Analysis of the role of Granzyme-A in the development of colorectal cancer stem cells

Trabajo de Fin de Máster

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Máster en Biología Molecular y Celular
Curso 2015-2016
07/06/2016

HOJA DE PRESENTACIÓN

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INFORMA:

Que SANTIAGO COSTAS RAMÓN ha realizado en el Centro de Investigación Médica de Aragón bajo su dirección el Trabajo de Fin de Máster descrito en la presente memoria, que lleva por título *"Analysis of the role of Granzyme-A in the development of colorectal cancer stem cells"* y que el trabajo realizado cumple los objetivos recogidos en las Directrices TFM en Biotecnología.

Zaragoza, 2016-06-067

Firmado:

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Index

0. Abstract

1. Introduction
    a. Cancer stem cells
        i. Cancer stem cell theory
        ii. Signalling pathways implicated in CSC development
        iii. CSCs origin and epithelial-to-mesenchymal transition
        iv. Clinical relevance of CSCs
    b. Colorectal cancer, inflammation and CSCs
        i. Colorectal cancer
        ii. Inflammation in CRC
        iii. Cytotoxic mechanisms of immune cells
        iv. Inflammation and CRC-associated CSCs

2. Antecedents and Objectives

3. Materials and Methods
    a. Materials
        i. Cell lines
        ii. Cell culture maintenance materials
        iii. Antibodies and reagents used for CSCs analysis and sorting
        iv. Mice strains and colorectal cancer induction materials
        v. Tumour disaggregation reagents
        vi. Other reagents
        vii. Analysis software
    b. Methods
        i. Cell culture maintenance
            1. Cell lines maintenance and manipulation
            2. Determination of cell number viability
            3. Cell freezing and thaw
        ii. Analysis of CSC-related cell surface markers and transcription factors in cell cultures
            1. Extracellular protein staining
            2. Intracellular protein staining
        iii. Analysis of the side population, ALDH activity and its correlation with extracellular and intracellular CSC-related markers in cell cultures
            1. Side population analysis
            2. ALDH activity analysis

iv. Cell sorting using anti-CD44 antibodies and differentiation and clonogenic assays
    1. Cell sorting using anti-CD44 antibodies
    2. Differentiation assays
    3. Clonogenic assays
    4. Limiting dilution assays
v. Enrichment of CSC population using PTX and doxorubicin drugs
    1. MTT assays
    2. Transcription factors and extracellular CSC-related markers analysis after the drug treatment
vi. Production of chemoresistant cell lines and CSC-related markers analysis
    1. Production of chemoresistant cell lines
    2. CSC-related markers analysis in chemoresistant cell lines
vii. Development of *in vitro* 3D cultures using the hanging drop method and CSC-related marker analysis
viii. Development of colorectal cancer in mice model using AOM/DSS protocol
ix. Analysis of the influence of the Granzyme-A in the CSC-related markers in inflammation-induced colorectal cancer mouse tumours

4. Results and Discussion
    a. Expression of CD44, CD166 and transcription factors (Nanog, Oxt3/4 and Sox2) in colorectal cancer cell lines.
    b. Correlation between CD44, CD166 and transcription factors expression with the ALDH activity and/or the side population
    c. Analysis of the differentiation and colony formation
    d. Use of the PTX and doxorubicin to enrich CSCs population
    e. Development of chemoresistant cell lines and analysis of their CSC-related characteristics
    f. Development of 3D cell cultures in order to get a CSC-enriched cell culture
    g. Influence of the Granzyme-A in the expression of CSC-related markers

5. Conclusions

6. Bibliography

7. Annex

Abbreviations

3D: 3 dimensional
7-AAD: 7-aminoactinomycin D
ABC transporters: ATP-binding cassette transporters
AOM: azoxymethane
ALDH: aldehyde dehydrogenase
APC (gene): adenomatous polyposis coli
APC: allophycocyanin
B6: C57Bl6 mice
CD: cluster of differentiation
CRC: colorectal cancer
CSC: cancer stem cells
CTL: cytotoxic T lymphocyte
DMEM: Dulbecco's Modified Eagle Medium
DMSO: dimethyl sulfoxide
DSS: dextran sulphate sodium
EDTA: ethylenediaminetetracetic acid
EMT: epithelial-to-mesenchymal transition
FACS: fluorescence-activated cell sorting
FBS: fetal bovine serum
FITC: fluorescein isotiocyanate
Fz: Frizzled
GzmA: granzyme-A
Hh: Hedgehog
IC50: half minimum inhibitory concentration
IFN-γ: interferon γ
IL: interleukin
iPS cell: induced pluripotent stem cell
MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltatrazolium
MDRs: multidrug resistant proteins
NF-κB: nuclear factor-κ-light-chain-enhancer of activated B cells
NK cell: natural killer cell
NOD/SCID: non-obese diabetic/severe combined immunodeficiency
PBS: phosphate buffered saline
PE: phycoerythrin
PS: phosphatidylserine
PTX: paclitaxel
STAT3: signal transducer and activator of transcription 3
TGF-β: transforming growing factor-β
TNF-α: tumour necrosis factor-α
WT: wild type

0. Abstract

Colorectal cancer is the third leading cause of cancer death worldwide with nearly 150.000 new cases diagnose annually. New therapies have been developed for the last years targeting different proliferation and survival signalling pathways but few advances have been done to avoid relapse in patients. Cancer stem cells are proposed as the main mechanism of resistance in current therapies. These cells have been described as a subset among all the tumour cell populations which have stemness properties such as self-renewal and differentiation capabilities. These features allow them to survive after antitumor treatments and regenerate the entire tumour mass. Also, cancer stem cells have been proposed as the responsible of metastasis.

Inflammation in colorectal cancer is a sign of poor prognosis and it has been proposed as the main driver of the tumour progression. Its relation with cancer stem cells has not been classified yet but pro-inflammatory molecules such as TNF-α or IL-1B are able to activate cancer stem cells-related signalling pathways such as Wnt or Notch pathways. Granzyme-A, a protease released by immune cells, is involved in implication during like bacterial sepsis or arthritis. Our preliminary data indicate it is involved in development of colorectal cancer.

The current study analyse the implication of Granzyme-A in the activation of CSCs during inflammation-associated colorectal cancer in a mouse model.

0. Resumen

El cáncer colorectal es la tercera causa de muerte por cáncer en todo el mundo con cerca de 150.000 de nuevos casos cada año. Durante los últimos años se han desarrollado nuevas terapias para tratar este tipo de cáncer pero relativamente pocos avances se han conseguido debido a la alta recurrencia en los pacientes. Las células madre tumorales se han propuesto como una de las principales explicaciones para esta alta recurrencia. Estas células se han descrito como un tipo de población celular dentro de la heterogeneidad de un tumor que presentan unas características similares a células madre encontradas en tejidos como la capacidad de autorenovación y la capacidad de diferenciación. Estas características las hace únicas para mantener la integridad del tumor y conseguir superar las actuales terapias antitumorales. Además, se han propuesto que son las responsables de la metástasis tumoral.

La inflamación en cáncer colorectal es un signo de mal pronóstico y se ha propuesto como uno de los principales motores de la progresión tumoral. Su relación con la aparición de células madre tumorales aún no está clara del todo pero moléculas proinflamatorias como TNF-α e IL-1B son capaces de activar rutas de señalización intracelular relacionadas con células madre como las rutas de Wnt o Notch. La Granzima-A, una proteasa liberada por las células inmunes, está bajo estudio debido a su implicación en enfermedades inflamatorias como la sepsis bacteriana o la artrosis. Estudios preliminares de nuestro laboratorio indican que está involucrada en el desarrollo de cáncer colorectal.

El presente estudio analiza la implicación de la Granzima-A en la activación de las células madre tumorales durante el desarrollo de carcinoma colorectal asociado a inflamación en modelo murino.

1. Introduction

CANCER STEM CELLS

The first time scientific community heard about cancer stem cells was around mid-1800's. "*The only point on which I lay stress is that the cause of the subsequent tumour is to be sought in a fault or irregularity of the embryonic rudiment*" Julius Cohnheim, 1889. With this statement, Julius presented to the scientific community a new way to understand cancer, the cancer stem cell theory (Conheim 1867). However it took over a hundred years to see the first evidence of this theory in a seminal paper from Lapidot (Lapidot T 1994).

It is well known that tumour cells within a tumour mass are very heterogeneous, overall in solid tumours, in terms of morphology, metabolism, proliferative rate, ability to metastasise and other features. This heterogeneity has been proposed for many years as an evident consequence of the genome instability and genetic reorganization produced in cancer cells. However, Lapidot et al. and a few years later Bonet et al., showed that these different cells found within a tumour did not have the same ability to produce tumours in mice (Lapidot T 1994, Bonnet D 1997). In these studies, different populations were identified based on the expression of different CDs (CD34 and CD38). The different populations were isolated using flow cytometry sorting and injected in NOD/SCID mice (severe combined immunodeficient mice) at the same cell concentration. After few weeks, just one particular cell population, $CD34^{+}$ and $CD38^{-}$, was able to produce tumours. After this discovery, researchers' attention was attracted again and cancer stem cell theory was put on the table and reviewed carefully.

Cancer stem cells theory

Cancer stem cell (CSC) hypothesis assumes that tumours are organised hierarchically, analogous to a normal tissue. In this cellular structure, CSCs would be on the top and through differentiation, other cell types would emerge.

The previous theory, the stochastic model or clonal evolution, describes heterogeneity in tumours as a consequence of high speed growing in tumour cells. As all of them are considered equal in terms of proliferation and tumour-forming potential, all different populations should have the same capability of initiating tumours. However, as Lapidot and Bonnet described, tumours might not be just a cluster of cells which grow quicker than other ones. Some cancer cells have the intrinsic ability to create an entire tumour mass with all the different populations meanwhile others only can divide themselves. Both models are shown in *Image 1*.

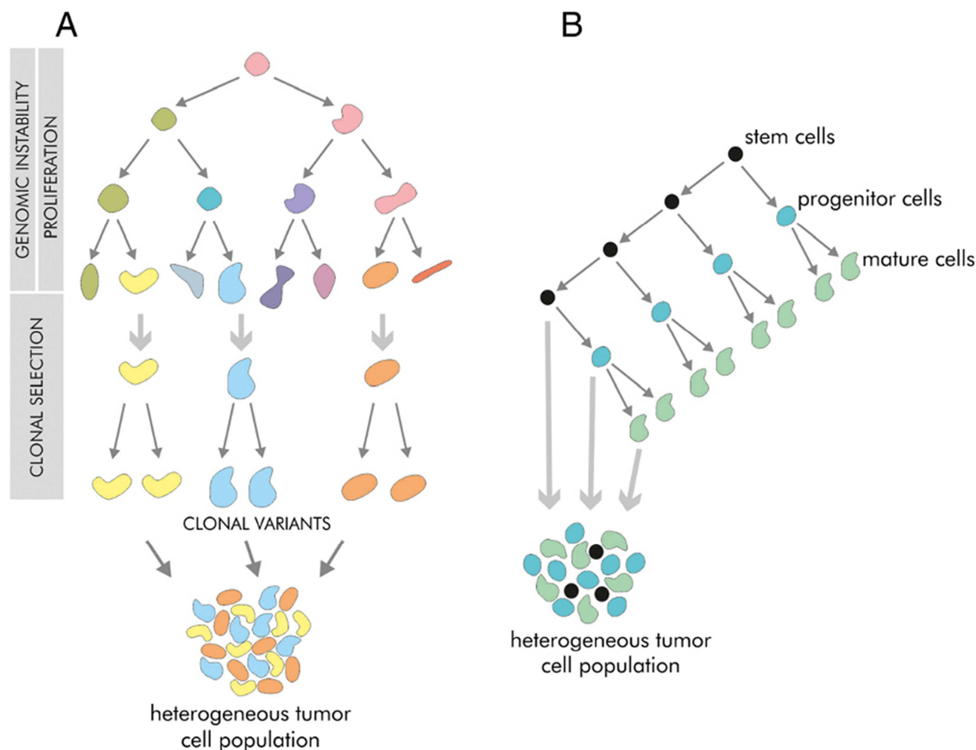

***Figure 1*. Tumour heterogeneity models**. A) Clonal evolution model. High proliferative and genomic instability result in a large number of cells differing in genotype and thus phenotype. The best fitted cells are selected by Darwinian processes to generate clonal variants of the tumours. B) CSC model. CSC population is capable to unlimited number of divisions. Tumour heterogeneity results from existence of phenotypically diverse populations of different stages of cell maturation (Fulawka L 2014).

In normal tissues, three different populations can be found regarding their differentiation state: stem cells, progenitor cells and mature cells. Stem cells are low proliferative cells which have the potential of differentiating into any cell of the tissue; although they are a minor population, they are able to divide throughout the lifespan of the organism. Progenitor cells come from the stem cells and have a lower differentiation potential. They grow faster than stem cells but do not have an “infinitive” division capability. Finally, mature cells are the descendants of the progenitor cells and have no differentiation potential. Dying mature cells are replaced by new-born mature cells derived from progenitors, so then stem cells are responsible of the maintenance of the tissue. CSC model propose that tumours are equally organised than normal tissues. CSCs would be the responsible of maintaining all the tumour populations and would be able to divide themselves and survive at least as much as the normal stem cells.

CSCs have two **main characteristics** that are vital in the maintenance of the tumour: self-renewal and differentiation capabilities. These features are share with normal stem cells and actually define themselves as stem cells. Self-renewal capability is the ability of stem

cells to produce daughter cells with the same characteristics as the parental cells. In this way, stem cell population is continuously renovating itself and never disappears. That feature is vital in order to keep a residual stem cell niche within the tissue and, in the case of the CSCs, to survive against antitumor mechanisms. In the division process, stem cells also generate other daughter cell types which have different features and lack of self-renewal capability, the cancer mature cells. This process is called cell differentiation (Fulawka L 2014). The term "stemness" has been adopted to denote both characteristic that define CSCs as well as normal stem cells.

Signalling pathways implicated in CSC development

The signalling pathways implicated in self-renewal and differentiation processes have been investigated for many years in order to elucidate the mechanisms by CSCs survive and keep the stemness. Notch, Hedgehog (Hh) and Wnt pathways are proposed as the best candidates to maintain the stemness and they likely are the most crucial for the tumorigenic potential of CSCs (Takebe N. 2015). **Notch signalling**, similar to the Wnt and Hh pathways, is a primordial, evolutionarily conserved cell-fate-determination pathway that has a great relevance in biology, from angiogenesis to CSCs or tumour immunity. It is involved in the communication between contiguous cells. The signalling pathway is show in *Image 2*. This pathway can be activated with 5 canonical ligands (DLL1, DLL2, DLL3, Jagged1 and Jagged2) which interact with 4 receptors (Notch1-4). Some clinical trials are underway to inhibit this pathway as it has been related to poor prognosis in cancer. **Hh signalling pathway** controls tissue polarity, patterning maintenance and stem-cell maintenance during the embryonic development. Hh is a protein which is released from a cell through the transporter Dispatched after acylation. Released Hh binds to the receptor Ptch1 to initiate its signalling pathway, shown in *Image 2*. Hyperactivation of this pathway has recently been recognized to cause tumorigenesis in a wide variety of tissues and its inhibition has shown to inhibit epithelial-to-mesenchymal transition (discussed below). Finally, **Wnt signalling pathway** is involved in embryogenesis and tissue self-renewal. Wnt proteins consist of a family of glycoproteins that serve as ligands for Frizzled (Fz), a transmembrane receptor. Fz receptors signalling converge in the transcription factor β-catenin, which is able to translocate into the nucleus thanks to the receptor signalling. Deregulation of this pathway have been associated to cancer and CSC activity, and β-catenin has been descripted to be necessary to maintain the CSC phenotype (Takebe N. 2010, Takebe N. 2015).

CSCs origin and epithelial-to-mesenchymal transition

In several kinds of cancer, CSCs have been proposed to originate from normal stem cells through tumour transformation (Sell 2004). However, some publications for over last decade have revealed than differentiated epithelial cancer cells can also serve as source of CSCs by reactivation of a latent developmental process called epithelial-to-mesenchymal transition or EMT.

EMT originally defined a process of cellular reorganization essential for embryonic development. This multi-step process results in the loss of cell-to-cell adhesion and the gain of invasive and migratory properties. During embryogenesis, EMT allows progenitor cells to migrate to distant sites within the embryo and generate the different tissues. Also, EMT is reversible in order to allow mesenchymal cells to differentiate and form the specific tissues and organs.

EMT can also occur during tumorigenesis. It was first proposed as a contributor to cancer due to a remarkable concordance between the mesenchymal phenotype and characteristics observed in metastatic tumour cells. They need the ability to get out from tumour mass and extracellular matrix, migrate through the circulatory system and colonize a new site (Chang J. T. 2013). During the EMT, cells downregulate E-cadherin, a glycoprotein involved in cell adherence, and its loss has been linked to metastasis and poor prognosis. Wnt, Notch and Hh are known as EMT inducers as well as TGF-β cytokines and other niche factors. These factors have been related to cancer-associated fibroblasts and their co-culture with tumour cells induce EMT (S. Lamouille 2014).

Clinical relevance of CSCs

Increasing evidences support the role of CSCs during tumour recurrence. Traditional chemotherapy targets cells which high proliferation rate and so, CSCs might resist this kind of treatment thanks to their intrinsic characteristics, and regenerate the whole tumour. CSC-related markers have been found to be predictors of clinical outcomes (Pece S 2010). High expression of ATP-binding cassette (ABC) superfamily of active drug transporters and multidrug-resistance proteins (MDRs) have been found in CSC population (Wong, 2011) . Also, we have already discussed the implication of EMT in the metastatic process and its relationship with CSCs.

All these data suggest that targeting CSC would increase the favourable responses. Inhibitors of stemness signalling pathways are on clinical trials such as γ-secretase inhibitors, which inhibit the Notch pathway, or Porcupine inhibitors targeting the Wnt signalling pathway (Porcupine in involved in the synthesis of Wnt proteins) (Takebe N. 2015).

