of necrosis evaluated on paraffin-embedded sections of xenografts, untreated or treated as in A. **C)** Representative Azan-Mallory staining on paraffin embebbed xenografts untreated (PBS) or treated as in A. **D)** Size of subcutaneous CRC xenografts derived from injection of sphere cells. Mice were untreated (PBS) or treated intraperitoneally with BMP7v (50ug/Kg), 5-FU plus Oxaliplatin alone or in combination with BMP7v. The arrows indicate the starting point of treatment. Data were obtained on three different cell lines .**E)** Immunohistochemical analysis of CD133 and TUNEL (dark blue) revealed by AEC (red) on paraffin-embedded sections of xenografts obtained as in D. Nuclei are revealed by hematoxylin staining (blue). Arrowheads indicate TUNEL positive cells. **F)** Percentage of $CD133^+$ cells evaluated on paraffin-embedded sections of tumors, untreated (PBS) or treated as in D. **G)** Percentage of TUNEL positive cells evaluated on paraffin-embedded sections of tumors untreated (PBS) or treated as in D. **H)** Immunohistochemical analysis of CD166, beta-catenin and Lgr5 revealed by AEC (red) on paraffin-embedded sections of xenografts obtained as in D. Nuclei are revealed by hematoxylin staining (blue). Arrowheads indicate CD166, beta-catenin and Lgr5 positive cells. **I)** Immunohistochemical analysis of Ki67 revealed by AEC (red) on paraffin-embedded sections of xenografts obtained as in D. Nuclei are revealed by hematoxylin staining (blue). Arrowheads indicate Ki67 positive cells. (upper: low magnification; lower: high magnification) **J)** Percentage of Ki67 positive cells evaluated on paraffin-embedded sections of tumors untreated (PBS) or treated as in D. **K)** Immunohistochemical analysis of CK20 revealed by AEC (red) on paraffin-embedded sections of xenografts obtained as in D. Nuclei are revealed by hematoxylin staining (blue). (upper: low magnification; lower: high magnification) **L)** Percentage of CK20 positive cells evaluated on paraffin-embedded sections of tumors, untreated (PBS) or treated as in D.

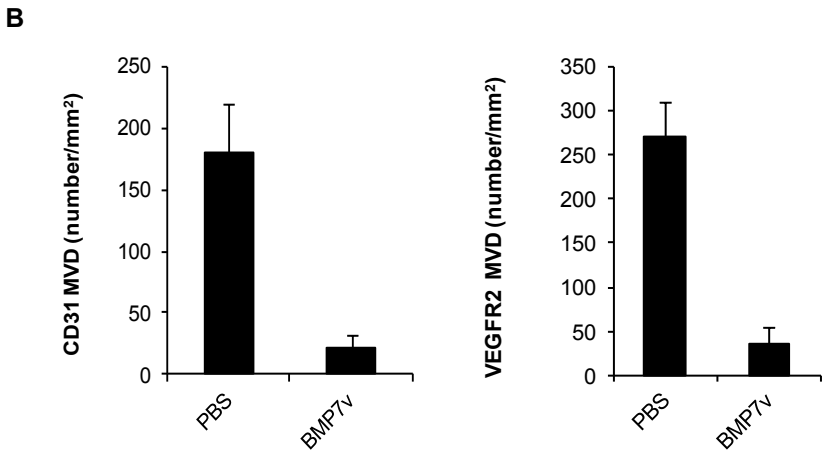
BMP7v has a strong anti-angiogenic effect *in vivo*

