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TESI

"Epithelial to mesenchymal transition (EMT) and cell plasticity in human colorectal cancer"

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List of abbreviations

CRC	Colorectal cancer
EMT	Epithelial to mesenchymal transition
TFs	Transcription factors
MET	Mesenchymal to epithelial transition
GSK-3β	Glycogen synthase kinase 3 beta
LiCl	Lithium Chloride
HNPCC	Hereditary nonpolyposis colorectal cancer
FAP	Familial adenomatous polyposis
MMR	Mismatch repair
CIN	Chromosomal instability
MSI	Microsatellite instability
CIMP	CpG island methylator phenotype
CSCs	Cancer stem cells

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Abstract

Colorectal cancer (CRC) was ranked third and second of all cancers affecting men and women, respectively, with metastases representing the first cause of death and defining the stage IV of the disease, characterized by a relatively short overall survival. It has been recently demonstrated that epithelial to mesenchymal transition (EMT), a physiological mechanism involved in embryonic development and tissue remodelling, could also play a critical role in invasion and metastasis of many types of cancer, including CRC. During EMT, epithelial cancer cells acquire not only mesenchymal traits, but also a stem cell-like phenotype, with ability of self-renewal, unlimited proliferation and resistance to apoptosis.

On this point of view, it is of significant and primary interest for cancer biomedical research to identify genes and proteins mainly involved in EMT and stemness, since they could represent good potential targets for therapy and predictive/prognostic molecular biomarkers.

During this work we isolated primary mesenchymal colorectal cancer cells from CRC patients, and demonstrated that they expressed epithelial (E-Cadherin and Cytokeratins), mesenchymal (N-Cadherin and Vimentin) and stemness markers, together with high level of epithelial-mesenchymal transition-transcription factors (EMT-TFs) (Snail and Twist). These primary cell cultures were isolated from adenocarcinomas, which are epithelial tumours, thus our data suggested that these cells were epithelial cells undergone EMT.

GSK-3β is a multifunctional serine/threonine kinase and an important regulator of cell survival by regulating PI3K, MAPK and WNT pathways. It may act as anti- or pro-apoptotic factor in a cell-specific manner; in colon and pancreatic cancer cells, GSK-3β activation confers a selective growth advantage to these cells, thus acting as a tumor promoter. Therefore, we investigated GSK-3β as a new druggable target and LiCl as new drug for cancer therapy.

We demonstrated that LiCl was able to induce mesenchymal-epithelial reverting transition (MET) and cellular differentiation, affecting migration in our cell cultures.

Spherical cancer models represent one of the major 3D *in vitro* models that have been described over the past 4 decades. These models have gained popularity in cancer stem cell research using tumorospheres. They contribute to chemoresistance, radioresistance, tumorigenicity, invasion and migration studies.

We demonstrated that our cell cultures were able to aggregate to form spheroids in suspension and this capability was affected when cells were

incubated with LiCl. Furthermore, the inhibition of GSK-3β by LiCl, strongly down-regulated the expression of stem cell markers, such as Oct4, Sox2, Nanog, ALDH1 and LGR5, in cancer spheroids.
Finally, we demonstrated that LiCl affected cellular plasticity, an important feature in tumor progression and metastasis; indeed, after LiCl treatment cancer cells were unable to switch from one phenotype to another.
In conclusion, we set up an experimental system of primary mesenchymal colorectal cancer cell cultures to study the role of EMT in CRCs, and molecular basis of cell plasticity during cancer progression and acquisition of resistance to the therapy. Furthermore, we observed that LiCl induced MET and altered dynamic of cancer spheres formation, indicating differentiated state that correlates with expression of undifferentiation/differentiation markers, suggesting that GSK-3β and LiCl could represent an eligible target and a potential drug to set up therapy able to interfere with CRCs progression.

I- Molecular basis of colorectal cancer

Colorectal cancer (CRC) is the third most frequent cancer worldwide and the fourth cause of cancer related deaths, implying a significant impact on public health especially in economically developed countries (Ferlay *et al*. 2010, Ferlay *et al*. 2015, Karsa *et al.* 2010).

During the development of colorectal adenocarcinoma, epithelial cells from gastrointestinal trait acquire mutations in specific oncogenes and/or tumor suppressor genes, conferring them a selective advantage on proliferation and self-renewal (Pancione *et al.* 2012, Ewing *et al.* 2014). Therefore, normal epithelium turns into a hyperproliferative mucosa and subsequently gives rise to a benign adenoma, which evolves into carcinoma and metastasizes in about 10 years. This model to explain CRCs progression is known as adenoma-carcinoma sequence (Volgestein *et al.* 1988). Sporadic CRCs, due to somatic mutations, account for about 70% of all CRCs. Familial CRCs, a group of diseases in which patients present a familial predisposition to develop cancer, are about 10-30%, whereas hereditary diseases are about 5-7% (Burt 2000).

The main hereditary CRC syndromes are hereditary nonpolyposis colorectal cancer (HNPCC) and adenomatous polyposis (FAP) syndrome (Rustgi 2007).

HNPCC, or Lynch syndrome, is an autosomal dominant disease which is caused by a germline mutation of DNA mismatch repair (MMR) genes, such as MLH1, MSH2, MSH6 and PMS2 (Aaltonen *et al.* 1998).

FAP is a hereditary autosomal dominant syndrome associated with mutations of the tumor suppressor gene APC (adenomatous polyposis coli), which is located on chromosome 5q21-q22 (Groden *et al.* 1991, Kinzler *et al.* 1991).

At molecular level, CRCs are a very heterogeneous group of diseases. Multiple genetic events are required for tumor progression, and genomic instability facilitates the acquisition of these mutations. Three mechanisms of genomic instability have been described in CRCs: Chromosomal instability (CIN), microsatellite instability (MSI) and aberrant DNA methylation (CpG island methylator phenotype- CIMP) (Goel *et al.* 2007, Boland and Goel 2010, Pino and Chung 2010) (Fig. 1).

- CIN is observed in 85% of sporadic CRCs and involves in chromosomal translocations, rearrangements of parts of chromosomes and genes multiplications or deletions. CIN can lead to imbalances in chromosome number (aneuploidy) and loss of heterozygosity (LOH) (Pino and Chung 2010).

- MSI is detected in 15% of sporadic CRCs and in HNPCC patients; it is caused by alteration of the DNA mismatch repair mechanism. Microsatellites are repeated sequences of one to six DNA nucleotides units and MSI consequence is a variation in the number of these units (Horvat and Stabuc 2011). Based on instability degree, it is possible to distinguish two different MSI phenotypes: MSI high (MSI-H) and MSI low (MSI-L) (De la Chapelle and Hampel 2010).
- CIMP is founded both in CIN and MSI CRCs, and is characterized by aberrant methylation of promoter CpG island sites, leading to alterations of several genes involved in tumor onset and progression (Lao and Grady 2011).

II- Signaling pathways altered in CRCs

It has been suggested a multistep model for the development of CRC, according to this model genetic and epigenetic mutations in oncogenes and tumor suppressor genes are required for the transition from benign adenoma to malignant carcinoma (Volgestein *et al.* 1988).

Therefore, alterations of several pathways are involved in CRCs onset and progression, such as Wnt/APC/β-catenin, Ras/Raf, PI3K/AKT, NF-κB, TGF-β/Smad, p53 and mismatch repair (MMR) signaling (Fig. 1).

These alterations confer individual susceptibility to cancer, and are responsible for responsiveness or resistance to antitumor agents (Colussi *et al.* 2013, Markowitz and Bertagnolli 2009).

