UNIVERZITA KARLOVA V PRAZE
FARMACEUTICKÁ FAKULTA V HRADCI KRÁLOVÉ
Katedra biochemických věd

CHARAKTERIZACE A REGULACE FENOTYPU KMENOVÝCH RAKOVINNÝCH BUNĚK V BUŇKÁCH LIDSKÉHO KOLOREKTÁLNÍHO KARCINOMU

Diplomová práce

Vedoucí diplomové práce: Prof. RNDr. Lenka Skálová, Ph.D.
Školitel specialista: Dr. Frédéric Hollande

Hradec Králové 2012 Jana Trávníčková

CHARLES UNIVERSITY IN PRAGUE
FACULTY OF PHARMACY IN HRADEC KRÁLOVÉ
Department of Biochemical Science

CHARACTERISATION AND REGULATION OF THE CANCER STEM CELLS'PHENOTYPE IN HUMAN COLORECTAL CANCER CELLS

Diploma thesis

Supervisor of Diploma thesis: Prof. RNDr. Lenka Skálová, Ph.D.
Specialized supervisor: Dr. Frédéric Hollande

Hradec Králové 2012 Jana Trávníčková

PROHLÁŠENÍ

Prohlašuji, že tato práce je mým původním autorským dílem. Veškerá literatura a další zdroje, z nichž jsem při zpracování čerpala, jsou uvedeny v seznamu použité literatury a jsou v práci řádně citovány.

DECLARATION

I declare that this thesis is my original author work. All literature and other sources, which I used, are given in the list of references and they are regularly quoted in the text.

11.5.2012 ……

Jana Trávníčková

ACKNOWLEDGEMENTS

I would like to use this part of the thesis to thank a lot all the people who helped me with this work. First of all I would like to thank my supervisor in Montpellier, Dr. Frédéric Hollande for accepting me in his laboratory, enabling me to participate on the project and all his advices and guidance through the project.

I would like to express great thanks to Fanny Grillet, PhD student for her help and guidance, teaching me all the methods, cooperation in part of the project and especially for her patience and friendship during my stay in Montpellier.

Much attention belongs in this part to my family and my loved ones for their never-ending support through my study.

I would also like to thank my supervisor in the Czech Republic, Prof. RNDr. Lenka Skálová, Ph.D., for her advices and remarks and help with my project.

Last, I am very grateful for having the opportunity to participate on the project in Montpellier, so I would like to thank a lot the Erasmus programme for enabling me to spend 6 months in this beautiful city in the great collective in laboratory IGF.

Thank you all

Jana

ABSTRAKT

Univerzita Karlova v Praze
Farmaceutická fakulta v Hradci Králové
Katedra biochemických věd

Kandidát: Jana Trávníčková
Školitel: Prof. RNDr. Lenka Skálová, Ph.D.
Školitel specialista: Dr. Frédéric Hollande
Název diplomové práce: Charakterizace a regulace fenotypu kmenových rakovinných buněk v buňkách lidského kolorektálního karcinomu

Kolorektální karcinom je v současnosti řazen mezi nejčastější život ohrožující onemocnění na světě. Pokud prozkoumáme a pochopíme příčiny a vývoj této nemoci, především na její molekulární úrovni, mohli bychom výrazně přispět k pokroku její léčby. Jednou z možných příčin rezistence tumoru a jeho relapsu jsou nyní považovány rakovinné kmenové buňky (CSC), jejichž podrobné prozkoumání by mohlo vést k vývoji nové cílené terapie.

U CRC1, nově odvozené kolorektální rakovinné buněčné linie vytvořené v laboratoři IGF v Montpellier, byla potvrzena jednoduchá morfologie buněk. Dále bylo také potvrzeno, že obsahuje vyšší množství buněk s vlastnostmi buněk kmenových. Tato linie vykazuje pozitivní značení markery, které se vyskytují u buněk s vlastnostmi kmenových, jako například CK-18 a EphB2. Také její morfologie pozorovaná v elektronovém mikroskopu se vyznačuje jako primitivní, protože buňky obsahují velké jádro a jen velmi nízký počet ostatních organel.

Za další možnou příčinu kolorektálního karcinomu je považována změna exprese Claudinu 2 (CLDN2). Jeho zvýšená exprese je spojována se vznikem a vývojem tumoru. Ovlivnění exprese CLDN2, buď transfekcí plazmidem nebo infekcí virové částice, vyústila ve změny v morfologii buněk, stejně jako ve změnu v tvorbě sfér, která souvisela s úrovní exprese CLDN2. V každém případě, k potvrzení těchto výsledků je zapotřebí provedení dalších kontrol, případně použití další buněčné linie k zopakování experimentu.

U výzkumu CSC je v současné době pozornost soustředěna na LGR5, který by mohl být markerem rakovinných kmenových buněk. Nedávné studie však způsobily dohady o stálosti kmenových markerů v průběhu času. Vyvinuli jsme model kultivace buněk, při níž jsou buňky kultivovány v různých skupinách vytvořených na základě jejich exprese LGR5. Fenotyp těchto buněk se měnil v průběhu času při kultivaci za 3D podmínek. Z toho vyvozujeme, že tyto buňky jsou schopny pomocí změny svého fenotypu vytvořit vždy rovnováhu, která napodobuje jejich přirozený stav v živém organismu.

Všechny výsledky získané v tomto projektu nám napomáhají, abychom lépe porozuměli kolorektálnímu karcinomu na molekulární úrovni.

ABSTRACT

Charles University in Prague
Faculty of Pharmacy in Hradec Králové
Department of Biochemical Sciences

Candidate: Jana Trávníčková
Supervisor: Prof. RNDr. Lenka Skálová
Specialized supervisor: Dr. Frédéric Hollande
Title of diploma thesis: Characterisation and regulation of the cancer stem cells'phenotype in human colorectal cancer cells.

Colorectal cancer nowadays represents one of the most common life threatening malignancies worldwide. Well describing and understanding the causes and development of this illness, especially on the molecular basis would provide a progress in the treatment. Defining cancer stem cells (CSC) that are considered as a potential cause of the tumour resistance and relapse would result in the development of a new targeted therapy.

CRC1, colorectal cancer cell line recently established by the laboratory IGF in Montpellier was confirmed to possess the primitive morphology and to contain a larger amount of stem-like cells. This cell line is positive of stem-like-cell markers such as CK-18 and EphB2 and its morphology observed in electron microscope was defined as primitive with big nuclei and rare presence of other organelles.

Change of Claudin 2 (CLDN2) expression is considered as another potential cause of colorectal cancer. Its increased expression is connected to the tumour initiation and development. Influencing the CLDN2 expression by plasmid transfection or viral construct infection resulted into the morphological changes observed by the electron microscope and in changed sphere formation correlated with the level of CLDN2 expression. Nevertheless, these results require additional controls and using of another cell line to confirm this observation.

Regarding the CSC, LGR5 was investigated as possible marker of these cells. The recent studies suggest that stem cell markers may do not stay stable. We established a model of cell culture of various subpopulations of cells sorted according to the LGR5 expression. Their phenotype does not remain stable over the time in 3D culture and the cells appear return to the equilibrium simulating their *in vivo* state.

All the results obtained in this project contribute to the understanding of molecular basis of colorectal cancer.

TABLE OF CONTENTS

1. INTRODUCTION

Colorectal cancer is one of the leading malignancies worldwide in the present. Though large interest is focused on the etiology and treatment of the tumor, we have to face complications including metastasis progression and relapse. Better understanding and defining the colorectal carcinogenesis would help to find new strategies in the therapy and eliminate the number of ill patients.

Hierarchical theory of tumor progression defines a subpopulation of cells responsible for the formation of the whole tumor. These cells are called cancer stem cells and are likely to be resistant to the conventional treatment. With determining the marker of this subpopulation we could influence these cells and find the new potential targets of therapy.

LGR5 is the most discussed marker of intestinal stem cells and cancer stem cells during the last years. Several studies already confirmed its appearance at the bottom of the intestinal crypt at the same place, where stem cells are supposed to be found. Nevertheless, potential of this marker to be phenotypically unstable and to change in dependence on the outer conditions attracts the attention, especially after the recent article concerning the breast cancer and stem cells (Gupta P.B. et al., 2011).

The research of cancer stem cells requires colorectal cancer cell lines containing stem cell and being well described. The recently established cell line CRC1 may enable to focus on cancer stem cells, especially thanks to its advantages such as suspension culture and the recency of the line.

Finally, colorectal cancer is highly connected with loss of the cell-cell connection and the increased membrane permeability. Molecular changes in tight junctions, mainly in its important transmembrane proteins, Claudins may become a crucial point of interest in cancer research. Among the TJ' proteins, Claudin 2 shows an interesting target of attention because its expression is increased a lot in colorectal cancer comparing to healthy intestine.

2. THEORY

2.1 INTESTINE AND ITS STRUCTURE

The intestine is a segment of canal connecting the stomach to the rectum and consisting of 2 anatomical and functional parts, small and large intestine. The small intestine is the upper section and the longest part of the intestinal tract, with a length ranging from 3 to 5 metres. Anatomically, we can subdivide the small intestine into the duodenum, jejunum and ileum and the large intestine into caecum and colon (ascending, traversing, descending and sigmoid). The inner part of the intestine is structured into lateral folds, which range from 6 to 8 mm. In the small intestine they contain about 10 to 40 villi per 1 mm^2. The villi are composed of enterocytes, goblet cells, enteroendocrine cells, M-cells and tuft cells. Between the villi other structures are found, called crypts. Crypts are formed by epithelial invaginations into the gut mucosa and are located in all parts of the small intestine; they are inserted as almost straight and narrow tubular glands that terminate close to the muscular layer. They contain enteroendocrine cells, Paneth cells, tuft cells and stem cells. The large intestine is very similar to the small one apart from the absence of villi and Paneth cells (Scoville D.H. et al, 2008, Čihák R. et al., 2002).

The intestinal wall consists of 4 layers. In the order from inner space outwards we find the mucosa, submucosa, smooth muscle layer and serosa. All the structures and types of cells mentioned above are localised in epithelial columnar layer so as the epithelium represents the target of attention in colorectal cancer research (Čihák R. et al 2002, Leushacke M. et al, 2011).

The intestinal epithelium is developed during gastrulation and is established due to epithelial-mesenchymal interactions. The epithelium, formed from the endoderm, invaginates to form villi and intervillus regions called crypts. The whole process is controlled by several signaling pathways (see chapter 2.2) (Scoville D.H. et al, 2008).

2.1.1 Types of cells

The intestinal cells can be divided to 2 major groups: differentiated cells and stem cells and progenitors. The epithelium renews every 2-5 days and the stem cells generate progenitors which by increased amplification and proliferation give birth to differentiated cells

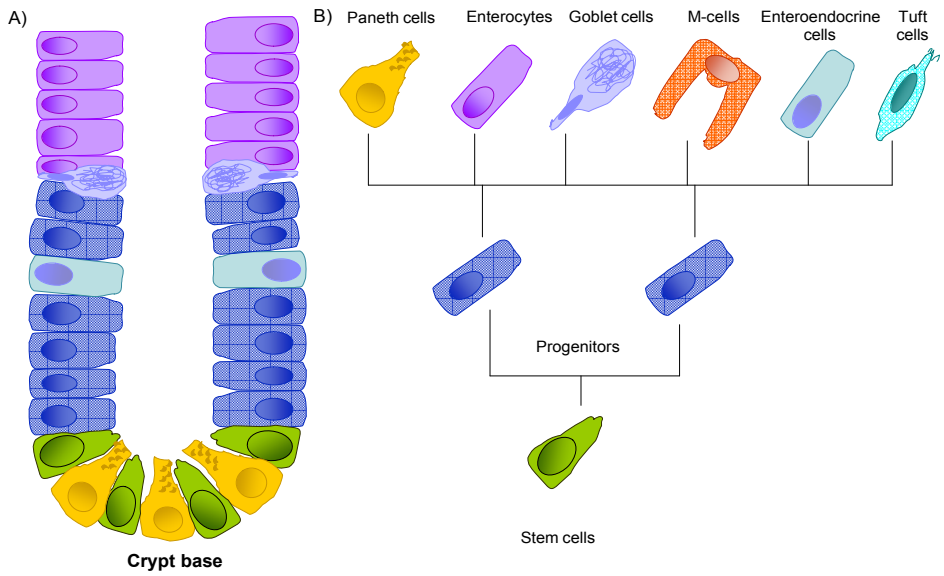


Fig.4.1: The structure of the intestinal crypt and cells adapted from Braker N. et al., 2010; A) The structure of the intestinal crypt demonstrating the distribution of cells. B) Types of cells and their differentiation.

2.1.1.1 Differentiated cells

Enterocytes are the most abundant cells in the small intestine. Their shape is thin, high, cylindric with many 1-2 µm high microvilli (up to 3000 on the top of each cell). Due to these structures, the surface of the cells and the whole intestinal surface are enlarged. The role of enterocytes is to enable the absorption of substances from the lumen.

M-cells (membranous cells) are located in places where lymphatic tissue is found under the mucosa. The cells are able to form a pocket which enables the passage of lymphocytes towards the lumen. M-cells also selectively search macromolecular antigens and pass them to the lymphocytes.

Goblet cells are situated among enterocytes. They contain droplets of mucus (made of a complex with mucin), which make up half of the cell cytoplasm. The droplets of mucus repress other organelles and nucleus towards the bottom of cells. This process explains the "goblet" shape of these cells. The mucus is then secreted in the lumen where it forms a protective layer on the top of the epithelium and lubricates it.

Tuft cells are also interspersed among the enterocytes. On the top they contain tufts of the microvilli which climb up over the top of the neighbor cells. Their function has not been described well yet.

Enteroendocrine cells are located in the crypts´ epithelium, and occasionally in the villi. They produce serotonin (responsible for stimulation of contraction of smooth

muscle) and various peptide hormones. The influence of these cells can be divided into 2 parts: endocrine (hormones such as gastrin, GIP, secretin) and paracrine with direct influence (somatostatine).

Paneth cells can be found in groups in the basal part of crypts. Their shape is typical for exocrine cells, they contain many eosinophil secretion granules. They are responsible for the secretion of peptidases and cooperate in the regulation of intestinal microflora (Čihák et al., 2007, Scoville D.H. et al., 2008).

2.1.1.2 Stem cells and progenitors

Stem cells in general (particularly embryonic) are characterized as cells with omnipotent characteristic. According to their capacity we can divide stem cells into embryonic stem cells, holding omnipotency, and adult stem cells with pluripotent characteristic which are actually found in renewing tissues such as the intestine. The intestinal stem cells (ISC) represent less than 1% of all intestinal cells and possess two essential characteristics that enable them to generate and maintain the whole tissue over a lifetime. Firstly, they ensure their existence by producing other daughter stem cells during division. In addition, they are the source of all types of differentiated cells appearing in intestinal tissues (Leushacke M. et al., 2011). This ability is provided by their specific mechanism of cell division. The ISC possess ability to divide either symmetrically or asymmetrically depending on environmental conditions. Symmetrical division means that 1 ISC give birth to 2 identical ISC, so it leads to an elevation of ISC number. The second, less frequent situation represents the division where 1 ISC give birth to 2 non-stem daughter cells which results into formation of differentiated cells and reduction of the stem cell pool. The asymmetrical division produces 1 ISC and 1 daughter cell which differentiates and becomes one of the above-mentioned cells. Apart from ISC, there exist also progenitors, cells which are formed from ISC by asymmetrical division and leads to an appearance of differentiated cells. Daughter cells formed in ISC division are called progenitors and they proliferate largely to increase the cell number. The newly formed cells then differentiate further into fully differentiated cells (Zeki S.S. et al., 2011, Alison M.R. et al., 2010).

An additional level of complexity is provided by the fact that ISCs appear divided in 2 different subpopulations. The first subpopulation is represented by quiescent cells, meaning that they do not divide in physiological conditions. These cells represent a reservoir in case of emergency, such as injury, in which case these cells activate, start

dividing and replace the lost cells. They are localized in the crypt at the position +4 counting from the bottom, just above the Paneth cells, and their number ranges from 4 to 6 cells in each crypt. The second subpopulation is formed by actively cycling cells which are responsible for routine regeneration of the intestine (every 2-5 days) and maintenance of its homeostasis. The actively cycling stem cells are likely to be placed directly at the bottom of the crypt, in between Paneth cells, and are also called columnar base cells (CBCs) (Shaker A. et al., 2010).

For better description and examination of stem cells it is necessary to identify reliable stem cell markers that can serve for their recognition. During the last several years the scientists try to find a reliable stem cell marker, a molecule that would be typical just for ISC and would not be expressed in other types of cells. Among the most discussed possible stem cell markers we range LGR5, BMI1 and ALDH1 (Barker N. et al., 2007, Sangiorgi E. et al., 2008, Carpentino J.E. et al., 2009).

BMI1 or B cell-specific Moloney murine leukemia integration site 1 is a protein localized in most human tissues. We can find it in brain, esophagus, kidney, lungs and other organs. BMI1 was suggested to play an important role during proliferation of adult stem cell and progenitors in liver, hematopoietic and maybe also in intestinal stem cells (Siddique H.R., et al., 2012). A recent study proposes a connection between BMI1 and LGR5 and their expression. BMI1 seems to appear in a subpopulation of adult stem cells called quiescent stem cells which are activated to multiply themselves after injury; LGR5 on the other hand seems to mark the subpopulation of the actively cycling stem cells located at the bottom of the crypt – see below (Yan K.S. et al., 2012).

ALDH1 or Aldehyde dehydrogenase is a metabolic enzyme in last years largely associated with adult stem cells and progenitors. It has been found and identified in small number of cells localized at the bottom of the crypt (Carpentino J.E. et al., 2009).

LGR5 or leucine-rich repeat containing G-protein-coupled receptor is a large 7-transmembrane protein. It is a member of the G-protein coupled receptors' family together with LGR4, LGR6 and other proteins and has similarities to hormonal proteins such as pituitary gland-regulating hormones. Apart from the gastrointestinal tract, the LGR5 marker has also been found in hair follicles and skin. In the intestine, this protein is expressed at the bottom of the crypt, suggesting that LGR5 really marks stem cells (Leushacke M et al., 2011, Barker N., et al., 2010). This idea is supported by another

experiment where whole crypts were grown from 1 murine Lgr5^{pos} cell. The crypt contained all intestinal cells, such as enterocytes, Paneth cells, goblet cells and others. (Sato T. et al., 2009). LGR5 marks one subpopulation of stem cells, actively cycling stem cells which are located at the bottom of the crypt and responsible for the basal renewal of the intestinal cells (Yan K.S. et al., 2012).

2.1.1.3 Cancer stem cells

With the discovery of ISCs and their potential there appeared discussions of the potential implication of stem cells in cancer initiation and progression. The term cancer stem cells (CSC) was established and the role of these cells is now closely examined to understand tumour formation (see chapter 2.3.3). Stem cells could be involved in colorectal cancer development and could be a cause of tumour relapse and metastasis formation. The CSC (called also tumour-initiating cells) may represent a group of cells which forms a tumour *de novo,* with only 1 cancer stem cell able to induce a tumourigenesis. The CSC population has not been well defined yet; several markers are studied to be confirmed as cancer stem cell markers (Vermeulen L. et al., 2008).

CD133 was the first marker to have potential to label CSC but was not found to be selective enough. The other more important markers are now LGR5, BMI1 and ALDH1, which are considered as marker of ISC (Zeki S. et al., 2011, Huang E.H. et al, 2009).

ALDH1 marker fulfills all the assumptions of being CSC marker. It is expressed at the bottom of the crypt and the isolated ALDH1^{pos} cells were found to be able to generate a colorectal tumour. Nevertheless, the connection between this marker and the other potential markers has to be well examined (Huang E.H. et al., 2008). **BMI1** as a potential marker of adult ISC could be also considered as a CSC marker but as the other molecules need to be confirmed and better described.

LGR5 is now discussed the most often as a potential CSC marker, especially after the experiment of formation of tumour from single cell Lgr5^{pos} (Vermeulen L. et al., 2008). The expression of LGR5 is also elevated in colorectal cancer and the LGR5 receptor is connected with the Wnt signaling pathway.

2.2 SIGNALLING PATHWAYS

During the development of the intestinal tract and formation of villi and crypts, cells are controlled mainly by 4 signalling pathways: Wnt, Hedgehog (HH), BMP and Notch.

The first pathway involved in tissue and organ development is bone morphogenetic protein (**BMP**) and its antagonists, gremlin 1, 2 and chordin-like inhibitor. They may play also an important role in controlling the stem cells. BMP is part of the transforming growth factor β superfamily and located generally at the top of the colonic crypt where its expression is highest. On the other hand Gremlin 1 and 2 are situated mainly at the bottom of the crypt, even in smooth muscle cells connected to the crypt where they are expressed at high levels.

BMP was found to antagonise Wnt signalling pathway via the PTEN tumour suppressor protein and to thereby restrict proliferation. This fact highlights the capability of BMP to repress de novo crypt formation.

Notch and sonic hedgehog (SHH) signals potentially play a role in stem cell maintenance. The Notch pathway is crucial for cell to cell communication. It controls a wide spectrum of activities in the intestine. Especially, Notch1 has been detected in crypt epithelial cells and is likely to influence cell proliferation. Activation of the Notch pathway also increases cell differentiation along the enterocyte lineage. This pathway also cooperates with Wnt signals to influence stem cells (Scoville D.H. et al., 2008).

Indian hedgehog (HH) is found in the intervillus region and is involved in embryonic development and formation of several tissues. The misregulation of this pathway can lead to tumourigenesis, but is more connected to pancreatic cancer and leukaemia (Curtin J.C. et al., 2010)

2.2.1 Wnt signalling pathway

The Wnt signalling pathway ranges among the most important pathways in intestinal development. The name is an abbreviation of 2 words: Wingless, segment gene found in Drosophila species and Int-1, the murine proto-oncogene. The Wnt pathway involves approximately 20 genes. The central role in this pathway is played by β-catenin, a cytoplasmic protein, the stability of which is regulated by complex of proteins including protein kinases. When Wnt signaling is not engaged, cytoplasmic β-catenin is bound to APC, axin and 2 kinases, Casein kinase and GSK3β. These enzymes phosphorylate specific Ser and Thr residues of β-catenin, which is then targeted by ubiqutin and degraded. On the other hand, upon activation of the Wnt pathway, APC, axin and kinases no longer form the connection with β-catenin and the protein remains unphosphorylated. In that form it can bind to E-cadherin at the cell membrane or enter the nucleus to bind proteins of the Tcf/Lef family, connected to the DNA to regulate

gene expression (Reya T., 2005). The Wnt signaling pathway controls and regulates the development of the intestine and is responsible for crypt formation. It also controls the expression of the EphrinB/EphB-receptor complex which by complex network determines the localization of the cells. EphB2 is moreover expressed at the bottom of the crypt so it could serve as a potential marker of stem cells (Zhang X., 2011, Reya T. et al., 2005, Merlos-Suárez A. et al., 2011)

Wnt signalling pathway plays a crucial role in cancer development. Among the most known and described signalling changes we range *APC* gene mutation. Inactivation of the *APC* gene leads to development of intestinal polyposis and cancer. The unbalance of β-catenin level in the cells leads to the uncontrolled proliferation of cells. The Wnt signalling pathway can also be activated by other mechanisms, e.g. β-catenin mutation which are not all known yet. After the understanding the Wnt pathways and its influence we may be able to design a target therapy to focus on this signal. Such treatments are already in development or even phase I of tests and gives a hope to many patients (Curtin J.C. et al., 2010, Reya T. et al., 2005, Caldwell G.M. et al., 2008).