Differentiation therapy emerged following the evidence of CSCs could come from normal stem cells. This kind of therapies tries to differentiate CSCs in order to force them to become mature cells. Mature cells do not have stemness characteristics and so, they would not be able to overcome traditional therapies (Sell 2004).

COLORECTAL CANCER, INFLAMMATION AND CSCs

Colorectal cancer

Colorectal cancer (CRC) is the third leading cause of cancer death worldwide with nearly 150.000 new cases diagnoses annually and the 5-year relative survival rate is only 8%

despite diagnostic and therapeutic advances (Thomassen I et al, 2013). Sporadic CRC arises as a consequence of lacking homeostatic control of proliferation and apoptosis within colon epithelial cells, driving the cells towards immortality and increased proliferation. This deregulation is caused by genetic and epigenetic alterations such as p53, PI3K or EGFR. Wnt-signalling pathway is the major driver of CRC initiation and progression. As we have already discussed, upon activation of the Wnt signalling pathway, β-catenin is translocate into the nucleus where it associates with transcription factors and different genes are activated. One of the most frequently Wnt-pathway related mutated genes in early transformation is *APC* (*adenomatous polyposis coli*). APC mutations generally result in defective β-catenin degradation and, as a consequence, the accumulation of β-catenin in the nucleus producing a perpetual transcription of Wnt target genes. Other genes associated to Wnt signalling pathway and commonly mutated in CRC are *TP53* and the *KRAS* oncogene (Lise et al, 2015). All these events are grouped in three stages which are shown in *Figure 3*. In inflammation-associated CRC, the progression starts on normal tissue which is affected by the environment or mutagens. These agents produce an injury which derives into a transformation of some cells in the colon crypts. Inflammation drives this stage in which signalling pathways are affected. These pathways evolve towards a low-grade adenoma, high-grade adenoma and finally a carcinoma, the final stage of the disease. Tumour recurrence and metastasis are two critical survival-influencing factors of CRC so that, identification and targeting of CSCs is an active field in CRC research (Brenner et al., 2014).

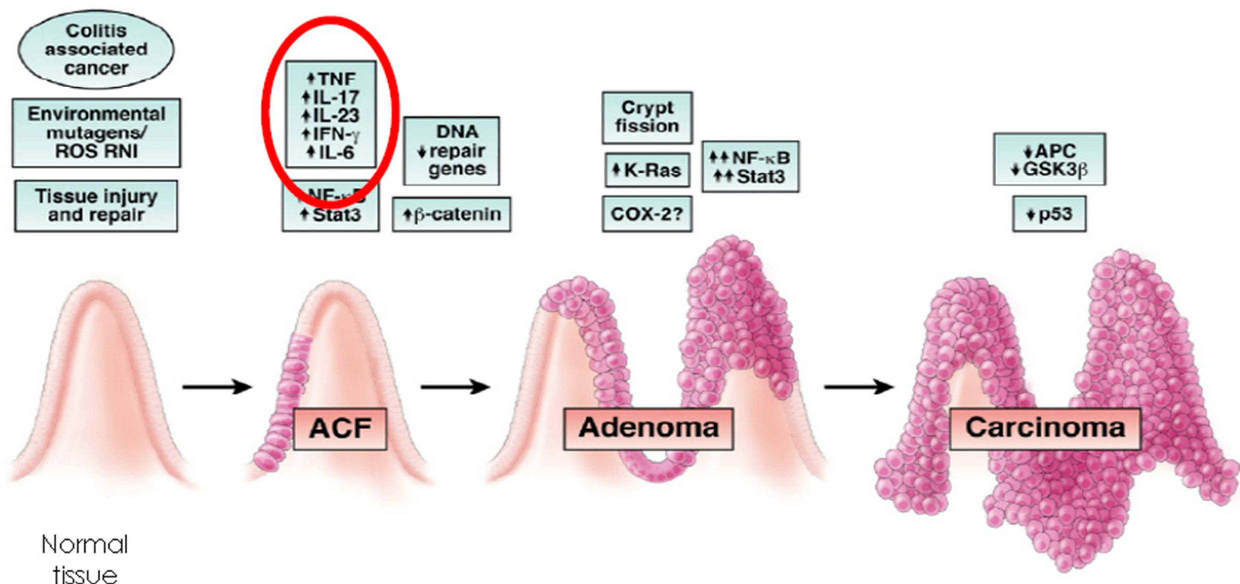

Figure 3. Scheme of the CRC progression. On the top of the image, it is shown the key events of each stage on the development of inflammation-associated CRC. The progression goes from normal tissue to carcinoma, passing through aberrant crypt and adenoma, and it is marked in red the inflammatory molecules implied in this progression.

ACF: aberrant crypt formation

Inflammation in CRC

Tumour progression depends on the interaction between tumour cells and all the components of the tumour microenvironment including macrophages, B and T cells even fibroblasts. Tumours have the ability to remodel the stroma and establish a permissive microenvironment which includes growing factors and inflammatory cytokines that recruit inflammatory cells. Although chronic inflammation may be involved in all three stages of tumour development (initiation, promotion and progression), it appears to play a major role in promotion and progression (Karin, 2009).

Inflammation is driven by soluble factors such as cytokines and chemokines which are produced by tumour cells as well as the cells which are recruited by them. Depletion of mast cells or macrophages resulted in a profound remission in intestinal polyps in mice confirming the implication of immune cells in the progression of cancer. Several inflammatory cytokines have been involved in colorectal carcinoma progression like TNF-α, IL-8 and IL-6 (Lidija Klampfer, 2013).

Recently, it has been described a serine-protease protein family produced by immune cells called granzymes (Gzm) which could be a link between cellular immunity and inflammatory processes such as CRC.

Cytotoxic mechanisms of the immune cells

Granzymes are mainly expressed inside cytosolic granules within cytotoxic B lymphocytes (CTL) and Natural Killer cells (NK cells).

CTLs and NK cells use IFN-γ, death cell ligands (TRAIL and FasL) as well as exocytosis of these granules in order to control intracellular pathogens and transformed cells. The granule exocytosis is a process leaded by the recognition of the immune cells and target cells, which produces the release of the granules. These granules contain perforins and Gzms (Martínez-Lostao et al, 2015).

Perforin is a membrane-pore forming protein which is essential for the Gzm cytotoxic mechanism. Perforin produce pores which are not able to kill cells on their own but allow Gzms to enter into the cells (Voskoboinik I et al, 2015). Perforin-deficient mice lose the cytotoxic capability of their lymphocytes and cannot control the viral infection as much as wild type mice do. Also, its role in tumour control is being clarified. Actually, perforin-deficient mice develop lymphoma spontaneously (Arias et al, 2014).

On the other hand, Gzms are a serine-protease protein family located within cytotoxic granules of the NK cells and lymphocytes. There are 5 Gzms known in humans (A, B, H, K and M) and 10 in mice (A, B, C, D, E, F, G, K, M and N) (Ewen et al, 2011). GzmA and GzmB are the most abundant and better characterised. Each Gzm has different cut

specifications and substrates. GzmA and B in addition to perforin constitute one of the main mechanism in antiviral and antitumor activity (Pardo et al, 2009).

GzmB can act in the extracellular medium independently of perforin, regulating processes such as inflammation, coagulation an aging (Pardo et al, 2007). Inside the cells, GzmB is able to cut a wide variety of substrate but mostly caspase 3 and Bid proteins. Once they are processed, these proteins are able to produce apoptosis in the cell.

The role of the GzmA in the cytotoxic process is still in controversy. The original articles when GzmA was described as well as its cytotoxic capability used high concentration of the protein which is almost impossible to find physiologically. Also, GzmA from different animals were used in those experiments (Hayes et al, 1989). However, experiments which use Gzms and perforins from the same specie showed that GzmA was not able to kill target cell on the contrary to GzmB at low concentrations (Susanto et al, 2013). Although this controversy in the role of GzmA in cytotoxic events, its influence in the inflammation is more clear. GzmA is able to process pro-IL 1β as well as induce the release of IL-1β, TNF-α and IL-6, all pro-inflammatory cytokines. Confirming this role in inflammation, GzmA-deficient mice are more resistant to sepsis than wild type (Arias et al, 2014).

Inflammation and CRC-associated CSCs

All the CRCs produce a local inflammatory response in a high or low degree, which actually means that immune cells are infiltrated within the local environment and produce the inflammation. Studies with mouse models indicate that the elimination of cytotoxic lymphocytes CD8 reduce the aggressiveness of the colitis (an inflammatory colon disease) and the incidence of colitis-associated CRC (Philippe et al., 2006). Immune cells are a reserve of all these pro-inflammatory cytokines so their elimination contributes to a better prognosis. In fact, most of the carcinoma has a constitutive activation of transcription factors such as NK-κB and STAT3 (Ditsworth et al, 2004).

Inflammation is a key step in the development of CRC and it has been demonstrated that pro-inflammatory cytokines in tumours predict a poor diagnostic. CSCs have been proposed as an explanation of this increase of the tumorigenesis. Li Y et al found that TNF-α was able to activate the Wnt signalling pathway through the activation of NK-κB, and its activation produced more amount of cancer-initiating cells (cells which are able to create a tumour mass and are related to CSC characteristics). These authors found that this cytokine can influence in the development and invasion of tumour cells by CSC renewal and EMT activation. IL-1B produced the same effects due to the fact that this cytokine activated the same pathway (Li et al, 2012). Also, indirect evidences of the relation among CRC and inflammation come from epidemiologic studies, in which administration of non-steroidal anti-inflammatory drugs is inverse related with the incidence even mortality (Flossmann et al, 2007).

2. Antecedents and Objectives

CSCs have been typically identified and isolated based on the expression of one or multiple cell surface markers associated to cancer stemness such as CD133, CD44 or different transcription factors associated to CSCs. *Image 3* show the hallmark of CSCs and the goal in CSC identification and isolation.

CD133 is also known as prominin-1. It belongs to a pentaspan transmembrane glycoproteins family which specifically localize to cellular protrusions. Although its function is unknown yet, it is believed to be associated with tumorigenesis and tumour progression. The up-regulation of CD133 in colorectal cancer correlates strongly with poor prognosis and synchronous liver metastasis (Spiegelberg et al, 2014).

CD44 is the hyaluronic acid receptor. It has a role in facilitation of cell to cell and cell-matrix interactions and in the assembly of growing factors on the cell surface; it presents cytokines and chemokines to their complementary receptors on the cell membrane. CD44 has been used extensively as a CSCs isolating marker in so many cancers such as breast, prostate, pancreas and colorectal cancer. As few as $CD44^+$ cells can promote tumorigenesis in mice and display stemness properties such as self-renewal and differentiation. Some contradictory papers can be found in the bibliography in order to validate this marker as a real CSC marker so controversy is still present (Jaggupilli et al, 2012).

CD24 protein has been associated to CSC properties too. It is a small cell surface protein anchored by glycosyl-phosphotidyl-inositol and involved in cell-to-cell and cell-matrix interactions. CD24 was discovered in mice as a heat-stable antigen and was used as a marker to differentiate hematopoietic and neural cells before knowing its implication in cancer. CD24 expression in tumours has been associated with poor prognosis and increased metastasis (Jaggupilli et al, 2012).

CD166 is another novelty cell surface marker used in the identification for CSCs in colorectal cancer. Its expression has been pathologically correlated with aggressive disease and high-expressing cells isolated in tumours are able to generate tumorigenesis in mice at low numbers, a hallmark of a CSC population (Levin et al, 2011)

In addition to cell surface markers, other features can be analysed in order to identify CSCs in tumours in general and in particular in colorectal cancer. One strategy is to identify **transcription factors associated to pluripotency** such as Oct3/4, Sox2 or Nanog. These factors were first descripted as the transcription factors necessary for induced pluripotential cells (iPS cells). Their expression can be detected in CSCs and it is a sign of poor prognosis in many types of cancer, including colorectal carcinoma, as well as of relapse, distant recurrence and chemoresistance (Amini et al, 2014)(Liu et al., 2013).

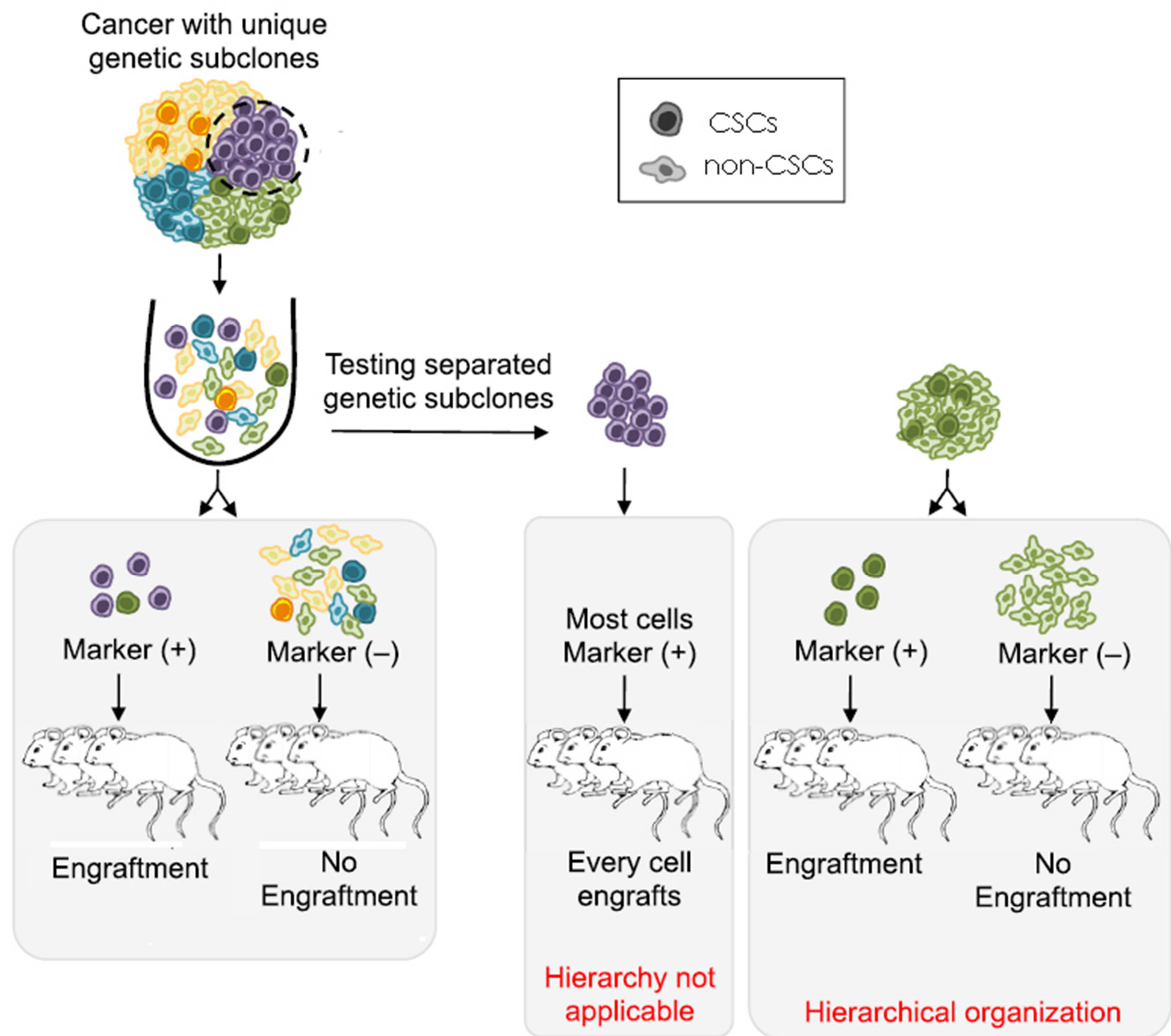

Figure 3. **Typical way to isolate and identify stemness markers.** Cells with a high expression of one particular CSC-related marker are isolated by flow cytometry and injected into NOD/SCID mice at low number. If these cells are able to engraft, the marker analysed might be a CSC-related marker.

ALDH1 activity is one of the widest markers used to identify CSCs, from now named as ALDH activity. ALDH1 (aldehyde dehydrogenase) belongs to a family of dehydrogenases which are involved in detoxification processes. ALDH activity has been used to detect and isolate both cancer stem cells and normal stem cells. Immunostaining data show that rare epithelial cells at the base of the normal crypt express ALDH activity (normal stem cells in their stem cell niche). In cancer, this activity is not limited to the base of the crypt and that expression expands in the epithelia of the cancerous colon. As few as 25 primary colon cancer cells expressing high levels of ALDH are able to generate tumours in NOD/SCID mice confirming their CSC properties of these cells (Shenoy et al, 2014).

Another marker typically used for the identification of stem cells, and later CSCs, is the **ABC transporter activity**, which can be analysed using the Hoechst 33342 expulsion test. This test has been used to evaluate the capability of cells of ejecting colorants thanks to their efflux

pumps. These pumps are ABC transporters involved in the excretion of diverse compounds and they are descripted as a detoxification mechanism. Cells with have a high expression of these transporters are commonly known as side population and are associated with stemness properties (Dean, 2009).

IMPLICATION OF GRANZYME-A IN INFLAMATION AND COLORECTAL CANCER

Although our data are not published yet, we have strong evidence that GzmA is involved in the development of inflammation-induced colorectal cancer. We use a mouse model of chronic inflammation leading colorectal cancer which consists in one first injection of a mutagenic compound, AOM, and one-week cycles of DSS treatment in drinking water (in detail description in *Material and Methods* chapter) which induce a chronic inflammation. These compounds together generate inflammation in colon which finally derivate into colorectal cancer. Our results have shown that GzmA-deficient mice have a lower incidence of cancer than wild type ones. Wild type mice show signs and symptoms of a high inflammation in colon and normal colon structure has completely destroyed after the treatment whereas GzmA do not show that signs.