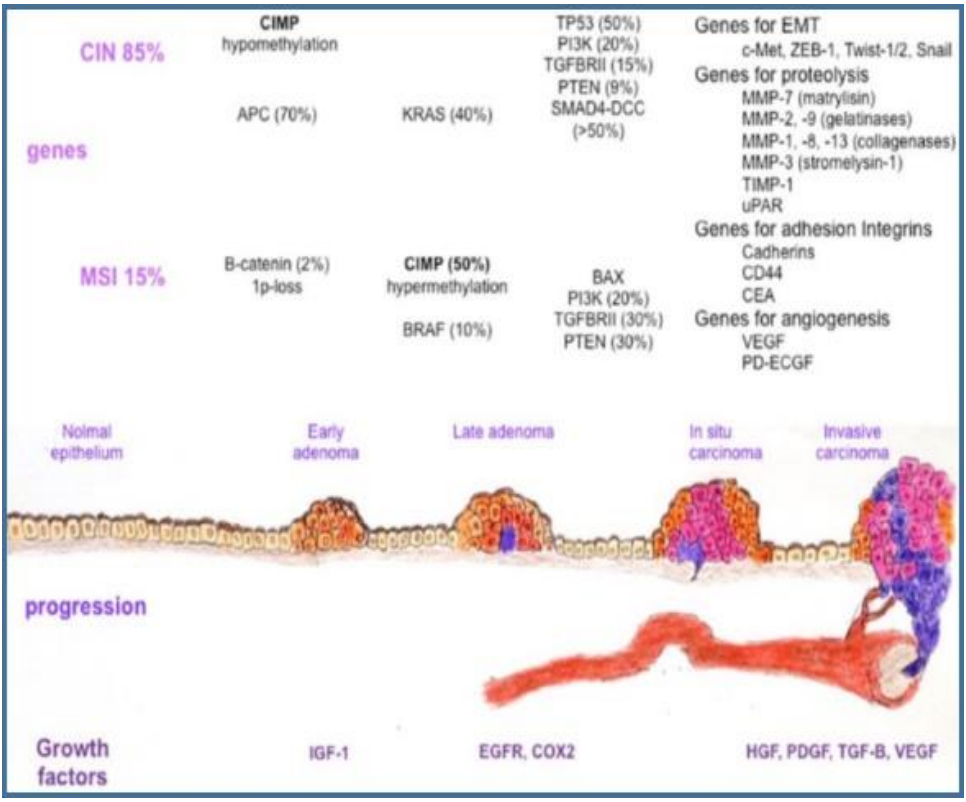

Figure 1. Molecular heterogeneity of CRCs. Several molecular mechanisms are involved in the onset and progression of CRCs. Several mutations in specific oncogenes and/or tumor suppressor genes are implicated into transition from adenoma to carcinoma. (Adapted from De Rosa et al. 2016).

Wnt/β-catenin pathway

The key event in the onset of the majority of sporadic CRCs (about 70% of CIN CRCs) is the mutation of APC tumor suppressor gene, which is responsible also for FAP syndrome due to germline mutations (Colussi *et al.* 2013).

APC protein plays an essential role in Wnt/β-catenin pathway. When Wnt ligand, a secreted glycoprotein, binds to its Frizzled (Fz) receptor, the multifunctional kinase GSK-3β is inactivated and β-catenin is stabilized and accumulates in the cytoplasm. Finally, β-catenin translocates into the nucleus where it interacts with members of the lymphoid enhancer factor (LEF)/T-cell factor (TCF) and activates specific target genes. In the absence of Wnt-signal, casein kinase 1 (CK1) and the APC/Axin/GSK-3β-complex phosphorylate β-catenin, targeting it for ubiquitination and proteasomal degradation (MacDonald *et al.* 2009).

Wnt/β-catenin pathway is involved in many cellular processes (proliferation, differentiation, migration and stem cell self-renewal); indeed β-catenin regulates transcription of several genes such as Cyclin D1, CD44, c-myc oncogene and matrix metalloproteinase MMP-7 (Arber *et al.* 1996, Wielenga *et al.* 1999, He *et al.* 1998, Brabletz *et al.* 1999).

Ras/Raf pathway

Ras/Raf signaling is involved in mitogen-activated protein kinase (MAPK) activation, a group of serine/threonine kinase proteins which mediate signal transduction from plasma membrane to the nucleus in response to several extracellular stimulations, such as growth and mitogenic factors (Malumbres and Barbacid 2003, Scaltriti and Baselga 2006). Ras genes (H, K and N) encode for small proteins with GTPase activity bound to the plasma membrane. Ras mutations enhance GTP levels, leading to Raf proteins (A, B and C) phosphorylation and activation (Hallberg *et al.* 1994). Raf proteins transduce signals between MEK (1 and 2) and ERK (1 and 2) proteins, inducing transcription of genes involved in cell cycle and transcription regulation, such as c-myc and cyclin-D/CDK (Liebmann 2001, Shao *et al.* 2014).

In CRCs, the early mutations are followed by events that promote cancer progression. It has been demonstrated that in 40% of CRCs the transition from adenoma to carcinoma is promoted by mutations of KRAS gene (Volgestein *et al.* 1988). These mutations lead to a permanently active state of RAS pathway that permits cells to escape apoptosis and acquire a growth advantage (Malumbres and Barbacid 2003).

BRAF is frequently altered in several tumors and its mutations are mutually exclusive to KRAS mutations. In sporadic CRCs, BRAF has been found mutated in 10% of cases, specifically in MSI tumors (Parsons *et al.* 2012). In detail, the V600E mutation of BRAF gene represents a hotspot mutation, which lead to a constitutively active status of the Ras/RAF/MEK/ERK pathway (Di Nicolantonio *et al.* 2008).

PI3K/AKT pathway

The PI3K pathway is often dysfunctional in both sporadic and hereditary CRC; it activates cell growth, inhibits apoptosis and regulates cellular transformation, adhesion, motility and survival (Cantley 2002, Fruman *et al.* 1998) in response to several extracellular signals, such as growth factors, cytokines, hormones, heat and oxidative stress, hypoxia, and hypoglycaemia (Testa and Bellacosa 2001, Martini *et al.* 2014, Vivanco and Sawyers 2002, Castellano and Downward 2011).

Growth factor binds its receptor, and this event induces the activation of receptor by its self-phosphorylation. Consequently, PI3K is recruited at the plasma membrane and activated. The active form of PI3K converts phosphatidylinositol (4,5)-bisphosphate (PIP2) into phosphatidylinositol (3,4,5)-trisphosphate (PIP3). PIP3 binds AKT anchoring it to the membrane and leading to its phosphorylation and activation via phosphoinositide-dependent kinase-1 (PDK1) (Shaw and Cantley 2006, Fresno Vara *et al.* 2004, Testa and Bellacosa 2001). AKT acts in many cellular processes (control of metabolism, translation, apoptosis and cell cycle) by phosphorylating several target proteins, such as BAD (BCL-2 antagonist of cell death), caspase-9, mTOR (mammalian target of rapamycin), GSK3β and β-catenin (Martini *et al.* 2014, Porta *et al*. 2014, Lee *et al.* 2010).

PI3K/AKT pathway is negatively regulated by PTEN, which dephosphorylate PIP3 down-regulating its levels (Simpson and Parsons 2001). Alterations of PTEN tumor suppressor gene are involved in several sporadic and hereditary CRCs. Somatic mutations in this gene, such as small quantitative alterations of its expression, have been founded to be associated to different types of cancer (Salmena *et al.* 2008). Furthermore, its germ-line mutations cause PTEN hamartoma tumors (PHTS) syndrome, a hereditary disorder predisposing patients to onset of multiple neoplasms (Manfredi 2010, Eng 2003, Galatola *et al.* 2012). Recently it has been suggested that PTEN protein acts in a quantitative manner (Alimonti *et al.* 2010), and quantitative changes in its expression levels could have a role in phenotypic variability shown by PHTS patients (Paparo *et al.* 2013).

NF-κB pathway

NF-κB plays a key role in many physiological processes such as immune response, cell proliferation, cell death, and inflammation. NF-κB family is composed of five members: RelA (p65), RelB, cRel (Rel), NF-κB1 (p50 and its precursor p105) and NF-κB2 (p52 and its precursor p100), which are able to form homo- and heterodimeric complexes. The activity of these proteins is regulated by the IKK complex, consisting of two catalytic (IKKα and IKKβ) and one regulatory (IKKγ) subunits. In absence of signals, NF-κB dimers are sequestered in the cytoplasm by IκB proteins. Following stimulation by several signals, such as TNF-α (tumor necrosis factor- alpha) or IL-1 (interleukin- 1), IκB molecules are phosphorylated by the IκB kinase complex (IKK) leading to their ubiquitination and proteasomal degradation. IκB degradation releases NF-κB proteins in the cytoplasm, allowing them to translocate into the nucleus and activate transcription of specific target genes (Ghosh and Karin 2002). It has been demonstrated that NF-κB is constitutively activated in 60-80% of CRCs (Lind *et al.* 2001), and this promotes proliferation of cancer cells and rescues them from cell death (Hassanzadeh 2011).

TGF-β/Smad pathway

The TGF-β/Smad signaling pathway is involved in several biological processes, including cell proliferation, differentiation, migration and apoptosis. TGF-β binds to type II TGF-β receptors (TGFBR2), leading to recruitment and phosphorylation of the type I TGF-β receptor (TGFBR1) by TGFBR2. This event activates TGFBR1 protein kinase activity on two downstream transcription factors: SMAD2 and SMAD3. Phosphorylated SMAD2 and SMAD3 bind to SMAD4 and these SMAD complexes translocate into the nucleus to regulate the expression of specific target, such as genes involved in cell cycle checkpoint. Therefore, TGF-β induces cell cycle arrest and apoptosis, acting as tumor suppressor (Elliott and Blobe 2005, Massague *et al.* 2000).