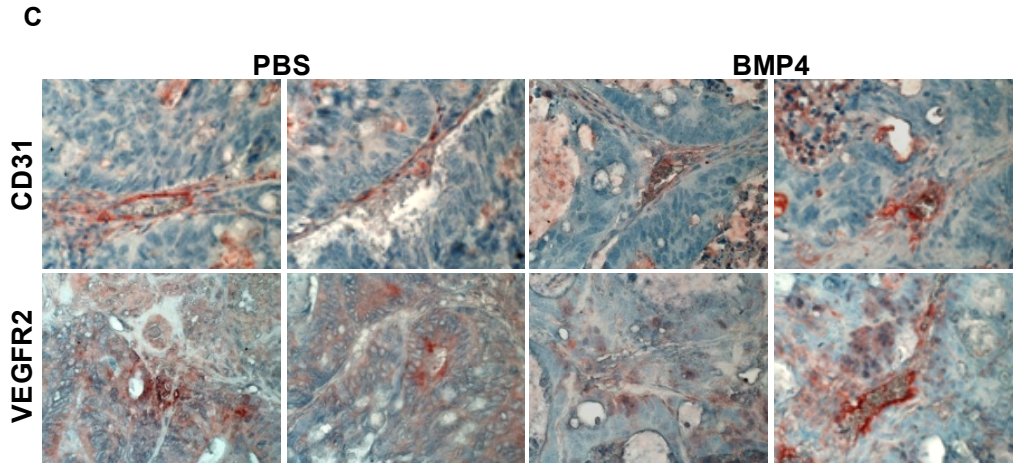
Since BMP7v treatment resulted in a considerable growth delay associated with a potent necrotic effect, we investigated further its anti-angiogenic potential.

Xenografts were exposed to BMP7v or BMP4, another member of the BMP family with an anti-tumour activity.

Histological examination showed a significant reduction of human CD31 and VEGFR2 expression following BMP7v, but not BMP4 treatment (Fig.11A-C).







We also performed an endothelial tube formation assay using endothelial cells from the umbilical vein (HUVEC): BMP7v affected the ability to form vessels after 48 hrs of treatment, while this was not observed for BMP4 (Fig.11D-F).

These findings confirm a specific anti-angiogenic effect of BMP7v in colorectal cancer xenografts.