2.3 COLORECTAL CANCER

Colorectal cancer (CRC) is the third most common cancer in the world. Every year more than 1,000,000 new cases are diagnosed worldwide (Jemal A. et al., 2011). The tumours are represented in 90-95% by adenocarcinoma, which is a tumour with epithelial origin. The incidence of this illness grows with age, and the highest is between 60 and 75 years. It ranges among well described tumours and several factors contribute to tumour formation such as heredity, diets rich in lipids and poor in fibre, methods of food preparation, alcohol and increased secretion of bile acids. There exist several significant predisposing factors which contribute to the development of the tumour:

Adenomatous polyps in patient etiology

Ulcerative colitis or Crohn's disease

Previously treated colorectal carcinoma or radiation in the area of the small pelvis

Colorectal cancer can be divided into 2 groups: sporadic, representing 85-90% of cases and hereditary, the remaining 10-15%. The most important molecular events

involved in the initiation of sporadic and hereditary cancers are mutations of the *APC* gene.

2.3.1 Metastasis

Metastasis represents the most life threatening situation in patients with cancer. The process of metastasis formation is composed of several steps called metastatic cascade. The cascade can be divided into these steps: invasion, intravasation, extravasation, implantation and growth in the target organ. Invasive tumour cells separate from the primary tumour following alterations of cell-cell adhesion. The process is also connected with a change of expression of proteins responsible for the control of motility and migration. The loss of cell adhesion and the increased motility is generally associated to a process called epithelium-mesenchymal transition (EMT). The invasive cells then enter the circulatory system and through it they move to distant parts of the body.

2.3.2 Treatment

Colorectal cancer is usually treated by conventional therapy, either chemotherapy (such as cisplatin) or ionizing radiation (mostly for rectal cancer). This treatment is efficient for the bulk of tumour but not for cancer stem cells, which are usually more resistant to the treatment (Curtin J.C. et al., 2010). One of the possibilities for colorectal cancer treatment seems to be a combination of several chemotherapeutics, called Folfiri. It contains 5-fluorouracil (5-FU), leucovorin (LV) and irinotecan (CPT-11). This combination offers several advantages thanks to different mechanism of effect of each agent. 5-FU inhibits proliferation by interfering with DNA synthesis, CPT-11 inhibits topoisomerase, an enzyme important for DNA replication and transcription and LV enhances the effect of 5-FU. This treatment showed to be more efficient than use of individual chemotherapeutics; it decreased the level of tumour and prolonged survival (Ishihara Y. et al, 2010).

In last years there was developed a new type of treatment, the targeted therapy. Understanding the signaling pathways and its activation enables to formulate new biological medicament promising the longer survival. Antibodies against VEGF and EGF receptors are members of the targeted therapy. Nevertheless, even with the new created treatment and huge progress in applied research we have to face resistance and relapses appearing during the therapy (Saltz L., 2008). The hope is now orientated to targeted therapy against cancer stem cells, respectively a combined therapy,

conventional and targeted. CSC represents new potential targets which could solve the issue of resistance and relapse (Curtin J.C. et al, 2010).

2.3.3 Molecular basis of colorectal cancer

The initiation and development of tumours is not still well described from the cellular perspective. There exists in general 2 theory of tumour development, the stochastic and the hierarchical theories.

According to the stochastic theory, a tumour is formed by a group of cells with the same characteristics as the other cells but which randomly underwent a series of somatic mutations. In a result of the mutations the cells start uncontrolled proliferation which ends in newly arising tumour. On the other hand, the hierarchical theory predicts that a subgroup of cells, called cancer stem cells, are able to initiate a tumour on their own by uncontrolled proliferation and to generate all other cell types found in a tumour (Odoux C. et al, 2008). Up till now, it is not sure which of these theories the right one is. Nevertheless, the hierarchical theory explains better all the processes which happen during cancerogenesis. In fact, it is currently thought that the most plausible theory mixes parts of the two initial theories described above.

2.4 CELL TO CELL CONNECTIONS

Each multicellular vertebrate organism is formed in compartments. Polarized endothelial and epithelial cells form barriers to separate individual organs from each other. Barriers play crucial role in living organism. They arrange separation of organs, compartmentalization to subparts and isolation from external environment. Nevertheless, barriers cannot separate units absolutely.

Selective permeability must be provided to allow communication between cells. Among communication we range movement of water, ions and proteins across the monolayers. The movement of ions and proteins between cells is called paracellular permeability and is regulated by cell-cell contact known as junctions (Aijaz S. et al., 2006).

The intestinal epithelium is formed by actively dividing cells and renew every 3-5 days. The cells move to the upper part of the epithelium by newly divided cells. These cells need to remain in connection and communicate with each other. These processes are provided by cell to cell connections.

These connections are mostly provided by three types of structures: desmosomes, adherent junctions and tight junctions. The group of these connections is usually called junctional complex (Farquhar M.G. et al., 1963, Aijaz S. et al., 2006).

Adherent junctions are found in proximity of tight junctions. Their characteristic and location are very close to TJ (Aijaz S. et al., 2006). AJ possesses the strongest link between the neighbouring cells. The most important protein expressed in AJ is considered to be E-cadherin (Hollande F. et al., 2005). E-cadherin is a transmembrane glycoprotein which is part of the family of cadherins together with N- or P-cadherins, and is responsible for cell to cell adhesion by calcium dependent connections. During colorectal tumourigenesis, several changes of intercellular communication have been observed. The modified expression of E-cadherin is considered as one of these changes involved in cancer. In most of the case the expression of E-cadherin is decreased or redistributed from the membrane to the cytoplasm (Dorudi S. et al., 1993, Buda A. et al., 2011).

Desmosomes are located along the lateral membrane. They are not observed in endothelial cells where TJ and AJ are found (Aijaz S., 2006). They are characterised by the widest intercellular space of the three types of junctions and are formed by two dense discs at each side of the cell contact and with a leaflet of lower density (Farquhar M.G., 1963).

2.4.1 Tight junctions

Tight junctions (TJ) are localised at the apical end of the lateral membrane. They seem to create very close contact between membranes of neighbouring cells (Aijaz S. et al., 2006). Actually, the intercellular space varies between 0.2 and 0.5 μm (Harhaj N.S. et al., 2004). There exist several functions of TJ in organisms. The main role of TJ, also the most known and described, is based on their name. They connect the cells and are responsible for separation of compartments. The TJ are crucial for the induction of the chemical or electrical gradient between the 2 sides of the membrane. By this they control the permeability of ions and other substances between neighbouring cells. Among other functions possessing the same importance we range the modulation of cell signalling (Martin T.A. et al., 2009, Aijaz S. et al., 2006), allowing them to regulate cellular processes including differentiation, gene expression and growth of cells.

TJ possesses a characteristic molecular architecture; they contain different types of proteins that modulate their structure and function. They can be divided into 3 subtypes:

The integral transmembrane proteins – occludin, claudins and junctional adhesion molecules (JAM)

The peripheral plaque proteins – proteins with PDZ motifs – Zonula occludens (ZO) 1-3, MAGI-1

Other TJ-associated proteins – α-catenin, symplekin (Martin T.A. et al., 2009)

The TJs were confirmed to play a crucial role during cancer development. Several studies have confirmed the correlation between loss of barrier function and tumour appearance (Harhaj N.S. et al., 2003). Colorectal cancer is associated with the change of expression of Claudin 1, 2 and 4, occludin and ZO1. Decreased expression of Claudin 4 has been detected in colorectal cancer including in metastatic lesions. In contrast, overexpression of Claudin 1 was confirmed to be involved in cancerogenesis (Martin T.A., 2009).

2.4.1.1 Claudins

The claudin family is part of the transmembrane proteins localized in tight junctions. The word Claudin comes from latin Claudere which means “to close”. The primary and physiological role of claudins is to regulate paracellular selectivity of small ions. The mechanism whereby claudins regulate the permeability is not fully understood. However, two main ways of regulation have been already described (Findley M.K. et al., 2009).

Claudins form in a structure by which they are able to charge selective pores and so promote specific ion permeability. The second way shows that claudins do not regulate selection only in a negative sense but can also increase paracellular permeability.

Until now 27 human claudins have been identified and described (Mineta K. et al., 2011). Claudins’ size ranges between 20 and 27 kDa and their structure is quite conserved. They are transmembrane proteins passing the barrier four times and forming two extracellular loop domains. Both ends are oriented towards the cytoplasm. The function of the N-terminus, a short 7-aminoacid chain, has not been discovered yet. The C-terminus, whose structure is more variable, divers from 21 to 63 amino acids and differs in terms of sequences. One domain is very similar for all members, the PDZ binding motifs that enable direct interaction with other tight junction proteins such as

ZO-1, -2, -3. Specially, interaction with cytoplasmic proteins ZO-1 and ZO-2 contributes to the stabilization of the TJ by linking claudins to the actin cytoskeleton.

The first extracellular loop contains approximately 52 residues and influences paracellular charge selectivity. This loop has a signature motif: Gly-Leu-Trp-x-x-Cys (8-10 aa)-Cys which is highly conserved and found even in closely related proteins. The cysteines integrated in this motif may bind together and form a disulfide bond which increases the stability of the protein conformation (this fact has not been proven yet).

The second extracellular loop is shorter, consists of 16 to 33 amino acids, and less characterized. Some studies suggest that the second loop forms into helix-turn-helix conformation which might participate into claudin-claudin interaction (Findley M.K. et al., 2009, Angelow S. et al., 2008).

CLAUDIN-CLAUDIN INTERACTION

Epithelial and endothelial cells usually express multiple claudin isoforms in an organ-specific manner. Interaction between individual isoforms probably plays role in controlling barrier permeability.

Claudins interact in 2 different manners:

1) laterally in the plane of the membrane (heteromeric interactions)
2) head-to-head binding between adjacent cells (heterotypic interactions)

Heteromeric binding is very little described. Claudin homomultimers consist of up to 6 monomers. Heteromeric and heterotypic interactions are very strictly determined. For example, Claudin 3 and Claudin 4 are heteromerically compatible when expressed in the same cell but they do not interact heterotypically despite very similar extracellular loop domains (both are highly conserved) (Findley M.K. et al., 2009).

On the other hand, Claudin 3 heterotypically interacts with other claudins, such as Claudin 1 and 5. It was defined that a single mutation of extracellular loop of Claudin 3 (amino acid 44 Asn – Thr – which is located in Claudin 4) change the ability of Claudin 3 to bind heterotypically to Claudin 4 without any changes of binding capacity to Claudin 1 and 5.

So far, only Claudin 3 was found as a protein capable to form heterotypic bonds, it seems that homotypic interactions are more common between claudins. Detailed analysis has not been performed yet though (Findley M.K. et al., 2009).

2.4.1.1.1 Claudin 2

Claudin 2 (CLDN2) is one isoform of the claudin family firstly isolated in 1998 by Tsukita's research group (Furuse, 1998). This 22 kDa-big protein passes the membrane four times as well as other claudins. CLDN2 plays an important role in ion transport control, by forming a cation-selective channel. CLDN2 was not only proven to control cation-transport, but it also handles the water transport ability. CLDN2 is expressed mainly in small intestine, liver, pancreas or loop of Henley in kidneys (Rosenthal R. et al., 2010, Aung P.P. et al., 2005). The change of expression of CLDN2 evidently plays a role in gastrointestinal tumour progression. In normal tissues, CLDN2 is expressed in the stomach and small intestine, nevertheless the level of the expression is not high, and it is barely detectable in the colon. In the small intestine, CLDN2 is localized at the bottom of the crypt and its expression decreased with higher differentiation (Dhawan P. et al., 2011). On the other hand in samples of colorectal cancer CLDN2 was found in much higher quantity than in healthy tissue or in gastric cancer samples (Aung P.P. et al., 2006, Dhawan P. et al., 2011). It is not clear whether the change of CLDN2 expression is a cause of cancerogenesis or an event that plays a role in tumour progression, though it is already proven that its expression is connected to the colorectal cancer growth. The increased expression was measured also in inflammatory intestinal diseases such as Crohn's disease or ulcerative colitis. Furthermore, cells modified to express more CLDN2 and injected to the mouse cause larger and more serious tumours than control cells. These results suggest that the change of CLDN2 expression may be considered as a possible promoter of colorectal cancer. (Dhawan P. et al., 2011).

Regarding the problematic of colorectal cancer I concentrated in my project to 2 main parts, the cancer stem cells and their identification by biological laboratory methods and the change of paracellular permeability in cancer development, concretely the role of Claudin 2 (see chapter 3).

3. AIM OF WORK

My research can be divided into 3 main parts:

- Characterisation of CRC1 cell line, a recently established colorectal cell line
- Characterisation of various colorectal cancer cell lines in terms of Claudin 2 expression, Influence of Claudin 2 expression in cell lines
- Plasticity of the *LGR5* marker

4. METHODS

4.1 MATERIALS AND CHEMICALS

MATERIALS:

Aerodisc syringe filters (Pall Corporation, France)

Biorad set for WB -boxes, combs, glass plates (Biorad, USA)

Blot paper Protean (Biorad, USA)

Light Cycler Capillaries (Roche, Germany)

Cell culture flasks 25 cm^2 and 75 cm^2 with filter BD Falcon (BD Biosciences, USA)

Cell culture inserts 0.4 μm Falcon (BD Biosciences, USA)

Cuvettes macro PS Fisherbrand (Fisher Scientific, France)

Falcon tubes 15 ml, 50 ml (BD Biosciences, USA)

FACS polypropylene tubes 5 ml Falcon (Falcon Becton, France)

Microscope slides superfrost (Menzel-Glaser, Germany)

Microtubes 0,2 ml; 0,5 ml; 1,5 ml (several producers)

Nitrocellulose membrane Protran BA85 (Whatman, Germany)

Pipette tips with and without filter Sorensen (Sigma Aldrich, USA)

Serological pipettes 5 ml, 10 ml, 25 ml (Falcon Becton, France)

Tissue culture plates with 6, 12 and 24 wells (Falcon Becton, France)

ULA cell culture flasks 25 cm^2 and 75 cm^2, Corning (Sigma Aldrich, USA)

ULA tissue culture plates with 96 wells,Costar (Sigma Aldrich, USA)

INSTRUMENTS :

Biophotometer (Eppendorf, France)

Cell sorter Aria FACS (BD Biosystems, USA)

Cell sorter Astrios (Beckman Coulter, USA)

Centrifuge 5417C (Eppendorf, France), Carousel (Roche, Germany)

Electron microscope Hitachi 7100 (Hitachi, Japan)

Fluorescent microscope AxioImager.Z1 (Zeiss, Germany)

Hood (Seroa, Monaco, ADS Laminaire, France)

Lightcycler 1,5 (Roche, Germany)

Mastercycler personal (Eppendorf, France)

Micoscope Eclipse TS100 (Nikon, UK)

Microspectrophotometer Thermo Scientific Nanodrop 2000 (Nanodrop, USA)

Odyssey Infrared Imaging system (Licor, USA)

PowerPac HC Power supply (Biorad, USA)

Transblot SD System – semi-dry transfer cell (Biorad, USA)

CHEMICALS AND SOLUTIONS:

2-mercaptoethanol (Sigma Aldrich, USA)

2-propanol (Fisher Scientific, USA)

7AAD (Invitrogen, USA)

Accumax (Sigma Aldrich, USA)

Acrylamide/bisacrylamide 40% (Euromedex, France)

APS (Sigma Aldrich, USA)

D-glucose (Sigma Aldrich, USA)

DAKO Fluorescent mounting medium (DAKO, USA)

DAPI (Invitrogen, USA)

Donkey serum (ABCam, USA)

DPBS 10x (Lanzo, Belgium)

EGF (R&D Systems, UK)

Ethanol (VWR International, France)

FCS (Eurobio, France)

FGF (R&D Systems, UK)

Glucose (Fluka Biochem, Sigma Aldrich, USA)

Glutamine (Lonza, Switzerland)

Insulin (Sigma Aldrich, USA)

N2 suplement (Gibco, Invitrogen, USA)

PFA (Sigma Aldrich, USA)

Prestained Protein ladder (Euromedex, France)

TEMED (Sigma Aldrich, USA)

TG-SDS 10x buffer (Euromedex, France)

Triton X-100 (Sigma Aldrich, USA)

Tween 20 (Euromedex, France)

ENZYMES :

MMLV (Invitrogen, USA)

Superscript Reverse Transcriptase (Invitrogen, USA)

Sybr green supermix (Qiagen, France)

Trypsin EDTA (Gibco, USA)

MEDIUMS:

DMEM (Lonza, Switzerland)

DMEM/F12 (Gibco, USA)

ANTIBIOTICS:

Penicillin (Invitrogen, USA)

Streptomycin (Invitrogen, USA)

Doxycyclin (Sigma, USA)

Puromycin (Invivogen, Toulouse, France)

G418 (Invivogen, Toulouse, France)

KITS:

FastStart DNA Master SYBR Green PCR kit

M-MLV RT kit (Invitrogen, USA)

PKH26 Red fluorescent cell linker kit (Sigma Aldrich, St. Louis, USA)

Qiagen RNeasy Microkit (Qiagen, France)

Qiagen RNeasy Minikit (Qiagen, France)

Superscript (Invitrogen, USA)

4.2 CELL CULTURE

For the experiments I used three lines of human colorectal cancer cells: CRC1, DLD1 and SW480.

CRC1 is a recently established colorectal epithelial cell line derived by the workers of the laboratory IGF from colorectal adenocarcinoma. This cell line was derived in 2008 directly in suspension. CRC1 as a new cell line is not much described.

DLD1 is a colorectal epithelial cell line derived from colorectal adenocarcinoma, classed as a type C (according to Duke's classification).

SW480 is a colorectal epithelial cell line derived from colorectal adenocarcinoma, classed as a type B (according to Duke's classification).

The cell lines were grown in two different conditions, as adherent cells (2D) or in suspension (3D). Apart from these wild-type cell lines, I also used cell lines modified by plasmid transfection, or viral construct infection.

Cells	Plasmid	Selection ATB	Activation ATB
CRC1 shCLDN2, NS	P.TRIP.Z	Puro 1 µg/ml	Dox 1µg/ml
DLD1 shCLDN2, NS	P.TRIP.Z	Puro 1 µg/ml	Dox 1µg/ml
SW480 Ctrl, C2, C2*	PTER	G418 500 µg/ml	Dox 1µg/ml

The number of cells and the duration of cell culture differed according to the proposed experiment:

Plate/dish	Cell number	Volume/ medium	Cultivation days	confluence	Type of experiment
6 well plate	200,000	2 ml	2	100 %	Western blot
6 well plate	100,000	2 ml	2	40%	Western blot
12 well plate	50,000	1 ml	3	40%	IF
12 well plate	100,000	1 ml	2-3	100%	IF
24 well plate – inserts	50,000	0.25ml /0.75ml	3-4	100%	Electron microscopy
10 cm diameter dish	1,200,000	10 ml	2	100%	Western blot
96 well plate ULA	100/50/25	100 µl	7	------	Cell counting
75 cm2 ULA flask	60,000	20 ml	7-14	-------	WB, EM

4.2.1 Culture of adherent cells

Adherent cells are cultured in flasks with adherent cover. These cells grow in monolayer in the bottom of the flask where they are attached one to the other by strong bonds. For culture I used DMEM medium (Dulbecco´s modified Eagle´s medium) supplemented with:

Antibiotics: Streptomycin/Penicilin 1:1	1%
Glutamax or Glutamine	1%
FCS	10%

In general, the flasks were used with the surface 75 cm^2 with 10 ml of the medium. Cells were incubated in 37°C/5% CO_2 in incubation box. To passage cells twice a week, the medium was aspired, flasks were washed with 1X PBS and cells were detached by using 0.05% trypsin. Then I suspended cells in DMEM complete medium, counted and used appropriate number of the cells according to the proposed experiment or for preserving the culture.

4.2.2 Culture of cells in suspension

Cells in suspension are cultured in special flasks with ultra low cell attachment surface (Corning), size 25 or 75 cm^2. Cells grow separately without attaching the surface; they bind just to one another and form aggregates or spheres. In suspension cells cannot be too concentrated, the usual concentration of cells is between 1000 to 4200 cells/ml. For culture I used medium M11 which contains DMEM and F12 in ratio 1:1 supplemented with:

Insulin	0.2%
EGF	0.01%
b-FGF	0.01%
D-glucose	1%
Antibiotics: Streptomycin/Penicillin, 1:1	1%
N2 Supplement	5 ml (1%)

For culture, flasks with surface 75 cm^2 were used, medium was changed once a week, cells were passed at the moment when they stopped forming spheres and started forming aggregates or they were multiplied enough to be passed.

It is possible to change 3D cells to 2D just by changing the flask and medium, as well from 2D to 3D. In general, it is necessary to let the cells grow at least one week under specific unchanged condition to obtain homogenous characteristics.

4.2.3 Sphere counting

For culture of the cells for sphere counting, I used ULA plates with 24 or 96 wells. These plates enable to use small amount of cells, various concentrations and better observation of the spheres in the microscope.

Cells were grown 1 week under the conditions characteristic for 3D culture, concentrations varied from 100 to 1000 cells/ml. 96-well-plates contained 100 µl of medium and just the 60 inner wells were fulfilled by cells. The outer wells around the border of the plate are not used for sphere counting but only contain medium without cells, because results obtained with these wells are usually not reliable.

After 1 week I observed the wells using an optical microscope Nikon eclipse TS100, counted spheres in every well and documented their numbers and size with photos.

4.3 WESTERN BLOT

Western blot (WB) is a method to detect and quantify expression of individual proteins in cells or tissues. It is based on separation of proteins according to their size by current in polyacrylamide gel.