TGFBR2 contains microsatellite sequences, which have been found altered in CRCs, especially in MSI tumors (Takayama *et al.* 2006, Grady *et al.* 1999).

Furthermore, it has been demonstrated that CIN CRCs are often characterized by the LOH of chromosome 18q, where Smad2, Smad4 and DCC genes are located and these mutations affect the response to TGF-β signalling (Lanza *et al.* 1998, Xu and Pasche 2007).

p53 pathway

p53 is a tumor suppressor gene that regulates cell cycle arrest in response to DNA damage, inducing cell apoptosis. p53 protein acts by promoting transcription of target genes involved in cell cycle checkpoints. A major player in the p53 signaling is p21, which inhibits the formation of cyclin E-cdk2 complexes responsible for cell cycle G1/S phase transition.

p53 is also able to induce cell cycle arrest in G2 phase, by promoting the expression of proteins which retain CDC25C in cytoplasm. CDC25C is required for the activation of cyclin B-CDC2 complexes in the nucleus, which are essential for the G2/M phase transition (Harris and Levine 2005).

Mutations of p53 have been found in about 50% of CRCs, furthermore p53 is located on the short arm of chromosome 17 (17p), a region found deleted in more than 75% of CRCs. It has been demonstrated that these mutations are involved in CRCs progression and are late events, which lead to adenoma-carcinoma transition (Volgestein *et al.* 1988).

MMR pathway

MMR pathway corrects errors occurring during DNA replication and the main components of this system are MSH2, MSH3, MSH6, MLH1 and PMS2 proteins. MSH2 forms heterodimeric complexes with MSH6 or MSH3 proteins to recognize base mismatches or insertions/deletions, respectively; while MLH1 binds PMS2 and the resulting heterodimer manages the interaction between MSH2-MSH6/MSH3 complex and other factors required for repairs, such as DNA polymerases, helicases, exonucleases and single strand binding proteins (Kolodner and Marsishcky 1999).

Mutations of MMR proteins are characteristic of MSI CRCs, in particular epigenetic silencing of MLH1 gene has been often observed (Herman *et al.* 1998). Defective MMR system lead to a 100-fold increase in the mutation rate in colorectal cancer cells, thus promoting tumor progression (Thomas *et al.* 1996).

GSK-3β regulates cross talk between pathways involved in CRCs

GSK-3β is a multifunctional serine/threonine kinase and an important regulator of cell survival that may act as anti- or pro-apoptotic factor, in a cell-specific manner. Its activity is regulated by site-specific phosphorylation. Full activity of GSK-3β generally requires phosphorylation at tyrosine 216 (tyr216), whereas phosphorylation at serine

9 (Ser9) inhibits its activity (Doble and Woodgett 2003, Jope and Johnson 2004).
More than 100 proteins are substrates of GSK-3β, among which β-catenin and NF-κB inhibitor IκB are the most well-known. The role of GSK-3β in cancer cells is controversial because this kinase is able both to interfere with cell proliferation, by inducing the degradation of β-catenin, and to contribute to cell survival by regulating NF-κB (Shakoori *et al.* 2005).
In colon and pancreatic cancer cells, GSK-3β positively regulate NF-κB and its activation confers a selective growth advantage to tumor cells (Li *et al.* 2014).
A cross talk exists between NF-κB, Wnt and other adhesion proteins such as E-cadherin. E-cadherin directly binds to β-catenin and NF-κB at the plasma membrane level, subtracting them from the nucleus. NF-κB promotes the transcription of Wnt5A, which regulates several target genes including Snail. Snail down-regulates E-cadherin expression, leading to the release of β-catenin and NF-κB from the plasma membrane and making them available to translocate into the nucleus. GSK-3β mediates the cross regulation between Wnt and NF-κB Pathways (Du and Geller 2010).

III- Epithelial-mesenchymal transition (EMT), cell plasticity and cancer progression

EMT and its reverse process mesenchymal-epithelial transition (MET) are physiological mechanisms occurring during embryonic development and tissue remodelling. It has been suggested that EMT is also involved in cancer progression and metastasis in several tumors, including CRCs (Loboda *et al.* 2011).
During EMT, epithelial cells lose epithelial features and acquire mesenchymal phenotype; these events are regulated by a group of transcription factors (EMT-TFs), including SNAIL, SLUG, ZEB1 and TWIST. The contribution of each EMT-TF is not homogeneous in all the cells, but it depends on cell type. Furthermore, the EMT-TFs influence each other and they can cooperate to regulate the expression of target genes (De Craene *et al.* 2013).
A key event of EMT is the disruption of cell-cell junction characteristic of epithelial phenotype. To this aim, E-cadherin is cleaved and cannot interact with β-catenin to form adherens junctions; moreover, its expression is down-regulated by the EMT-TFs (David and Rajasekaran 2012, De Craene and Berx 2013).

On the other hand, the EMT-TFs induce the expression of N-cadherin, normally localized at the membrane level of mesenchymal cells, conferring to the cells migratory properties. This "cadherin switching" is crucial in cancer cells, since they acquire the ability to detach from primary tumor and migrate into surrounding tissues (Wheelock *et al.* 2008).
During EMT a cytoskeleton reorganization occurs. Specifically, the cell intermediate filament status changes from a keratin-rich network that takes part in adherens junctions and hemidesmosomes to a vimentin-rich network taking part in focal adhesions (Kokkinos *et al.* 2007).
EMT programme can be induced by several signals, among which TGFβ signaling is the main pathway involved in the regulation of EMT. Other pathways able to induce EMT are Wnt, Notch, HGF, FGF, IGF1, EGF and VEGF; by promoting the expression of the EMT-TFs, manly SNAIL and TWIST (Lamouille *et al.* 2014).

In the last few years, it has been demonstrated that a link exists between EMT and stemness. Indeed, EMT program induces the expression of stem cell genes, conferring to the cells the ability of self-renewal (Mani *et al.* 2008). Thus, EMT in tumors can lead to generation of "cancer stem-like cells", which have the capability to initiate new tumors (Reya *et al.* 2001).
EMT could be a biological process common to several tumors, so it could represent a target mechanism to interfere with cancer progression.
In CRCs, EMT has been observed at the invasive front producing single migratory cells, which could initiate the metastatic process. (Brabletz *et al.* 2001).
Metastases are the first cause of death in CRC, as well as in many other solid tumors. During metastases development, the cells detach from primary tumor and acquire the ability to invade the basal membrane, thus migrating into neighbouring tissues. Later, they interact with endothelial cells and enter in lymphatic and blood vessels, in a process known as intravasation. At last, the cells survived in the lumen of the vessels, come out from the vases (i.e. extravasation) and disseminate into the adjacent organs.
Thus, EMT confers to the epithelial cancer cells all the features needed to complete this process, such as an increase of migratory and invasive ability and resistance to apoptosis (Thiery *et al.* 2009) (Fig. 2).
The EMT-TFs are involved in the resistance to apoptosis of cells undergone EMT. It has been demonstrated that TWIST1 inhibits cell cycle arrest and apoptosis by impaired activation of p53 target genes (Vichalkovski *et al.* 2010). Moreover, SNAIL is able to suppress TGF-β induced cell death, and

regulates components of the early to late G1 transition and the G1/S checkpoint, including the repression of Cyclin D2 transcription and the increase in p21 (Yang *et al.* 2006, Vega *et al.* 2004).
The expression of N-Cadherin at the membrane level of cancer cells undergone EMT, facilitates their interaction with endothelial cells, which express not only VE-cadherin, but also N-cadherin, thus promoting intravasation (Wheelock *et al.* 2008).
Once in a new distant site, MET program is induced, allowing cancer cells to grow, colonize the new tissue and generate metastases (Brabletz 2009) (Fig. 2).