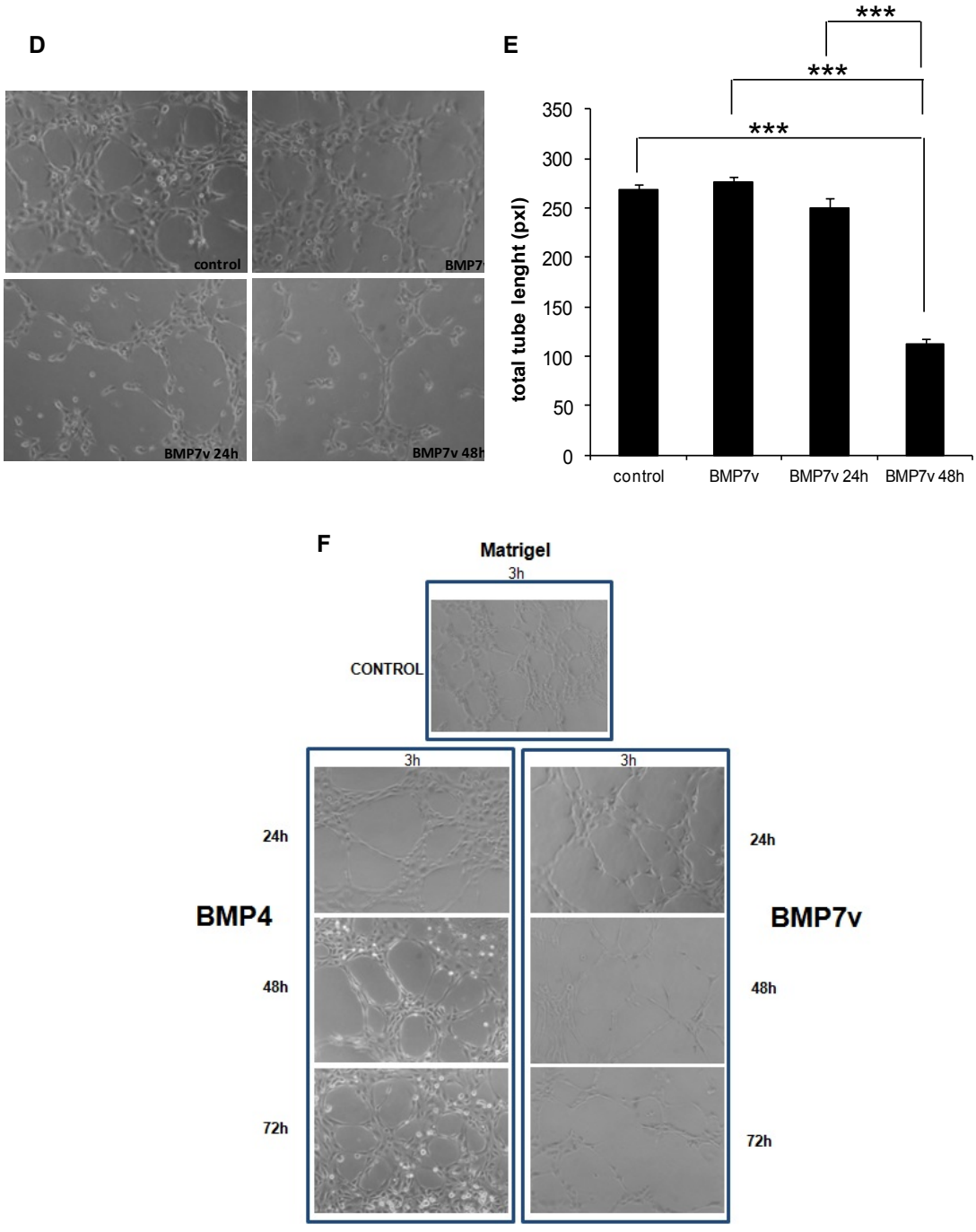


Fig.11. BMP7v has a strong anti-angiogenic effect. A) Immunohistochemical analysis of CD31 and VEGFR2 revealed by AEC (red) on paraffin-embedded section of xenografts obtained after PBS or BMP7v treatment (50ug/Kg). Nuclei are revealed by hematoxylin staining (blue). **B)** Percentages of CD31 and VEGFR2 expression evaluated on paraffin embedded sections of tumors

treated as in A. **C)** Immunohistochemical analysis of CD31 and VEGFR2 revealed by AEC (red) on paraffin-embedded section of xenografts obtained after intra-tumoral injection of PBS or BMP4 loaded beads. Nuclei are revealed by hematoxylin staining (blue). **D)** Representative images of phase-contrast microscopy of endothelial tube formation assay. Huvec cells, untreated (EGM medium as control) or pretreated with BMP7v (100ng/ml) at different time points. **E)** Measure (pxl) of total tube length obtained with Huvec treated as in D. **F)** Representative images of phase-contrast microscopy of endothelial tube formation assay. Huvec cells untreated (EGM medium as control) or pretreated with BMP4 (2nM) and BMP7v (100ng/ml) at different time points.

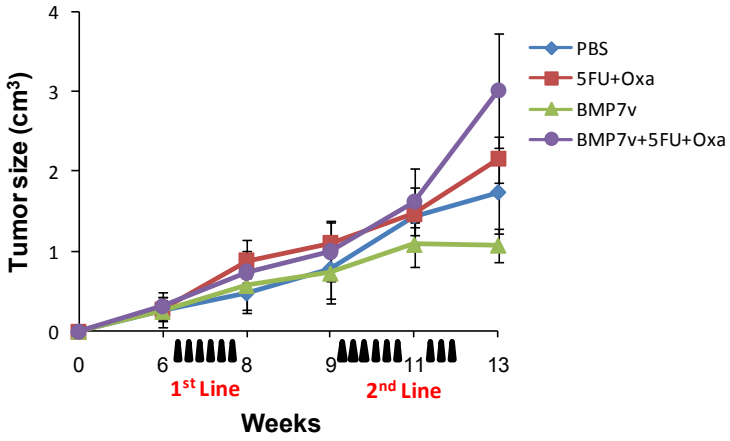
BMP7v as second-line treatment shows significant anti-tumor activity in xenografts refractory to chemotherapy

To test the activity of BMP7v as second line treatment, first line treatment with 5FU plus Oxaliplatin was administrated for two weeks. After tumour re-growth, mice were treated with: PBS, 5FU+Oxaliplatin, BMP7v or BMP7v+5FU+Oxaliplatin, for three weeks. BMP7v alone showed greater efficacy compared to combined treatment in tumor xenografts previously treated with standard chemotherapy (Fig.12A).