4.3.1 Cultivation and lysis

For WB, cells were cultured under 2 conditions, in suspension and monolayer. In monolayer, cells were grown in Petri dishes or cell culture plates (various sizes) until desired confluence. Then, medium was removed and monolayers were washed twice with 1X PBS. For lysis, an appropriate volume of RIPA was used for 20 minutes and lysis was facilitated by mechanical means (scratching knife) and vortexing several times. Samples were centrifuged for 15 min at 15,000 rpm to remove rest of nucleic acids and other undesired material.

4.3.2 Dosage of proteins

The amount of protein in samples was dosed by using microLowry method. The Lowry assay is one of the most common methods for quantification of soluble proteins. The protein concentration can be determined with a calibration or standard curve. The content of samples and calibration curve is written below.

	Blank (µl)	Standards (µl)						Samples (µl)
BSA	0	1	2.5	5	10	15	20	2 of sample
1X RIPA	4	4	4	4	4	4	4	2
H_20	20	19	17.5	15	10	5	0	20
Sol. A	125	125	125	125	125	125	125	125
Sol. B	1000	1000	1000	1000	1000	1000	1000	1000

For WB the samples containing 40 µg of proteins were prepared (same volume of each sample was achieved by adding 1X RIPA). Proteins were denatured and disulfide bonds were lyzed by adding 4X Laemmli buffer into the samples ($^1/_3$ of volume of sample) and incubating for 4 min at 95°C in a heating block.

4.3.3 Gels

For WB in general 2 gels were used: gels containing 10 or 12% of acrylamide. The gel for the WB contains 2 parts: the running gel, which is responsible for protein separation, and the stacking gel, which is situated above running gel and enables the loading of the samples. Gels were prepared according to the table below, poured between glass slides and wells were formed with plastic combs (all parts of the Biorad set).

Running gel:

Content for 2 gels	10%	12%
Demineralised water	5 ml	4.5 ml
Buffer pH 8.8 SDS 0.4%	2.5 ml	2.5 ml
Acryl/bis 40%	2.5 ml	3 ml
APS	40 µl	40 µl
TEMED	12 µl	12 µl

Stacking gel:

Content for 2 gels	Volume
Demineralised water	3.2 ml
Buffer pH 6.8 SDS 0.4%	1.25 ml
Acryl/bis 40%	0.56 ml
APS	40 µl
TEMED	12 µl

Gels were prepared just before use or 1 to 2 days before and stored at +4°C in a humid environment.

4.3.4 Migration and transfer

Samples were loaded into gel by using tips with flexible top. Gels were placed into box with the 1X SDS-TG buffer. As a protein ladder I used Prestained protein ladder 10-170 kDa (Euromedex). Migration was performed at 40 mA-60 mA.

For transfer, a nitrocellulose membrane measuring 8.5x5.5 cm was cut and 2 soft plates were humidified in the transfer buffer (absolute ethanol and 1X SDS-TG buffer in ratio 1:4). Soft plate, nitrocellulose membrane, gel and soft plate were placed into the transfer cell (Biorad) as a sandwich. The transfer was performed at 160 mA for 50-55 min.

4.3.5 Blocking and antibody incubation

After the transfer, the membrane was dyed by Ponceau red to control the efficiency of the transfer and membrane was cut in parts if necessary for use of several antibodies (Ab) at the same time. Membrane was washed and then incubated for 1 hour in non-fat dry milk in 1X PBS (5%) to block the protein binding places and increase the selectivity of the Ab.

Then the membrane was incubated in primary Ab in TPBS overnight at +4°C, then washed 5 times with demineralised water and twice with 1X PBS for 10 min. Membrane was incubated in the secondary Ab in 1X TPBS for 1 hour at RT in the dark and then washed 5 times with distilled water and twice with 1X TPBS for 10 min. The membrane was scanned on a Licor and quantified with the Odyssea software.

Primary Ab	Producer	dilution	Secondary Ab	Producer	Dilution
Claudin 2	Invitrogen	1/250	Anti-mouse IRDye 800	Millipore	1/50 000
E-Cadherin	BD Biosciences Pharmigen	1/2500 1/5000			
ß-catenin a.	BD Biosciences Pharmigen	1/1000			
Actin	Sigma	1/5000			

4.4 ELECTRON MICROSCOPY (EM)

For EM cells were cultured under 2 conditions, in suspension or as monolayers. In suspension, 60,000 cells were grown in 20 ml (75 cm^2 flask ULA) M11 medium for 1 week in 37°C/5% CO_2. After 1 week and sphere control in optical microscope, suspension was centrifuged for 5 min at 300 x g, supernatant removed and pellet washed with 1X PBS. Then, 250 µl 2.5% glutaraldehyde in PHEN buffer was added to

the pellet. The next day, 2.5% GTA was replaced by 0.5% GTA and samples stored at 4°C.

In monolayer, cells were cultured in special cell inserts with 0.4 µm pores in DMEM complete medium until 100% confluence. Then, wells were washed with 1X PBS and 250 µl 2.5% GTA in PHEN buffer was added. The next day, 2.5% GTA was replaced by 0.5% GTA.

Samples were then prepared for electron microscope by the workers responsible for electron microscope. After two rinses in Sorensen's buffer, the cells were dehydrated in a graded series of ethanol solutions (30-100%). The cells were embedded in EmBed 812 using an Automated Microwave Tissue Processor for Electronic Microscopy, Leica EM AMW. Thin sections (70 nm ; Leica-Reichert Ultracut E) were collected at different levels of each block. These sections were counterstained with uranyl acetate and observed using a Hitachi 7100 transmission electron microscope in the Centre de Ressources en Imagerie Cellulaire de Montpellier (France).

4.5 IMMUNOFLUORESCENCE STAINING

Solution	Ingredients	
PFA 4%	Paraformaldehyde 1X PBS	4% to 100%
Sodium azide 0,1%	Sodium azide 1X PBS	0.1% to 100%
Triton 0,5 %	Triton 1X PBS	0.5% to 100%
Blocking buffer (BB)	Triton 0.5% Donkey serum 1X PBS	20% 2% to 100%

Immunofluorescence (IF) is a method serving to detect specific proteins in cells or tissue and to determine their localization. It is based on binding specific primary Ab to proteins, followed by detection of this primary Ab with a secondary Ab coupled to a fluorophore. Thanks to this fact, the labeled protein can be detected and observed under a fluorescence microscope.

For IF, cells were cultured in monolayer on specific glass slides sterilized by UV radiation with DMEM complete medium under general conditions until the desired confluence was reached.

For the cells with low adherent ability to the surface, I used Matrigel as a polymer facilitating cell binding to the surface. Matrigel was suspended in cold DMEM medium (pure), added to the wells with glass slides and incubated for 30 minutes at RT. Then, the medium was removed and cells suspended in fresh medium could be added.

After growing the cells to the desired confluence, cells were fixed on slides with a 4% PFA solution (after washing with 1X PBS), washed with 1X PBS and stored in a PBS + 0.1% sodium azide solution at +4°C.

Before Ab labeling, cells were permeabilised with 0.5% Triton for 10 min to facilitate the passage of antibodies through the membrane, then washed with 1X PBS. Then, they were incubated for 45 min in blocking buffer for IF to achieve the highest possible specificity of Ab labeling.

Primary Ab diluted in BB were used separately or in pairs (with different secondary Ab) overnight at +4°C. After 3 PBS washes, secondary Ab, also diluted in BB, are applied, for 1 hour in the dark at RT. Lastly, cells were stained with DAPI (nucleus marker) for 5 minutes, after 3 washes with PBS for 5 min in dark at RT.

After washes with PBS and demineralised water, slides were fixed onto microscope glass slides by using DAKO fluorescent mounting medium and stored in the dark at RT.

Primary Ab	Producer	Dil.	Secondary Ab	Producer	Dilution	DAPI
Cytokeratin-18, cl.DC10	Neomarkers	1/100	Alexa 488 Ab mouse green	Molecular Probes, USA	1/500	Invitrogen USA dilution 1/5000
Cytokeratin-20	Neomarkers	1/100				
activated ß-catenin	BD Biosciences	1/50				
E-cadherin	BD Biosciences	1/100				
Claudin 2	Invitrogen	1/100				
Mucin-2, Ccp58	Santa Cruz	1/50				
Ephrin B2	R&D systems	1/20	Alexa 568 Ab goat red	Molecular Probes, USA	1/500	
Ephrin B3	R&D systems	1/20				

4.6 LABELING FOR PLASTICITY EXPERIMENT AND FACS

Flow cytometer is a special machine which is able to detect cells with fluorescent characteristics. These cells can be transfected with genes producing fluorescent proteins or labeled by Ab coupled to fluorophores. Cytometer is then able to separate them according to these characteristics into groups. Detection of several proteins using

several fluorescent markers can be performed simultaneously if their emission peaks differ.

4.6.1 PKH26 labeling for FACS

PKH26 is a red fluorescent membrane dye which is generally used to stain the cell membrane. The labeling is performed according to manufacturer's instructions with changes of incubation time and concentration in the case of DLD1 cells, according to the protocol used by Engstrom J.U. et al., 2007.

Cells were detached by using Accumax (trypsin can decrease the efficiency of the subsequent staining) and washed with 1X PBS. Cells were resuspended in Diluent C (part of the kit) and incubated with PKH26 in diluent C for 5 min. Staining was stopped with 1% BSA in PBS and cells were washed 3 times with the same solution.

4.6.2 LGR5 labeling before FACS

For LGR5 labeling cells were detached or released from spheres by Accumax, centrifuged and suspended in labeling buffer (5%FCS in PBS). Cells were incubated with LGR5 Ab for 20 min on ice with vortexing every 10 min, then washed 3 times with labeling buffer and incubated 20 min with APC Ab in the dark on the ice again with vortexing, then washed 3 times. Finally, cells were filtered and resuspended in 7AAD in labeling buffer within FACS tubes, and stored on ice in the dark until FACS was performed.

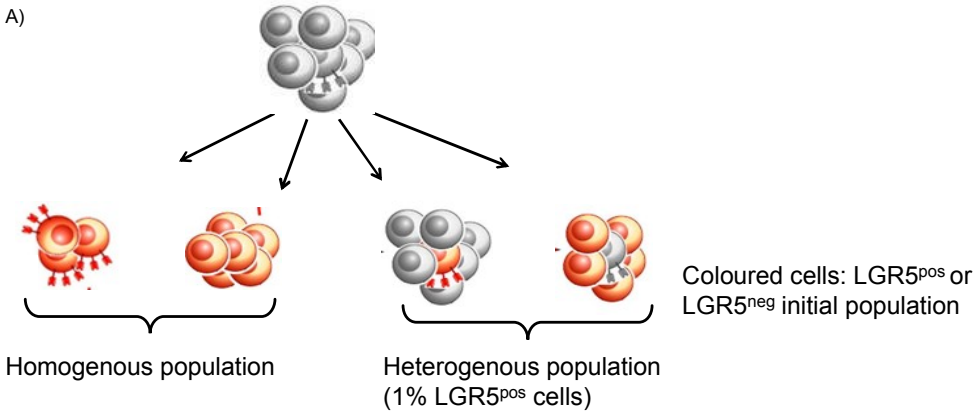
Marker	Producer	Dilution
LGR5/GPR 49 N-term Ab	Interchim	1/25 25µl for 1 million cells or 25 µl for 50,000 at analysis
APC antirabbit Ab	Invitrogen, USA	1/250 500 µl for 30 million cells
7AAD	Invitrogen, USA	1/400

4.6.3 Marker plasticity examination

For experiment to examine plasticity of LGR5 marker we cultured the cells in 4 specified conditions with selected subpopulation of cells. In general, cells were grown in 2D, collected and part of the cells labeled by PKH26 (for the DLD1 cell line). Then, all the cells were stained with LGR5/GPR49 N-term primary Ab (see above), APC secondary Ab and 7AAD as a marker of cell death. All the cells were sorted on a FACS Aria machine for DLD1 cells or on a MoFlo Astrios for CRC1 cells and divided into 4 individual groups, 2 containing homogenous populations coloured by PKH26, and two where these populations were mixed respectively with unstained $LGR5^{neg}$ or

LGR5^{pos} cells, in proportions that respect the original percentages of each subpopulation in the parental cell line (see table below).

A)



B)

	DLD1	CRC1
Coloured population	PKH26	CRC1-GFP
FACS	Aria	Astrios
Amplification	2D DMEM	
Homogenous or heterogeneous growth	3D M11	
	4 days	7 days

C)

Name of condition	LGR5^{pos}	LGR5^{neg}
LGR5^{pos}	125 000	----------
LGR5^{neg}	---------	300 000
Mix LGR5^{pos} labelled, LGR5^{neg}	5000 coloured	495 000
Mix LGR5^{pos}, LGR5^{neg} labelled	5000	495 000 coloured

Fig. 4.1.: Scheme of marker plasticity analysis, A) Drawing of used groups of cells, B) Defined conditions of culture and analysis

These 4 sample groups were cultured in 3D form for 4 days (for DLD1 cells) or 7 days (for CRC1 cells). After this time, cells were stained with the LGR5 Ab again and analyzed with the same cell sorter as previously.

4.7 RNA EXTRACTION

To obtain RNA, 2 types of cells were used: directly from cell culture (in monolayer as well as in suspension) or after cell sorting. The cells from culture were washed in 1X PBS, trypsinised (if in monolayer), washed in 1X PBS and resuspended in RLT/βME 1%. Cells from cell sorting were centrifuged for 5 min at 300 x g to remove labeling buffer and resuspended in RLT/βME 1%. Then, the protocol is same for both conditions.

RNA extraction was performed with Qiagen RNeasy Mini or Microkit (depending on the number of cells), following the manufacturer's instructions (Qiagen handbook). RNA was precipitated by 70% ethanol, washed several times with various wash buffers and dissolved in RNase free water. The whole process was done in minispin columns.

After extraction, the RNA was quantified with a Nanodrop (260/280 nm absorption).

4.8 REVERSE TRANSCRIPTION (RT)

Reverse transcription is a method used to obtain cDNA from RNA. In this experiment we used the M-MLV RT kit, or the Superscript II kit for samples with low amount of RNA. M-MLV kit uses Moloney Murine Leukemia Virus Reverse Transcriptase as an enzyme, oligoDT as primers, mix of bases (dNTP mix) and as buffers, DTT and 5X first strand buffer. In general 1µg of total RNA is used (this volume can be decreased to 250 ng if needed).

The amplification process is done in Mastercycler (Eppendorf). The program used includes heating the mix of RNA, oligoDT and dNTP in water for 5 min at 65°C, cooling to 37°C for 2 min, adding mix of buffers and enzyme, incubating 50 min at 37°C and heating to 70°C for 15 min to inactivate reaction. The cDNA can be used as a template for qPCR.

4.9 QUANTITATIVE POLYMERASE CHAIN REACTION (QPCR)

qPCR is a quantitative method enabling the detection of specific genes in cells and letting the comparison among individual samples. By multiplying the gene in cycles until detectable concentration it allows to quantify the gene expression in comparison with other cells. As a control of RNA content, we used the GAPDH gene, the expression of which is detectable in high quantity in all cells and is not thought to vary.

To perform the qPCR, cDNA is mixed with specific primers (sense and antisense) and Sybrgreen enzyme in capillaries and then placed into Lightcycler (Roche). The program used includes heating up to 95°C to denature the DNA structure, cooling to 55°C to enable binding of primers and reheating to 75°C to arrange the highest efficiency of the enzyme, which runs in cycles (usually 40 to 50 cycles) Mix without DNA is used as a negative control.

Primer	Sens	Antisens	Producer
GAPDH	GGT-GGT-CTC-CTC-TGA-CTT-CAA-CA	GTT-GCT-GTA-GCC-AAA-TTC-GTT-GT	Eurogentec
CLDN2	GAG-GGA-TTA-GAG-GTG-TTC-AAG-G	AGG-GAC-TGC-TCC-CTT-GTC-TT	Invitrogen
LGR5	TCA-GTC-AGC-TGC-TCC-CGA-AT	CGT-TTC-CCG-CAA-GAC-GTA-AC	Zymed
BMI1	AAA-TGC-TGG-AGA-ACT-GGA-AAG	CTG-TGG-ATG-AGG-AGA-CTG-C	Eurogentec
ALDH1	TCC-TGG-TTA-TGG-GCC-TAC-AG	CAA-CAG-CAT-TGT-CCA-AGT-CG	Eurogentec

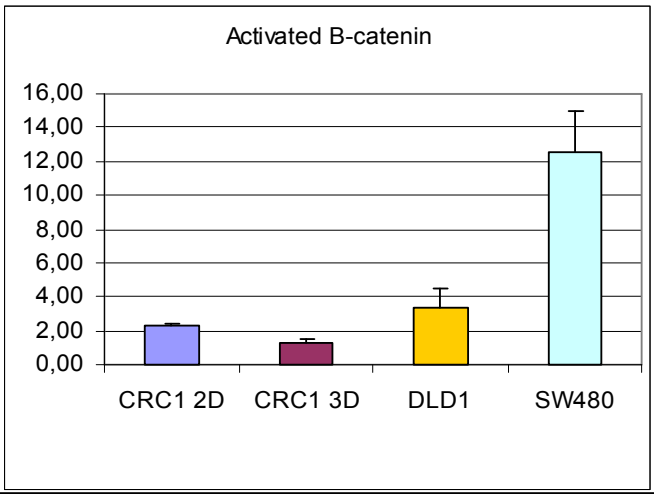
5. RESULTS

5.1 CHARACTERISATION OF THE CRC1 CELL LINE, A RECENT COLORECTAL CELL LINE

The CRC1 cell line is a recent colorectal cell line derived from a colorectal carcinoma sample by this laboratory. As a relatively new cell line it brings several advantages. It has not been changed by many mutations during the time it has been cultured *in vitro*. Also, the CRC1 cell line was derived directly in 3D form (suspension), which favours the growth of stem cells.

To characterise the CRC1 cell line I focused on the expression of several proteins in comparison to other cell lines. As the first I chose *activated* β-catenin, a protein involved in the Wnt signalling pathway. Mutations of genes involved in this pathway are considered as one of the primary causes of colorectal cancer. Cyoplasmic β-catenin can bind to a multiprotein complex containing axin, APC, and GSK-3β, which phosphorylate it to promote its ubiquitination and its subsequent degradation by the proteasome machinery. The *activated* form of β-catenin is a dephosphorylated form, which is thus not targeted towards degradation and can interact either with E-cadherin at the cell membrane or with Tcf4 to activate transcription in the nucleus.

A)



B)

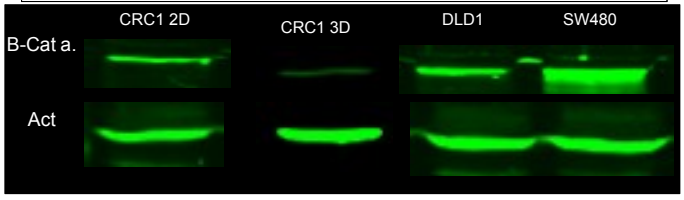


Fig.5.1: Expression of *activated* β-catenin in CRC1 cell line in comparison with other cell lines. A) Quantification of expression of β-catenin in WB showing the differences in individual cell lines. B) The data are represented as mean value ± SEM. B) Western blot of protein lysates from cell culture in monolayer (or in suspension for 1 week for 3D). For migraton I used 1.0 mm thick gel with 10% acrylamide. Actin expression was used as a loading control.

This antibody against activated β-catenin should bind mainly to the protein located at the membrane or in the nucleus of the cells. As we can see in Fig. 5.1, *activated* β-catenin expression in CRC1 cells is the weakest of all three lines.

To confirm this fact I also performed immunofluorescence staining to check the localisation of total β-catenin.

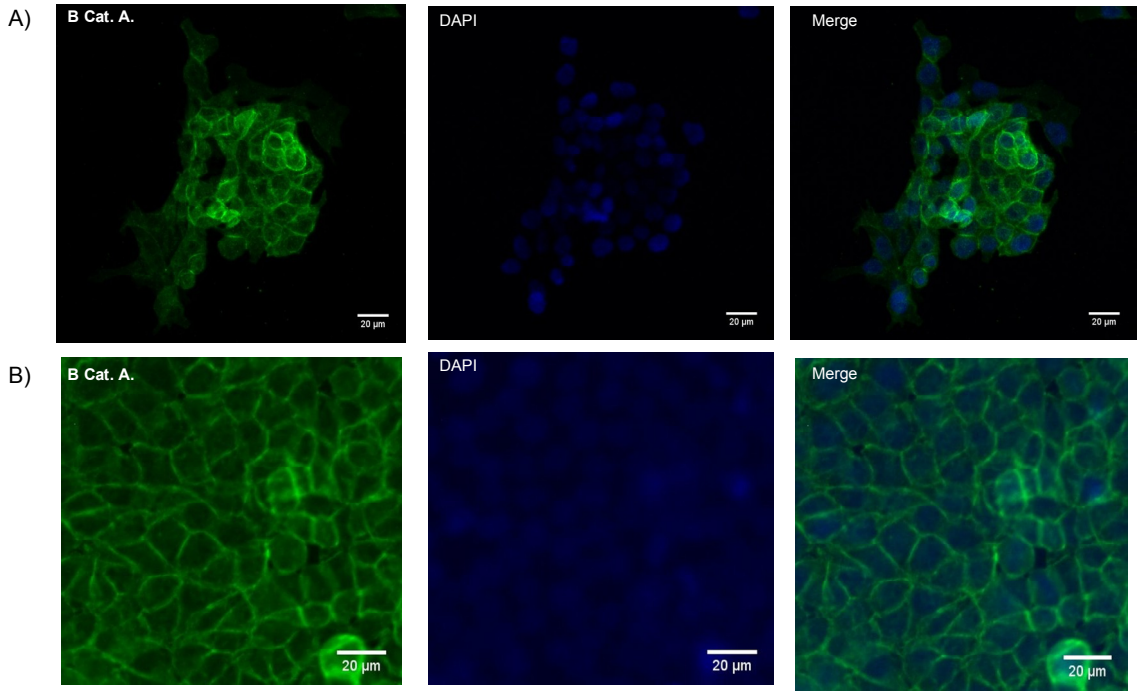


Fig.5.2: Expression and localisation of *activated* β-catenin by immunofluorescence staining. Images at confocal microscope, β-catenin at dilution 1/100. Scale bar in the picture represents 20 µm. A) Expression of β-catenin in cells grown until 40% confluence. B) Expression of β-catenin in cells grown until 100% confluence.

Fig.5.2 shows that β-catenin is mostly localised at the membrane, not in the nucleus. This localisation implies that it is probably bound to E-cadherin at adherens junctions.

Therefore, I also checked the expression and localisation of E-cadherin, in the CRC1 cell line, in comparison with its expression in DLD1 and SW480 cell line, similar to what was done for the β-catenin comparison.