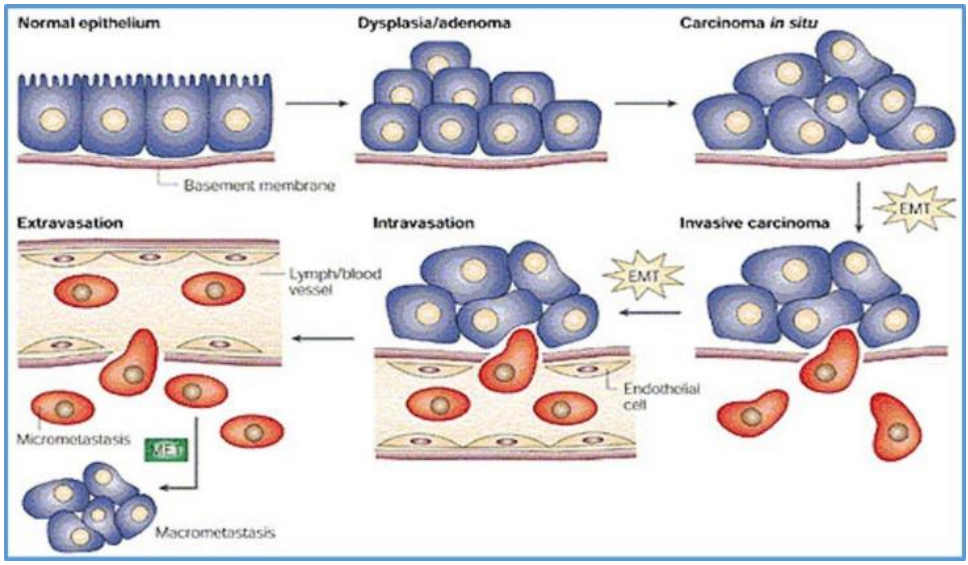

Figure 2. EMT and MET in metastasis development. Epithelial cancer cells undergone EMT acquire characteristics, which allow them to detach from tumor, entry and survive into lymphatic and blood vessels, come out from the vases and reach new tissues. EMT is reverted and MET allow cancer cells to grow and generate metastasis. (Adapted from Thiery 2002).

It has been demonstrated that cellular plasticity plays a crucial role in tumor progression and metastasis. Indeed cancer cells that are able to switch from epithelial to mesenchymal phenotype and *viceversa*, show metastasis-initiating potential; while disseminated cancer cells undergone EMT that lose the phenotypic plasticity are ineffective in seeding metastatic colonies. Several studies demonstrated that EMT and phenotypic plasticity are

associated with the acquisition of resistance to therapy (Chaffer *et al.* 2016, Doherty *et al.* 2016).

IV- Cancer stem cell (CSCs)

In the last decades, several authors proposed a new hierarchical model about how cancer growth is sustained. According to this hierarchy theory, tumors are heterogeneous and they are composed of several types of cells among which only a few are able to initiate the tumor. These cells show stem cell properties, such as self-renewal and multilineage differentiation capacity, and for this reason, they are called cancer stem cells (CSCs) (Vermeulen *et al.* 2008).

The CSCs play a key role in cancer progression and metastases, and they are probably responsible for both relapse and resistance to therapy (Gangemi *et al.* 2009, Fanali *et al.* 2014). It is still unclear if CSCs derive from differentiated cancer cells, carriers of a somatic mutation, that can de-differentiate, or alternatively they arise from healthy stem cells that accumulate genetic mutations and acquire a neoplastic phenotype (O'Brien *et al.* 2007, Greaves and Maley 2012). As mentioned above, EMT program induces the expression of stem cell specific markers, so cancer cells undergone EMT acquire both mesenchymal and stem cell-like phenotype. Therefore, EMT might be a source of CSCs or CSC-like cells (Reya *et al.* 2001, Fan *et al.* 2012).

CSCs derived from different tumors are characterized by the expression of specific surface markers; in particular, CD133 is one of the most used markers to isolate colorectal CSC. It has been demonstrated that $CD133^+$ cells are able to generate a tumor, similar to the original lesion, when they are xenotransplanted in immunodeficient mice (O'Brien *et al.* 2007). However CD133 expression has been found both in undifferentiated and differentiated cancer cells, suggesting that there is not an exclusive link between its expression and cancer cell stemness and tumorigenicity (Lugli *et al.* 2010).

It has been suggested that other markers, such as CD44, CD166, EpCAM, CD24, CD29, LGR5 and ALDH1, have a role in stemness, invasiveness and metastasis in colorectal cancer (Vermeulen *et al.* 2008, Lugli *et al.* 2010).

CD44 gene is composed of 19 exons and it encodes for different isoforms, generated by alternative splicing of exons 6-14 (also known as v2-v10). All these proteins have the same N- and C-terminus domain. The N-terminus domain (encoded by exons 1-5) interacts with hyaluronic acid, while the C-terminus (encoded by exons 15-19) is a trans-membrane domain that sticks

out into cytoplasm. Depending on which exons are included in the final transcript, the isoforms differ for the extracellular membrane proximal insert (Gotley *et al.* 1996).
CD44 overexpression is associated with CRCs progression, and in detail, it has been proposed that the CD44v6 isoform is involved in the migration of colorectal CSCs and generation of metastases (Du *et al.* 2008, Todaro *et al.* 2010).
Aldehyde dehydrogenase (ALDH) protein is involved in alcohol metabolism, and its overexpression is correlated to CRCs aggressiveness and metastases (Hou *et al.* 2013). Moreover, it has been demonstrated that $ALDH^{high}$ cancer cells show stem cell properties and elevated tumorigenic potential (Shenoy *et al.* 2012).
The leucine-rich-repeat-containing G-protein coupled receptor 5 (LGR5) gene encodes the R-spondin receptor, which is an agonist of the canonical Wnt pathway. LGR5 is expressed in stem cells at the crypt base of intestinal epithelium and it has been suggested that LGR5-positive cancer cells might become CSCs in tumors (Barker *et al.* 2007).

Given the role of CSCs in cancer metastasis and drug resistance, it becomes relevant to study their molecular biology to develop therapies able to target these cells.
CSCs can be isolated from cancer cell cultures and propagated *in vitro* to test their ability of self-renewal. As mentioned above, one of the main feature of CSCs is the ability to differentiate leading to the generation of the different lineages of cancer cells, which constitute the whole tumor. *In vivo* experiments are necessary to test tumorogenicity of CSCs, through transplantations in immunodeficient non-obese diabetic (NOD)/severe combined immunodeficient (SCID) mice (Tirino *et al.* 2012).

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References

Chapter 1

Aim of study

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Metastases are the main cause of death in patients affected by CRCs, thus the understanding of the molecular basis of this mechanism is useful to set up therapies and interfere with cancer progression. Recently, it has been suggested that EMT, which is a physiological process, is borrowed by cancer cells to carry out invasion and metastases. EMT program is orchestrated by a group of transcription factors (EMT-TFs), including SNAIL, SLUG, ZEB1 and TWIST. During EMT, the cells lose the expression of E-Cadherin, that guarantees the epithelial phenotype, and acquire mesenchymal characteristics.

EMT program provides to the epithelial cells all the traits that are needed to drive on metastases development such as an increase of migratory and invasive properties, resistance to apoptosis and stem cell-like features allowing cancer cells to generate a new tumor in a distant site. Moreover, several studies suggest that EMT is involved in the acquisition of pharmacological resistance by cancer cells.

Cellular plasticity is the ability to switch from one phenotype to another, and it is a crucial feature of cancer cells in metastasis development.

Genomic instability facilitates the accumulation of multiple mutations during the development of CRC, chromosomal instability (CIN) is observed in 85% while microsatellite (MSI) is detected in 15% of sporadic CRCs. Molecular mechanisms underlying CRC progression is still poorly understood, especially for MSI CRCs.

GSK-3β is a key regulator of the cross talk between different signaling pathways involved in CRCs development. GSK-3β is a multifunctional serine/threonine kinase and an important regulator of cell survival that may act as anti- or pro-apoptotic factor, in a cell-specific manner. In colon and pancreatic cancer cells, GSK3β acts as oncogene since it positively regulates NF-κB and its activation confers a selective growth advantage to tumor cells.

Given all these data, we decided to investigate genes and mechanisms responsible for EMT and stemness in CIN and MSI CRCs, with the main purpose to shed light on metastases development and mechanisms for cancer relapses and resistance, thus identifying new targets for cancer therapy and new prognostic and predictive biomarkers.

We also elucidate the role of GSK-3β and LiCl (its specific inhibitor) as eligible target and a potential drug to develop therapy able to affect CRCs progression and metastases, respectively.