Hystological examination of xenografts, obtained 13 weeks after injection, revealed a strong necrotic effect. This was also confirmed with Azan Mallory staining (Fig.12B-C).

These results further support the possible use of BMP7v in colorectal cancer, providing evidence for its efficacy in pre-treated patients.

A



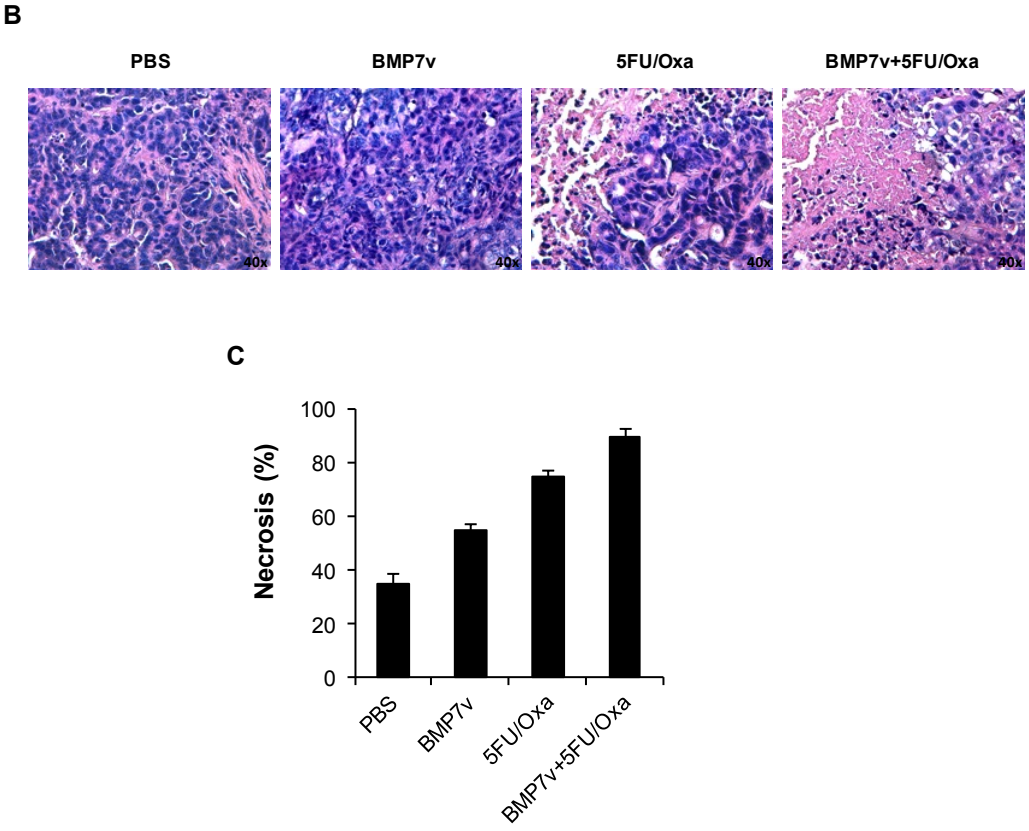


Fig.12. BMP7v shows an anti tumor activity as second line treatment in xenografts refractory to chemotherapy. A) Size of subcutaneous CRC xenografts derived from injection of sphere cells. Mice were untreated (PBS) or treated intraperitoneally with 5-FU+Oxaliplatin for two weeks. After tumor re-growth mice were treated with PBS, BMP7v (50ug/Kg), 5-FU+Oxaliplatin alone or combination. **B)** H&E staining of paraffin-embedded sections of xenografts obtained as in A. **C)** Percentage of necrosis evaluated on paraffin-embedded sections of xenografts untreated (PBS) or treated as in A.