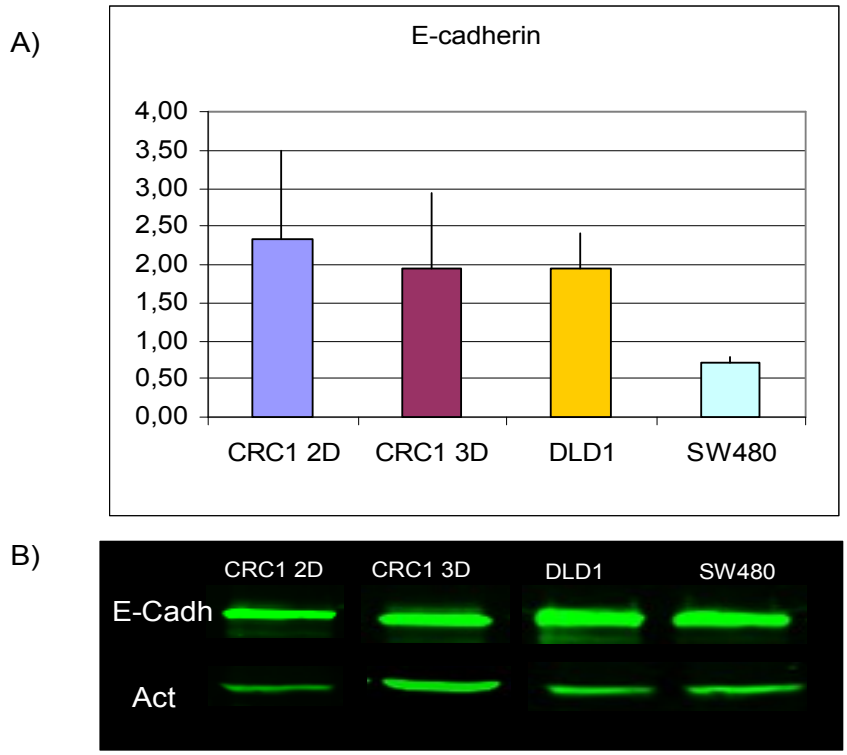


Fig.5.3: Expression of E-Cadherin in CRC1 cell line in comparison with other cell lines. A) Quantification of expression of E-cadherin in WB showing the differences in individual cell lines. The data are represented as mean value ± SEM. B) Western blot of protein lysates from cell culture in monolayer (or in suspension for 1 week for CRC1 3D). Migration was performed in 1.0 mm thick gel with 10% acrylamide. Actin expression was used as a loading control.

As fig.5.3 shows, expression of E-cadherin in CRC1 cell line is comparable to the other colorectal cell line, DLD1, but higher than in SW480.

In addition, E-cadherin is a main protein of adherens junctions and is localised in the cell membrane. Nevertheless, some results have shown the possibility of changed localisation of E-cadherin during cancerogenesis (Buda and Pignatelli, 2011). During colorectal cancer development, expression of E-cadherin and its complex with β-catenin at the membrane can be decreased or absent, and there can be a redistribution of the complex into the cytoplasm and localisation in vesicles. To control this possibility I observed the localisation of expressed E-cadherin by immunofluorescence staining.

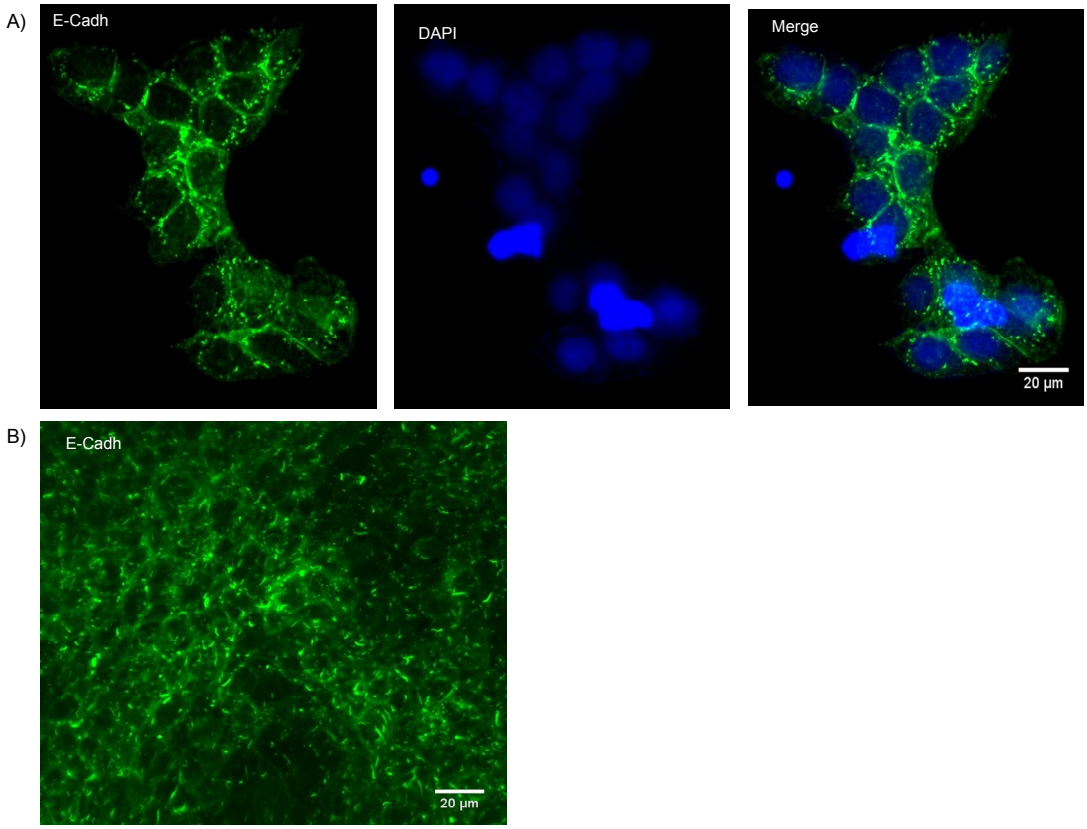


Fig.5.4: Expression and localisation of E-cadherin by immunofluorescence staining. Images at confocal microscope, E-cadherin at dilution 1/100. Scale bar in the picture represents 20 µm. A) expression of E-cadherin in cells grown until 40% confluence. B) Expression of E-cadherin in cells grown until 100% confluence.

As shown in Fig. 5.4, E-cadherin is partly well localised in the membrane, though part of this staining appears as small vesicles, confirming the observation of Buda and Pignatelli (2011).

In an attempt to better describe CRC1 cells, I then focused on the proteins that can be used as differentiation markers of the cells. Cytokeratin 18 is a marker of less differentiated cells (more stem-cells like) and on the other hand, Cytokeratin 20 is generally expressed in differentiated cells.

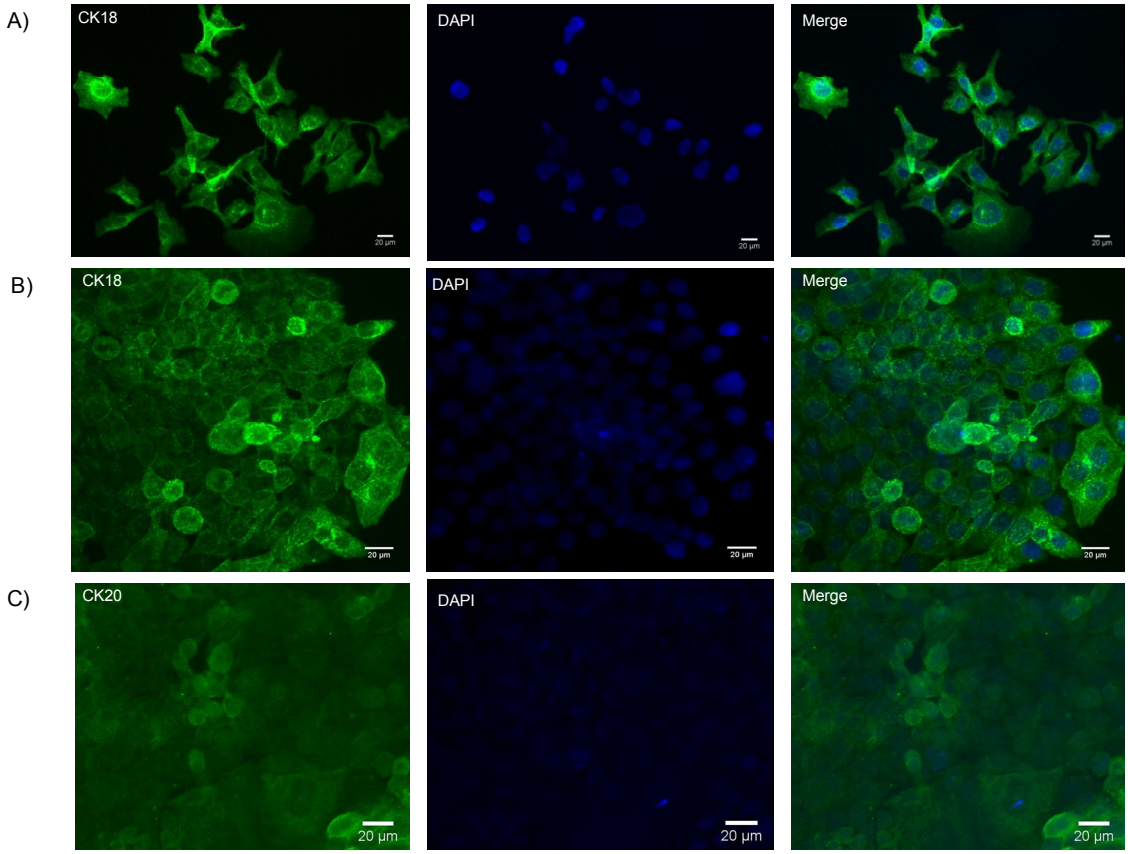


Fig.5.5: Expression and localisation of CK18 and CK20 by immunofluorescence staining; Images at confocal microscope; dilution of antibodies 1/100. Scale bar in the picture represents 20 µm. A) expression of CK18 grown until 40% confluence; B) expression of CK18 grown until 100% confluence; C) expression of CK20 grown until 100% confluence

Cytokeratin 18 staining was very positive and expressed in the whole cytoplasm, whereas cytokeratin 20 seems to be, despite increased exposure time, fairly negative. There is no significant difference between the labelling of cells grown until 40% confluence and 100% confluence, with the only differences being in the size and shape of cells. The positiveness of CK-18 expression reflects a more primitive phenotype, suggesting that CRC1 cells may have stem-like characteristic.

As a potential marker of stem cells I used EphB2, marker of cells in the bottom of the intestinal crypt and EphB3 to compare the labelling. After that, I observed also the expression of Mucin 2 in these cells, protein that is only expressed in Goblet cells.

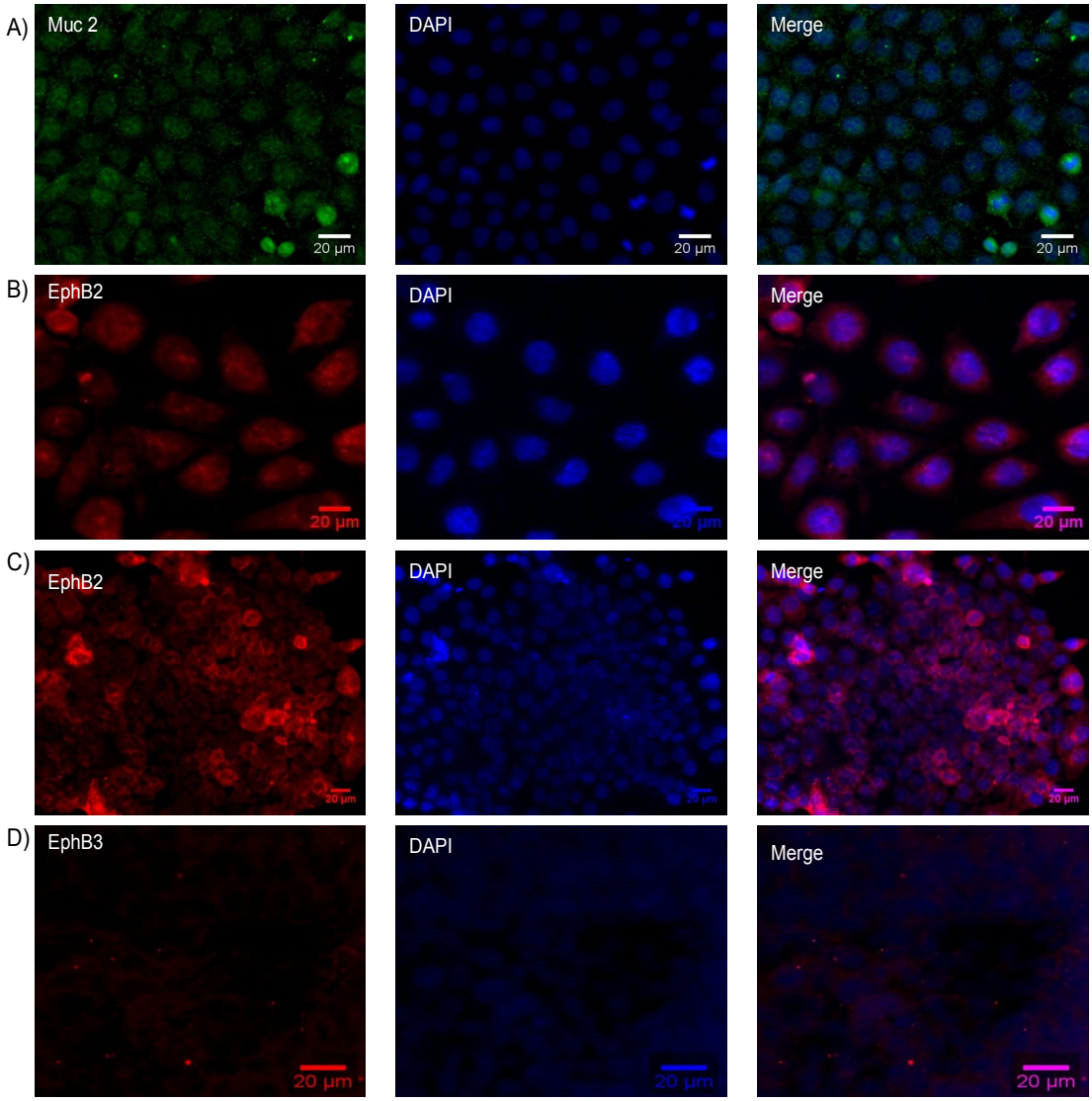


Fig.5.6: Expression and localisation of Muc2, EphB2 and EphB3 by immunofluorescence staining; Images at confocal microscope, dilution of Muc2 1/50, EphB2 and EphB3 1/20; Scale bar in the picture represents 20 µm. A) Expression of Muc2 at cells grown until 100% confluence with elevated exposure time; B) Expression of EphB2 at cells grown until 40% confluence; C) Expression of EphB2 at cells grown until 100% confluence; D) Expression of EphB3 at cells grown until 100% confluence

Mucin-2 is not strongly expressed in the CRC1 cell line. With increased exposure time, we can see differences between individual cells and some of appear positive. In these cells MUC2 is expressed in the cytoplasm as expected (see Fig.5.6.A). The expression of EphB2 is nicely visible and positive for both confluences, 40 and 100% and confirms the suggestion that the cells could have the stem-like characteristics.

As another method to find out CRC1 characteristics, I grew and fixed the cells under 2 conditions, as adherent cells grown in special cell culture inserts (diameter 0.4 µm) until full confluence, and in suspension, cultured in M11 medium in ULA flask.

I observed the cells in a Transmission Electron Microscope Hitachi 7100, described and compared the morphology of these 2 conditions.

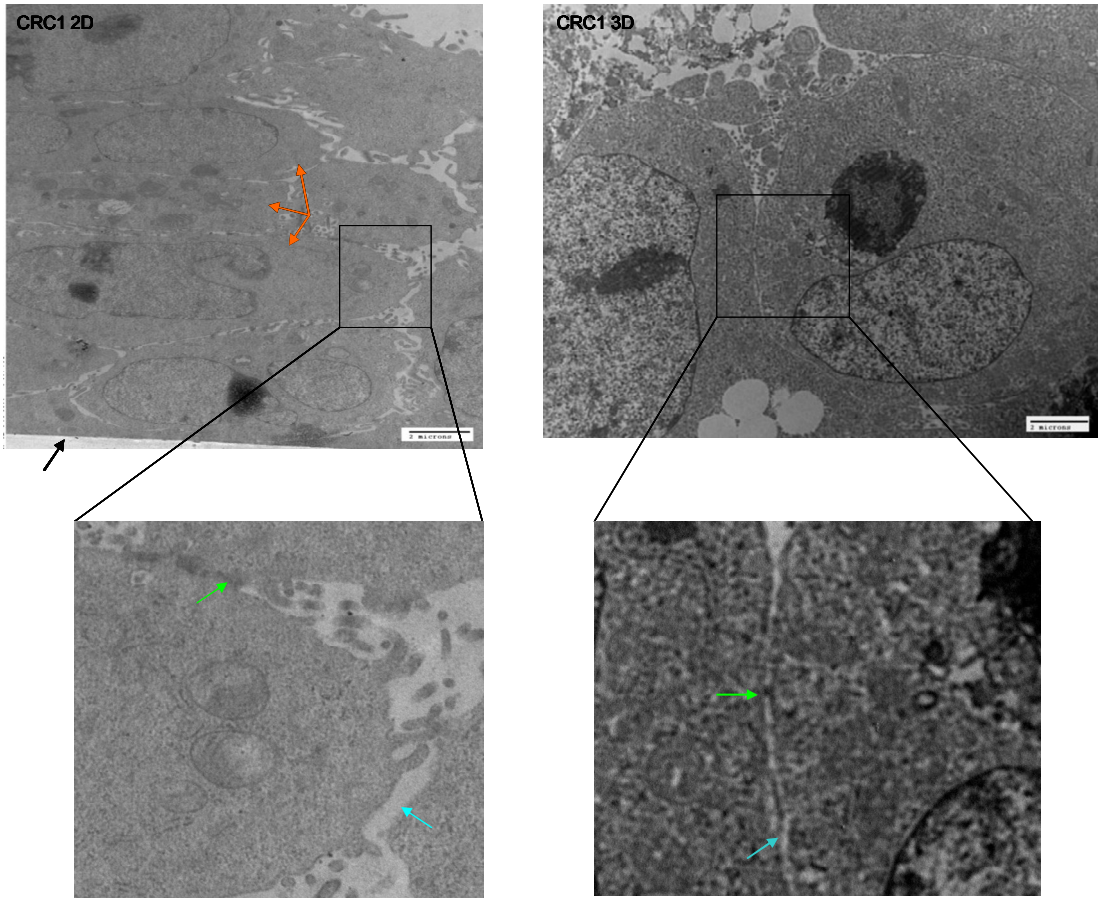


Fig.5.7: Comparison of morphology of CRC1 cell line grown under 2 conditions: on the left in monolayer for 5 days and on the right in the suspension for 2 weeks in TEM; scale bar represents 2 µm

CRC1 cells grown as adherent cells seemed to form several layers after achieving full confluence (see orange arrows), not just as a monolayer. The grid that supports the cells is showed on the bottom left corner (see black arrow).

The right side of fig. 5.7 shows that CRC1 cells grown in suspension have a very primitive morphology and that the cells grow close to each other with junctions between them. We can see quite big nuclei, few organelles and few or almost no structures that would reflect differentiation of the cells. The green arrows in both pictures show junctions between the cells and the light blue arrows point out the difference in the distance between cells if we compare 2D and 3D culture.

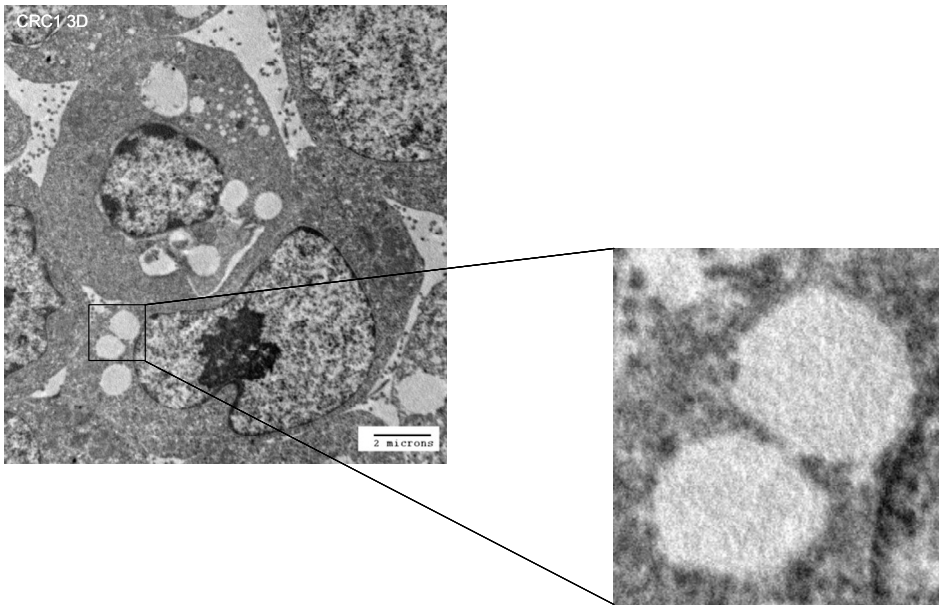


Fig.5.8: Detail of CRC1 3D cell line grown in suspension for 2 weeks by TEM; Orange arrow shows an untypical structure which appears in most of the cells; scale bar represents 2 µm

For better description I also added a detailed picture of CRC1 3D cell line, to show some unexpected structures detected in these cells. As you can see in the detail picture on the right side of the page, we could observe untypical structures in majority of the cells. This structure was round, white with smooth borders and diameter about 1-1.5 µm. This structure could be a mucus or lipid vacuole, but none of these structures shows the same morphology as the one we detected in CRC1.

5.2 CHARACTERISATION OF VARIOUS COLORECTAL CANCER CELL LINES ACCORDING TO CLAUDIN 2 EXPRESSION, INFLUENCE OF CLAUDIN 2 EXPRESSION IN CELL LINES

The first aim was to make a brief description of different colorectal cancer cell lines and to use several cell lines with various expression of CLDN2.

Claudin 2 was already examined by a student working on this project before me on the DLD1 cell line, which strongly expresses CLDN2 and other student working with SW480 cell line, which expresses low levels of CLDN2. The objective was to find another cell line whose expression of this protein ranges between the previously used cell lines.

I used CRC1, stable colorectal cancer cell line derived from colorectal adenocarcinoma by laboratory IGF. To compare the expression, I let the cells grow until 100% confluence to prepare protein lysates and WB.