MASTER DISSERTATION OBJECTIVES

In the light of these results, we proposed the hypothesis of GzmA was involved in the emergence and development of CSCs in inflammation-associated CRC. So that, we proposed the following main goal: to figure out the implication of GzmA in the development of colorectal carcinoma through the study of CSCs. Our results show how GzmA produce a higher tumour incidence and this project tries to unravel the molecular and cellular mechanisms involved in this process.

To get this main goal, we proposed the following secondary objectives:

1. Evaluate the presence of CD44, CD166, Nanog, Sox2 and Oct3/4 CSC-related markers in colorectal cancer mouse cell lines.
2. Evaluate the correlation between these markers and other CSCs-associated features such as ALDH activity and Hoechst 33342 expulsion (ABC transporter activity) and chemoresistance.
3. Development of *in vitro* cell cultures enriched in CSC population such as 3D cell cultures an chemoresistant cancer cell lines
4. Evaluate the presence of CSCs during CRC development *in vivo* in WT and GzmA-deficient mice.

3. Materials and Methods

3.1 - MATERIALS

3.1.1 – Cell lines

The next CRC cell lines were used in this study: CT26 was obtained from N-nitroso-N-methylurethane-induced colorectal carcinoma in BALB/c mice, and MC38 and PJ01 were obtained from DSS-and-AOM-induced colorectal carcinoma in C57Bl/6 mice. CT26 and MC38 cell lines were gently donated by Dr. Ignacio Melero, Clinical University of Navarra. PJ01 cell line was generated in our lab following the DSS/AOM-induced colorectal cancer-induction protocol as described below. These cell lines grow as adherent cells and have similar morphology to epithelial cells (Wang M et al, 1995) (Eisenthal A et al, 1993).

3.1.2 – Cell culture maintenance materials

Dulbecco's modified Eagle medium (DMEM) from Lonza (Germany) was used as culture medium for all the cell lines. Fetal bovine serum (FBS) was from the same commercial company.

Cell culture flasks, 15 ml and 50 ml conical tubes (Falcon tubes), 96-well plates and the rest of disposable sterile material were provided by Corning Company (USA). 5, 10 and 50 ml pipettes were supplied by Corning too. Cryogenic tubes to freeze cells were provided by Nunc (Netherlands).

L-glutamin, penicillin, streptomycin, dimethylsulfoxide (DMSO) and trypsin-EDTA were provided by Sigma (Spain). Paraformaldehyde (PFA) was obtained from Scharlau (Spain). Different powders to make the buffers such as calcium phosphate, calcium chloride or sodium chloride were provided by Panreac (Spain).

3.1.3 – Antibodies and reagents used for CSCs analysis and sorting

To measure the expression of CD44, CD166, Nanog, Sox2 and Oct3/4, antibodies against these proteins were used. These antibodies are conjugated with different fluorophores in order to measure the union to the targeted proteins by flow cytometry (fluorophores used shown in *Table 1*). As a negative control, IgG1 isotypes conjugated with the same fluorophores were used (REA control). All antibodies were provided by Miltenty Biotech (Germany). The protein Annexin V was expressed and purified in our lab and conjugated with the fluorophores APC and FITC. 7-AAD reagent was provided by InmunoStep. The fluorescence maximum emission peak of Annexin V-FITC protein is at 519 nm while Annexin V-APC's one is at 660 nm. 7-AAD's is at 647 nm.

Hoechst 33342 was provided by Sigma and its maximum emission peak is at 461 nm. The ALDEFLUOR kit (ALDH enzyme activity detection kit) was provided by STEMCELL Technologies. The kit contains the reagent ALDEFLUOR, ALDEFLUOR buffer, ALDEFLUOR DEAB, HCl and DMSO. The ALDEFLUOR reagent emits fluorescence at 488 nm.

Sorting was performed using FACS Aria sorting flow cytometer (BD Bioscience) at the flow cytometry service at CIBA.

Antibody	Fluorophore conjugated	Excitation peak wavelenght (nm)	Emission peak wavelenght (nm)	Clon	Isotype
Anti-CD44	PE	565	573	REA664	IgG1
Anti-CD166	APC	650	660	REA370	IgG1
Anti-Nanog	APC	650	660	REA297	IgG1
Anti-Sox2	APC	650	660	REA320	IgG1
Anti-Oct3/4	APC	650	660	REA622	IgG1

***Table 1*. Technical information about antibodies used.** Fluorophore conjugated, excitation peak wavelength, emission peak wavelength, hybridome clon and Isotype data are shown in this table.

3.1.4 – Mice strains and colorectal cancer induction materials

Inbred C57Bl/6 (B6) and mouse strains deficient for Granzyme A (GzmA -/-) bred on the B6 background were bred at Centro de Investigación Biomédica de Aragón (CIBA). Their genotypes were periodically analysed as described (Pardo et al., 2008). Mice of 8-12 weeks of age were used in all experiments and were performed in accordance with and approved by the local animal care commission.

DSS, dextran sodium sulphate, was provided by MP (Canada) and AOM, azoxymethane, was provided by Sigma. Syringes were from BD Bioscience (Spain) and cell strainer from Miltenyi Biotech (Germany).

Animal maintenance was at CIBA Animal Care Services.

3.1.5 – Tumour disaggregation reagents

Kit contains Predigestion solution (1xHBSS containing 5 mM EDTA and 1 mM DTT) and Digestion solution made of 0.05g of collagenase D (from Roche), 0.05 DNAse I (from Sigma (Spain)) and 0.3g of dispase II (from Roche) all dissolved in 100 ml of PBS.

3.1.6 – Other reagents

3-(4,5-dimethylthiazol-e-yl)-2,5-diphenyltetrazolium bromide (MTT) was provided by Sigma (Spain). The commercial solution is at 5mg/ml. Methocel (methylcellulose) was provided by Sigma (Spain) for developing *in vitro* 3D cultures.

3.1.7– Analysis software

All cytometry data were analysed using the software Weasel (v1.6) and statistical analysis were performed using GraphPad Prism 5 Software.

3.2 – METHODS

3.2.1 – CELL CULTURE MAINTENANCE

3.2.1.1 – Cell lines maintenance and manipulation

All cell lines were cultured in 25 or 75 cm^2 cell culture flasks depending on the necessity. The cell lines were cultured in DMEM medium supplemented with 10% of SFB, Glutamax 2 nM (glutamin) and antibiotics (penicillin 100 U/ml; streptomycin (100 µg/ml), known as complete DMEM medium. Cell lines were subcultured every 2 or 3 days depending on the cell proliferation rate. Cells were detached using a mixture of Trypsin-EDTA for 5-10 minutes (5-10’). After the incubation, cells were resuspended in complete DMEM medium and centrifugated at 525 xg for 5’. After discarding the supernatant, all cells were resuspended at the desired concentration in complete DMEM medium and placed into the corresponding culture flask. Cultures were performed in an incubator at 370C, 5% CO_2 and moisture saturated air.

Cell manipulations were performed into a vertical laminar flow hood (Telstar Bio All), which allow us to work in sterile conditions. Hand-made buffers were sterilised using 0,22 µm pore-size filters or using an autoclave depending on the stability of the reagents.

3.2.1.2 – Determination of cell number and viability

Cell counting and cell viability were determinated using a Neubauer haemocytometer and Trypan blue exclusion test. Trypan blue is a colorant able to get into the dead cells which have lost membrane integrity. To perform it, we mix an aliquot of the cell culture with the same volume of Trypan blue solution the blue and non-blue cells are count in the Neubauer chamber using an optical microscope (Nikon).

3.2.1.3 – Cell freezing and thaw

To freeze, cells were taken in a Falcon tube and were spun down 525 xg for 5’. After the centrifugation, supernatant was discarded and cells were resuspended in complete DMEM medium supplemented with 10 % of DMSO. Cell suspension was put into sterile freezing containers frozen at -80º C and subsequently stored in liquid Nitrogen.

To thaw, cell vial was taken out from the freezer and got warm little by little. As cell-containing ice is being defrost, liquid was taken and added to a Falcon tube with complete DMEM medium. When all cells were added to the tube, it was spun down 525 xg for 5'; the supernatant is discarded and cells were resuspended in complete DMEM medium and cultured as described above.

3.2.2 – ANALYSIS OF CSC-RELATED CELL SURFACE MARKERS AND TRANSCRIPTION FACTORS IN CELL CULTURES

The analysis of the surface markers was performed using specific antibodies targeting the proteins described in the literature as CSC-related proteins. We used antibodies against CD44, CD166, Nanog, Sox2 and Oct3/4; CD44 and CD166 are extracellular proteins while Nanog, Sox2 and Oct3/4 are intracellular transcription factors and different protocols were used for each protein type.

3.2.2.1 – Protein extracellular staining

After trypsinization and cell count, 150.000 cells were taken and add them into wells of a 96-well plate. The plate was spun down and cells are resuspended in 50 µl of PBS with 5% of FBS. Antibodies anti-CD44 and anti-CD166 were added diluted according to the optimised concentrations (CD44 at 1/200 and CD166 at 1/20) as well as 7-AAD and Annexin V (both at 1/10). 7-AAD is a reagent that enters the dead cells with the plasma membrane damaged. This reagent allows us to identify those cells which have some damage in the membrane and so, they are dead, and those which are supposed to be alive with the membrane intact. Annexin V is a protein that binds phosphatidylserine (PS); PS is a lipid exposed on the outer plasma membrane as an early event on the apoptosis development, so the identification of this lipid allow us to discriminate early apoptotic cells which have the membrane intact yet (and so 7-AAD is not able to get into them) and living cells. As negative isotype controls, IgG1 antibodies chains conjugated with APC and PE were used at the same concentration as the corresponding antibody. IgG1 correspond to the heavy chain of the antibodies so it is used as a measure of the non-specific joints. As proteins, antibodies can interact with other membrane proteins on the cells and fluorescence would be detected although there was not specific recognition; using the nonspecific region of these antibodies (heavy chains) linked with the same fluorophores, these nonspecific interactions can be detected and eliminated. Cells were incubated with the antibodies for 20' at 4º C in order to reduce the nonspecific interactions. Afterwards, plate was spun down at 2.850 xg for 2' and each well was resuspended in 100 µl of PBS with 5% of FBS, transferred to flow cytometer tubes and analysed using 4 colour flow cytometry in a Beckman Coulter GALLIOS flow cytometer. The cytometer is able to analyse each cell independently; as default, size and complexity are analysed for each cell but also, fluorescence can be measured. In this case, 4 fluorescence measures were analysed as well as size and complexity for each analysis.

3.2.2.2 – Protein intracellular staining

After trypsinization and cell count, 150.000 cells were taken for each analysis and added into wells of a 96-well plate. The plate was spun down and cells were resuspended in 50 µl of PBS with 5% of FBS. 7-AAD and Annexin V were added to the wells as described above and cells were incubated in the fridge at 4ºC for 20’. Afterwards, the plate was spun down and cells were resuspended in 50 µl of PBS with 4% PFA. PFA crosslinks the proteins and fix the cells. The fixation is a key step to keep the proteins and the cell cycle in the same state during the staining but obviously, cells are not alive anymore after this step. Cells were incubated with PFA for 20’ at 4º C. After the incubation, the plate was spun down at 2.850 xg for 2’ and cells were resuspended in PBS with 5% of FBS to wash the PFA and were spun down again. Antibodies targeting Nanog, Sox2 and Oct3/4 were added then in 50 µl of PBS supplemented with 0.1% of Saponin. Saponin is a compound which permeates cell and nucleus membranes and so, antibodies can get into the cells and reach their targets. REA Control is used as the isotype negative control. Cells were incubated with the antibodies for 45’ at room temperature. Then, the plate was spun down at 2.850 xg for 2’ to wash the antibodies and cells were resuspended in 100 µl of PBS with 1% of PFA. Cells were transferred to flow cytometer tubes and analysed using 4 colour flow cytometry in a Beckman Coulter GALLIOS flow cytometer.

3.2.3 – ANALYSIS OF THE SIDE POPULATION, ALDH ACTIVITY AND ITS CORRELATION WITH EXTRACELLULAR AND INTRACELLULAR CSC-RELATED MARKERS IN CELL CULTURES

3.2.3.1 – Side population analysis

After trypsinization and cell count, 150.000 cells were taken in wells of a 96-well plate. Hoechst 33342 was added final concentration of 5 µg/ml and cells were incubated with the dye for 45’ at 37ºC. Hoechst 33342 is a dye which can attach to the DNA; to analyse the efflux pumps activity, all the DNA binding sites must be saturated in order to accumulate free dye in the cytosol. These conditions allow the dye to get into the cells and saturate the DNA joints. Once incubation was done, plate was spun down and cells were resuspended in 100 µl of complete DMEM medium without Hoechst 33342. The plate was incubated for 45’ at 37º C. As the DNA is saturated with the dye, efflux pumps can throw the Hoechst 33342 out the cell. Those cells which have a high expression of efflux pumps will be able to release more dye and then, a less stained cell population can be found using flow cytometry. This population is known as side population and it is related to CSC characteristics. After the incubation in complete DMEM medium without dye, the plate was spun down and cells were resuspended in PBS with 5% of FBS. Further analysis such as intracellular or extracellular staining can be performed after this protocol.

3.2.3.2 – ALDH activity analysis

To analyse the ALDH activity by flow cytometry, ALDEFLUOR kit was used. This kit includes ALDEFLUOR buffer, ALDFLUOR reagent, ALDEFLUOR DEAB reactive, HCl (concentrated at 2N)

and DMSO. ALDEFLUOR reactive must be activated as it is provided in inactive form by the commercial company. To activate the reagent, 25 µl of DMSO were added to the vial of lyophilised ALDEFLUOR reagent. After 1’, 25 µl of 2N HCl were added and it was mixed thoroughly. The mixture was incubated for 15’ at room temperature. After the incubation, the reagent was ready to be used.

To analyse the ALDH activity, 300.000 cells were transferred into an eppendorf tube with 1 ml of ALDEFLUOR buffer. 5 µl of activated ALDEFLUOR reagent was added and, after mixing well, 500 µl of the mixture were transferred into another eppendorf tube containing 5 µl of DEAB reagent was added previously. Both tubes are kept within an incubator for 45’ at 37 ºC. ALDEFLUOR reagent is highly fluorescence and the background is too high to measure fluorescence differences properly. DEAB is an inhibitor of the ALDH enzyme so, in this way, each analysis tube has its own negative control in which DEAB reagent inactivates the enzyme ALDH and background fluorescence can be measured.

After incubation with the ALDEFLUOR reagent, tubes were spun down at 525 xg for 5’ and cells were resuspended in 100 µl of PBS with 5% of FBS to wash the ALDEFLUOR reactive. Finally, cells were spun down again and were resuspended in 200 µl of PBS with 5% of FBS to their analysis by flow cytometry in 50 µl of PBS with 5% of FBS. Further analysis such as intracellular or extracellular staining can be performed afterwards.

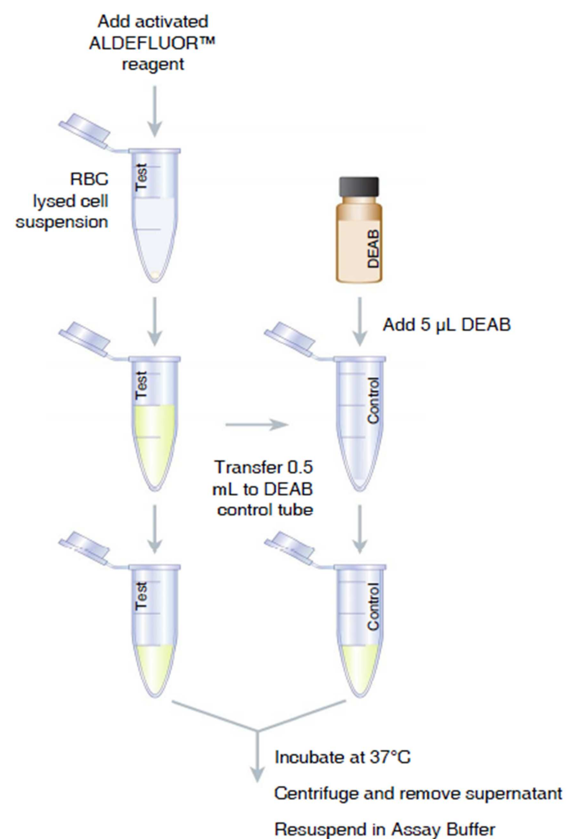

Figure 4. Schematic diagram which summarise the ALDH activity analysis protocol.

To analyse all the markers on the same cells, Hoechst 33342 staining protocol must be performed at first in order to preserve ALDH reagent fluorescence as much time as possible. After that, ALDH activity analysis protocol was performed. As cells are going to be split in two tubes on ALDH protocol, double amount of cells must be used at the beginning. After ALDEFLUOR staining, extracellular staining was performed, adding antibodies anti-CD44 and anti-CD166 as well as 7-AAD. Some experiments were done using only 7-AAD to distinguish living and dead cells due to the limitation of fluorescence filters which restrain the use of Annexin-V as additional cell death marker.

3.2.4 – CELL SORTING USING ANTI-CD44 ANTIBODIES

In order to analyse the differentiation and colony formation capabilities of potential CSCs, cells were sorted using a sorter flow cytometer. This equipment allows us to isolate different cell populations in different tubes or plates. Cells can be sorted according any characteristic analysed by the flow cytometer such size, complexity and, the most common, fluorescence staining.

Cells were stained with anti-CD44 antibodies following the protocol to stain extracellular proteins. Intracellular protein staining cannot be used as a sorting tool due the fixation step what kill the cells.