DISCUSSION AND CONCLUSIONS

Current therapies mostly hit the differentiated cells, sparing the tumorigenic population which is responsible for the tumor relapse. Although clinical protocols are traditionally directed against the bulk of tumor cell population, increasing evidence suggests that curative therapies can be established only by targeting the subpopulations of tumor cells with tumorigenic potential. Several reports indicate that chemotherapy is more active on differentiated cells and generally ineffective against the tumorigenic population. In fact, traditional debulking agents often fail to produce long-term clinical remission and tumor relapses as a result of the inability to target cancer initiating cells (151).

The induction of differentiation affecting the self-renewal ability of CSCs represents an alternative way to inhibit tumor growth and to sensitize CSCs to conventional chemotherapy drugs.

According to recent publications, the tumor-initiating cells have stem like characteristics such as abilities of self-renewal, differentiation and invasion. Several research groups have identified tumorigenic populations with stem-like features in CRC (28-30, 73, 76, 80). These cellular subpopulation is able to promote tumor growth and is considered as an optimal cellular target to obtain effective therapies.

Many reports suggest that the balance between self-renewal and differentiation in normal stem cells results deregulated in CR-CSCs.

Indeed, the activation of Wnt signaling plays a key role in maintaining the normal stem cell population in the gut and promoting self-renewal of CR-CSCs (153-156) but also the cytokines, released in the microenvironment, contribute significantly to maintain the undifferentiated status and clonogenic activity of the tumorigenic cells (157).

Thus, the differentiation therapy could represent a considerable therapeutic option for the treatment of colon cancer, inhibiting CSCs self-renewal ability and eradicating the tumor-driving cell population. Although this approach does not directly kill the cancer cells, it could make the conventional therapies more effective in the eradication of the tumor bulk.

For years retinoic acid has been used in the treatment and differentiation of promyelocytic leukemia, thus validating this concept (158). The pro-differentiative effect of Bone Morphogenetic Protein 4 (BMP4) has been already proposed as a therapeutic option for human glioblastoma and colorectal cancer (147, 158-160)

Moreover these treatments enhanced the antitumor activity of chemotherapeutic drugs, whose concomitant administration is able to induce a complete therapeutic response, also after treatment interruption. Thus supporting the combined use of differentiative and cytotoxic agents for cancer therapy. Accordingly, the induction of differentiation, affecting the self-renewal ability of CSCs, represents an alternative way to inhibit tumor growth and to sensitize CSCs to conventional chemotherapy.

On the basis of these data, this work proposed to investigate the role of a stable BMP7 variant (BMP7v) on CSCs purified from CRC sample, in order to make these cells more sensitive to conventional chemotherapy drugs and to develop a new treatment protocol easily tested in preclinical models to design a future appropriate clinical trials.

This study identifies, the use of BMP7v, as new potential therapeutic approach that activates CRC differentiation program. As previously described, the pro-differentiation activity of BMPs, is perfectly detectable in normal gut in which these molecules are expressed following a decreasing gradient from the intestine lumen up to the crypt, thus limiting the stem cell expansion at the bottom of the crypts promoting the intestinal epithelial cell differentiation along the upper part of the crypts (113, 114).

The present data show that all CRC specimens analysed, widely express BMP7, compared to their normal counterpart and its expression is limited to the differentiated progeny of CRC epithelial cells, which constitute the major population of the tumor mass. Here we demonstrated that BMP7 is localized along the upper part of the crypt, with the exception of the base of the crypt where stem cells reside. On the contrary, its expression is undetectable in the $CD133^{+}$ CR-CSCs fraction. BMP7 results exclusively expressed by $CD133^{-}$ cells or in their differentiated counterpart, the sphere-derived adherent cells (SDACs). Although both CRC sphere cells and SDACs express BMPR1A, BMPR1B and

BMPR2. These results suggest a correlation between BMP7 expression and differentiation of CRC cells, and therefore a possible role in CSCs differentiation.