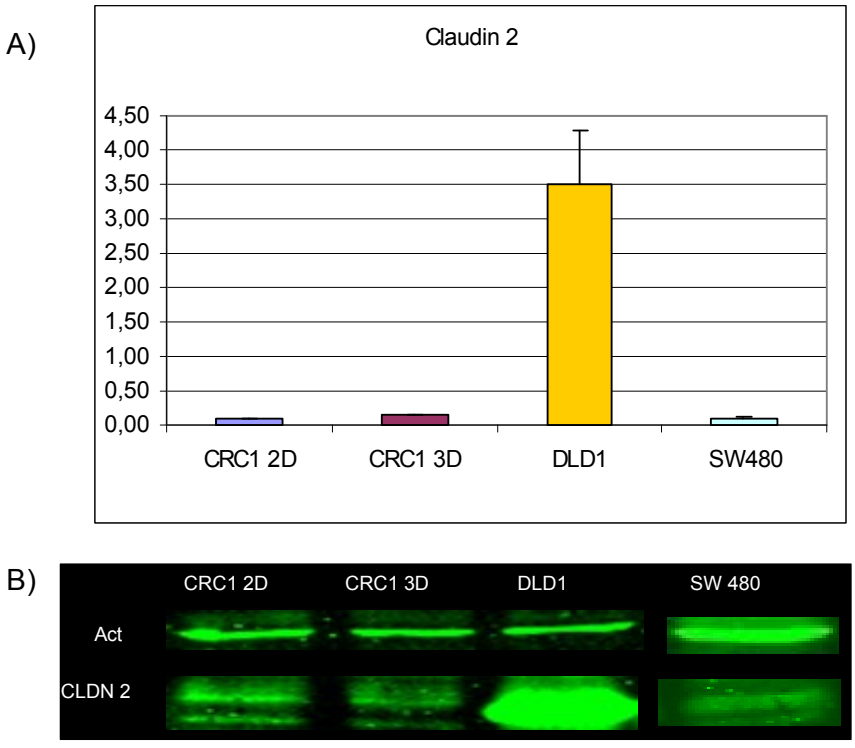


Fig 5.9: Expression of CLDN2 in different cell lines (CRC1, DLD1, SW480). A) Quantitative analysis of expression of CLDN2 showing the differences in expression of this protein. The data are represented as mean value ± SEM. B) Western blot of protein lysates from cell culture in monolayer (or suspension for 1 week for 3D). For migration I used 1.0 mm thick gel with 12% acrylamide. Actin expression was used as a loading control.

The results in fig. 5.9 show that expression of CLDN2 in CRC1 is really between the expression of DLD1 and SW480. Nevertheless, the level of the expression is much closer to SW480.

Claudin 2, protein of TJ, is expressed differently according to the confluence and conditions used for culture. To compare the CLDN2 expression I used 3 various conditions: 100% confluence, 40% confluence and 3D culture.

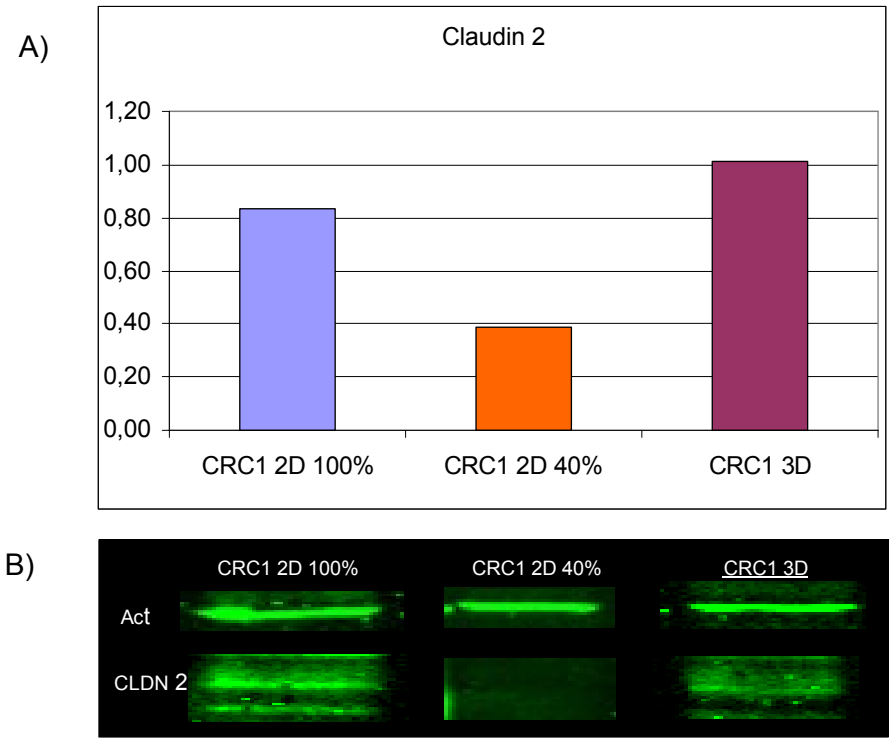


Fig 5.10: Expression of CLDN2 in CRC1 cell line cultured under different conditions (2D in 100%, 40% confluence and 3D) A) Quantitative analysis of expression of CLDN2 showing the differences in expression of this protein. The data are represented as mean value ± SEM. B) Western blot of protein lysates from cell culture in monolayer (or suspension for 1 week for 3D). For migration I used 1.0 mm thick gel with 12% acrylamide. Actin expression was used as a loading control.

As shown in fig.5.10, I confirmed that the TJs' protein Claudin 2 appears in larger concentration at 100% confluence and even more at cells grown under the condition which favours stem cells.

After control of CLDN2 expression in various cell lines, I used the cells with modified expression of CLDN2 by transfection. I used the SW480 cell line expressing a Control vector (Ctrl), Claudin2 (C2) and Claudin2*(C2*), which overexpresses a chimera with extracellular loops of Claudin 4 and the rest of Claudin 2. These cells were prepared by a previous Master student by co-transfection of pTER CLDN2 and pcDNA-G418 and then selected by G418.

The cells were observed and examined by various methods to describe the differences between the lines. I used electron microscopy to observe the morphological changes in cells after overexpressing Claudin 2 or the Claudin 2* chimera. Cells were grown in suspension, fixed by GTA and observed in electron microscope (Hitachi 7100) to determine morphological differences.

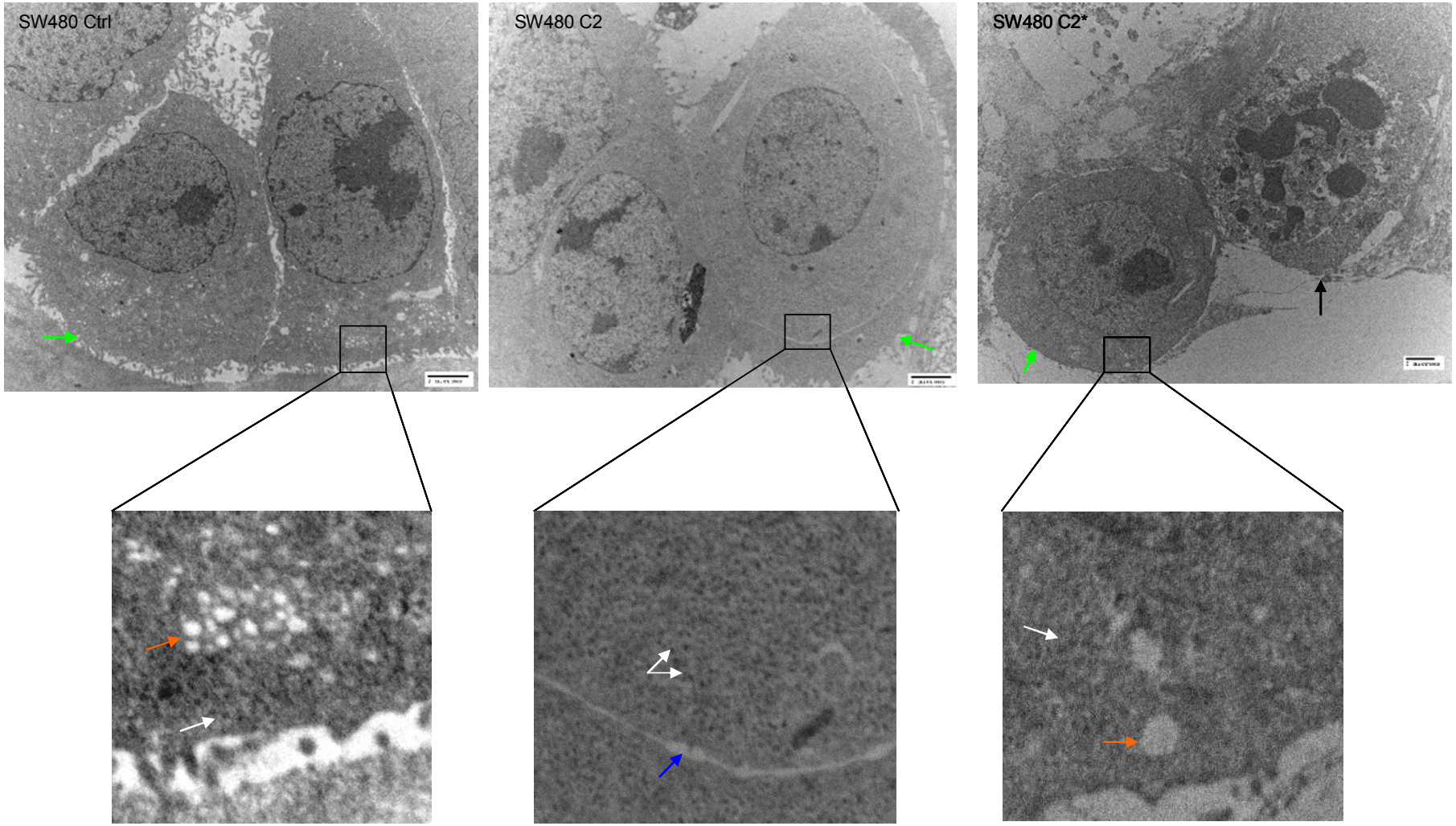


Fig.:5.11. Comparison of morphology in SW480 cell lines: control (Ctrl), C2, C2* in TEM. Cells were grown in suspension for 2 weeks. The scale bar represents 2 µm

Figure 5.11 shows several differences among the cell lines with influenced expression of Claudin 2 pointed out by arrows of various colours. In the control line, cells are poorly attached to each other, a large distance separates them, there are a lot of vacuoles (orange arrow) and not many ribosomes (white arrow). In C2 cell line, cells exhibit more ribosomes and less vacuoles and form waves of membrane. The C2* cell line appears less resistant to the fixation procedure; there exist more apoptotic or exploded cells (black arrow). The cover of cells is the less furrowed comparing to the other cell lines (green arrow).

To compare the behaviour of modified cell lines, I cultured them under the conditions for 3D cells. I used 3 concentrations of cells (250, 500 and 1000 cells/ml) and after 1 week culture I observed and counted the formed spheres.

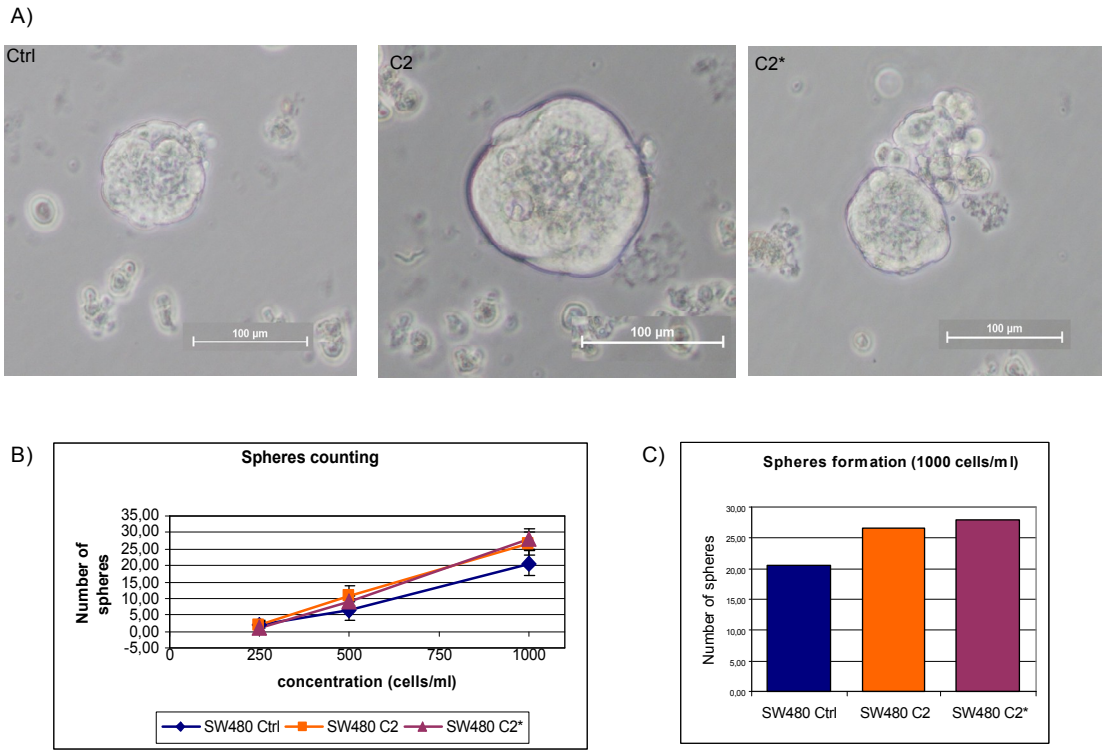


Fig.5.12: Sphere counting and observation of SW480 Ctrl, C2 and C2* cell line grown in suspension in 3 concentrations. Cells observed in microscope Nikon Eclipse TS100, focus 10x A) Comparison of spheres formed by SW480 Ctrl, C2 and C2* cells after 2 weeks of culture in suspension. Scale bar represents 100 µm, B) Sphere counting after 7 days in culture in suspension in 96-well-plate with 100 µl of medium. 3 concentrations were used: 250, 500 and 1000 cells/ml, C) Comparing of number of spheres in well with the highest concentration (1000 cells/well)

Figure 5.12.A shows an example of the difference in size between the spheres formed by the various lines. SW480 C2 forms bigger spheres than the other 2 lines. The spheres of SW480 C2* are also bigger than the control but the difference is not as important. The number of spheres, shown in fig. 5.12.B and C, also varies, the control forms less

spheres than C2 and C2* cell line. Nevertheless, the difference is not as big as we predicted and as was observed by previous student working on this project.

I also extracted RNA and performed RT-qPCR. I checked the efficiency of transfection and also controlled it by immunofluorescence staining.

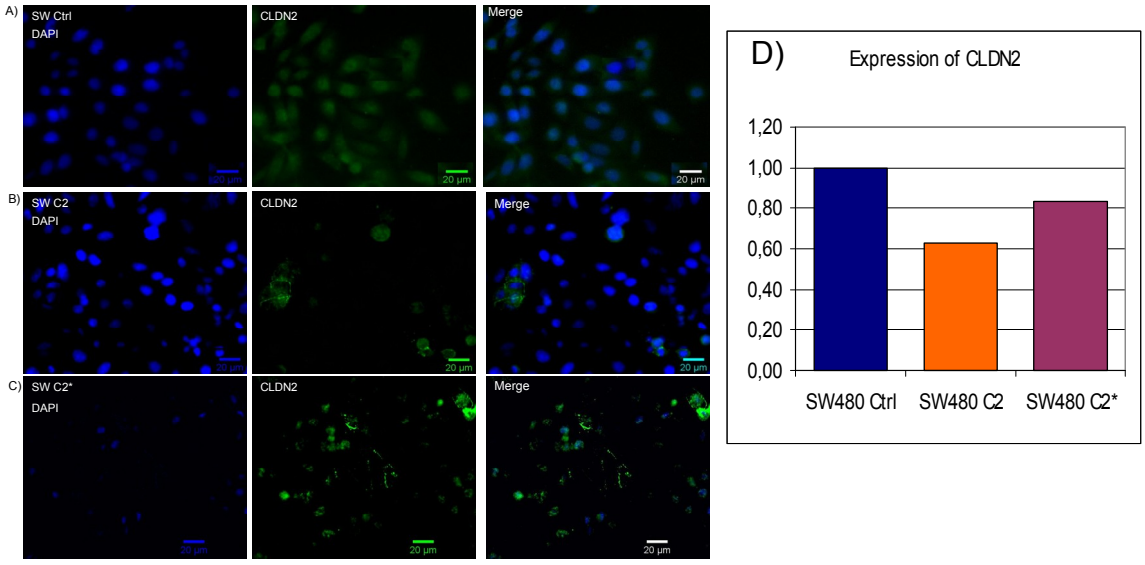


Fig.5.13: Expression and localisation of CLDN2 in SW480 cell line transfected by pcDNA3 CLDN2 in comparison to control. Staining observed at confocal microscope, dilution of CLDN2 1/100; Scale bar in the picture represents 20 µm. A) Expression of CLDN2 in SW480 Ctrl, B) Expression of CLDN2 in SW480 C2, C) Expression of CLDN2 in SW480 C2*; D) Comparison of CLDN2 gene expression in SW Ctrl, C2 and C2* by qPCR

The immunofluorescence staining confirmed the differences in expression of CLDN2 between Ctrl and cell lines transfected to overexpress Claudin 2 or the C2* chimera. Nevertheless the difference was found to be very weak. The control is largely negative (Fig.5.13.A), in the checked cell lines there are few cells positive (Fig. 5.13.B and C). The control of efficiency of transfection by qPCR seems to be even negative (Fig.5.13.D).

CLDN2 expression was also altered in the CRC1 cell line. Cells infected by virus with PTRIP.Z shCLDN2 plasmid and control (P.TRIPZ NS) were kindly provided by Pascussi J.M., IGF. I selected cells with Puromycin. After activation by Doxycyclin at different times, I checked the expression of CLDN2 by WB.

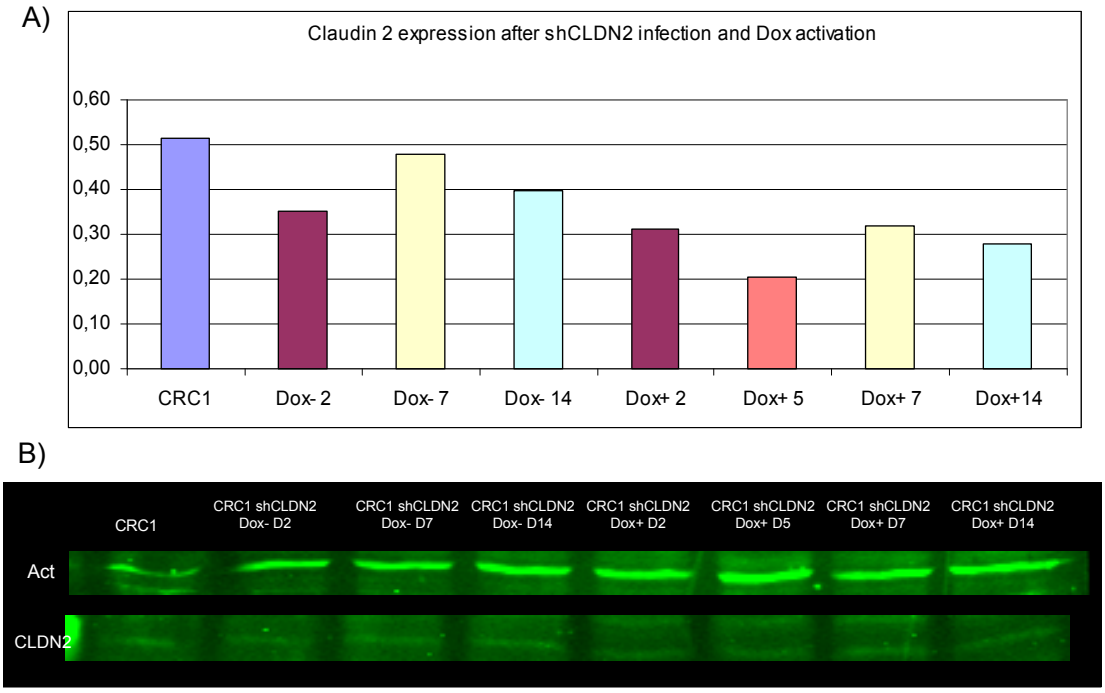


Fig.5.14: Expression of CLDN2 in CRC1 cell line activated or not activated by Dox during different times (days) A) Quantitative analysis of expression of CLDN2 showing the differences in expression of this protein. B) Western blot of protein lysates from cell culture in monolayer activated or not activated by Dox for 2, 5, 7 or 14 days. For migration I used 1.0 mm thick gel with 12% acrylamide. Actin expression was used as a loading control.

Unfortunately, the WB did not show any statistically significant changes in CLDN2 expression between CRC1 shCLDN2 Dox^+ and Dox^-. As a control I also extracted RNA to check the difference among the cells at the level of mRNA expression.

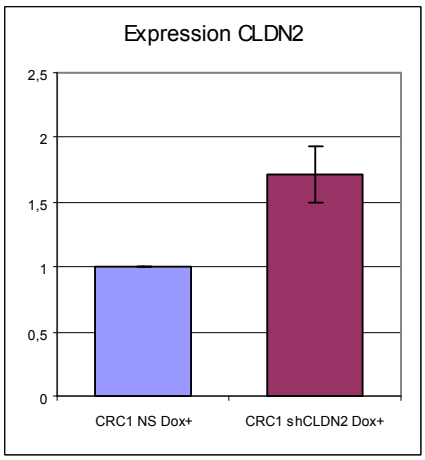


Fig.5.15: The difference in gene expression between CRC1 NS and CRC1 shCLDN2, both activated by doxycyclin.

However, the RT-qPCR did not show higher expression of CLDN2 in control cells, but contrary in cells with decreased expression of CDLN2.

At the same time I cultured the cells in 3D form to compare the growth of cells shCLDN2 to control. After one week of culture I observed cells at optical microscope (Nikon) and counted the formed spheres.