To realize these main objectives we fulfil the following aims:

a) Biological samples collection from CRC patients
We sampled pairs of normal colorectal mucosa and its matched colorectal cancer tissues from patients with sporadic and hereditary colorectal cancer and frozen them in liquid nitrogen. We also collected peripheral blood, sampling DNA, RNA, proteins and serum. Primary cell cultures were established from healthy mucosa and cancer tissues as previously described (Costabile *et al.* 2015).

b) Genetic characterization of primary colorectal cancer cell cultures
To achieve this objective, primary colorectal cancer cell cultures were analysed for MSI and CIN status, and cancer cells were assessed for co-expression of epithelial and mesenchymal markers, by using immunofluorescence assay (Costabile *et al.* 2015).

c) Investigation of genes and mechanisms involved in the EMT and stemness of CIN and MSI CRCs
To this aim, two mesenchymal primary colorectal cancer cell cultures, established from CIN and MSI CRCs, were grown as spheres in serum-free stem cell medium. Indeed, it is reported that only stem cells and/or stem-like cells, but not differentiated cancer cells, are able to survive and grow in these conditions.
Both cell cultures, adherent cancer cells and spheroids, were analysed for expression of the main EMT-TFs, such as Twist1 and Snail, and other EMT specific proteins, such as E-cadherin, N-cadherin and Vimentin, by using immunofluorescence assay, Western Blot and Real Time RT-PCR. We also evaluated the expression of the main stem cell biomarkers, such as LGR5, ALDH1, Oct-4, Sox-2 and Nanog.

d) Investigation of the effect of GSK-3β inhibition on EMT
To shed light on the role of GSK-3β in EMT and CRC progression, we evaluated effects of LiCl incubation on the expression of mesenchymal and stemness biomarkers both in adherent cancer cells and spheroids. We also assessed cell response to GSK-3β inhibition on cell migration and plasticity, by using wound healing assay and hanging drop assay.

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Chapter 2

Materials and methods

2.1 Patients

Blood samples, normal colorectal mucosa and colorectal cancer tissues have been obtained from patients with sporadic colon cancer operated in the "Istituto Nazionale Dei Tumori- Pascale" in Naples (Italy). Tissues have been frozen in liquid nitrogen to realize a bio-bank.
Samples from all patients have been collected after authorization from the "Comitato Etico per le Attività Biomediche- Carlo Romano" of the University of Naples Federico Ⅱ, with protocol no. 120/10. This authorization has been obtained once the study received ethics approval, the participants have been informed and written consent has been signed.

2.2 Cell cultures

Samples of colorectal cancer have been washed overnight at 4˚C in PBS containing 300 U/ml penicillin, 300 µg/ml streptomycin and 2.5 µg/ml amphotericin B (all from Gibco BRL- Karlsruhe, Germany). Then they have been finely minced with scissors and incubated in 2 ml 0.1% collagenase II (Boehringer Mannheim- Mannheim, Germany) for 1 h at 37˚C and 5% CO2. Cell suspension has been collected by centrifugation, washed twice with PBS, and subsequently cultured in DMEM/F12 (1:1) containing 10% FBS medium, 100 U/ml penicillin, 100 µg/ml streptomycin and 2.5 µg/ml amphotericin B.
Colorectal cancer cells have been selected by differential sedimentation.
To inhibit GSK-3β activity, cells have been incubated in the same medium containing 30 mM LiCl for 1h, 24h and 10 days.
To evaluate cell migration, we performed wound-healing assay as described by Rodriguez *et al.* (*Methods Mol Biol*, 2005).

2.3 Hanging drop assay

Cells have been diluted at a final concentration of $3.7x10^4$ cells/ml in DMEM/F12 (1:1) containing 2% FBS medium, 100 U/ml penicillin, 100 µg/ml streptomycin, 2.5 µg/ml amphotericin B, 10µg/ml bFGF and 20 µg/ml EGF. 27µl drops have been seeded on the cover of the plate and incubated at 37˚C and 5% CO2 for 72h. In these conditions, cells aggregate to form 3D spheroids.

2.4 Cytogenetic analysis

Cytogenetic analysis has been performed by the cytogenetic service of the Department of Molecular Medicine and Medical Biotechnologies of University of Naples, Federico II.

Metaphase chromosome analysis has been performed on cell cultures from colorectal cancer tissues, by using high-resolution G-banding (550 bands) according to standard procedures. Multicolor-FISH (M-FISH) has been carried out using MetaSystems' 24Xcyte color kit (MetaSystems GmbH). FISH analysis has been performed using whole chromosome painting (WCP) probes for chromosomes 20 and 22 and locus specific DiGeorge probe mixture (MetaSystems GmbH) containing a Spectrumorange probe located at 22q11.2, a Spectrumgreen LSI probe mapping the 22q13.3 region and subtelomeric probes for the p (green) and q (red) arms of chromosomes 20. Multicolor chromosome banding (MCB) has been performed using the multicolor banding DNA probe kit based on microdissection derived region specific libraries for chromosome 22 (MetaSystems GmbH) according to standard protocols (Liehr *et al.* 2002).
FISH experiments have been performed on metaphase spreads and fluorescent images have been analysed using a fluorescence microscope (Axio Imager.Z1 mot; Carl Zeiss Microscopy, LLC) with ISIS software imaging system (MetaSystems gmbh) for image capturing and processing.

2.5 RER assay

The MSI status has been confirmed with a fluorescent multiplex system including six mononucleotide repeats (BAT-25, BAT-26, BAT-40, NR21, NR24, and TGFBR II) and four dinucleotide repeats (D2S123, D5S346, D17S250, and D18S58) as described earlier (Duraturo *et al.* 2013), using the CC-MSI kit (AB Analitica s.r.l), according to the manufacturer's instructions. PCR products have been analysed by capillary electrophoresis analysis using an ABI Prism 3130 Genetic Analyzer (Applied Biosystems).

2.6 RT-PCR analysis

Total RNA has been extracted from primary colorectal cancer cells and tissues of CRC patients, after homogenization, using Qiazol reagent (Qiagen) according to the manufacturer's instructions. 1µg of total RNA has been treated with DNAse (Invitrogen Life Technologies) and cDNA has been synthesized by mixing 500ng random hexamers, 1mM dNTPs, 1µl SuperScriptIII Reverse Transcriptase, 4µl 5X RT buffer, 1µl DTT 0.1M and nuclease-free water to 20µl (Invitrogen Life Technologies).
The reaction has been run in a thermocycler for 60 min at 55˚C, heated to 70˚C for 15 min and quickly chilled on ice. 1µl cDNA has been amplified by RT-PCR using CTK20, CTK18 and E-cadherin primer pairs (Table 2.1).

All oligonucleotides have been obtained by using Primer-BLAST Software (http://www.ncbi.nlm.nih.gov/tools/primer-blast/).

Table 2.1 Oligonucleotide sequences

Gene	Primers	Accession no.
CTK 18	F:5'-AGACTGGAGCCATTACTTC-3' R:5'-GCTCTGTCTCATACTTGACTC-3'	[NM_199187.1; start +441] [NM_199187.1; start +563]
CTK 20	F:5'-CTGCAAATTGATAATGCTAA-3' R:5'-GGTCATCAAAGACCTTATTC-3'	[XM_005277792.1; start +485] [XM_005277792.1; start +586]
E-Cad	F:5'-TCCTGGGCAGAGTGAATTTT-3' R:5'-CCGTAGAGGCCTTTGACTG-3'	[NM_004360.3; start +276] [NM_004360.3; start +381]
Snail 1	F:5'-GCGAGCTGCAGGACTCTAAT-3' R:5'-TCCCAGATGAGCATTGGCAG-3'	[NM_005985.3; start +143] [NM_005985.3; start +269]
Twist1	F:5'-CCTTCTCGGTCTGGAGGAT-3' R:5'-TCCTTCTCTGGAAACAATGACA-3'	[NM_000474; start +908] [NM_000474; start +1004]
Vim	F:5 '-TCTGGATTCACTCCCTCTGG-3' R:5'-GGTCATCGTGATGCTGAGAA-3'	[NM_003380; start +1694] [NM_003380; start +178]
COX2	F: 5'-CAGCACTTCACGCATCAGTT -3' R:5'-CGCTGTCTAGCCAGAGTTTCA -3'	[NM_000963.3; start 702] [NM_000963.3; start +820]
GUS	F:5'-GAAAATATGTGGTTGGAGAGC-3' R: 5'-CCGAGTGAAGATCCCCTTTTT-3'	[XR_242233.2; start +1695] [XR_242233.2; start +1774]

2.7 Real-time PCR quantification analysis

Real-time PCR quantification analysis has been performed on 0.5µl cDNA, using Twist1, Snail, Vimentin and Cyclooxygenase-2 (COX2) primer pairs (Table I). Relative expression has been calculated with the comparative Ct method and normalized against the Ct of glucuronidase (GUS) mRNA. Quantitative Real-time assay has been performed by using the Bio-Rad iCycler iQ Real-Time PCR Detection System (Bio-Rad laboratories) as previously described (Galatola *et al.* 2012, Galatola *et al.* 2013).