The activation of the BMP signalling pathway was confirmed by the prevalent p-Smad1,5,8 nuclear translocation following BMP7v treatment, as confirmed by analysis of downstream protein targets. Accordingly, BMP7v treatment increased PTEN levels inhibiting PI3K/AKT survival pathway confirming its differentiative role on CR-CSCs (161).

BMP7v treatment induced a rapid differentiation of CR-CSCs, morphologically evaluated by plastic adherence and acquisition of the typical differentiated phenotype, into large and polygonal colonic cells. Consequently, this treatment on CR-CSCs reduced the percentage of $CD133^{+}$ subpopulation increasing the CK20 expression, a typical markers of epithelial colonic differentiation.

BMP7v, also reduce the sphere forming capacity, and maintain this capacity also after a first step of treatment, showing that this second treatment reduces the colony forming efficiency even more drastically. Moreover BMP7v not only decreases CR-CSCs self-renewal but also potentiates the effect of standard chemotherapy.

Taken together, these *in vitro* results indicate that this cytokine drives the different cell subpopulations present in sphere cultures to exit from the quiescent state, characteristic of stem cells, thus making them more sensitive to standard chemotherapies, as showed by cell cycle analysis. The BMP7v treatment in fact increased the percentage of cells in the G2/M phase, reducing the cells in G0/G1, confirming that BMP7v treatment is able to reduce CR-CSCs stemness trait.

According to these data the treatment reduces CR-CSC motility and invasiveness, underlining the link between CSCs, epithelial-mesenchymal transition (EMT) and metastasis, as showed by the analysis of putative EMT markers: BMP7v suppressed the mesenchymal trait, inducing E-cadherin expression and cytoplasmic localization of beta-catenin, reducing Vimentin expression.

To confirm the correlation between BMP and Wnt pathways suggested by western blotting analysis a Wnt signaling trascriptional profile was performed on CR-CSCs after 48 hours of BMP7v treatment: BMP7v induced up-regulation of Wnt inhibitors such as SFRP2 and Axin2, associated with down regulation of stem cell markers, such as SOX2 e Nanog. In addition, the treatment reduced expression of

migration regulator genes, such as Met and MMP2 and 7. These results confirm the induction of differentiation *via* the Wnt pathway.

The clinical results obtained in leukemia, sustain the combination of a pro-differentiation agent with chemotherapy, suggesting the synergic action of BMP7v in combination with conventional drugs used in the management of CRC disease. BMP7v in combination with standard chemotherapy reduced CR-CSCs tumor growth *in vivo* as showed by strong reduction of tumor size, and by lower expression of CD133, CD166, beta-catenin, Lgr5 and Ki67, with a significant increase of CK20 compared to control, suggesting anti-proliferative and pro-differentiative effects *in vivo.*

Our findings confirm the rationale for combining a pro-differentiation agent with chemotherapy drugs, supporting the use of BMP7v in colorectal cancer patients.

Since BMP7v treatment resulted in a notable necrotic effect, paraffin embedded xenografts sections were analyzed for CD31 and VEGFR2 expression.

The histological examination showed a significant reduction of human CD31 and VEGFR2 expression following BMP7v treatment, but not BMP4 treatment. This anti-angiogenetic effect was confirmed through an endothelial tube formation assay. These findings confirm a specific anti-angiogenic effect of BMP7v in colorectal cancer xenografts

BMP7v also showed a significant anti-tumor activity in xenografts refractory to chemotherapy, revealing a strong necrotic effect and a greater efficacy compared to combined treatment in tumor xenografts previously treated with standard chemotherapy.

These results also support the possible use of BMP7v in colorectal cancer, providing evidence for its efficacy also in pre-treated patients.

Concluding, the clinical benefit obtained by the combination of a prodifferentiative agent with chemotherapy led us to propose the combination of BMP7v with current standard chemotherapy regimens for CRC further supporting the usefulness of CSCs differentiation as a CRC therapy.

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