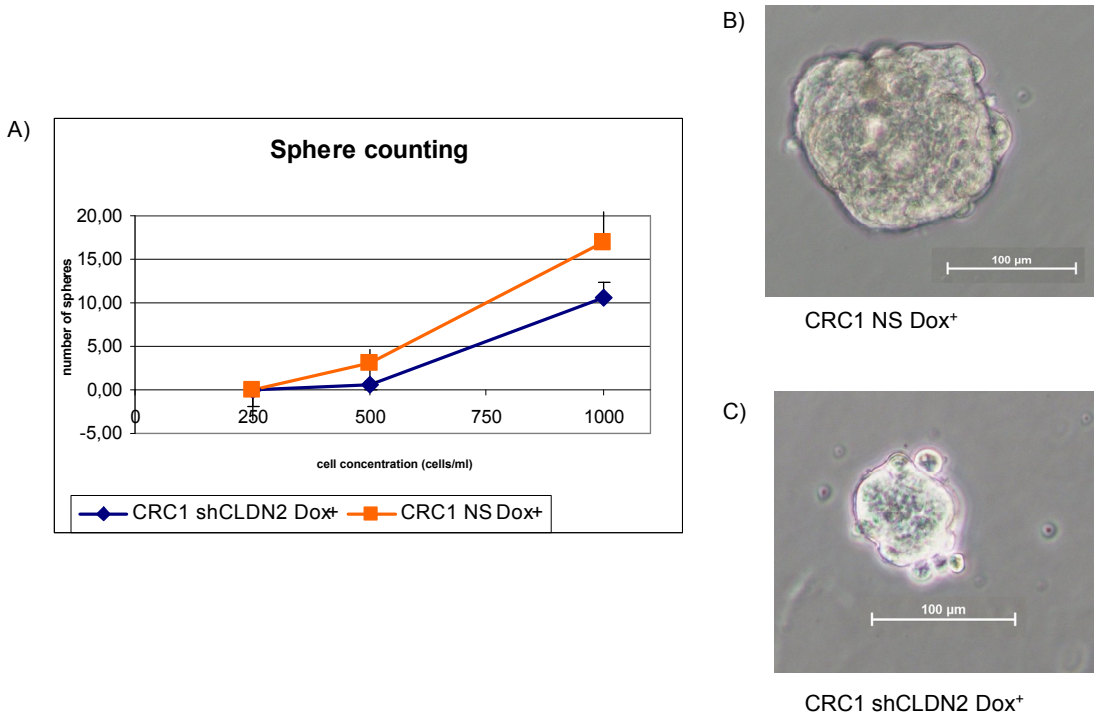


Fig.5.16: Sphere counting of CRC1 shCLDN2 and NS after 7-day-culture. A) comparison of number of spheres after 7-day-culture in 3 concentrations: 250, 500 and 1000 cells/ml; B) photo of sphere of CRC1 NS dox$^+$ cells, optical microscope Nikon, focus 10x; C) photo of sphere of CRC1 shCLDN2 dox$^+$ cells, optical microscope Nikon, focus 10x

The sphere counting (fig.5.16.A) as well as photos of spheres (fig.5.16.B and C) showed the difference between CRC1 shCLDN2 and NS. The control cell line formed more spheres with bigger diameter than the cell line with decreased expression of CLDN2.

5.3 PLASTICITY OF THE LGR5 MARKER

Before my arrival, my colleagues examined the relation between LGR5 expression in cells expressing different amounts of CLDN2 (DLD1, DLD1 shCLDN2) and tumour formation. Even though this experiment showed interesting results *in vitro* as well as *in vivo* in a mouse model, it raised the suggestions that expression of LGR5 may not remain stable and that $LGR5^{pos}$ or $LGR5^{neg}$ cells may not permanently maintain their respective phenotypes.

We established an experiment to grow cells under 4 different conditions (only LGR5 positive, only negative, or $LGR5^{pos}$/$LGR5^{neg}$ mixes where one of the two phenotypes is marked with a cytoplasmic or membrane colouring marker). Cells were labelled with a rabbit LGR5 N-term antibody and sorted by FACS into individual groups (see fig.5.21).

5.3.1 Analysis of the LGR5 plasticity on DLD1 cells

The first cell line chosen for the LGR5 phenotypic stability test is represented by the DLD1 cell line. The cells labelled by LGR5 primary Ab, APC secondary Ab and the nuclear marker 7AAD were analysed with a FACS Aria machine (BD Biosystems).

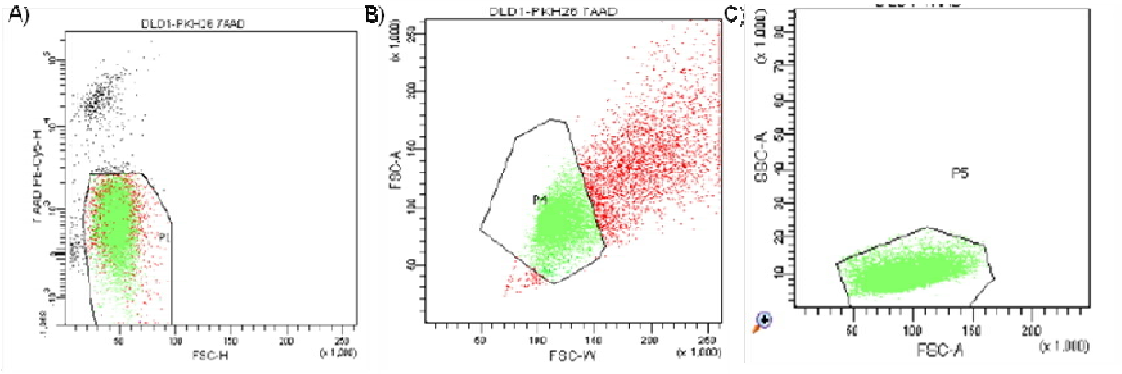


Fig.5.17: Conditions of cells' characteristics chosen for cell sorting; DLD1 cell line; A) living cells (nucleus labelling 7AAD); B) singlets (elimination of aggregates); C) morphologically common cells

Fig.5.17 shows the characteristics of cells chosen for cell sorting. The polygon drawn in each diagram defines the subpopulation of cells used for culture. Panel A shows the determination of living cells. Only dead or dying cells are labelled by the nuclear marker 7AAD, which cannot penetrate the outer membrane of living cells. Panels B and C indicate how single and homogenous cells were chosen for the analysis.

Then two groups of cells underwent the sorting, one of them represented by cells marked with the LGR5 antibody, the other with LGR5 and by PKH26 dye solution.

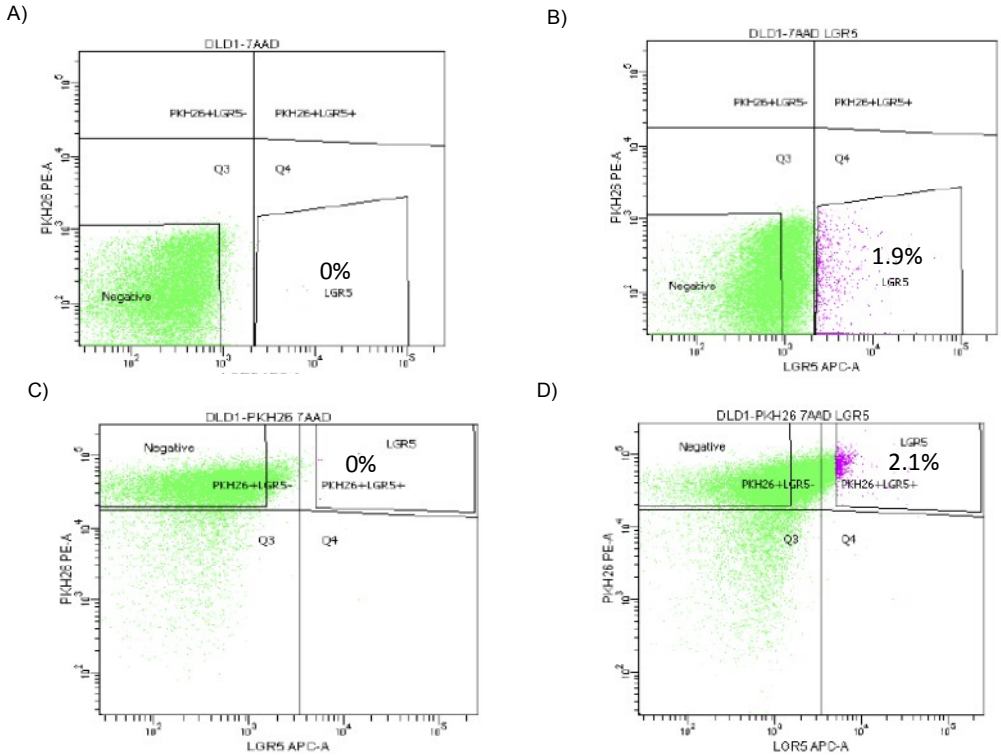
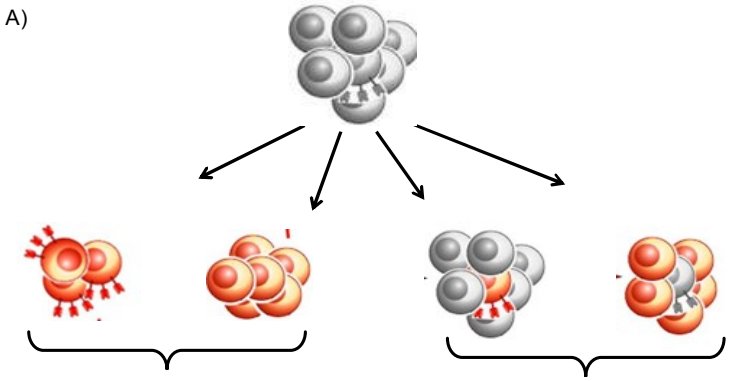


Fig.5.18: Cell sorting of DLD1 cell line for cell culture under specified conditions; A) negative control with only secondary antibody (APC); B) Cell sorting of DLD1 with percentage of $LGR5^{pos}$ cells; C) negative control for DLD1 cell line labelled with PKH26 dye marker with only secondary antibody (APC); D) Cell sorting of DLD1 PKH26 labelled cells with percentage of $LGR5^{pos}$ cells

Fig.5.18 B and D demonstrate the percentage of positive and negative cells at sorting and the small table summarizes the percentage of cells that we actually collected. Fig.5.18 A and C show negative controls, in which the APC-labelled secondary Ab was used without prior incubation with a primary Ab. The percentage of $LGR5^{pos}$ cells varied from 0.8 to 1.6% between experiments.

After cell sorting cells were despatched and cultured under 4 conditions, as described in Fig.5.19 (and in methods chapter 4.5.3).

A)



B)

	DLD1	CRC1
Coloured population	PKH26	CRC1-GFP
FACS	Aria	Astrios
Amplification	2D DMEM	
Homogenous or heterogeneous growth	3D M11	
	4 days	7 days

C)

Name of condition	LGR5pos	LGR5neg
LGR5pos	125 000	----------
LGR5neg	---------	300 000
Mix LGR5pos labelled, LGR5neg	5000 coloured	495 000
Mix LGR5pos, LGR5neg labelled	5000	495 000 coloured

Fig.5.19: Scheme demonstrating the culture conditions for LGR5 marker plasticity; A) 4 conditions of homogenous and heterogeneous populations cultured in 3D conditions as described in table C; B) technical details of culture, labelling and FACS for individual cell lines.

After 4 days in culture the cells were labelled again with the LGR5 antibody and analysed with the FACS Aria for DLD1 cells. As the part of cells was labelled with PKH26 dye solution we should be able to observe also the possible number of the cell divisions due to the signal divided into layers. The PHK26 marker binds to the lipid membrane and during cell division it divides between the daughter cells, which means that with every division the intensity of labelling is weaker and we can observe an approximate number of cell division in layers in FACS machine divided according to the intensity of signal.

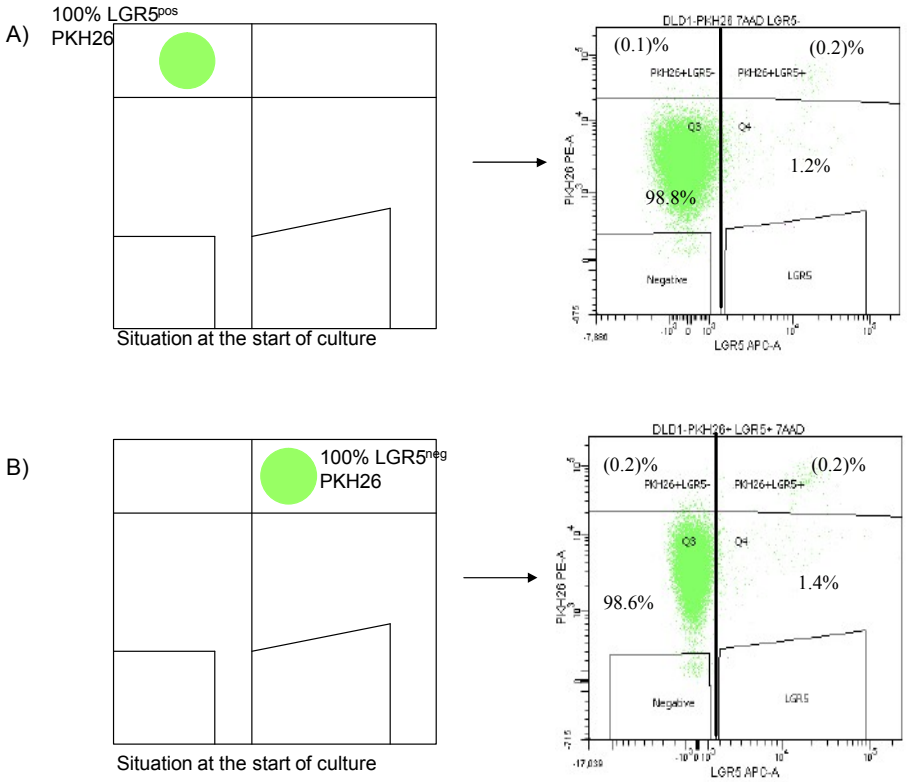


Fig.5.20: Analysis of DLD1 cell line LGR5^{pos} and LGR5^{neg}. Figures on the left side show the situation after 1st cell sorting. Figures on the right side show the ratio of LGR5^{pos} and LGR5^{neg} cell after 4-day-culture

Figure 5.20.A shows the results obtained with the first group of cells, LGR5^{neg} cells grown by themselves. On the left side you can see a drawing showing the initial situation after the first sorting step. The picture in the right side shows the analysis performed after 3D culture. The percentage of LGR5^{pos} cells after 4-day-culture is 1.2%, although no LGR5^{pos} was seeded at the beginning of the experiment. The small populations on the upper corners represent cells that are highly PKH26 positive (0.2% LGR5^{pos} in the right, 0.1% LGR5^{neg} in the left), indicating that they probably did not divide over the time. Figure 5.25.B represents the same situation with LGR5^{pos} cells. Looking at the difference between the initial situation drawn in the picture on the left side and analysis shown on the right side, we can observe that 1.4% LGR5^{pos} cells remains from an initial proportion of 100%. These results suggested that the LGR5 phenotype may in fact not remain stable during the culture. To obtain more information about this suggestion we analyzed also other 2 conditions, mixes with one subpopulation of cells stained.

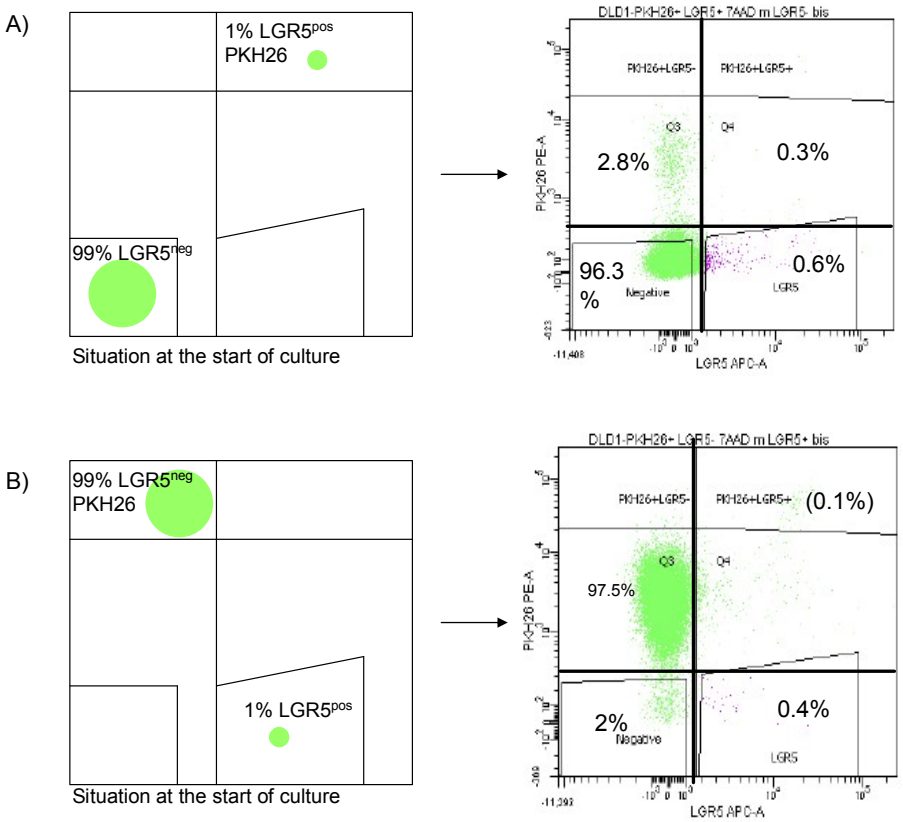


Fig.5.21: Analysis of mixes LGR5^{pos} and LGR5^{neg} cells. Figures on the left side show the situation after 1st cell sorting. Figures on the right side show the ratio of LGR5^{pos} and LGR5^{neg} cell after 4-day-culture

Figure 5.21.A shows the third sample group represented by 1% LGR5^{pos} PKH26^{+}/ 99% LGR5^{neg} PKH26^{-} cells as initiate condition upon seeding. After 4 days in culture, similar general percentages are detected: 0.9% LGR5^{pos} and 99.1% LGR5^{neg}. Nevertheless looking at the proportion of marker in individual subpopulation we observe a shift in each group. We obtained 0.3% LGR5^{pos} and 2.8% LGR5^{neg} cells coloured with PKH26 (whereas only LGR5^{pos} cells were stained with PKH26 at the start of the experiment), which have a lower level of signal of PKH26 marker than the cells at the start indicating that these cells divided. The rest of the cells is represented by 96.3% LGR5^{neg} and 0.6% LGR5^{pos} cells (both uncoloured).

Figure 5.23.B demonstrates the opposite situation; the coloured cells were LGR5^{neg} in the beginning. The general proportion stays almost unchanged: 0.6% LGR5^{pos}/99.4% LGR5^{neg}. We found out by analysis 97.5% LGR5^{neg} and 0.5% LGR5^{pos} coloured cells and 2% LGR5^{neg} cells (and no positive cells) uncoloured. The signal of PKH26 was weaker again suggesting cell divisions.

These results confirm our previous suggestion about the phenotypic plasticity of the LGR5 marker. The cells cultured in the closest condition to the real situation let us hypothesize that LGR5 cells change phenotype with time in culture.

5.3.2 Analysis of LGR5 plasticity on CRC1 cells

Similar experiments were performed with the CRC1 cell line, with 1 exception. As the PKH26 dye solution was found to be toxic for this cell line, we used 2 populations of CRC1 cells, one transfected with GFP and one without GFP, to distinguish the $LGR5^{pos}$ and $LGR5^{neg}$ cells while mixing them.

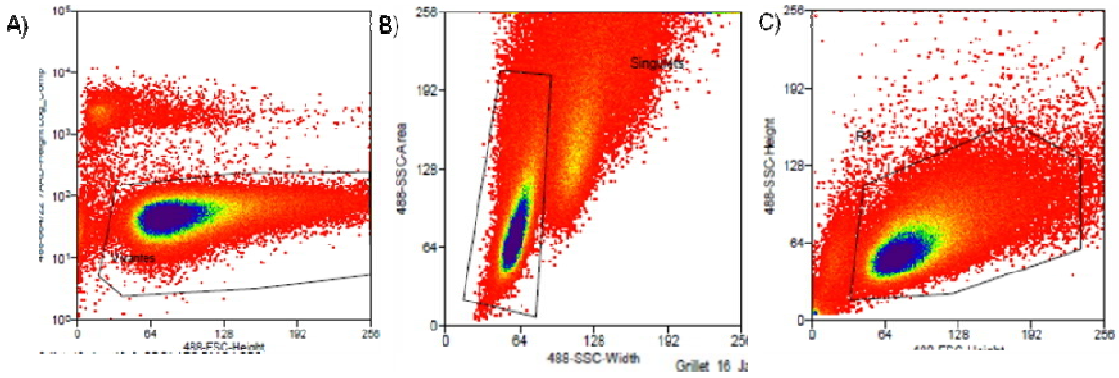


Fig.5.22: Conditions of cells' characteristics chosen for cell sorting; CRC1 cell line; A) living cells (nucleus labelling 7AAD); B) singlets (elimination of aggregates); C) morphologically common cells

The CRC1 cells were selected upon the same criteria as DLD1. We selected living cells, singlets and morphologically homogeneous cells. The cell sorting was performed immediately after selection.

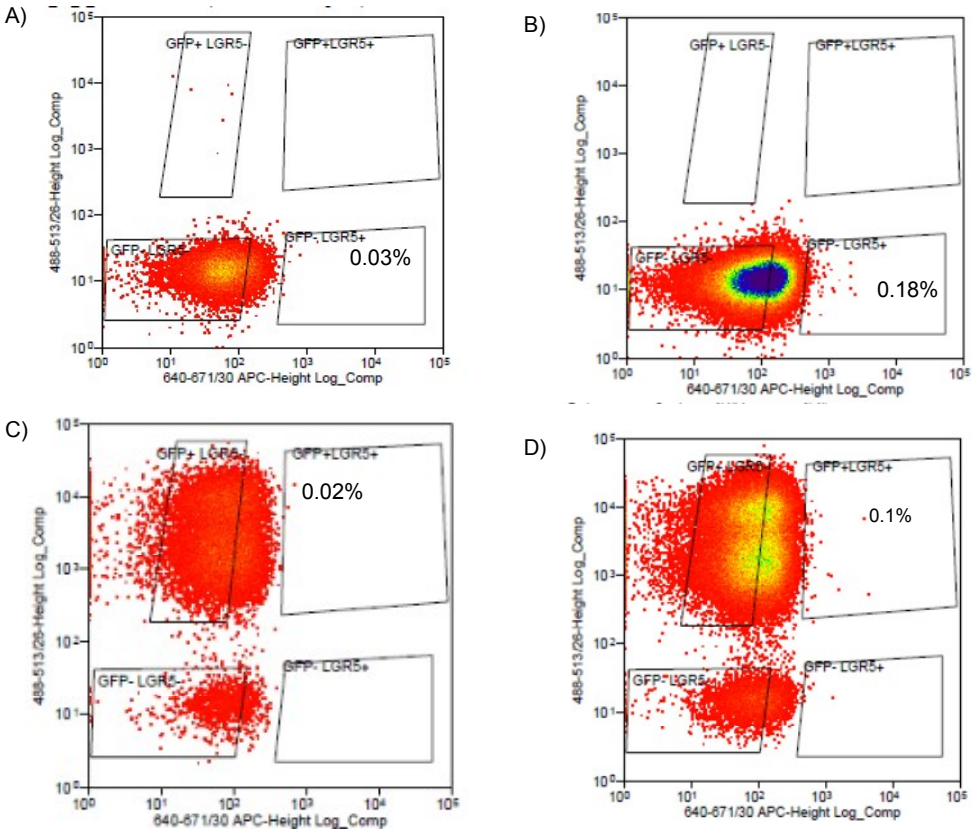


Fig.5.23: Cell sorting of CRC1 cell line for cell culture under specified conditions; A) negative control with only secondary antibody (APC); B) Cell sorting of CRC1 with percentage of $LGR5^{pos}$ cells; C) negative control for CRC1 cell line transfected with GFP with only secondary antibody (APC); D) Cell sorting of CRC1-GFP cells with percentage of $LGR5^{pos}$ cells

Fig.5.23 demonstrates the percentage of $LGR5^{pos}$ and $LGR5^{neg}$ cells in comparison to negative controls. The percentage of $LGR5^{pos}$ cells varies from 0.1 to 0.8%. The CRC1 cell line thus appears to contain less $LGR5^{pos}$ cells than DLD1 cells, but another reason could play a part in the difference of percentages. Indeed, the CRC1 cell line was sorted with a MoFlo Astrios cytometer (Beckman coulter), with different settings and significantly different sensitivity compared to the FACS Aria (BD Biosystems) used for DLD1 cells.