To sort cells, at least $1x10^6$ cells must be stained following the extracellular protocol previously described. The basis of the technique is: in a previous flow cytometry analysis, sorting regions are selected; fluorescence measures of these regions are registered by the sorting software and cells with the same fluorescence are electrically charged positively. Cells with different fluorescence are charged negatively. Using electric fields, both cell populations can be separated in different tubes or added into different wells. Cells were sorting depending on their CD44 expression. Cells with high expression of CD44 are considered as $CD44^{high}$ and cells which have low expression are considered as $CD44^{low}$. Both cell populations were sorted in different plates and analysed as described below.

3.2.5 – DIFFERENTIATION ASSAYS

$CD44^{high}$ and $CD44^{low}$ were incubated in different 12-well plates at 30.000 cells/ml and 10.000 cells/ml. Wells with 30.000 cells/ml were incubated for 48h and then, was analysed by flow cytometry. Wells with 10.000 cells/ml were incubated for 1 week and then, CD44 expression was analysed as the same way.

3.2.6– CLONOGENIC ASSAYS

100 cells per each population were added to a 24-well plate in 2 ml of complete DMEM medium and incubated at 37ºC. After 10 days, the number of colonies was analysed using violet crystal staining. Cells were incubated for 20’ at room temperature with violet crystal solution which contains 6% of glutaraldehyde. After the staining, solution was recovered from the wells carefully and the colorant excess is washed using PBS. Colonies are now counted and pictures were taken.

3.2.7 – LIMITING DILUTION ASSAY

Cells were sorted by flow cytometry but now one cell was collected per well in a 96-well plate and incubated in complete DMEM medium for 2-3 weeks. If a single cell is able to create a colony, a white dot will be observed after the incubation. The numbers of the colonies formed are counted in each plate as well as the type of colony concerning its size.

3.2.8 – ENRICHMENT OF CSC POPULATION USING PACLITAXEL AND DOXORRUBICIN DRUGS

3.2.8.1 - MTT assays

To find out the IC50 of each drug (the drug concentration that inhibit cell proliferation at 50%) in each cell, MTT assays were used. On a 96-well plate, 5.000 cells were added in a 96-well plate and incubated in 50 µl complete DMEM medium overnight. The following day, drugs were added at different concentrations in 50 µl of medium. Cells were incubated with the drugs for 48h and afterwards, 10 µl of MTT (5 mg/ml) was added to each well and incubated at 37º C for 1h. MTT is reduced by living cells generating an insoluble compound known as formazan that accumulates inside living cells as a purple coloured aggregate. On this way, the proliferation rate in medium with different drug concentrations can be measured after 48h. After the incubation with MTT reagent, the plate was spun down at 2.850 xg for 30', supernatant was discarded and cells were resuspended in 100 µl of DMSO in order to dissolve the formazan crystals. Once dissolved, absorbance was measured at 540 nm.

3.2.8.2 – CSC marker analysis after the drug treatment

The IC50 concentrations determinated in the previous experiments were used to enrich CSC population in cell lines. CSCs are supposed to be more resistant to drugs than differentiated cancer cells so they should be enriched after treating cell cultures with adequate concentrations of drugs.

100.000 cells were added in a 12-well plate in 500 µl of complete DMEM medium. After 24h, drugs were added and incubated for 48h. Then, supernatant was transfer to an eppendorf tube and cells attached to the plate were trypsinised in order to take all the cells, living as well as dead cells (which would be floating in the supernatant). When all the cells (living and dead) were taken in an eppendorf tube, it was spun down at 525 xg for 5' and cells were stained following the protocols previously described. As control, cells with no drugs were used.

3.2.9 – PRODUCTION OF CHEMORESISTANT CELL LINES

Chemoresistant cell lines to PTX and Doxorubicin were generated using CT26, MC38 and PJ01 cell lines to find out if CSCs can reconstitute the starting cancer cell populations.

To create chemoresistant cell lines, cells were cultured in the presence of drugs as it has been described (Dallas et al, 2011). IC50 was considered again as a reference; this was the starting concentration. Cells were treated for 48h with drugs and then, medium was replaced by a fresh medium. Cells were incubated in medium without drug until reaching the confluence; cells were subcultured and then, drug was added again. This procedure was followed at least for one month until cells were able to grow in the medium containing drugs. At this point, the length of drug treatment was increased to 4 days.

3.2.10 – DEVELOPMENT OF 3D IN VITRO CELL CULTURES

This procedure is based on Methocel reagent (methylcellulose). Cells were added as drops in culture medium and methylcellulose on a Petri dish lid. When the lid was placed back on the Petri dish, drops did not fall down thanks to the high superficial tension created in the drop. As the drops were hanging, cells go down towards the bottom of the drop and were forced to stay together. If the cells have enough adhesion molecules, cells start to grow as a spheroid which actually is considered a 3D culture.

The method was optimised to make 1.000-cell-containing spheroids. A mixture of Methocel, complete DMEM medium and cells was placed in a Falcon tube. The proportions (V/V/V) of these compounds were: 10% of cells in complete DMEM medium, 20% of Methocel and 70% of complete DMEM medium. 25 µl were placed on a Petri dish lid forming drops. Once all the volume was added, the lid was turned over and drops were incubated 24h at 37ºC. The bottom of the Petri dish was covered with water in order to prevent evaporation of cell culture medium.

Once the spheroids had been formed, the drops were taken out and added in a 96-well plate. In order to analyse the CSC-related markers by flow cytometry, at least 50.000 cells were needed so around 10 spheroids were added per well. Spheroids were trypsinised using 100 µl of Trypsin-EDTA for 10’ and individual cells were stained as described above.

3.2.10 – AOM/DSS MOUSE COLORECTAL CANCER

C57Bl/6 mice were treated with a single injection of AOM plus 3 DSS cycles for 56-60 days. AOM is a carcinogenic agent which in combination with a potent inflammatory stimulus induces colorectal cancer in mice. DSS administrated in drinking water produces a chronic intestinal inflammation. The combination of a single AOM injection with 3 cycles of DSS in drinking water produces inflammation-induced colorectal cancer in mice. AOM is administrated at 10 mg/kg of weight per mouse in 100 µl of PBS and DSS is administrated dissolved in drinking water at 2,5% (V/V). Each DSS cycle is followed by a two-week rest cycle during which water does not contain DSS. The scheme of the process is shown in *Figure 5*.

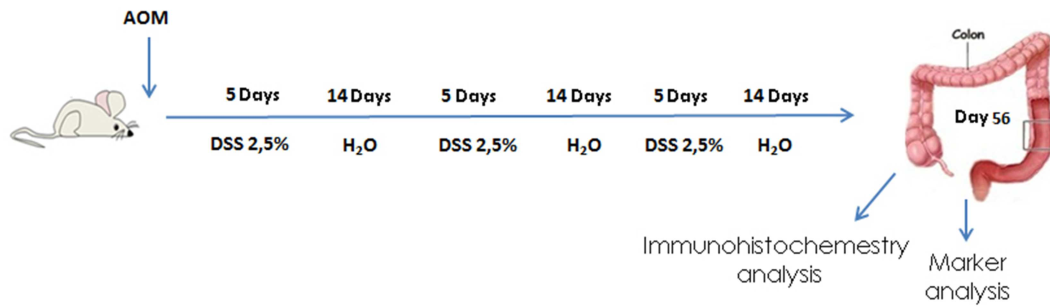

Figure 5. **Scheme of the AOM/DSS mouse colorectal cancer model.** Mice were treated with a single injection of AOM plus 3 DSS cycles for 56-60 days. Each DSS cycle is followed by a two-week rest cycle during which water does not contain DSS. AOM is administrated at 10 mg/kg of weight per mouse in 100 µl of PBS and DSS is administrated dissolved in drinking water at 2,5% (V/V). After 56-60 days, mice were sacrificed and tumours were extracted from the distal colon in order to analyse the CSC-related markers.

3.2.11 – ANALYSIS OF THE INFLUENCE OF THE GRANZYME A IN THE CSCs MARKERS IN INFLAMATION-INDUCED COLORECTAL CANCER MOUSE TUMOURS

GzmA-deficient (GzmA -/-) mice and WT mice were treated with the protocol described above for. After 56-60 days, mice were sacrificed in accordance with the local animal care procedures and tumours were extracted from the distal colon. Tumours were kept in complete DMEM medium and digested following a previously described protocol (Weigmann et al, 2007). Tumours were incubated with 5 ml of Predigestion solution for 20’ at 37 ºC under slow rotation (around 40 xg). Subsequently, pieces were passed through a 100 µm cell strainer and smashed carefully on it. Then, pieces were incubated again with 5 ml of Predigestion solution for 20’ at 37ºC under slow rotation and passed through the cell strainer. The remaining tissue is washed with 1x PBS and transfer to a tube containing 5 ml of Digestion solution. The tissue was incubated for 20’ at 37ºC under slow rotation and afterwards, passed through the cell strainer. This last step was done as many times as the tissue requires to be completely disaggregated. Once all the cells were recovered, the tube is spun down at 525 xg for 5’ and cells are resuspended in 5 ml of complete DMEM medium.

4. Results

4.1 – EXPRESSION OF CD44, CD166 AND TRANSCRIPTION FACTORS (NANOG, SOX2 AND OCT3/4) IN COLORECTAL CANCER CELL LINES

According to the bibliography, CD44 and CD166 are proteins whom expression is related to CSCs characteristics. Our first aim was to identify these markers in the *in vitro* models of colorectal cancer. Three different cell lines were used: CT26, MC38 and PJ01, and the CSC-related markers CD44, CD166 and the transcription factors Nanog, Sox2 and Oct3/4 were analysed. Although these markers have been described as CSC-related markers, its use is still controversial.

These markers were analysed by flow cytometry as described in *3.2.3 Materials and Methods*. After optimizing the staining protocol, the results of representative experiments are shown in *Figure 6*.

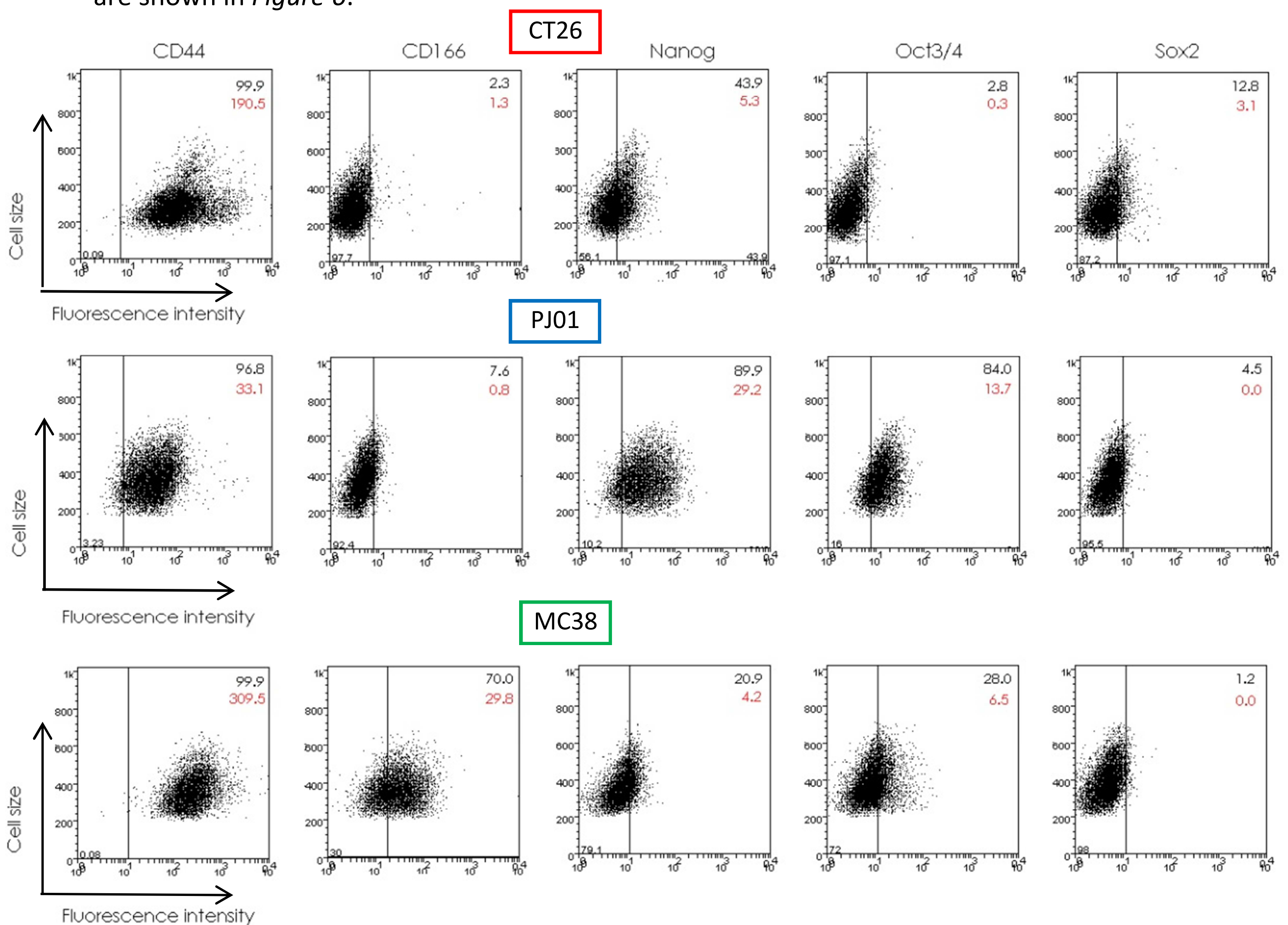

Figure 6. **CD44, CD166, Nanog, Oct3/4 and Sox2 expression in CT26, PJ01 and MC38 cell lines.** Cells were incubated with antibodies anti-CD44, anti-CD166, anti-Nanog, anti-Oct3/4 or anti-Sox2 and fluorescence was analysed using flow cytometry. Living cells were selected by 7-AAD exclusion. Results are shown as dot plots in which cell size and fluorescence intensities are shown. The vertical bars within each plot represent the fluorescence of the isotype negative control. Black numbers within each dot plot indicate the percentage of positive cells and red numbers indicate the mean of the fluoresce intensity.

As shown in *Figure 6*, all cell lines expressed high amount of CD44. Almost all cells expressed CD44 but some differences were found between them. CD44 expression in CT26 cells was higher than in PJ01 and MC38 cells. Also, huge differences were found in CD44 expression in CT26 cell line. The difference between different cell populations was almost 2 orders of magnitude, which means that some cells expressed 100 times more of CD44 than others. Regarding MC38 and PJ01 cells, expression levels were more homogenous than in CT26 cells.

In contrast to CD44, CD166 expression was not observed in all cell lines. CT26 cell line had a few numbers of cells as well as PJ01; however, MC38 cell line showed a higher expression of CD166.

Regarding transcription factors, some differences were found too. Nanog expression was easily detectable in all cell lines in contrast to Oct3/4 and Sox2. A little Oct3/4 positive population was found in CT26 cell line whereas PJ01 and MC38 cells showed a higher expression of this marker. Sox2 positive cells were almost not found in the three cell lines. A few positive cells were detected in CT26 cells but they were not found in PJ01 and MC38 cell lines.

4.2 – <u>CORRELATION OF CD44, CD166 AND TRANSCRIPTION FACTOR EXPRESSION WITH ALDH ACTIVITY AND/OR THE SIDE POPULATION</u>

After analysing marker expression in each cell line, we were interested in knowing if these markers correlated with other characteristics associated to CSCs such as high ALDH activity or high efflux pumps activity in cell membrane. To analyse this correlation, the protocol indicated in *3.2.3 Material and Methods* was performed. The analysis scheme is shown in *Figure S1* (*Annex)*. As shown in *Figure S1*, living cells were selected by their cell size and complexity as well as by 7-AAD exclusion. On selected living cells, CD44, CD166 and Nanog expression, ALDH activity and Hoechst 33342 retention was analysed by flow cytometry. The correlation of the high expression of these markers with the rest of them was analysed by histograms using flow cytometry software. As anti-CD166 antibody was conjugated with the same fluorophore as anti-Nanog's, their correlation with CD44 and ALDH was analysed independently. *Figure 7* shows all these results in CT26 cell line and *Figure S2* (*Annex*) shows the same results for the other cell lines.

As shown in *Figure 7*, expression of the markers is quite similar to the results shown in *Figure 6*. The populations expressing high and low levels of each marker were gated and the rest of the markers were analysed. CD44 high-expressing cell population does not show big differences in comparison with low-expressing cell population. Regarding ALDH, some differences were found, overall in CD166 expression. Cells with high ALDH activity express more amount of CD166 than cells with low ALDH activity as the maximum peak displacement shows. Same differences were found regarding CD166 and Nanog expression; positive cells in both markers showed peak displacements in comparison with negative cells

despite the fact that there were a few number of positive cells. These results indicate that CD44, CD166 and Nanog high-expressing cells show a high ALDH activity.