Healthy mucosa (HM) has been used as a control to measure the relative expression in colorectal cancer cells and tumor tissue of the same patient.

All statistical analyses have been computed using Prism software. The unpaired two-tailed *t* test with confident intervals of 99% was calculated. A value of $p < 0.005$ was considered statistically significant.

2.8 Western blot assay

Total proteins have been extracted from colorectal healthy mucosa (HM) and tumor tissue (TT) of patients, and from primary colon cancer cultures, using Qiazol reagent (Qiagen) following the manufacturer's instructions. Proteins concentration has been determined by using Bradford assay (Bio-Rad laboratories), adopting bovine serum albumin standards. 30µg of proteins have been separated by SDS-polyacrylamide gel electrophoresis and blots have been prepared on an Amersham Hybond-ECL nitrocellulose membrane (Amersham Pharmacia Biotech). Primary antibody against β-catenin (rabbit polyclonal anti-human; no.9581), E-cadherin (rabbit monoclonal anti-human; no.3195), CD44 (mouse monoclonal anti-human; no.5640), Snail (rabbit monoclonal anti-human; no.3879) and Vimentin (rabbit monoclonal anti-human; no.5741) have been from Cell Signalling technology. Primary antibody against Actin (polyclonal, rabbit anti-human; sc-1615) has been from Santa Cruz Biotechnology, Inc. The membrane has been probed with a secondary antibody against peroxidase-conjugated rabbit or mouse immunoglobulin g, and immunoreactivity has been detected using the enhanced chemiluminescence Immobilon Western HRP Substrate (Millipore).

2.9 Immunofluorescence

Primary colorectal cancer cells have been seeded and grown in 12-well cultivation chambers (ibidi). After fixation in 4% paraformaldehyde (PFA)-PBS for 10 min, cells have been permeabilized in 0.1% Triton X-100-PBS and then have been blocked in 10% FBS- 0.1% Triton X-100-PBS for 45 min. Cells have been incubated overnight with primary antibodies (Table 2.2) and with secondary antibodies (Alexa Fluor 546 donkey anti-rabbit A10040, Alexa Fluor 488 donkey anti-mouse A21202; Thermo Fisher) for 1h and with DAPI (SIGMA-Aldrich) for 30min to label nuclei. Negative controls without primary antibodies have been also included and no staining has been observed. Immunofluorescence has been visualized under a fluorescence confocal microscope Zeiss LSM 700 and image have been captured.

Table 2.2 Primary Antibodies used for immunofluorescence analysis.

Antibodies	
Pan-cytokeratin (no.74384347, Thermo Fisher)	E-cadherin (no.76055, Abcam)
Nanog (no.3580, CST)	N-cadherin (no.76057, Abcam)
Sox2 1:50 (no.4900, CST)	Snail (no.180714, Abcam)
Oct4 (no.2840, CST)	LGR5 (no.135238, Santa Cruz)
Vimentin (no. 5741, CST)	ALDH1 (no.24343, Abcam)
CD44 (no.5640, CST)	β-catenin (no.9581, CST)

References

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Chapter 3

Results

3.1 Isolation and molecular characterization of primary colorectal cancer cell cultures

We realized a tissue biobank by sampling pairs of healthy colorectal mucosa and its matched tumor tissues, freezing them in liquid nitrogen. From the same tumor tissues, we established primary colorectal cancer cell cultures. We decided to analyze two of these cell cultures, T88 and T93, isolated from tumor tissue of patient number 88 and patient number 93 of our bio-bank. The classification of these two tumors was T3N1 and T4N2, according to TNM staging. These cells were characterized by different morphologies, specifically T88 were elongated and resembled the mesenchymal phenotype, while T93 showed a cuboidal shape that is more characteristic of epithelial cells (Fig. 3.1).

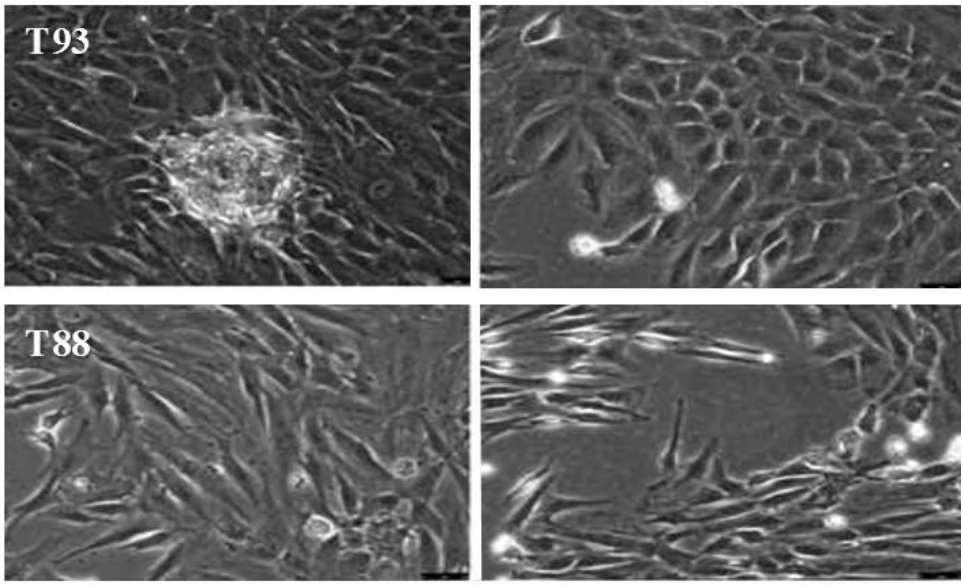


Figure 3.1. T88 and T93 cell cultures. Images in a bright field with x20 magnification (Scale bar 50µm).

To prove the cancer status of these cell cultures, we performed cytogenetic analysis. T93 cells showed a female karyotype with a reciprocal translocation between the q arm of chromosome 20 and the q arm of chromosome 22 in 95% of analysed karyotypes; no numerical aberrations were found (Fig. 3.2A). The high resolution MCB image of the normal chromosome 22 compared to the rearranged chromosome 22 showed that

the 22q13.2qter region of chromosome 22 was translocated to chromosome 20, while the translocated region of chromosome 20 includes the region 20q13 to 20qter (Fig. 3.2B). FISH analysis was performed by using subtelomeric probes for the p (green) and q (red) arms of chromosomes 20 to confirm the presence of this rearrangement (Fig. 3.2C). The complete chromosomal characterisation according to ISCN 2013 (Shaffer *et al.* 2013)was: 46,XX,t(20;22)(q13;q13)(20pter→20q13.2::22q13.2→22qter: 22pter→22q13.::20q13→20qter).

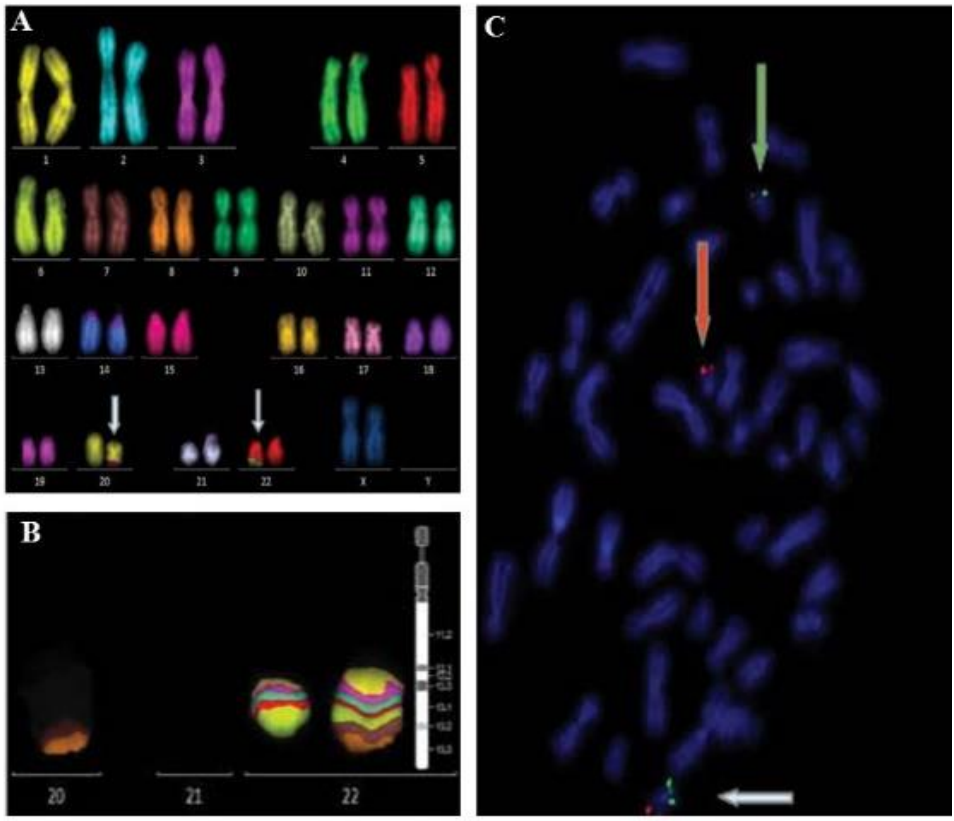


Figure 3.2. Cytogenetic analysis of T93 cell culture. (A) multicolor-FISH (M-FISH) showed a reciprocal translocation between chromosomes 20 and 22; (B) high resolution multicolor chromosome banding (MCB) image of the normal chromosome 22 compared to the rearranged chromosome 22; (C) FISH with subtelomeric probes for the p (green) and q (red) arms of chromosome 20.