After 7-day-culture cells were again labelled with LGR5 Ab and analyzed with the MoFlo Astrios cytometer. For better analysis we first set the controls.

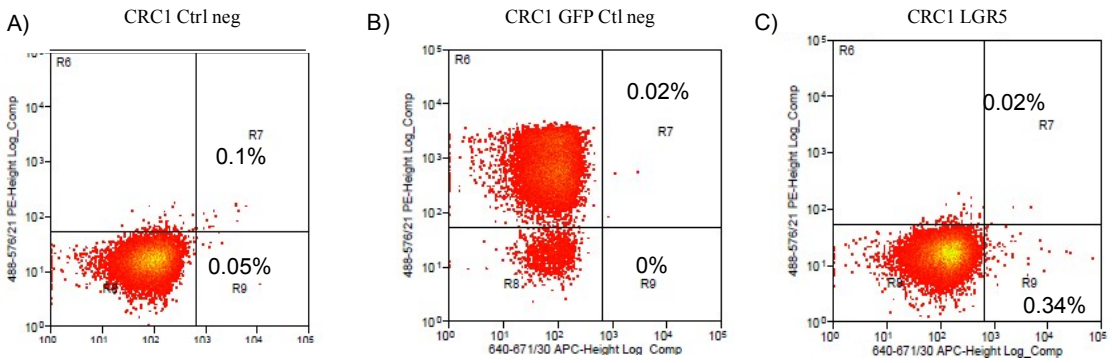


Fig.5.24: Controls at 2nd FACS, analysis after 7-day-culture. A) Negative control of CRC1 cell line with only secondary Ab, coupled to APC; B) negative control of CRC1-GFP cell line with only secondary Ab, coupled to APC; C) control analysis of CRC1 cell line grown for 7 days under 3D culture conditions.

As a negative control, cells were again stained with the APC-labelled secondary antibody without using prior LGR5 labelling. In figures 5.24.A and B you can see the negative controls of CRC1 and CRC1-GFP cell lines. Another control was performed by staining CRC1 cells that were cultured under the same condition as the analysed cells, but without the previous sorting. This control enables a better comparison of analysed cells because we measure the percentage of $LGR5^{pos}$ cells in the cells cultured in similar 3D conditions to those used for the sorted cells (see the scheme).

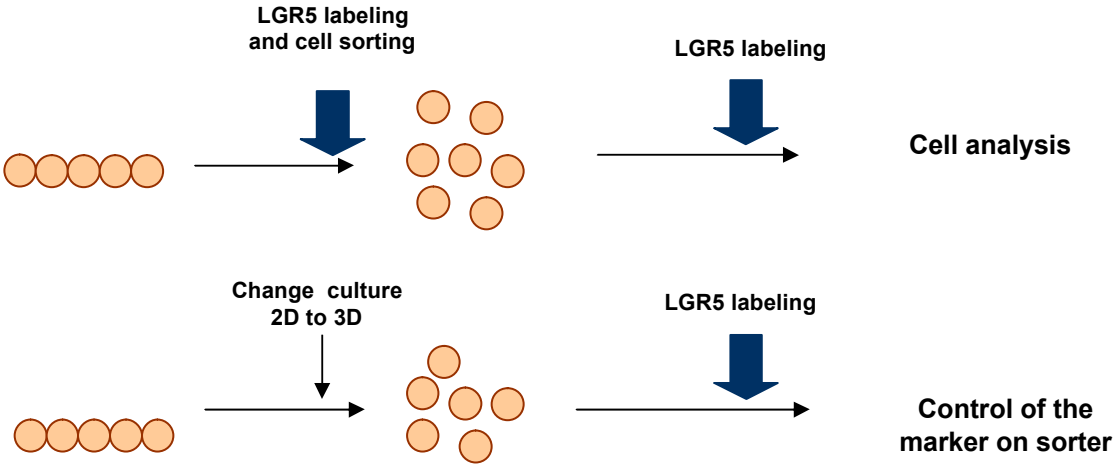


Fig.5.25: Scheme of LGR5 control of CRC1 cells grown in similar conditions as the analysed cells

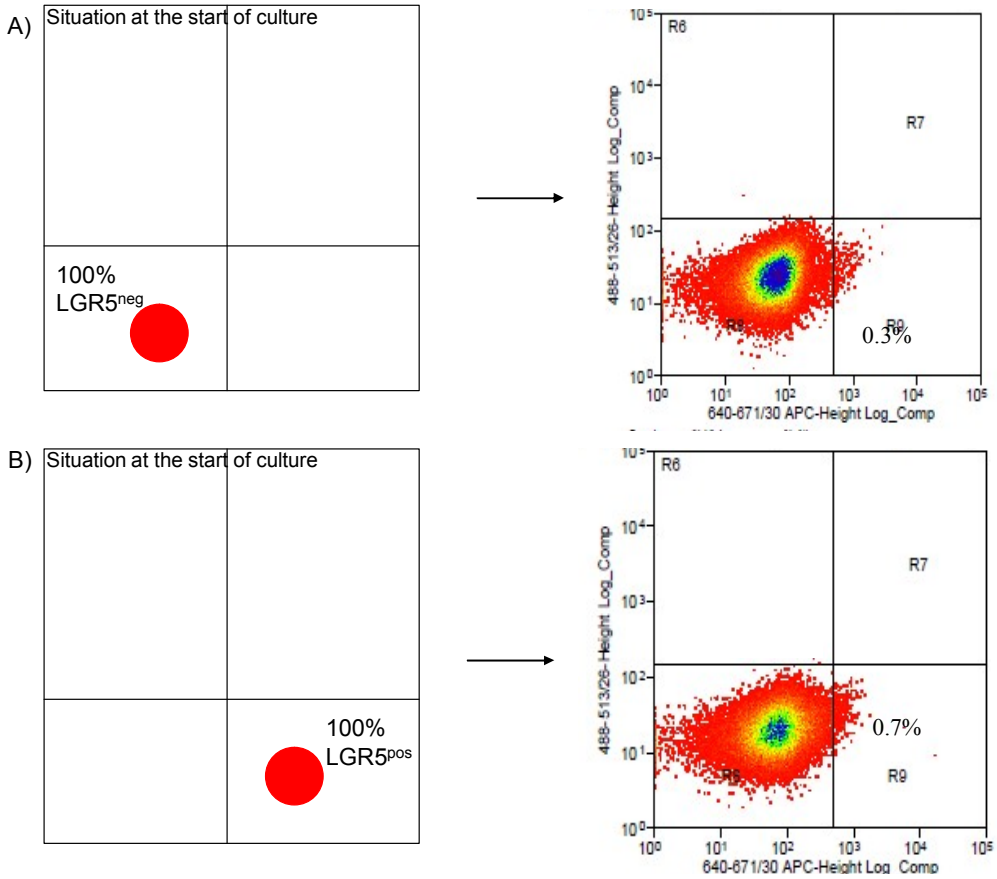


Fig.5.26: Analysis of CRC1 cell line LGR5^{pos} and LGR5^{neg}. Figures on the left side show the situation after 1st cell sorting. Figures on the right side show the ratio of LGR5^{pos} and LGR5^{neg} cell after 7-day-culture

Figure 5.26.A shows the first group of cells, LGR5^{neg} cells. Analysis after 7-day-culture shown on the right side demonstrates that we obtained 0.3% LGR5^{pos} cells although 0% was present at the start of the experiment, where we only used LGR5^{neg} cell as shown on the left side of the figure. Figure 5.26.B represents the same situation with LGR5^{pos} cells. The left panel shows the initial situation, the right side the analysis after culture demonstrating 0.7% LGR5^{pos} cells (from 100% at the beginning of the experiment). These results can be compared to the results obtained from the experiment with DLD1 cells and suggest that the LGR5 phenotype does not remain stable in the cell culture. From the obtained percentage of LGR5 marker we hypothesize that independently from the initial percentage of positive cells, cell population may revert back after some time to the situation that represents their natural equilibrium, equivalent to the initial proportion measured in the first analysis.

The same procedure was done with other 2 conditions, using mixes of two populations including one harbouring a fluorescent marker.

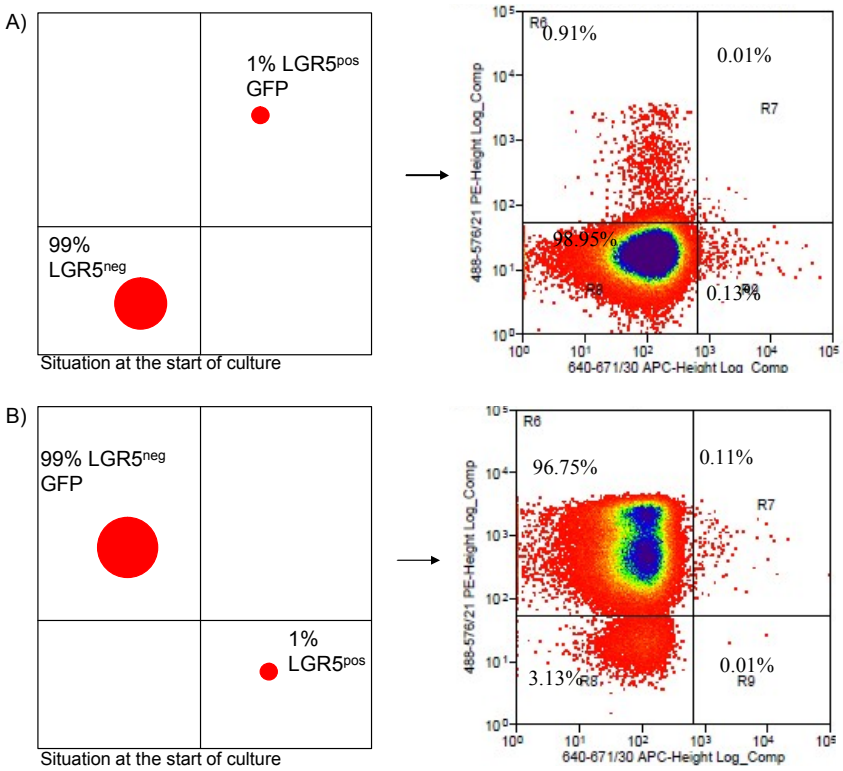


Fig.5.27: Analysis of mixes LGR5^{pos} and LGR5^{neg} cells. Drawings on the left side show the situation after 1st cell sorting. Figures on the right side show the ratio of LGR^{pos} and LGR5^{neg} cell after 7-day-culture

Figure 5.27.A shows the third group of cells represented by 1% LGR5^{pos}-GFP/99% LGR5^{neg} cells. The analysis demonstrates the proportion of 0.23% LGR5^{pos} and 99.77% LGR5^{neg} cells. Regarding the used subpopulations we can observe 0.91% LGR5^{neg} and 0.1% LGR5^{pos} cells both GFP^{+}. The rest is represented by 98.95% LGR5^{neg} and 0.13% LGR5^{pos} cells (non-GFP).

The same situation you can see in the figure 5.27.B, just in the initial ratio LGR5^{neg} cells were GFP^{+}. The analysis shows the final percentage after 7 days: 0.11% LGR5^{pos}-GFP, 96.75% LGR5^{neg}-GFP, 0.01% LGR5^{pos} and 3.13% LGR5^{neg} cells.

These results just confirm our suggestion about the phenotypic instability of the *LGR5* marker.

To control the efficiency of the cell sorting I collected part of the cells and by extraction and RT-qPCR I checked the expression of several genes. I examined not only *LGR5* as a control, but also expression of other potential stem cells' markers, such as *ALDH1* and *BMI1*, and also *CLDN2* to see the difference of the expression between LGR5 positive and negative cells.

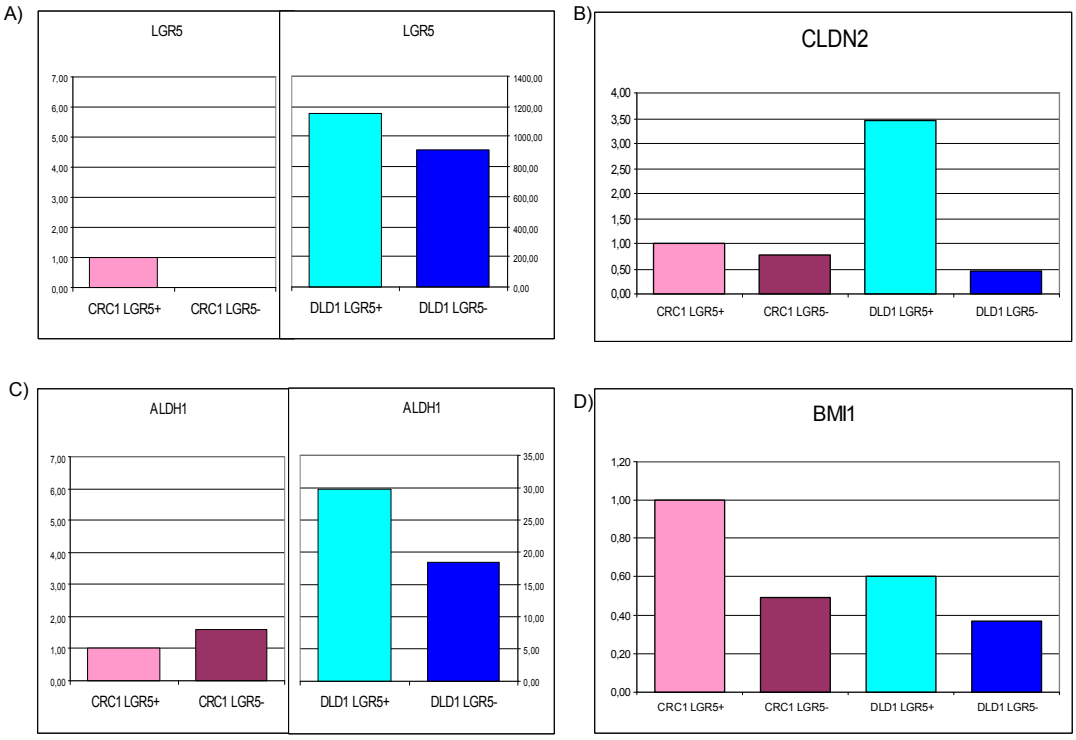


Fig.5.28: Expression of the several genes by RT-qPCR in LGR5^{pos} and LGR5^{neg} cells sorted by FACS. All the expressions are compared to the expression of CRC1 LGR5^{pos} cells A) Control of efficiency of cell sorting to LGR5^{pos} and LGR5^{neg} cells by checking the *LGR5* expression in individual groups; B) expression of *CLDN2* gene; C) Expression of *ALDH1* gene; D) Expression of *BMI1* gene

The cell sorting of CRC1 cell line on the Astrios separated the LGR5^{pos} cells from negative completely, on the other hand the sorting of DLD1 cell line on the Aria divided the cells, but not completely, the difference between positive and negative cells was not complete (Fig. 5.28.A). The difference in expression of *ALDH1* differs between CRC1 and DLD1 cell line (fig.5.28.C), the DLD1 cell line possesses the same difference between *LGR5* expression and *ALDH1* expression, but at CRC1 cell line, the situation is opposite (LGR5^{pos} cells express less *ALDH1* than LGR5^{neg}). The expression of the *BMI1* gene is similar in DLD1 as well as CRC1 cells and the difference is corresponding to *LGR5* expression (Fig.5.28.D). The expression of *CLDN2* is also corresponding to the expression of *LGR5* (Fig.5.28.B, F).

6. DISCUSSION

6.1 CHARACTERISATION OF CRC1 CELL LINE

CRC1 is a colorectal cell line recently established from a tumour sample of a patient with colorectal carcinoma. As a relatively new cell line it brings several advantages.

It has not been changed by many mutations during the time it has been cultured *in vitro*. Other cell lines have been derived since 1960's and 1970's so they are likely to have undergone several additional mutations in culture and as we do not know which genes have been changed, we do not know how different they are from "real" patient tumours. Thus, results obtained in CRC1 cells could be considered as closer the actual situation *in vivo*, especially while cultured in suspension (Debnath J. et al., 2005).

In addition, the CRC1 cell line was derived directly in suspension (in medium containing stem cell-promoting growth factors such as EGF but without FCS, because FCS promotes cell differentiation), conditions that favor primitive stem-like cells. Due to this fact we hope the cell line contains a higher amount of cancer stem cells.

Indeed, I confirmed by electron microscopy that CRC1 cells have a primitive morphology and do not contain many organelles, suggesting that they are poorly differentiated. The nucleus is big and very visible; it forms the majority of the cell. In the cytoplasm, I could observe a small amount of the crucial organelles needed for cell survival, such as Endoplasmic reticulum, mitochondria…

These suggestions and stem-like characteristic of cells were confirmed also by immunofluorescence staining, especially by positive labeling with CK18 Ab and EphB2 Ab, both of which are expressed in less differentiated primitive cells. The absence of staining for CK20, a marker of differentiated cells, just strengthens the theory.

In the pictures from electron microscope there was found another structure, not fully recognized. White round vacuolar structures with regular borders were observed only in CRC1 3D cells, not in other cells cultured under the same conditions, such as SW480 Ctrl, C2, or C2*. Due to this reason, the contamination or influence by substances used for cell culture are unlikely to be responsible for this phenotype. Even the comparison of CRC1 cultured in 3D vs. 2D form demonstrated the existence of this organelle just in cells grown in suspension.

After researching the histology/ultrastructure literature I ruled out the identification of the structure as lipids droplets. Indeed, lipid droplets are typical for very round, regular and well establish borders, which is slightly different from our structure. They were also too big to be the ordinary vacuoles.

One of the hypotheses was that these cells could be Goblet cells and that the detected structures would be vacuoles filled with mucin. However, this theory does not seem likely due to several facts. The morphology does not correspond to the morphology of Goblet cells and, except for these white organelles, the cells have a primitive structure. In addition, labeling with a Muc-2 antibody was performed with negative results, which rules out goblet cells.

Another possibility currently seems to be the most plausible but is not fully confirmed yet. Thus, structures detected in CRC1 cells strongly resemble vacuoles invaginating the content of the cytoplasm, concretely vacuolization of endoplasmic reticulum (Ryabchikova E.I. et al, 2010). In that case the vacuoles could appear as a stress reaction for an external stimulus of unknown origin. The research of Ryabchikova addressed the reaction of cells intoxicated by TiO_2 nanoparticles and this structure appeared in a remarkable number of cells and led to other pathological changes ending in necrosis.

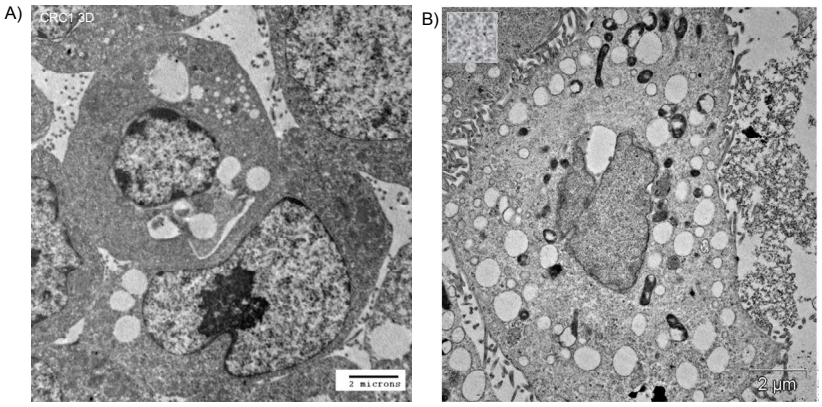


Fig. 6.1.: Comparison of the found structure in CRC1 3D cells in comparison to the literature. A) CRC1 cell line cultured in 3D form, fixation of cell after 2 weeks culture by GTA. Observed in electron microscope Hitachi 7100; B) vacuoles found in MDCK cells by Ryabchikova E.I. et al., 2010 after intoxication by nanoparticles of TiO_2; Figure 6.1.B was quoted from Ryabchikova E.I. et al., 2010

The morphology of CRC1 cells cultured in 2D also gave interesting suggestions. The cells observed in electron microscope seemed to grow in several layers. One of the reasons for this fact could be related to the preparation of the samples for microscopy (cutting). We can never be sure if the sections are really at a 90° angle to the grid. In that case, the cells may appear to be in layers just because the angle of cutting is

diagonal. Nevertheless, this explanation is not very likely if we look at the number of layers in the picture (fig.5.7).

By checking other important protein, β-catenin, I proved that the Wnt signaling pathway is not strongly activated in these cells. The DLD1 and SW480 cell lines both carry inactivating mutations of APC and therefore express high levels of Wnt target genes such as CyclinD1 or oncogenes such as c-myc. In contrast the moderate activation of the Wnt pathway in CRC1 cells could make these cells an interesting model to study the influence of Wnt signalling inhibition or activation.

We used an antibody against activated β-catenin, which only labels the dephosphorylated form of the protein that is involved in adhesion to E-cadherin and in cell proliferation (Fuchs S.Y. et al, 2005). In this case β-catenin was mainly located in the membrane which implicates the binding to E-cadherin with formation of complexes and no appearance in the nuclei.

We also checked E-cadherin, which is involved in modulating the Wnt signaling pathway and can form a complex with β-catenin. As the expression of E-cadh does not differ importantly from the other referent cell lines, it could serve as another cell line for confirming the results found at the research at already used lines. The only difference was found in the localization of the expressed protein. The IF staining demonstrated localization of E-cadh not only in the membrane, as is typical for adherens junctions' protein, but also in the cytoplasm within small vesicles.