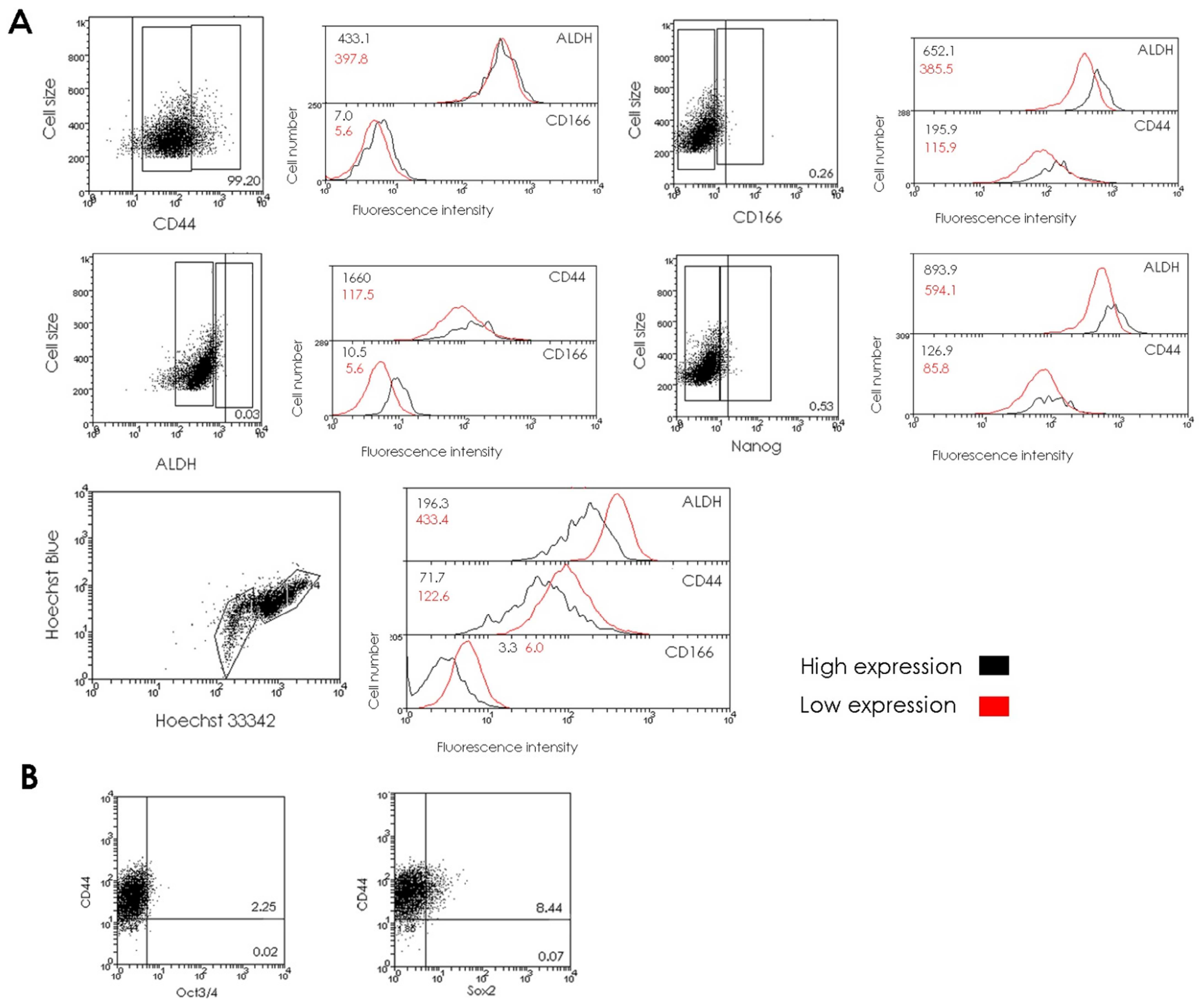

***Figure 7*. Correlation between CD44, CD166 and Nanog expression with ALDH activity and Hoechst 33342 retention in CT26 cell line.**

A. Cells were incubated with antibodies anti-CD44, anti-CD166, anti-Nanog and ALDH activity detection kit and Hoechst 33342 retention assays were used to analyse the correlation between all of these CSC-related markers. Living cells were selected by 7-AAD exclusion. The results for each marker are shown in dot plots within numbers which indicate the percentage of positive cells. For each marker, high and low-expressing population were selected with gates using specialised flow cytometry software and the rest of the markers were analysed over these selected populations by histograms. In red, it is shown the population which has a higher expression of one particular marker, and in black, the population which has a low expression. Within the dot plots, percentage of positive cells of each marker is shown.

B. In parallel, cells were incubated with anti-CD44 and anti-Oct3/4 or anti-Sox2 in order to analyse the correlation between these markers. The results are shown as dot plots within numbers show the percentage of positive cells of each quadrant. The detailed protocol used in these experiments is descripted in *Material and Methods 3.2.3* and the analysis scheme is indicated in *Figure S1* (*Annex*).

Although no differences were found between CD44 high-expressing and CD44 low-expressing cells regarding CD166 expression, great differences were found between CD166 high and CD166 low-expressing cells regarding CD44 expression. This apparent contradiction can be explained because of the number of the cells. Small CD166 positive cell population was found in contrast to the large CD44 high-expressing population. Within the CD44 high-expressing population there must be some cells which express high amount of CD166 and other cells which have low expression of CD166. The fluorescence peak show the fluorescence of all the cells; as the CD44 high-expressing population is large, CD166 high-expressing cells within this population are undercovered by CD166 low-expressing cells and as a result, no differences can be found. However, if CD166 high-expressing population is selected from the beginning, as it is smaller, differences can be found.

Hoechst 33342 retention test contradicted our previous results as well as the results found in the literature. Cells with high amount of efflux pumps are able to release Hoechst 33342 therefore a population with less Hoechst 33342 fluorescence can be identified by flow cytometry. This population has been related stemness characteristics and it is known as the side population (Dean, 2009). Hoechst 33342 fluorescence can be measured on two channels by a flow cytometer; the easiest way to find the side population is representing both channels in a dot plot as shown in *Figure 7.* A Hoechst 33342 low retention population was found as previously described (Mori et al., 2012). However, and as shown in *Figure 7*, Hoechst 33342$^-$ population express less amount of the other markers than its Hoechst$^+$ counterpart. Some authors include efflux pumps inhibitors in the Hoechst 33342 retention assay such as verapamil. This efflux pump inhibitor blocks their function and therefore, colorants are kept inside the cells. Using cells treated with verapamil as control, we would be able to observe differences in the Hoechst Blue-vs-Hoechst 33342 dot plot and confirm the identity of the side population. Although verapamil was not available in our lab, ALDH buffer contains different efflux pumps inhibitors so we decided to test if this buffer could be useful to confirm the side population in our cell lines. As *Figure 8* shows, the side population was enriched when ALDH buffer was not used confirming that the side population was properly identified. However, no positive results were found regarding the correlation with the other markers.

The correlation of Oct3/4 and Sox2 expression with ALDH activity was not analysed due to the low number of positive cells found and CD166 expression was not analysed because of both antibodies were conjugated with the same fluorophore. So, only the correlation between CD44 and transcription factor expression was analysed. However, a few numbers of double CD44 and Oct3/4 or Sox2 positive cells was found, as shown in *Figure 7.B*.

Regarding the other cell lines, results are shown in *Figure S2.A and S2.B* (*Annex*) and are quite similar to the results found in CT26 cell line. Slight differences were found between MC38 CD44^{high} and CD44^{low}-expressing population. High expression of ALDH correlated with

a high expression of CD166 and slightly with CD44; Nanog expression correlates with both higher expression of CD44 and ALDH. Large CD166$^+$ population was found as *Figure 1* shows but their correlation with high expression of ALDH and CD44 was not clear. Regarding Hoechst 33342, the same contradictory results as in CT26 cell line were found.

Results in PJ01 cells were found. In this cell line, ALDH activity correlated to CD44 and CD166 and also with Nanog expression. CD44 high expression slightly correlated with high expression of the other markers. The Nanog$^+$ and Oct3/4$^+$ populations have decreased a bit in these experiments in comparison with the results shown in *Figure 6*.

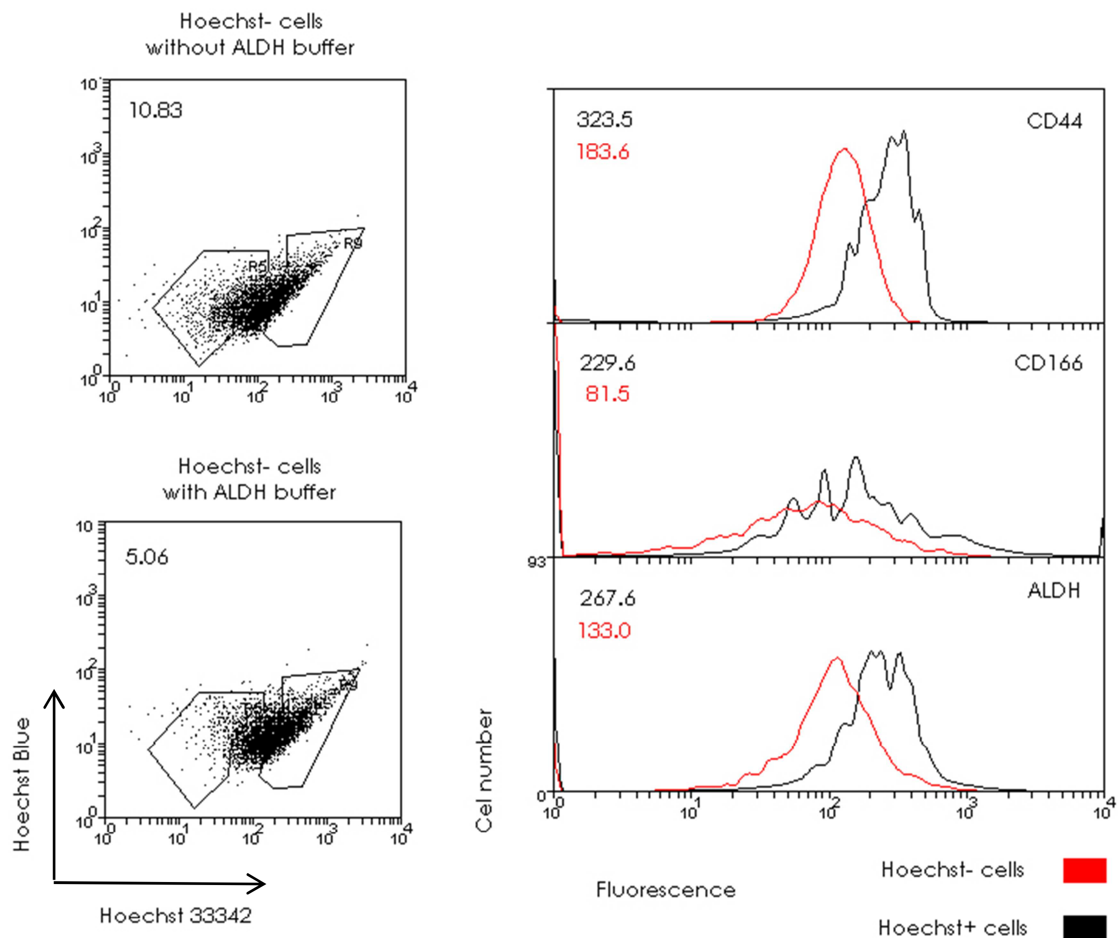

***Figure 8*. Effect of efflux pumps inhibitors in the Hoechst 33342 retention assay in CT26 cell line.** Cells were incubated with or without ALDH buffer during the Hoechst 33342 staining protocol and afterwards, the extracellular, intracellular staining and ALDH activity protocols were performed. The side population is identified in Hoechst Blue vs Hoechst 33342 dot plot as the population with less fluorescence. Numbers in the dot plots indicate the percentage of the side population cells. Side population and non-side population in the analysis without ALDH buffer was gated and CD44, CD166 and ALDH fluorescence was analysed as histograms. Red numbers within the histograms indicate the mean of fluorescence intensity in the side population and black numbers indicate the non-side population ones.

4.3 – ANALYSIS OF CELL DIFFERENTIATION AND COLONY FORMATION

After analysing the expression and correlation of CSC-related markers by flow cytometry, we were interested in analysing other features associated to CSCs. The main characteristics of CSCs, which actually define them as CSCs, are self-renewal and differentiation capabilities. As described in detail in the *Introduction*, both capabilities allow CSCs to regenerate the tumour mass by renewing CSC population as well as its non-CSC counterpart. If one of these CSC-related markers is essential to define CSCs, a population enriched in this marker should show these features.

In order to figure out and test this hypothesis, differentiation and colony formation assays were performed sorting the different populations found by flow cytometry. Flow cytometry allow us to get these populations using its tool named FACS.

30.000 cells of CD44^{high} and CD44^{low} populations were isolated from each cell line using FACS in order to perform **a clonogenic assay**. Cells were incubated in complete DMEM medium for 1 week and a half and afterwards, the number of colonies was counted. Results from CT26 cell line are shown in *Figure 9*.

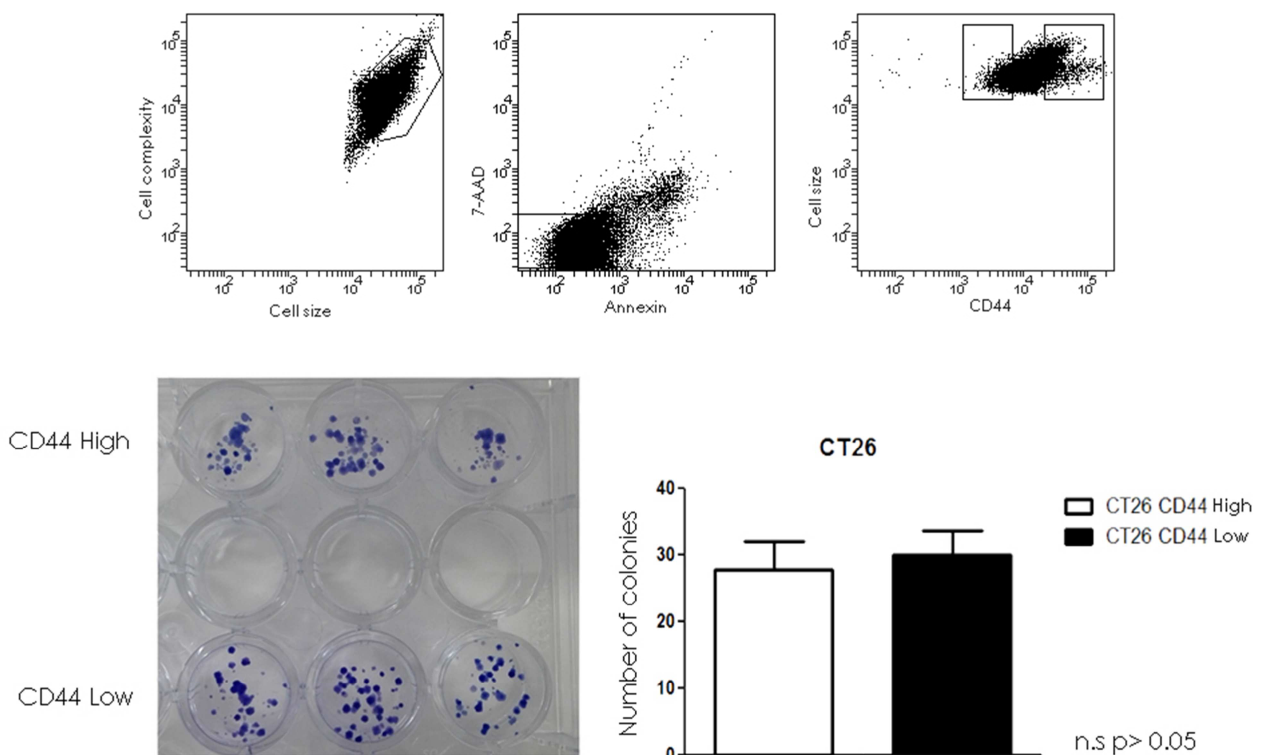

***Figure 9*. Clonogenic assay using the CD44^{high} population (CD44+ in the Figure) and CD44^{low} population (CD44-) from CT26 cell line.** Cells were incubated with anti-CD44 antibodies and isolated by flow cytometry using cell sorting as it is indicated in *Materials and Methods*. Living cells were selected using 7-AAD exclusion and Annexin V staining. 100 cells of the 10% top or low CD44-expressing cells were collected in 24-well plate with complete DMEM medium and incubated for 10 days at 37ºC. Afterwards, colonies were stained with crystal violet solution and counted. Chi-square statistical analysis was performed using GraphPad Prism Software.

As shown in *Figure 9*, sorted cells were able to create a large number of colonies so cell sorting not affect the clonogenic capabilities in CT26 cell line. However, no significant differences were found between CD44^{high} and CD44^{low} populations.

Results from PJ01 cell line are shown in *Figure S3* (*Annex*) whereas MC38 isolated cells were not able to form any visible colony. It should be emphasised that sorting is a quite stressful procedure for cells and not all cell lines are capable to bear it. After few days, sorted MC38 cells were not attached to the plate at all, indicating they were probably dead (data not shown). A few number of small colonies of this cell line were initially detected but after one week, there were no colonies anymore. In PJ01 (*Figure S3*), CD44^{+} population seems to form more number of colonies than its CD44^{-} counterpart but no significant differences were found. Indeed, results are not very reliable since the colonies were too big after the incubation and to count accurately. This indicates that PJ01 colonies were able to grow faster than CT26 ones.

Another way to analyse the colony formation capability, is to perform a **limiting dilution assay**. This assay consists of diluting the cell suspension to get a density as low as 1 single cell can be added in a 96-well plate. FACS facilitates the task so just 1 cell per well instead of thousands. Cells were sorted using anti-CD44 antibodies and the plates were incubated for 2 weeks in order to allow the colonies to grow. If the sorted cells have the intrinsic characteristic to grow on their own, cells form a visible colony. Results for these assays are shown in *Figure 10*.