Since patient 88 did not show chromosomal alterations, we tested his DNA for microsatellite instability. Thus, we performed MSI assay on DNA

extracted from his tumor tissue and peripheral blood sample, founding MSI-high (MSI-H) status, with instability at mononucleotide markers NR21, BAT40 and NR24 (Fig. 3.3).

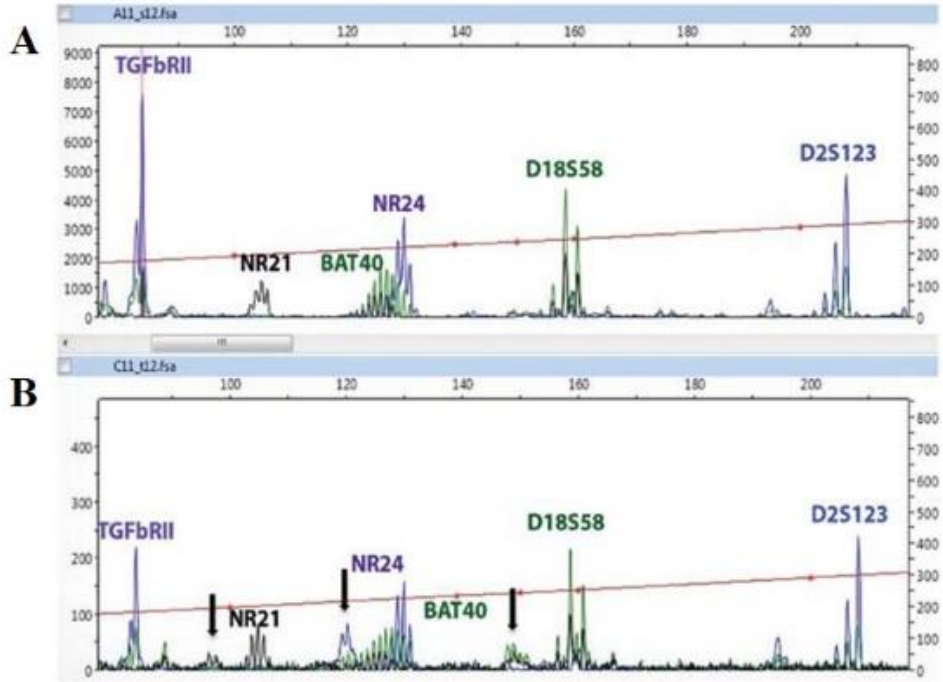


Figure 3.3. Microsatellite instability assay of patient 88. We performed this analysis on DNA extracted from (A) peripheral blood cells and (B) tumor tissue. Black arrows indicate altered microsatellites.

3.2 T88 and T93 cell cultures express EMT-TFs, mesenchymal, epithelial and stemness markers

We analysed the expression of several markers in T88 and T93 cells, by performing RT-PCR, Real-Time RT-PCR, Western Blot and immunofluorescence assay.

COX2 and EMT-TFs (Twist and Snail) messengers were upregulated in both cell cultures compared to tumor tissue (TT) and healthy mucosa (HM) of each patient, when investigated by Real-Time RT-PCR (Fig. 3.4 A and B). For relative quantification, each HM was adopted as control sample.

Western Blot results showed that protein expression of the epithelial marker E-cadherin was down-regulated in patient 88 and upregulated in patient 93 TTs compared to matched HMs, while it gave no hybridization signal in

primary tumor cell cultures, in this Western Blot assay and in our experimental conditions. On the contrary, the mesenchymal protein Vimentin and the EMT-TF Snail were upregulated in TTs, when compared to HMs, and resulted highly expressed in tumor cell cultures. Finally, CD44 was upregulated in both cell cultures and in TT from patient 88 but it gave no signal in TT and HM from patient 93, in our experimental conditions (Fig. 3.4 C and D). Then we performed RT-PCR to analyse the expression of Cytokeratin 18 and 20, normally expressed in epithelial cells. As it is shown in Figure 3.4 E and F, HMs and TTs expressed both Cytokeratins, while T88 and T93 tumor cells expressed only Cytokeratin 18, according to its specific expression in colorectal cancer cells.

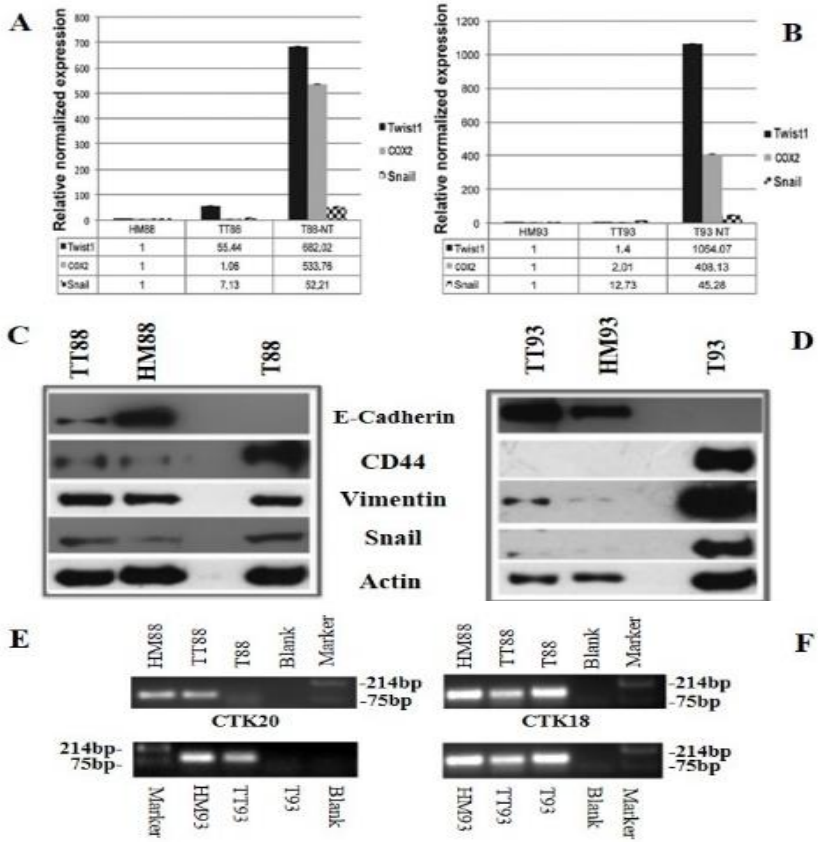
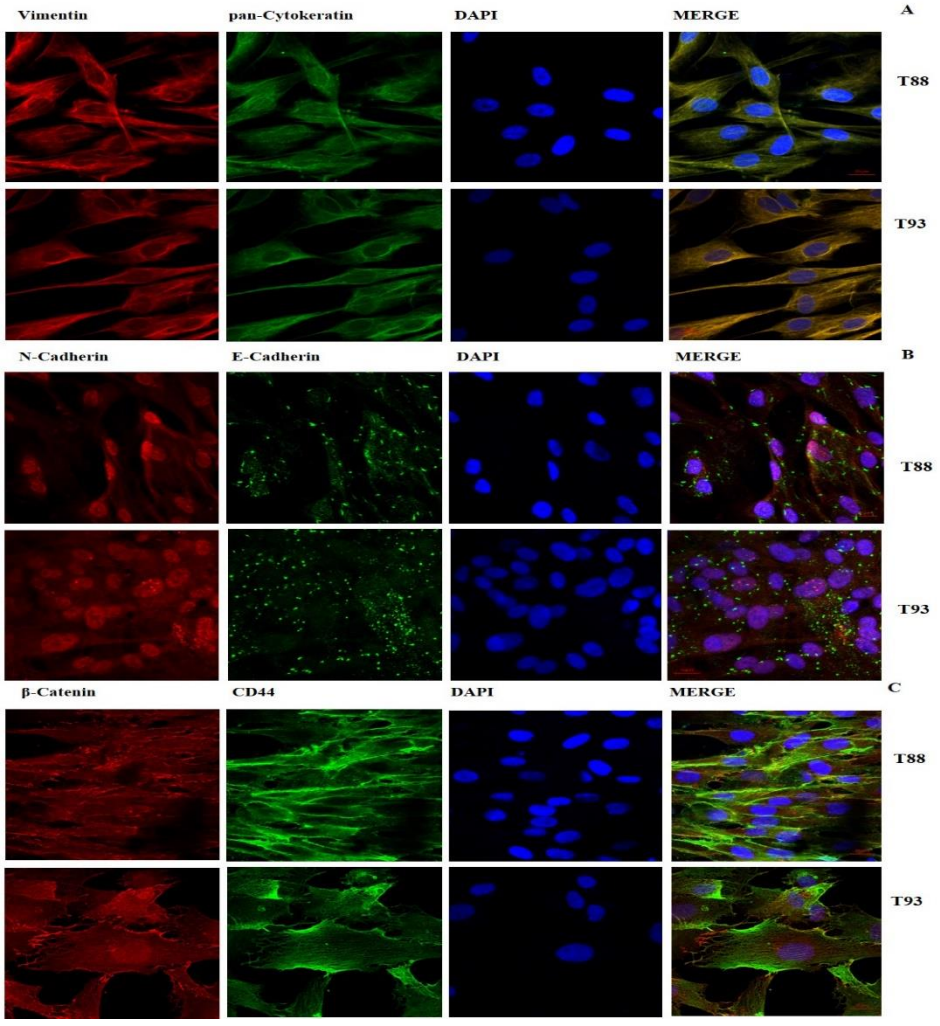