If the CRC1 is really a cell line largely composed of colorectal stem cells, it gives us several opportunities. Models of cancer stem cells are rare and these cells generally constitute a minority in cancer cell lines. Research on a cell line such as CRC1 could give us important answers in their role in tumour development and progression. They are thought to be an important player in drug resistance and metastasis (Curtin C.J. et al., 2010). If so, cancer stem cells can be considered as a promising target for colorectal cancer therapy. According to the hierarchical model of tumourigenesis a tumour is formed from cancer stem cells that do not undergo the terminal differentiation but instead generate progenitor cells that exhibit uncontrolled proliferation (Curtin C.J. et al, 2010). As we destroy the tumour by conventional cytotoxic treatment, it means chemotherapy or ionizing radiation we do not influence the stem cells, often resistant to this type of treatment. The surviving cancer stem cells can form the tumour *de novo*. If we focus on the cancer stem cells and destroy them by

targeted therapy, especially in combination with conventional therapy, we could eliminate the tumour and prevent tumour relapse.

6.2 CHARACTERISATION OF VARIOUS COLORECTAL CANCER CELL LINES ACCORDING TO CLAUDIN 2 EXPRESSION, INFLUENCE OF CLAUDIN 2 EXPRESSION IN CELL LINES

By western blot, I proved that Claudin 2 is expressed variously in different cell lines. Due to this fact we influenced the expression of CLDN2 in both senses by plasmid transfection or viral construct infection.

SW480 which expresses CLDN2 very little was transfected (resp. co-transfected by pcDNA3 CLDN2 and pcDNA3-G418 for selection) to overexpress Claudin 2, or. the chimera of Claudin 2 and Claudin 4. The target was to observe changes in cell line caused by changed expression of CLDN2, or the C2* chimera. The observation of spheres confirmed the difference in size between the cell lines, nevertheless the difference was not convincing. More important result we obtained from the sphere counting and the difference in number of spheres between the cell lines. C2 and C2* formed more spheres comparing to the control, but this difference was not convincing neither. This experiment was first performed before my arrival and the actual counting should have been just confirmation. After the control of the transfection by IF staining and RT-qPCR, I suggest the transfection has not been permanent.

The method of changing the level of expression in cells could be performed also by different ways. First of all, more certain results give DNA which already encodes the genes of resistance. In this case it would be beneficial to repeat the formation of plasmid and try to construct a new vector by cloning DNA containing the sequence encoding the protein of interest as well as a fragment encoding the drug-resistance gene, in this case G418-resistance gene (Lodish H., et al, 2000).

More efficiency could also be reached by infecting the cells with a viral construct instead of transfecting them. Infection of the cells can provide better and more stable integration of the gene and improve the long-term expression. Nevertheless, even if the cells are infected there does not exist certitude that the cells will not reject the DNA, but the possibility is much lower.

The second part of this project is represented by CRC1 cell line, which was infected by P.TRIP.Z plasmid to decrease expression of CLDN2. The western blot prepared to control the cell infection efficiency demonstrated that the difference between the shCLDN2 cells activated by Doxycyclin and non-activated (as control) is not convincing. Differences are detected, as control cells expressed more Claudin 2, but the

result in general cannot be considered as positive. Then, the control of Claudin 2 mRNA expression by RT-qPCR between shCLDN2 cells and control cells infected with an empty virus construction (both activated by dox) showed results as clearly negative. Nevertheless, the sphere formation of cell lines pointed out remarkable differences between the conditions in size as well as in number. The decreased expression of CLDN2 caused the diminution of sphere formation. The most possible explanation of this result could be based just on CLDN2 expression of CRC1 cell line before infection. CRC1 cell line expressed CLDN2 at the level between DLD1 and SW480 cell lines; however the level was much closer to the one of SW480, which means low. In that case, the infection could have been partially successful, but may be difficult to assess by western blot, just because differences of expression may not be big enough to be visible at bands. The other possibility, that the infection was not done successfully is also possible, but does not explain the difference in spheres formation between the cell lines. It may be caused by other change coming from the process of infection, but it seems to be unlikely.

This method brought many advantages in comparison with the previous one. The infection provides better integration of the gene into cell and also longer duration of the changed expression. The vector was prepared to be resistant to ATB directly, so no co-transfection neither co-infection was needed. The vector was inducible by doxycyclin which enables to start the effect of infection when needed. The proposal for future project is to find another cell line whose expression of CLDN2 is really in higher level than CRC1 but lower than DLD1. CRC1 in general presented another difficulty. As one of the very few cell lines it was found to be resistant to G418 antibiotics, derivate of neomycin.

6.3 PLASTICITY OF LGR5 MARKER

LGR5 is a protein that is considered as a stem cell marker in the healthy adult intestine. It was confirmed that a single cell $Lgr5^{pos}$ is able to form a whole crypt containing all types of cells typical for the intestinal crypt (Sato T. et al., 2009). In recent years it has been examined a lot to find out if it could be also a cancer stem cell marker. Vermeulen L. and his colleagues isolated cells from colorectal tumours positive for the LGR5 marker and by using these cells they created a newly formed tumour (Vermeulen L. et al., 2008). This convincing demonstration that LGR5 could be a CSC marker may bring very interesting consequences for colorectal cancer research. Nevertheless, this result was never confirmed by using a control of $LGR5^{neg}$ cells to observe if they are able to form tumours too. Next, there appeared doubts about the phenotypic stability of stem cell markers. On first examination, $LGR5^{pos}$ cells appear in phenotypic stable state *in vivo* as well as *in vitro* cultured cells. We ask this question as the result of Gupta's group experiments (Gupta P.B. et al., 2011). They cultured separately stem and non-stem cancer cells sorted by FACS, and after 6 days they observed the percentage of subpopulations in the cell groups. The experiment was done on breast cancer cell lines and at the end of experiment they obtained the original proportion of cells.

This experiment provided only partial results. Indeed, the cells were sorted and grown separately, which does not correspond to the natural state *in vivo*. The subpopulation if held separately in culture may behave as under stressful environment and may be forced to return to the original proportion.

We decided to do similar experiment on colorectal cancer cell lines and observe the phenotypic plasticity of LGR5 marker. Apart from culturing just separated positive and negative cells, we let grow the cells also in conditions imitating the natural state, which means in mix $1\%LGR5^{pos}/99\%LGR5^{neg}$ cells with one subpopulation of cells stained. Cell sorting was performed using 2 different FACS machines, Aria and Astrios. To control the efficiency of sorting I collected a sample of cells, extracted RNA and compared the expression by RT-qPCR.

Even though additional controls and replicates of experiments must still be performed, we obtained some very interesting results. Shown in the analysis of cell cultured in 3D form (figures 5.20.B and 5.26.B demonstrating the condition $LGR5^{pos}$ on their own),

LGR5^{pos} cells do not remain LGR5^{pos}. From 100% positive cells we obtained at the end just 0.7% (for CRC1 cells) or 1.4% (for DLD1 cells) of positive cells. Similar situation we can observe at isolated LGR5^{neg} cells (Fig.5.20.A and 5.26.A), we received 0.3% and 1.2% of positive cells from the condition where no positive cell was present at the beginning. The results obtained from these groups are similar to the Gupta's results with breast cancer cell lines. The equilibrium of the subpopulations equal to the original situation was observed after 4 or 7 days in 3D culture. Nevertheless it remains unsure if this plasticity is typical of cancer cells or a situation induced by artificial culture conditions.

After examining the behaviour of isolated cells (only positive or only negative) I did the same experiment with the mix of cells simulating the original ratio between positive and negative cells. Also in the mix of cells representing the original ratio of LGR5^{pos} and LGR5^{neg} cells, the populations do not seem to be phenotypically stable. The cells were cultured with one subpopulation (either LGR5^{pos} or LGR5^{neg}) stained with PKH26 or GFP to have the opportunity to observe the change of LGR5 expression in individual groups if they are cultured in the situation simulating *in vivo* state. Figures 5.21 and 5.27 demonstrate the change of the expression of LGR5 after culture in 3D form. The percentage of positive cells was close to the natural situation *in vivo*, so it did not changed much from the initial situation, but regarding the subpopulations the percentage equilibrated also in individual subpopulations. These results propose a suggestion that the expression of *LGR5* gene can change over time in 3D culture.

On figure 5.20 there appears also other part of cells, which attracts attention. Part of cells in each group stayed in the top of the chart with the strongest signal of PKH26 dying solution, the rest of the cells always shifted down to the middle. As PKH26 dying solution binds on the membrane and its signal becomes weaker with every cell division, this result suggests an idea that the cells did not divide at all or much slower than the rest of the cells. This fact is typical for quiescent, or slowly proliferating cells. Quiescence is a characteristic demonstrated by stem cells in several tissues. In one of the theory quiescent cells represents a subpopulation of stem cells, a reservoir that serves in condition when the pool of rapidly cycling cells is lost, for example because of injury (Buczacki S et al., 2011). The recent results obtained by Yan K.S.'s laboratory confirmed the existence of this subpopulation of stem cells and determined one of their marker as BMI1. To confirm if our cells could be quiescent cells the current co-labeling with BMI1 to see the positiveness of both markers together.

As classical chemotherapy usually influences cells just during their division, the quiescent cells could cause one of the reasons of tumour resistance and relapse.
The origin of the cells appearing in the top of the table still rests unsure and also doubts concerning the used method appeared. Either the sorter did not work right, the signals of the secondary Ab could interfere with other signals, either the antibody is not working right and does not mark separately $LGR5^{pos}$ cells or the primers of *LGR5* gene is not selective as well. On the other hand RT-qPCR of CRC1 cell line collected by Astrios showed the complete and successful separation. The sample of $LGR5^{neg}$ cell did not show any expression of the *LGR5* gene at all.

One of the other problems is a lack of positive control. As original experiment which could not rely to the existing articles, it needs to be performed multiple times and controls need to be added to be sure about the obtained results. The percentage of $LGR5^{pos}$ cells is very low. To be sure of the results, we need the positive control to check if the marker works well. One of these possibilities would be to use LS174T cells. Theses cells express more LGR5 than other cells. Nevertheless, the experiment with FACS has not been performed and as cancer cell line we could not be sure about the localisation and the structure of the protein in the cells. The characteristic of LGR5 marker plays an important role for future research. As LGR5 is strongly examined as a potential cancer stem cell marker it could be used for determination of CSC. CSC cells represent a new potential target of cancer treatment, ideally in combination with conventional therapy (see 6.1.). If the phenotypic plasticity of *LGR5* gene is confirmed, the results would influence this possible treatment. If a new treatment was established on targeting *LGR5* gene which would be found plastic, the treatment would prove unsuccessful if used alone. The destroyed cells would be replaced by $LGR5^{neg}$ cells with the capacity to turn into positive. This turn would then cause relapse of the tumour.

7. CONCLUSION

The aim of this work was to examine and describe two very important potential causes of the colorectal cancer: to characterise and examine cancer stem cells and discover correlation between Claudin 2 expression and tumour development.

In the first part of my project I characterized a recently established colorectal cancer cell line and proved its primitive morphology suggesting it contains higher number of cancer stem cells. This theory was supported by positive labelling of certain markers, concretely CK18 and EphB2, markers of stem-like cells.

Then, I focused on potential cancer stem cell marker and observed its phenotypic plasticity. In a complex experiment I cultured the cells separated on pos/neg cells and on groups simulating the natural situation in vivo with one of the subpopulation stained. The analysis after 3D culture demonstrated that the LGR5 marker may be able to change its phenotype regarding the environmental conditions.

At last, I determined Claudin 2 expression in various colorectal cancer cell lines and by influencing its expression I observed the changes. The sphere counting showed the difference but the control with RT-qPCR and western blot did not give the persuading results and for the certitude the experiment requires more controls.

8. LIST OF ABBREVIATIONS

2D	two-dimensional
3D	three-dimensional
5-FU	5-fluorouracil
7AAD	7- aminoactinomycin D
Ab	antibody
Act	actin
AJ	Adherens junctions
ALDH1	Aldehyde dehydrogenase 1
APC	adenomatous polyposis coli
APS	amminopersulfate
β-cat a.	activated β-catenin
βME	β-mercaptoethanol
BB	blocking buffer
BME	β-mercaptoethanol
BMI1	B cell-specific Moloney murine leukemia integration site1
BMP	bone morphogenetic protein
BSA	bovine serum albumin
c-myc	Avian myelocytomatosis virus oncogene cellular homolog
C2	Claudin 2
C2*	Chimera of Claudin 2 and Claudin 4
CK	cytokeratin
CLDN2	Claudin 2
CO_2	carbon dioxide
CPT-11	irinotecan
CSC	cancer stem cells
Ctrl	control
DAPI	4,6-diamidino-2-phenylindole
DPBS	Dulbecco's phosphate-buffered saline
DMEM	Dulbecco´s modified eagle medium
dNTP	deoxyribonucleotide triphosphate
Dox	doxycyclin

DTT	dithiotreitol
E-cadh	Epithelial cadherin
EGF	endothelial growth factor
EM	electron microscopy
EMT	epithelium-mesenchymal transition
Eph	ephrin
FACS	fluorescence-activated cell sorting
FCS	fetal calf serum
FGF	fibroblast growth factor
Fig	figure
GAPDH	glyceraldehyde-3-phophate dehydrogenase
GSK-3β	glykogen synthese kinase 3 β
GTA	glutaraldehyde
H2O	water
HH	Hedgehog
IF	immunofluorescence
IGF	Functional genomics‘institute
ISC	Intestinal stem cells
JAM	Junctional adhesion molecule
LGR5	leucine-rich-repeat-containing G-protein coupled receptor
LV	leucovorin
MMLV	Moloney Murine Leukemia Virus
Muc-2	Mucin-2
NA	nucleic acid
Neg.	negative
PBS	phosphate-buffered saline
PFA	paraformaldehyde
Pos.	positive
Puro	puromycin
qPCR	quantitative polymerase chain reaction
RIPA	radioimmunoprecipitation assay
RT	room temperature
RT	reverse transcription
SDS	sodim dodecylsulfate

SDS-PAGE	sodium dodecylsulfate-polyacrylamide gel electroforesis
SEM	standard error of the mean
Ser	serin
SHH	sonic hedgehog
Sol.	solution
TPBS	1% tween/phosphate saline buffer
TEM	transmission electron microscope
TEMED	tetramethylethylenediamine
TG-SDS	tris-glycine – sodium dodecylsulfate
Thr	threonin
ULA	ultra low attachment
UV	ultra violet
WB	western blot
ZO	zonula occludens

9. REFERENCES

Aijaz S., Balda M.S., Matter K., 2006, Tight junctions: Molecular architecture and function, *Int. Rev. Cytol.* 248, 261-297

Alison M.R., Islam S., Wright N.A., 2010, Stem cells in cancer: instigators and propagators?, *J Cell Sci 123*, 2357-2368

Angelow S., Ahlstrom R., Yu A.S.L., 2008, Biology of claudins, *Am J Physiol Renal Physiol* 295, 867-876

Aung P.P., Mitani Y., Sanada Y., Nakayama H. et al., 2006, Differential expression of Claudin-2 in normal human tissues and gastrointestinal carcinomas, *Virchows Arch* 448, 428-434

Barker N., Clevers H., 2010, Leucine-rich repeat-containing G-protein-coupled receptors as markers of adult stem cells, *Gastroenterology* 138, 1681-1696

Buczacki S., Davies R.J., Winton D.J., 2011, Stem cell, quiescence and rectal carcinoma: an unexplored relationship and potential therapeutic target, *BJC* 105, 1253-1259

Buda A., Pignatelli M., 2011, E-Cadherin and the cytoskeletal network in colorectal cancer development and metastasis, *Cell communication & adhesion* 18, 133-143

Caldwell G.M., Jones C.E., Soon Y., Warrack R. et al., 2008, Reorganisation of Wnt-response pathways in colorectal tumorigenesis, *B J Cancer* 98, 437-442

Carpentino J.E., Hynes M.J., Appelman H.D., Zheng T. et al., 2009, Aldehyde dehydrogenase-expressing colon stem cells contribute to tumorigenesis in the transition from colitis to cancer, *Cancer Res* 69, 8208-8215

Curtin C.J., Lorenzi M.V., 2010, Drug discovery approaches to target Wnt signalling in cancer stem cells, *Oncotarget* 1, 552-566

Čihák R., 2002, Anatomie 2, Grada publishing 2nd edition, Praha 83-115

Debnath J., Brugge J.S., 2005, Modelling glandular epithelial cancers in three-dimensional cultures, *Nature Cancer* 5, 675-688

Dhawan P., Ahmad R., Chaturvedi R., Smith J.J. et al, 2011, Claudin-2 expression increases tumorigenicitx of colon cancer cells: role of epidermal growth factor receptor activation, *Oncogene* 30, 3234-3247

Dítě P. et al., 2007, Vnitřní lékařství, Galén 2nd edition, Praha, 273-289

Dorudi S., Sheffield J.P., Poulsom R., Northover J.M.A. et al., 1993, E-cadherin expression in Colorectal cancer; An Immunocytochemical and In situ hybridization study, *Am J Pathol* 142, 982-987

Engstrom J.U., Kmiec E.B., 2007, Manipulation of cell cycle progression can counteract the apparent loss of correction frequency following oligonucleotide-directed gene

repair, *BMC Molecular biology* 8, 9-25

Farquhar M.G., Palade G.E., 1963, Junctional complexes in various epithelia, *The J Cell Biology* 17, 375-388

Findley M.K., Koval M., 2009, Regulation and roles for claudin-family tight junction proteins, *IUBMB Life* 61, 431-437

Fuchs S.Y., Ougolkov A.V., Spiegelman V.S., Minamoto T., 2005, Oncogenic β-catenin signaling networks in colorectal cancer, *Cell cycle* 4, 1522-1539

Furuse M., Hirase T., Itoh M., Nagafuchi A., et al., 1993, Occludin: A novel integral membrane protein localizing at Tight junctons, *J Cell Biology* 123, 1777-1788

Gupta P.B., Filmore C.M., Jiang G., Shapira S.D., et al., 2011, Stochastic state transitions give rise to phenotypic equilibrium in populations of cancer cells, *Cell* 146, 633-644

Harhaj N.S., Antonetti D.A., 2004, Regulation of tight junctions and loss of barrier function in pathophysiology, *IJBCB* 36, 1206-1237

Hollande F., Shulkes A., Baldwin G.S., 2005, Signalling the junctions in gut epithelium, *STKE* 277, 13-17

Huang E.H., Hynes M.J., Zhang T., Ginestier C., et al., 2008, Aldehyde Dehydrogenase 1 is a marker for normal and malignant human colonic stem cells (SC) and tracks SC overpopulation during colonic tumorigenesis, *Cancer Res* 69, 3382-3389

Ishihara Y. et al., 2010, The combination of 5-FU, Leucovorin and CPT-11 (FOLFIRI) prolongs survival through inhibition of metastasis in an orthotopic model of colon cancer; *Anticancer Research* 30, 403-408

Jemal A., Bray F., Center M.M., Ferlay J., et al., 2011, Global cancer statistics, *CA Cancer J Clin* 61, 69-90

Knoblich J.A., 2008, Mechanisms of asymmetric stem cell division, *Cell* 132, 583-597

Leushacke M., Barker N., 2011, Lgr5 and Lgr6 as markers to study adult stem cell roles in self-renewal and cancer, *Oncogene* 479, 1-14

Lodish H., Berk A., Zipursky S.L., at al., 2000, Molecular cell biology, 4th edition, W.H. Freeman and Company, New York, Chapter DNA cloning with plasmid vectors

Martin T.A., Jiang W.G., 2009, Loss of tight junction barrier function and its role on cancer metastasis, *Biochim Biophys Acta* 1788, 872-891

Merlos-Suárez A., Barriga F.M., Jung P., Iglesias M., et al., 2011, The intestinal stem cell signature identifies colorectal cancer stem cells and predicts disease relapse, *Cell stem cell* 8, 511-524

Mineta K., Yamamoto Y., Yamazaki Y., Tanaka H., et al., 2011, Predicted expansion of

the claudin mutigene family, *FEBS Letters* 585, 606-612

Odoux C., Fohrer H., Hoppo T. et al., 2008, A stochastic model for cancer stem cell origin in metastatic colon cancer, *Cancer Res* 68, 6932-6941

Reya T., Clevers H., 2005, Wnt signalling in stem cells and cancer, *Nature* 434, 843-850

Rosenthal R., Milatz S., Krug S.M., Oelrich B. et al., 2010, Claudin-2, a component of the tight junction, forms a paracellular water channel, *J. Cell Sci* 123, 1913-1921

Rubin D.C., 2007, Intestinal morphogenesis, *Curr Opin Gastroenrerol* 23, 111-114

Ryabchikova E.I., Mazurkova N.A., Shikina N.V., Ismagilov Z.R., 2010, The crystalline forms of Titanium dioxide nanoparticles affect their interactions with individual cells, *JMedCBR* 8,

Saltz L., 2008, Colorectal cancer tratment: What's next? (or: Is there life after EGFR and VEGF?), *Gastrointest Cancer Res 2 (suppl 2)*, S20-S22

Sato T., Vries R.G., Snippert H.J., van de Wetering M., et al., 2009, Single Lgr5 stem cells build crypt-villus structures *in vitro* without a mesenchymal niche, *Nature* 459, 262-266

Scoville D.H., Sato T., He X.C., Li L., 2008, Current view: intestinal stem cells and signalling, *JGastro* 134, 849-864

Shaker A., Rubin D.C., 2010, Intestinal stem cells and epithelial-mesenchymal interactions in the crypt and stem cell niche, *Translational Research* 156, 180-187

Siddique H.R., Saleem M., 2012, Role of BMI1, a stem cell factor, in cancer reccurence and chemoresistance: preclinical and clinical evidences; *Stem Cells* 30, 372-378

Tracey A.M., Jiang W.G., 2009, Loss of tight junction barrier function and it role in cancer metastasis, *BBA* 1788, 872-891

Vermeulen L., Todaro M., Sousa Mello F., Sprick M.R., et al., 2008, Single-cell cloning of colon cancer stem cells reveals a multi-lineage differentiation capacity, *PNAS* 105, 13427-13432

Yan K.S, Chia L.A., Li X., Ootani A., et al., 2012, The intestinal stem cell markers Bmi1 and Lgr5 identify two functionally distinct populations, *PNAS* 109, 466-471

Zeki S.S, Graham T.A., Wright N.A., 2011, Stem cells and their implications for colorectal cancer, *Nat.Rev.Gastroenterol.Hepatol.* 8, 90-100

Zhang X., 2011, EphB2: a signature of colorectal cancer stem cells to predict relapse, *Protein Cell* 2, 347-348