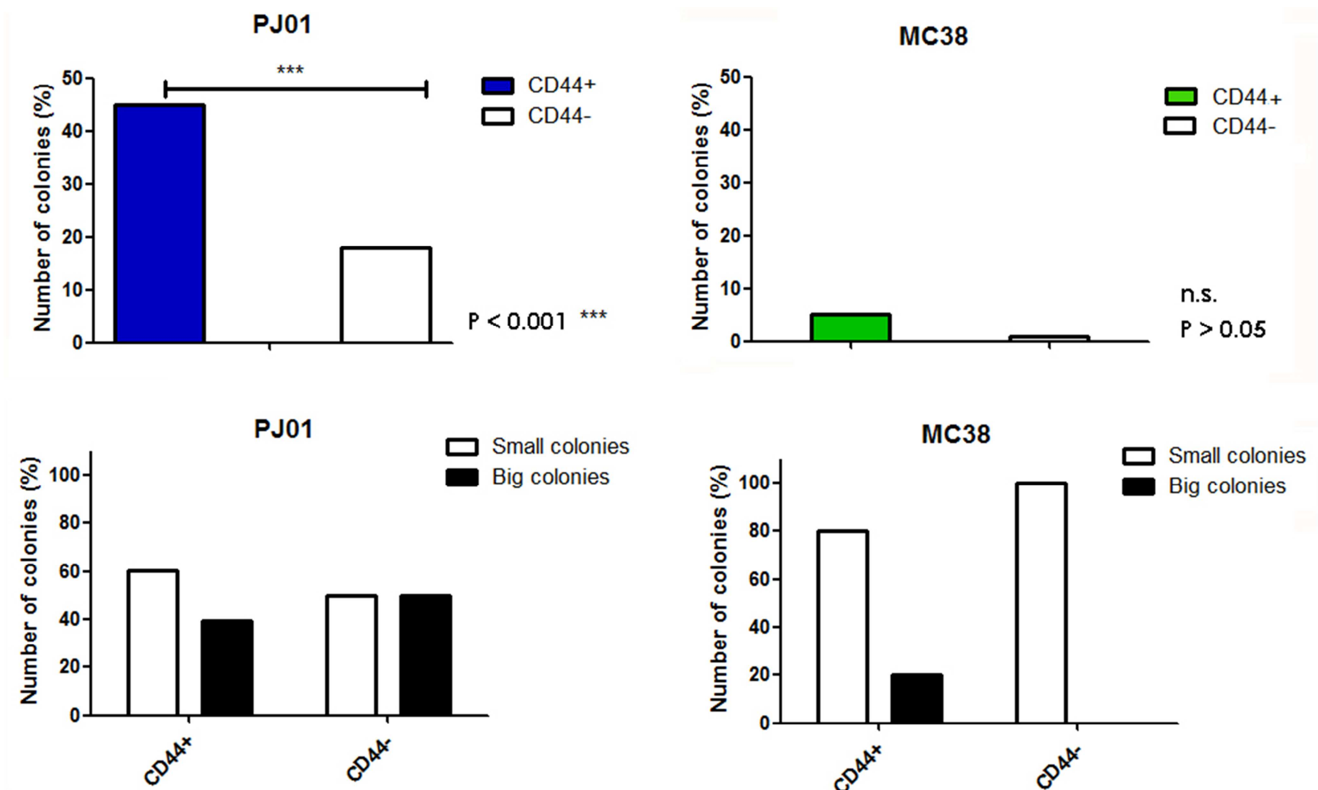

Figure 10. **Results of the limiting dilution assays after sorting the cells by cell sorting in PJ01 and MC38 cell lines.** Cells were incubated with anti-CD44 antibodies and living cells were selected by 7-AD exclusion and Annexin V staining. High CD44-expressing cells (CD44+) and low CD44-expressing cells (CD44-) were sorted using a cell sorting flow cytometer and 1 single cell was added per each well in a 96-well plate. Cells were incubated for 2 weeks and colonies were counted. The results are shown as histograms. Up histograms show the percentage of total colonies found in a 96-well plate and down histograms show the percentage of big and small colonies in the colonies formed.

Two different types of colonies were found: big colonies which even grew in several cell layers and small colonies which were composed by few cells growing together. So the percentage of these big colonies was quantified in reference to the total of colonies found. As shown in *Figure 10*, PJ01 $CD44^{high}$-expressing cells were able to form more colonies than $CD44^{low}$-expressing cells. Regarding the size of the colonies both sorted cell populations formed the similar rate of big colonies. MC38 cell line hardly grew in colonies and a very few was found. However, results shown in PJ01 cell line suggest that CD44 high-expressing cells form higher colony number although sorting protocol for this cell line must be optimised in order to improve the cell culture viability. CT26 was unable to follow the protocol due to a technical issue.

Regarding the **differentiation assay**, same settings than in the clonogenic assay were used and the same populations ($CD44^{high}$ and $CD44^{low}$) were isolated by cell sorting. We wanted to know if isolated $CD44^{high}$ population had the capability of differentiation, that is, if it was able to regenerate the $CD44^{low}$ population after the sorting. As previously described, CD44 is considered as a CSC-related marker (Dotse et al, 2014) but our results are not conclusive on this aspect. So the $CD44^{high}$ and $CD44^{low}$- expressing populations were isolated and incubated for 2 or 7 days. If the hypothesis is true, high-expressing population should be able to differentiate into low-expressing population and reproduce the parental culture. On the other hand, if $CD44^{low}$ population is not a CSC-like population as it was described, it would not be able to generate the $CD44^{high}$ population on the same conditions.

Results of these experiments are shown in *Figure 11* for CT26 and PJ01 cell lines. MC38 cells, like in the previous experiments, were not able to resist the sorting procedure. In this case, PJ01 was unable to either. After one week, cells were not attached anymore to the plate and the subsequent analysis of cell viability using Annexin V and 7-AAD staining revealed that, indeed, almost all the cells were dead.

As shown in *Figure 11*, after 48h both $CD44^{high}$ and $CD44^{low}$-expressing cells have almost the same expression of CD44 in CT26 cell line. Both populations seem to be able to reconstitute its counterpart in 48h. The same data are shown in histograms in which a slight displacement of maximum peak was found, which would indicate that $CD44^{high}$ cells express indeed more CD44 than $CD44^{low}$. After 7 days, differences become bigger. $CD44^{high}$ population shows slow growth in comparison with $CD44^{low}$ (data not shown) and also, they express more CD44. Regarding PJ01, $CD44^{low}$-expressing cells seem to express more CD44 after 48h of incubation than $CD44^{high}$. Nevertheless, a few number of $CD44^{low}$ cells were alive after sorting and this fact can distort the results. Actually, $CD44^{high}$-expressing cell culture looked better than $CD44^{low}$-expressing cells. All these results show in general that in PJ01, CD44 seem to select a cell population which exhibit characteristic associated to CSCs. However, in CT26 and MC38 cell lines is still unclear their relevance as a CSC marker.

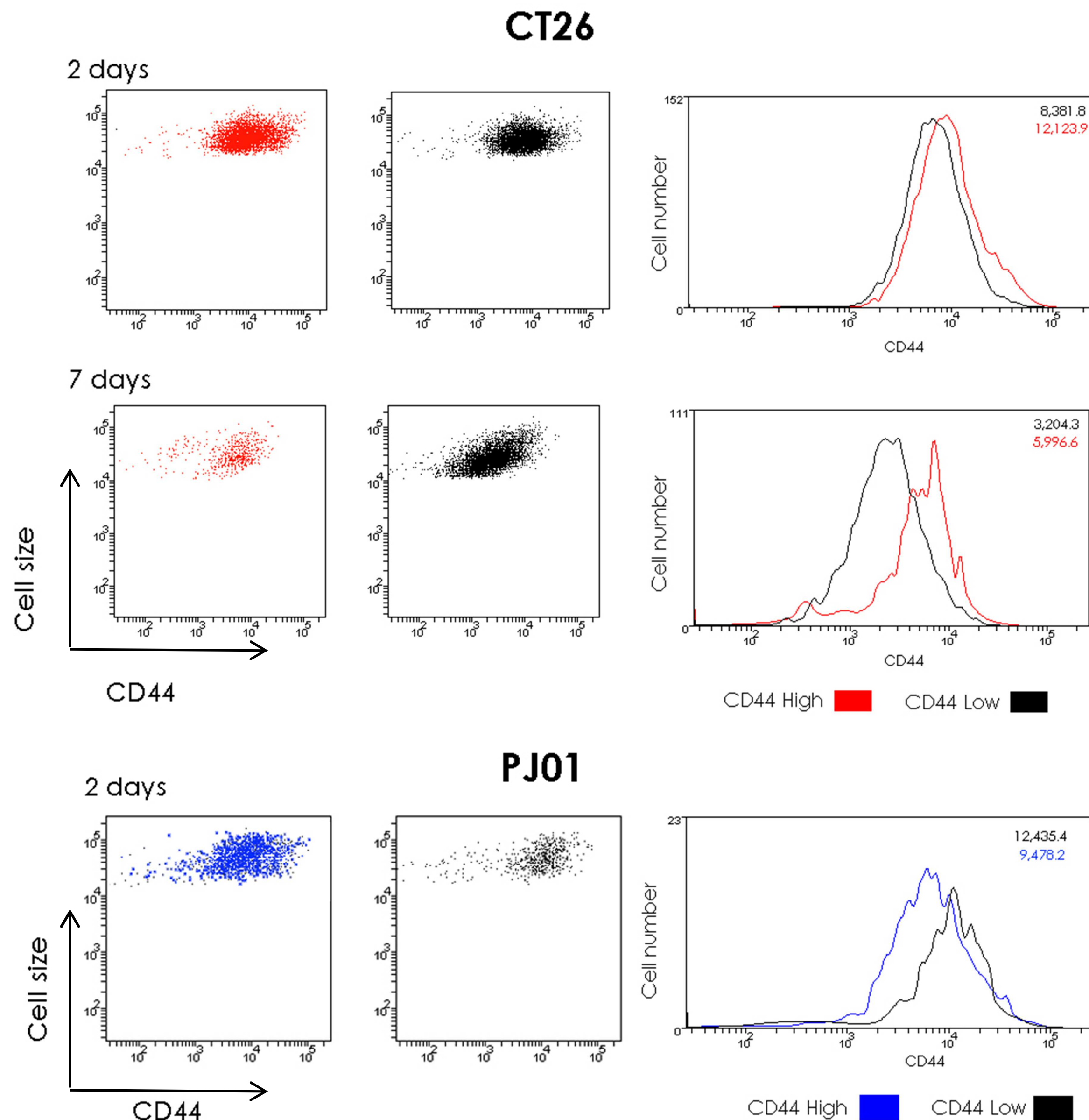

Figure 11. **Analysis of the differentiation capability of CD44^{high}-expressig and CD44^{low}-expressing cells in CT26 and PJ01 cells.** Cells were incubated with anti-CD44 antibodies and sorted according to CD44 expression. Living cells were selected by 7-AAD exclusion and Annexin V staining. CD44 10%-top and low-expressing cells were collected in 24-well plates with complete DMEM media and were incubated for 2 or 7 days. After the incubation, cells were trypsinised and CD44 expression was analysed again by flow cytometry. Results are shown as dot plots and histograms.

4.4 – USE OF PLACLITAXEL AND DOXORUBICIN TO ENRICH CSC POPULATION

In addition to self-renewal and differentiation, CSCs have been described as highly chemoresistant cells. In order to see if drug treatment can enrich the CSC population and therefore, confirm the validity of some of the markers analysed, paclitaxel (PTX) and doxorubicin were used. These drugs are widely used in cancer chemotherapy and in

particular, different formulations of these compounds against advanced colorectal cancer (Sam et al., 2010).

First, the half maximum inhibitory concentration (IC50) for PTX and doxorubicin were analysed in CT26, MC38 and PJ01 cell lines. MTT assay was used as described in 3.2.5 *Materials and Methods*. Results of these assays are shown in *Figure 12* and *Figure S4* (*Annex*).

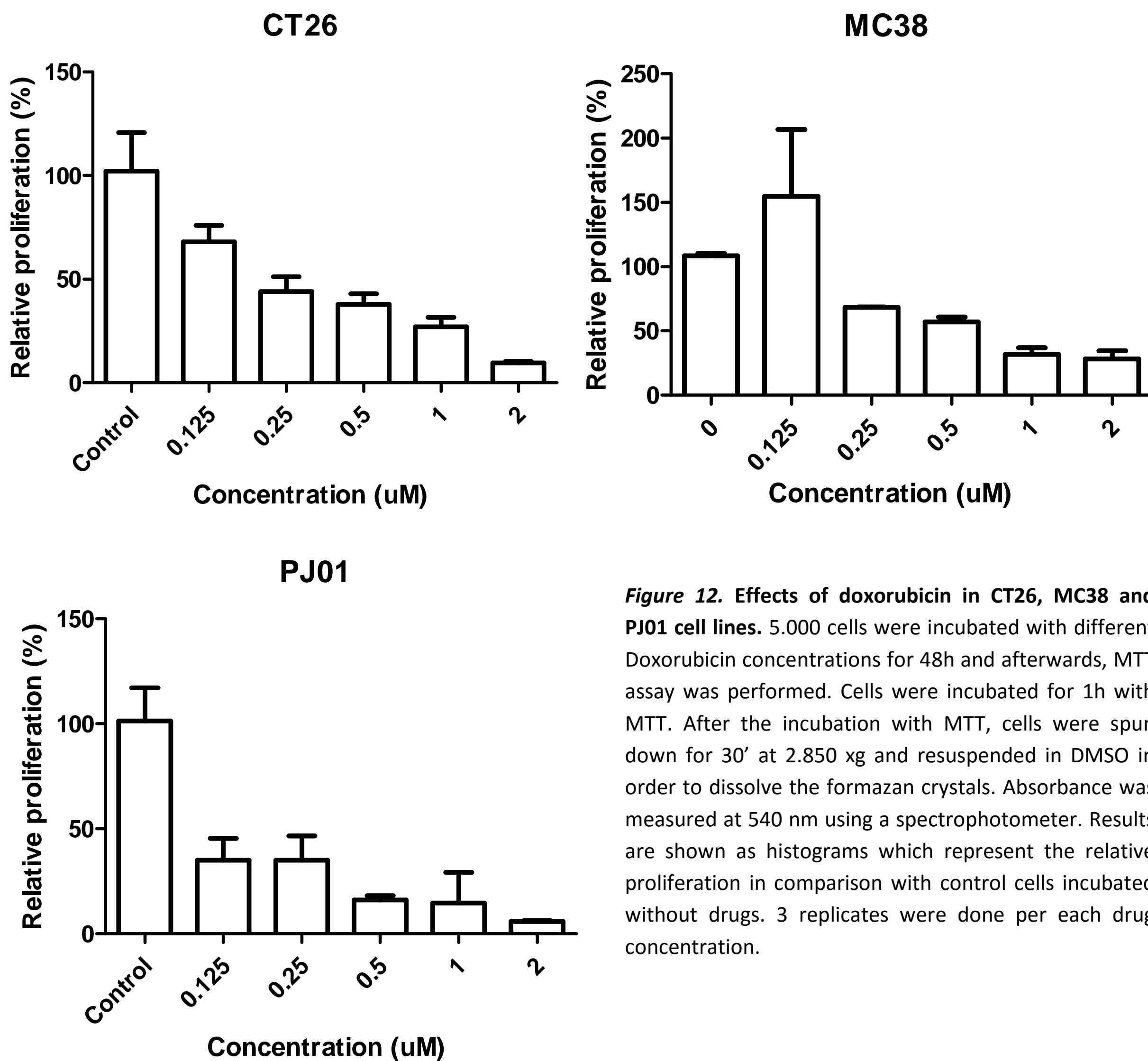

Figure 12. **Effects of doxorubicin in CT26, MC38 and PJ01 cell lines.** 5.000 cells were incubated with different Doxorubicin concentrations for 48h and afterwards, MTT assay was performed. Cells were incubated for 1h with MTT. After the incubation with MTT, cells were spun down for 30’ at 2.850 xg and resuspended in DMSO in order to dissolve the formazan crystals. Absorbance was measured at 540 nm using a spectrophotometer. Results are shown as histograms which represent the relative proliferation in comparison with control cells incubated without drugs. 3 replicates were done per each drug concentration.

The MTT results for doxorubicin are shown in *Figure 12* while the results for PTX are shown in the *Figure S4* (*Annex*). All the data are summarised on the *Table 2*. Regarding PTX, IC50 was not easy to find so the data in the table show the concentrations at which cell proliferation was the 50% of the control proliferation.

As shown in the table, IC50 for doxorubicin was correctly determinated in CT26 and MC38 cell lines. PJ01 cells were not able to proliferate above the 50% even at concentrations of 0.125 µM in comparison with the proliferation of the control.

	CT26	**MC38**	**PJ01**
Doxorubicin (IC50)	0.25 µM	0.5 µM	< 0.125 µM
PTX (IC50)	0.125 - 5 µM	-	0.125 - 2 µM

***Table 2.* Summary of the results of MTT assays**. Results of the IC50 for doxorubicin and PTX in CT26, MC38 and PJ01 cell lines.

These approximated values are in concordance with those found in the bibliography (Sam et al., 2010). Once IC50 were calculated in each drug and cell line, these concentrations were used in order to enrich the CSC population. Drug concentrations around the IC50 were used to this aim.

As above described in 3.2.5.2 *Materials and Methods*, cells were incubated with different drug concentrations and CSC-related markers were analysed by flow cytometry. Results of CT26 cell line are shown in *Figure 13* and *Figure S.5* (*Annex*) show them in more detail. Results for MC38 cell line are shown on the *Figure S.6* (*Annex*).

Once more time, maximum peak displacement indicates that one specific marker is express in a higher amount. As shown in *Figure 13*, CD44 expression is increased slightly after the drug treatment but almost all the drug concentrations tested produce the same fluorescence variation. The same results were obtained for CD166 expression. Some problems were found regarding the analysis of CD166 treated with 1 µM of doxorubicin, so the histogram for this one is missed. Nevertheless, as shown the drug treatment increases the CD166 expression in all case but again, no differences were found between different treatments.

Regarding transcription factors, some differences were found. Nanog and Oct3/4 expression were increased in accordance with the drug concentration; the much drug concentration was increased, the much fluorescence measure was detected. Differences between different drug concentrations for Sox2 are not so clear but the drug treatment increase the Oct3/4 expression for certain.

MC38 results are shown in the *Annex* and they are quite similar to the found in CT26 cell line. The CD44 expression hardly increases between different drug concentrations but it does increase in comparison with the control one. Transcription factors expression seems to increase according to the drug concentration.

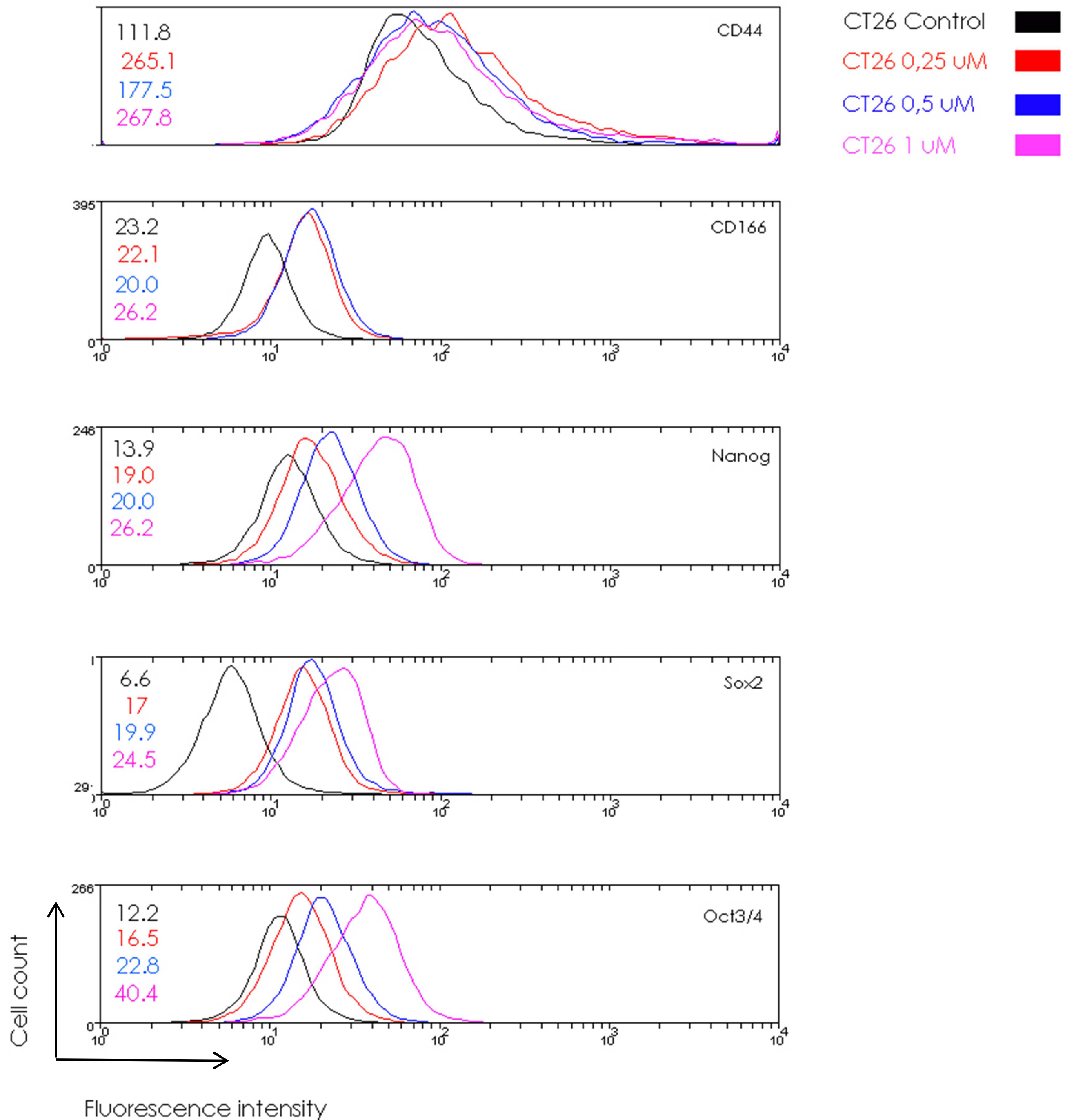

***Figure 13*. Analysis of transcription factor and extracellular CSC-related markers after treatment with different concentrations of doxorubicin.** 100.000 cells were incubated with 0.25, 0.5 or 1 µM of doxorubicin for 48h and afterwards, cells were stained with anti-CD44, anti-CD166, anti-Nanog, anti-Oct3/4 and anti-Sox2 antibodies as indicated in *Materials and Methods.* Results are shown as different histograms; each peak represents a different drug concentration as indicated. As anti-CD166, Nanog, Sox2 and Oct3/4 antibodies are conjugated with the same fluorophore, so different tubes were used for the analysis but anti-CD44 was added in all of them in order to see differences between replicates.

4.5 – DEVELOPMENT OF CHEMORESISTANT CELL LINES AND ANALYSIS OF THEIR CSC-RELATED CHARACTERISTICS

Since identification of CSCs using extracellular markers was a bit tricky, we were interested in knowing if CSC cell culture was feasible. As already discussed, chemoresistance has been related to CSC characteristics. In human colorectal cancer, some authors have found evidence that chemoresistant cell lines show CSC-related features being a good approach to study them (Bose et al, 2011). New cell lines were generated which were able to grow in presence of drugs.

A protocol used for years was followed in order to generate chemoresistant cell lines (Yang et al, 2006). This protocol is summarised in *Figure S7* (*Annex*). Briefly, cells were incubated with a sub-lethal drug concentration for some days, depending on the cell line, and then, cells were kept on fresh medium without drug in order to allow them to grow again. When surviving cells were able to proliferate reaching the confluence, cells were treated again. The same procedure was performed for at least three weeks or until cells were able to grow easily in drug-containing medium. When cells were able to grow under these conditions, the drug concentration was increased and the same procedure was repeated. The drug concentration was increased progressively until reaching clinically relevant doses.

6 cell lines chemoresistant to doxorubicin and PTX were generated: CT26-0,5 DoxR, CT26-1 DoxR, CT26-2 DoxR, CT26-1 PTXR, PJ01-50n DoxR and MC38-50n DoxR as it is indicated in *Table S1* (*Annex*).

Chemoresistant CT26 cell lines were relative easier to generate in comparison with PJ01 and MC38 cell lines. These cell lines were more susceptible to doxorubicin than CT26 as previously shown and lower concentrations (50 nM of doxorubicin) were tested in order to create a chemoresistant cell line in these cell lines. When the concentration was optimised, some cells were able to proliferate despite the drugs and chemoresistant cell lines were generated. After generation of these cell lines, chemoresistance was tested by MTT assay as previously described as well as cross-chemoresistance. Results are shown in *Figure 14*.

Only CT26-0,5 DoxR and CT26-1 PTX cell lines were tested due to they were the first cell lines generated. Doxorubicin chemoresistant cell lines were able to grow better than WT cell lines with statistically significant differences; although PTX chemoresistant cell line was resistant in drug-containing medium, no significant differences were found.

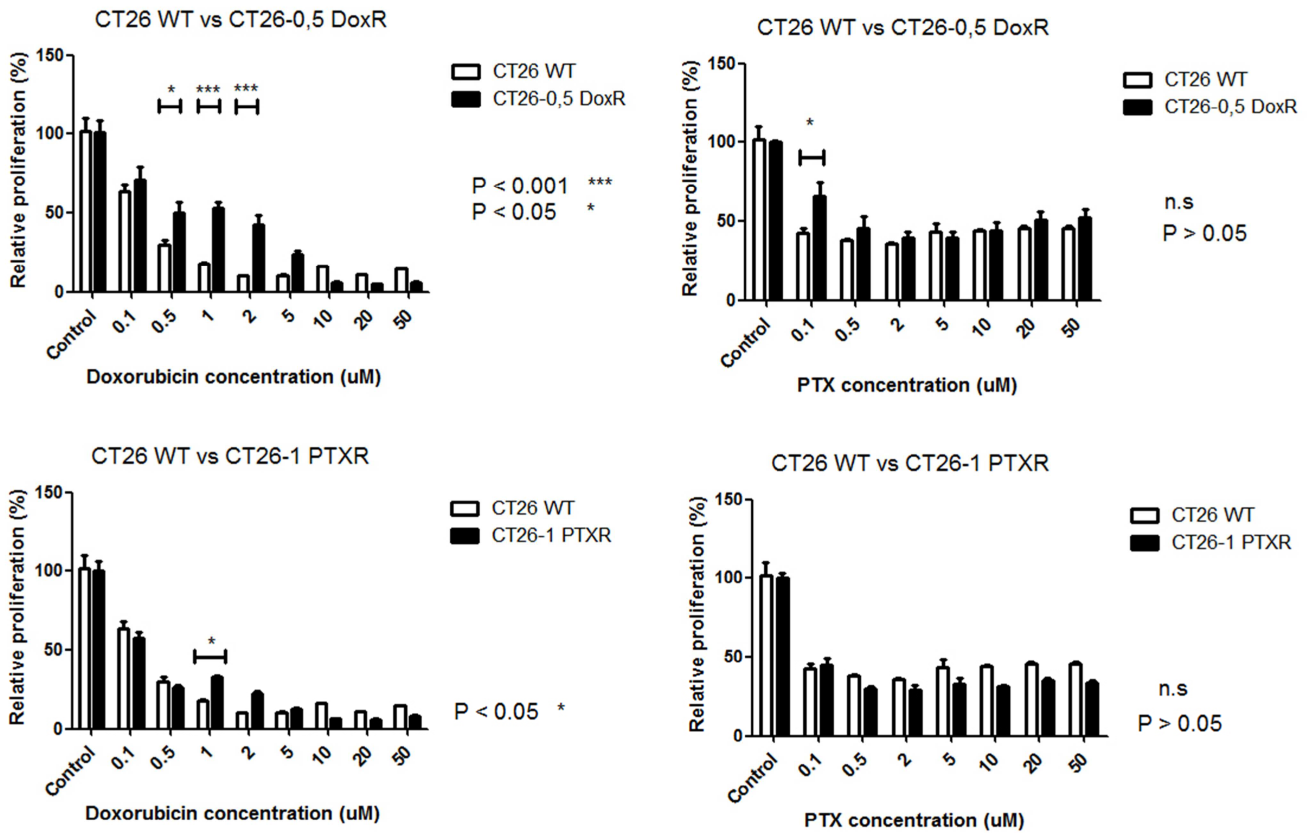

***Figure 14*. Results of the MTT assays in chemoresistant cell lines generated.** MTT assays were used in order to analyse the differential proliferation of the chemoresistant cell lines in comparison with the wild type ones. Cells were incubated with different drug concentrations for 48h. After the incubation, MTT assays were performed as indicated in *Materials and Methods*.

As shown *Figure 12*, MTT assays using PTX did not work as usual and no proliferation differences were found so this fact might explain these results. Also, no cross-chemoresistance was found between doxorubicin chemoresistant cell lines and PTX, and vice versa except for 0.1 µM of PTX in doxorubicin resistant cell line and 1 µM of doxorubicin in PTX resistant cell line.

CSC-related markers were analysed in the CT26-0,5 DoxR and CT26-1 PTXR cell lines. Results were shown in *Figure 15.* As shown in *Figure 15* because of the maximum peak displacement, doxorubicin and PTX seems to enrich all the CSC-related population. These results looks to indicate that a long-term exposition could increase the CSC-related features but further analysis of these chemoresistant cell lines must be done.

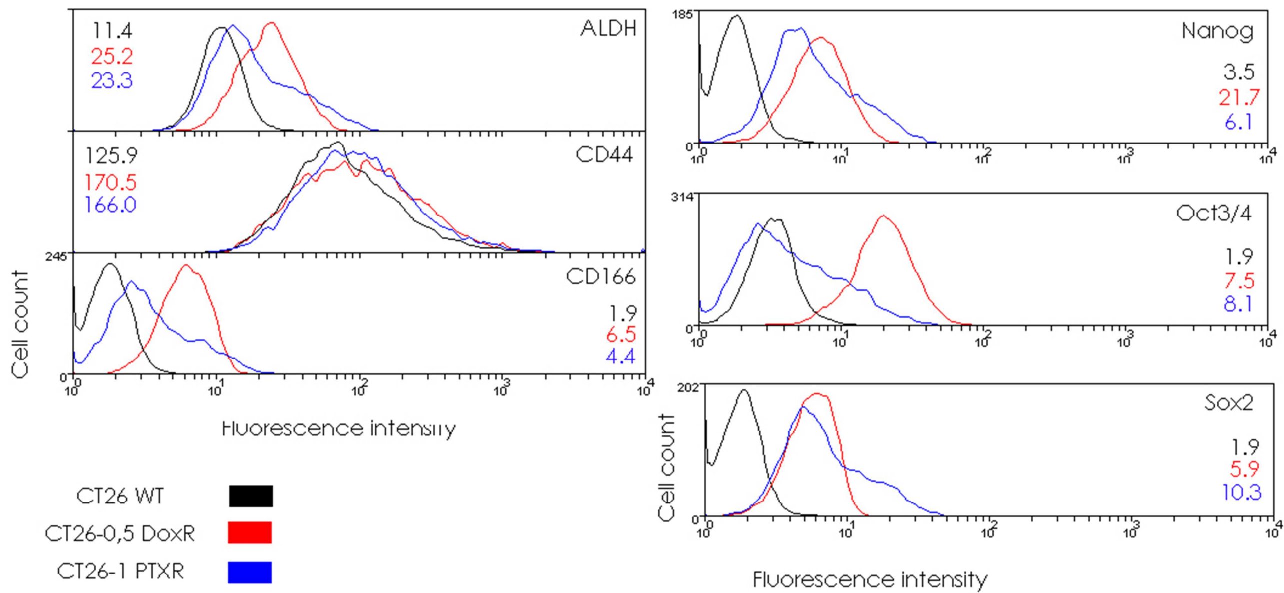

***Figure 15*. CSC-related markers on the chemoresistant cell lines.** CT26-0,5 DoxR and CT26-1 PTXT cell lines as well as CT26 WT were incubated with anti-CD44, anti-CD166, anti-Nanog, anti-Oct3/4 and anti-Sox2 antibodies as the intracellular and extracellular protocol indicate in *Material and Methods* . ALDH activity was measured in these cell lines too. Results of these experiments are shown as histograms where peak displacements indicate a higher expression of the marker.

4.6 – DEVELOPMENT OF 3D CELL CULTURES IN ORDER TO GET A CSC-ENRICHED CELL CULTURE

In order to get a cell culture enriched in CSCs to test different antitumor therapies against CSCs, a 3D cell culture protocol was optimised. It has been descripted that the architecture of the cell culture as well as the lack of oxygen and other vital nutrients can influence the CSC population (Yang et al, 2012).

As described in *3.2.7 Materials and Methods*, the hanging drop method was used to develop 3D cell cultures. Briefly, cells were mixed with a medium containing methylcellulose in order to increase the surface tension. The mixture was added as drops on the inner side of a Petri dish lip so when the lid is closed, cells rounded down towards the bottom of the drop and, being together, started to grow in a 3D cell culture known as spheroid. Spheroids were collected in a 96-well plate and trypsinised in order to analyse the extracellular markers. Results of these experiments are shown in *Figure 16*.

The peak displacements indicate that 3D culture is enriched in CD44-expressing cells in comparison with 2D cell culture in CT26 cell line; however, PJ01 and MC38 3D culture did not show the same enrichment for CD44. Regarding CD166, 3D cultures did not show big differences although the expression in the expression of CD166 was slightly higher indeed.

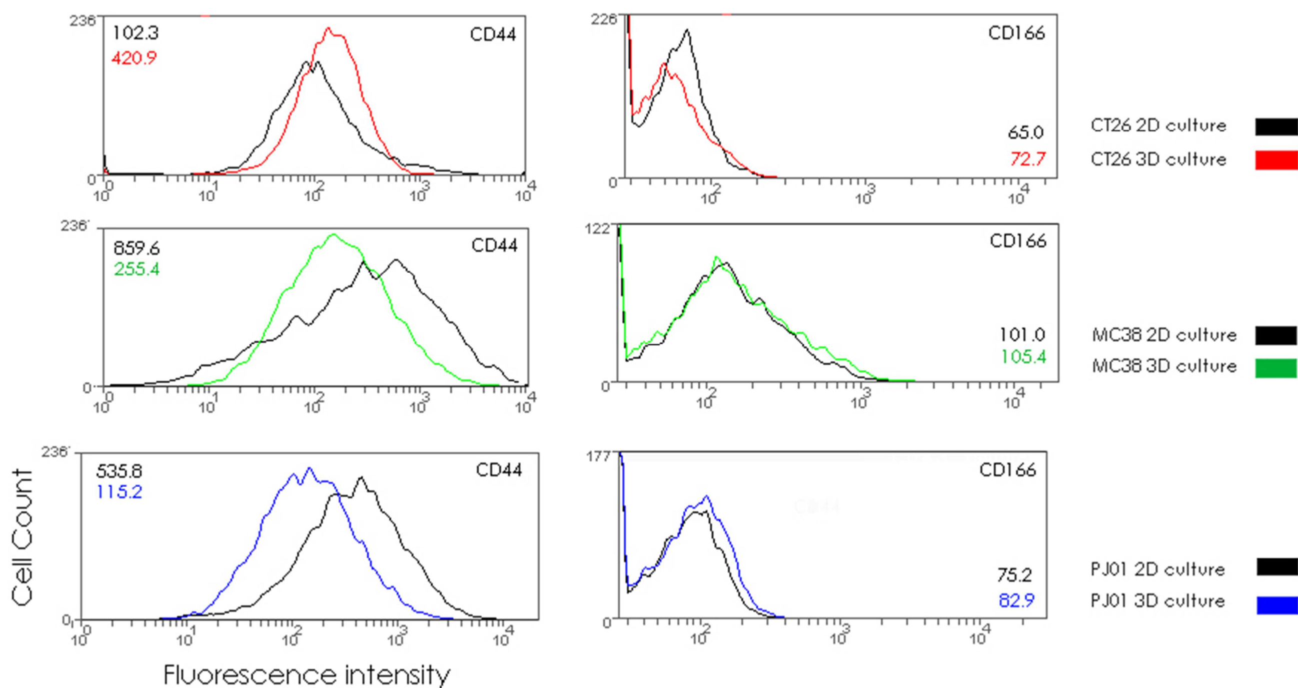

***Figure 16*. CD44 and CD166 expression in 3D cell culture of CT26, MC38 and PJ01 cell lines.** Cells were grown in 3D culture using the hanging drop method. CD44 and CD166 expression was analysed by flow cytometry and compared with cells in 2D cell culture. Results are shown in histograms.

4.7 – ANALYSIS OF THE INFLUENCE OF GRANZYME-A IN THE EXPRESSION OF CSC-RELATED MARKERS

The final aim of this study is to analyse the role of Granzyme-A in the emergence of CSCs during inflammation-associated colorectal cancer in mice. Our lab has optimised the protocol for developing colorectal carcinomas in the C57Bl/6 mice using the AOM/DSS protocol. As described in *3.2.8 Materials and Methods*, mice GzmA -/- (Gzm-A-deficient mice) and WT mice were treated following the AOM/DSS protocol. After treatment, mice were killed and tumours were extracted from the distal colon. Tumours were digested and individual cells were obtained in order to analyse the CSC-related markers. In this case CD44, CD166 and transcription factor expression were analysed as well as the ALDH activity. Results are shown in *Figure 17*.

It is a bit difficult to analyse the differences in the image but briefly, no differences were found among GzmA -/- tumours and WT tumours. All maximum peaks are around the same fluorescence measure and in the cases in which any peak is displaced, no correlation was found. For example for CD44, red and blue peak are displaced from the others indicating a low expression of CD44; however, red peak corresponds to a WT tumour while blue peak correspond to a GzmA -/- tumour.

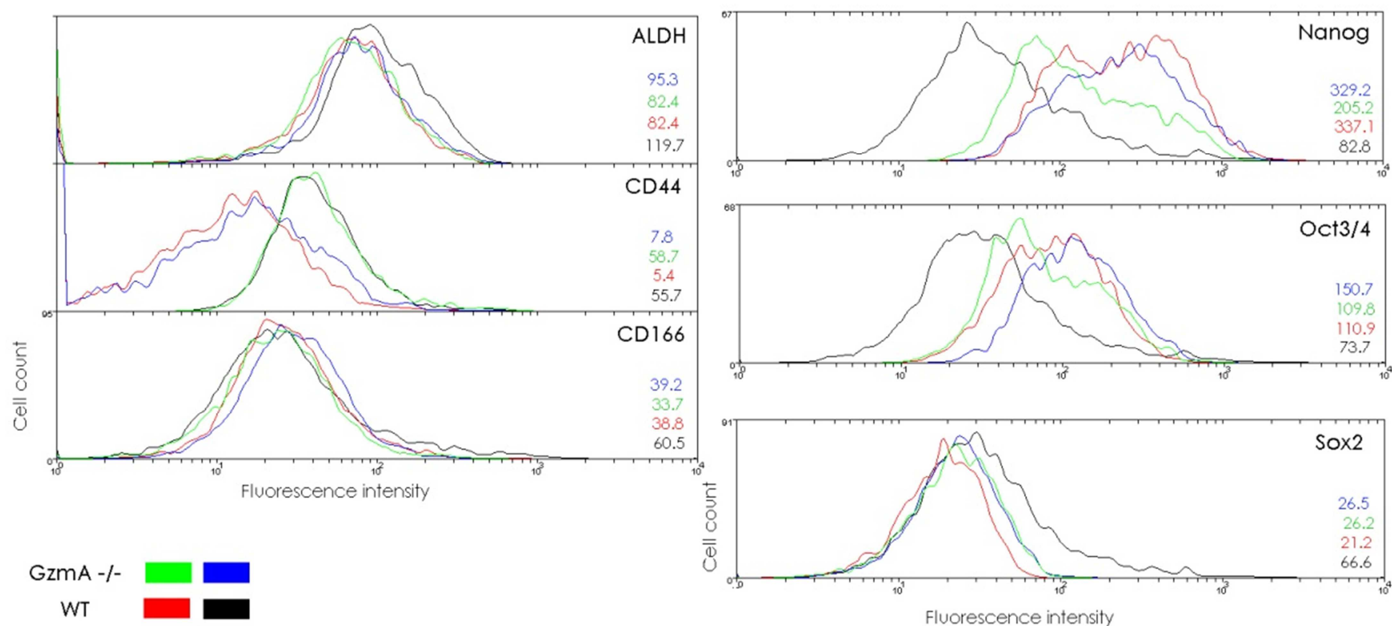

***Figure 17*. Analysis of the influence of GzmA in the presence of CSCs.** GzmA-deficient (GzmA -/-) and WT mice were treated with DSS/AOM protocol as described in *Material and Methods*. After treatment, mice were killed and tumours were extracted from the distal colon. Tumours were digested using a Digestion Kit as indicated in *Material and Methods* and individual cells were obtained. Then cells were stained using anti-CD44, anti-CD166, anti-Nanog, anti-Oct3/4 and anti-Sox2 antibodies and ALDH activity was measured by ALDEFLUOR kit and flow cytometry. Results are shown as histograms. Red and black histograms show the data from 2 independent WT tumours and green and blue ones show the data from 2 independent GzmA -/- tumours.

5. Discussion

In this study CD44, CD166, Nanog, Oct3/4 and Sox2 expression has been analysed in order to identify a CSC population in mouse cell cultures and then, to use these protocols to analyse the influence of Granzyme-A in the development of colorectal cancer in mouse model.

Our first goal was to analyse the **expression of these markers in cell cultures**. Few studies have been published regarding CSCs in mouse colorectal cancer cell lines. Transcriptome and proteome analysis has been described in order to characterise CT26 cell line and CD44 and Oct3/4 expression was highly found (Castle et al, 2014). Our results in CT26 cells are in agreement with these works as a major CD44 positive population was found. Expression of CD44 was not homogenous in CT26 cell line indicating that there were different subsets based on CD44 expression. CD166 and Nanog positive population were also identified in this cell line; however, no Oct3/4 and Sox2 positive population was found. Nanog is a protein which is able to be expressed in the cytoplasm and inside the nucleus while Oct3/4 and Sox2 are not described as cytosolic proteins (Amini et al, 2014), so our first hypothesis was that our protocol staining was only working for cytosolic proteins. However, this hypothesis was discarded due to the positive results found in the other cell lines. All these transcription factors have been described as stemness markers and their expression was used to identify stem cells in normal tissues (Miyoshi et al, 2009). They have been related to CSCs in human transformed cell lines but there are not articles in mouse cell lines (Amini et al, 2014). CD44 positive cells were found in PJ01 and MC38 cell lines but its expression was more homogenous than in CT26 cell line. Nanog positive cells were also identified in MC38 and PJ01 cell lines but this population was especially higher in PJ01 cells. A huge Oct3/4 positive population was found in PJ01 cell line and a smaller well-distinguished positive population was identified in MC38 cells. Regarding PJ01, obviously there is no article in the literature as it is a cell line generated in our lab. However, these differences between cell lines regarding CSC-related markers can be related with their sensitivity to antitumor drugs as it has been shown in human colorectal cancer cells as well as their capability to create colonies (Dolgova et al, 2014). These results are in accordance with the IC50 calculated in this study. CT26 and MC38 cell lines showed a higher expression of CD44, CD166 and ALDH activity than PJ01 and those cell lines showed a higher resistance to doxorubicin as the IC50 values showed.

It is difficult to find studies that analyse CSC presence in mouse cell lines and usually, analysed the expression of these proteins using qPCR or other genetic tools (Castle et al, 2014). Correlation between two or more CSCs-related markers has been proposed as the best way to find and isolate the CSC population in tumours, and in particular, in CRC tumours (Zhou et al, 2016). Some studies described CD44 positive cells as CSCs regarding their abilities to generate bigger and more aggressive tumours in mice after transplantation (Jinushi et al, 2011). This population is supposed to be enriched in CSCs but functional

analysis must be performed in order to confirm it. CSCs are defined by their functional features such as self-renewal, differentiation and chemoresistance so other characteristics should be analysed like **ALDH and ABC transporter activities**. These activities have been used to identify CSCs as well as normal stem cells so they were tested in order to find a correlation between them and CD44, CD166, Nanog, Oct3/4 and Sox2 expression. Our results show that a higher activity of ALDH correlates with a higher expression of CD44, CD166 and transcription factors. However, the Hoechst 33342 low-retention population was not correlated with a high expression of these markers. Side population, identified as a population with low retention of Hoechst 33342, has been used for years to identify CSCs (Dean, 2009). Some possibilities were considered to explain our results. It could be that the side population was an apoptotic population that retained less dye decause of DNA degradation. ALDH buffer containing efflux pump inhibitors was used in order to reveal this problem. The use of this buffer blocks the ABC transporter activity so side population can be identified easily. As shown in *Results*, the side population was identified when ALDH buffer was not used. Also, 7-AAD and Annexin V were used to select living cells so we concluded that side population was identified properly but it does not correlate with the CSC-related markers and it might not be a suitable approach to identify CSCs in mouse cell lines.

In order to analyse other CSC features like **clonogenic** and differentiation potential, cell sorting was used. Intracellular markers such as Nanog or Oct3/4 cannot be used for these assays since the protocol fix the cells. Few ALDH and CD166 positive cells were found in the three cell lines so cell sorting would compromise cell viability during the procedure if these markers were used. So finally, CD44 was chosen to sort cells and different assays were performed in CD44 high- and low-expressing cells. On one hand, clonogenic and limiting dilution assays were performed in order to analyse the ability of sorted population to grow and form colonies. On the other hand, differentiation assays were performed in order to analyse if sorted populations were able to regenerate the parental culture. Both features define CSCs.

Clonogenic assays showed that CD44 high-expressing cells were not able to form more colonies than low-expressing cells. However, limiting dilution assays showed a significant increase in the number of cell clones formed by the high-expressing cells. These apparently contradictory results can be explained regarding the procedure of both assays. Clonogenic assays evaluate the clonogenic capabilities adding hundred cells per well. The number of cells per well is so low that they are supposed to be isolated from the others and, if they are CSCs, they must be able to grow on their own. However, CSCs have been described to be able to influence to other cells through releasing soluble factors such as IL-8 or other growing factors. These factors activate proliferation signalling pathways like Wnt pathway (Pattabiraman et al, 2014)(Li et al, 2012). So, although cells were indeed isolated from the others in clonogenic assays, soluble factors might be present in the medium and

allow differentiated cells to grow. Only physical separation would be suitable for isolating CSCs and evaluating clonogenic capabilities in cell cultures as limiting dilution assays do. These assays add 1 single cell per well and so, cells are completely isolated from the other. If they are CSCs, after few weeks they will form a colony. Results for this assay showed that CD44 high-expressing cells were able to form more colonies than CD44 low-expressing cells indicating that this population was enriched in CSCs.

Two types of colonies were found in limiting dilution assays regarding the size. CSCs show a slower proliferation rate than mature cancer cells and proliferation tests proved that low proliferative cells did show CSC features such as chemoresistance or higher tumour incidence in mice (Dolgova et al, 2014). Large colonies might come from a specific CSC with higher differentiation capability but no differences were found among sorted populations regarding the number of big colonies. Also, no articles were found about this so further analysis must be done to unravel it.

Another feature that defines CSCs is the **differentiation ability**. Cell sorting was used in order to analyse this ability. However, both sorted populations were able to reconstitute the parental culture although some differences were found regarding CD44 expression and cell viability. These results can be explained in some different ways. On the one hand, cell sorting is usually used for isolating well-distinguished populations with at least 2 or 3 orders of magnitudes so maybe the sorting might not work properly on this isolation. Nevertheless, other authors were able to isolate and observe differences in colony formation ability between high and low-expressing cells for one or two different markers so a priori this should not be a problem (Zhou et al, 2016). Although cell sorting would work properly, some cells can escape to the sorting and they might grow and proliferate so finally, cell culture would be regenerated. However, during long-term incubation, differences were found and $CD44^{low}$ population was not able to grow as well as $CD44^{high}$ population did. It should also be noted that in both cell "types", a high number of 7-AAD and Annexin V positive cells were detected after one week indicating that a large amount of cells were dying and so, results might be distorted.

Some authors have claimed that cell line cultures could not be a good approach to analyse and isolate CSCs and propose primary cell culture or different culture medium as better options. In this study, drug treatment, chemoresistant cell lines and 3D cultures were used to try to get a CSC-enriched cell culture.

To overcome **chemoresistance** is one of the main goals to achieve in colorectal cancer research and CSCs have been hypothesised as the main responsible of this process. Drug treatments have been used in order to enrich CSC population in human cell but there are no studies regarding mouse cell lines (Choi et al, 2011) (Li et al, 2015). These papers show how drug treatments increase the Notch and Wnt signalling pathway, a pathway

related to CSCs characteristics. Doxorubicin is an anticancer drug which acts as a DNA intercalating agent and its cardiac toxicity is a serious limitation for its clinical use besides haematological and gastrointestinal disorders (Gianni et al 2001) whereas PTX mechanism of action involved interference with the normal breakdown of microtubules during cell division. These drugs were used in order to enrich CSCs populations and results showed that a higher expression of all CSC-related markers was found in cells treated with drugs. Similar results were found in human colorectal cancer cell lines although other CSC-related markers were studied (Dallas et al, 2011).

Chemoresistant cell lines were developed in order to analyse the influence of a long-term drug exposure in CSC-related markers. All markers were expressed in a higher amount in chemoresistant cell lines except for and Nanog or Oct3/4 in PTX-resistant cell line.

3D cell culture is a new approach to imitate real tumours and it is supposed to be a better *in vitro* model than typical cell culture in 2D. Some studies show that 3D cell culture can enrich CSC population (Chen et al, 2012); however, no all of them have shown an enrichment in CSC-related extracellular markers. Our results show that only CD44 was enriched in CT26 cell line whereas the rest of cell lines showed a similar CD44 expression and even a decrease of CD44 expression. However, CD166 showed a slight increase in 3D cultures. CD44 and CD166 are adhesion proteins which mediate the interaction among cells in tissues. However, in cancer they have been described as key molecules for migration and metastasis processes. Cells in spheroid culture grow as a tumour mass in which cells are strongly attached between them and so, nutrients such as oxygen or glucose might increase the expression of these molecules.

Finally, the **influence of Granzyme-A** in CSC-related marker expression was analysed. Tumour environment has been described as a key target in immunotherapies. Molecules such as TNF-α or IL-1B are mediators of chronic inflammation which has been related to poor prognosis and tumour development. GzmA has a key role in the development of colorectal cancer as a pro-inflammatory molecule and its presence is related with a higher tumour incidence in mice as our lab has shown (data not shown). CSCs might be behind this. However, our preliminary results did not show any difference among tumours from GzmA -/- and WT mice. It should be noted that some differences were found in comparison with previous experiments of the DD/AOM model. Usually GzmA -/- mice have less inflammation than WT mice. However, in this experiment a high inflammatory response was not found in WT mice as usual. Also, GzmA -/- mice used to have less tumour incidence than WT mice but in this experiment, they have around the same number of tumours than WT and similar size. All these data suggest that this experiment might not work properly and further analysis must be done in order to clarify if GzmA is not related to the emergence of the CSC population.

6. Conclusions

As a result of all the data analysed in the current study, the following conclusions were extracted:

- The high expression of CD44 and CD166 extracellular markers correlates with the expression of the transcription factors Nanog, Oct3/4 and Sox2 in CT26, MC38 and PJ01 cell lines
- High ALDH activity is related with CD44, CD166 and Nanog, Oct3/4 and Sox2 expression.
- Hoechst 33342 exclusion test was unable to identify a side population with a correlation with the other CSC-related markers.
- CD44 high-expressing cells were able to form more clones than CD44 low-expressing cells.
- Limiting dilution assay is more effective than clonogenic assay to analyse proliferating capabilities.
- Doxorubicin and PTX drug treatments enriched the CSC population.
- The expression of CSC-related markers was increased in chemoresistant cell lines.
- 3D cell cultures were optimised for the three cell lines and enrichment was found in CD166 expression and CD44 expression was increase only in CT26 cell line.
- DSS/AOM-induced colorectal tumours from GzmA-deficient mice did not show a higher expression of CSC-related markers in comparison with tumours from WT mice.

6. Conclusiones

Como resultado de todos los datos analizados en este estudio, se han extraído las siguientes conclusiones:

- La alta expresión de CD44 y CD166 mostró correlación con una alta expresión de los factores de transcripción Nanog, Oct3/4 y Sox2 en las líneas celulares CT26, MC38 y PJ01.
- La alta actividad de la enzima ALDH mostró correlación con una elevada expresión de CD44, CD166 y de los factores de transcripción Nanog, Oct3/4 y Sox2.
- El ensayo de exclusión de Hoechst 33342 no fue capaz de identificar una población lateral que correlacionara con una alta expresión del resto de marcadores analizados relacionados con CSCs.
- La población $CD44^{high}$ en la línea celular CT26 fue capaz de formar más clones que la población $CD44^{low}$.
- El ensayo de dilución límite es más efectivo para analizar las capacidades proliferativas de una línea celular.
- Los fármacos doxorrubicina y PTX fueron capaces de enriquecer la población positiva para los marcadores relacionados con CSCs analizados.
- Las líneas quimioresistentes desarrolladas en este estudio mostraron un incremento de los marcadores relacionados con CSCs analizados.
- Se optimizaron los cultivos en 3D para las líneas celulares utilizadas en este estudio y se encontró un ligero aumento de la expresión de CD166 en las 3 líneas celulares analizadas y sólo de CD44 para la línea CT26.
- Los tumores obtenidos en el protocolo de generación de cáncer colorectal inducido por DSS/AOM en ratones deficientes de Granzima-A no mostraron diferencias en comparación con los tumores obtenidos de la misma manera en ratones WT.

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