Figure 3.4. Expression analysis of several markers in T88 and T93 cell cultures. (A- B) Real-time RT- PCR analysis of Twist1, Snail and cyclooxygenase-2 (COX2), performed on healthy mucosa (HM), tumor tissue (TT) and untreated tumor cell cultures (T) from patient (A) 88 and (B) 93. (C- D) Western Blot assay of E- cadherin, CD44, Vimentin, Snail and Actin performed with 30μg of proteins extracted from HM, TT and untreated T from patient (C) 88 and (D) 93. (E-F) RT- PCR analysis of cytokeratin 20 (E) and 18 (F) performed on cDNA from HM, TT and untreated T from patient 88 and 93.

To understand if T88 and T93 cell cultures were heterogeneous cell populations or all cells expressed both epithelial and mesenchymal markers, together with high level EMT-TFs and stemness biomarkers, we performed immunofluorescence assay. As shown in Figure 3.5, we evaluated the expression of epithelial and mesenchymal biomarkers, such as Vimentin, pan-Cytokeratin, E- and N-Cadherin, the expression of the EMT-TF Snail, and the expression of CD44 and β-catenin, proteins playing a pivotal role in CRC development and progression (Fig. 3.5 A, B, C and D). We also analysed the expression of several stem cells specific markers such as Nanog, Oct4, Sox2, ALDH1 and LGR5. All the cells of both cultures expressed all these tested markers (Fig. 3.5 D, E and F).



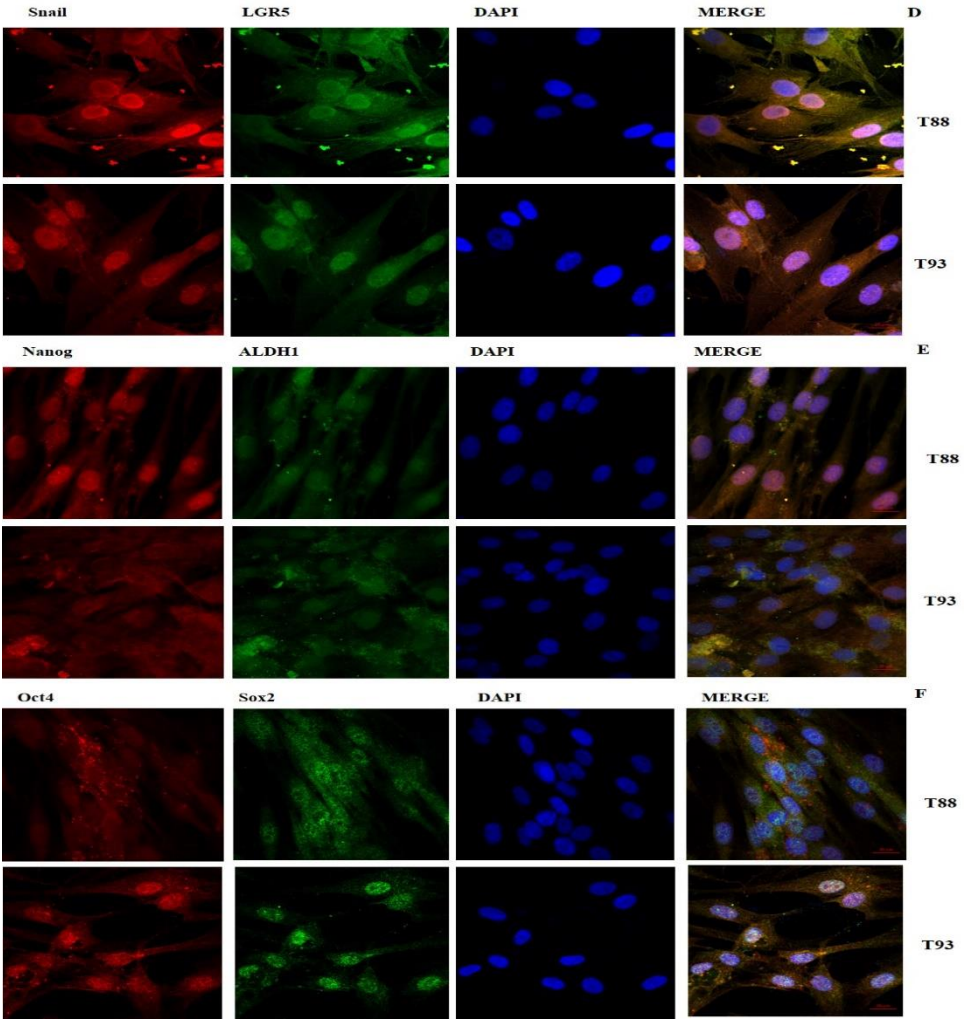


Figure 3.5. T88 and T93 cell cultures expressed epithelial, mesenchymal and stemness markers. Confocal microscopy images of immunofluorescence analysis on T88 and T93 cells performed with (A) Vimentin/ pan-Cytokeratin, (B) N-/ E-Cadherin, (C) β-Catenin/ CD44, (D) Snail/ LGR5, (E) Nanog/ALDH1 and (F) Oct4/ Sox2 antibodies.

As shown in Figure 3.5 A, Vimentin and Cytokeratin co-localized at cytoskeleton level in all analysed cells, while CD44 was predominantly expressed at plasma membrane level (Fig. 3.5 C). As expected, β-Catenin localized into the nucleus and at cytoplasm and plasma membrane level (Fig. 3.5 C). We also observed that T88 and T93 cells expressed both E- and N-Cadherin proteins. E-cadherin, which did not give a signal when analysed

by Western Blot assay, as previously reported (Fig. 3.4C and D), showed a low immunofluorescence hybridization signal, while interestingly, N-cadherin showed an unexpected nuclear localization (Fig. 3.5B). As expected, Snail and LGR5 localized mainly into the nucleus but they were also expressed at the cytoplasm level (Fig. 3.5D). Concerning about the analysed stem cell biomarkers, both T88 and T93 cell cultures expressed ALDH1, Nanog, Oct4 and Sox2; however, Nanog was more expressed into the nuclei of T88 cells, and Oct4 and Sox2 into the nuclei of T93 cells (Fig. 3.5F and G).

3.3 GSK-3β inhibition, by Lithium Chloride, induces Mesenchymal to Epithelial transition in T88 and T93 cell cultures

We studied the role of GSK-3β protein during the EMT of CRC cells, by incubating T88 and T93 cells with Lithium Chloride (LiCl), a specific GSK-3β inhibitor. After 10 days of treatment, cells changed their morphology and differentiated (Fig. 3.6).

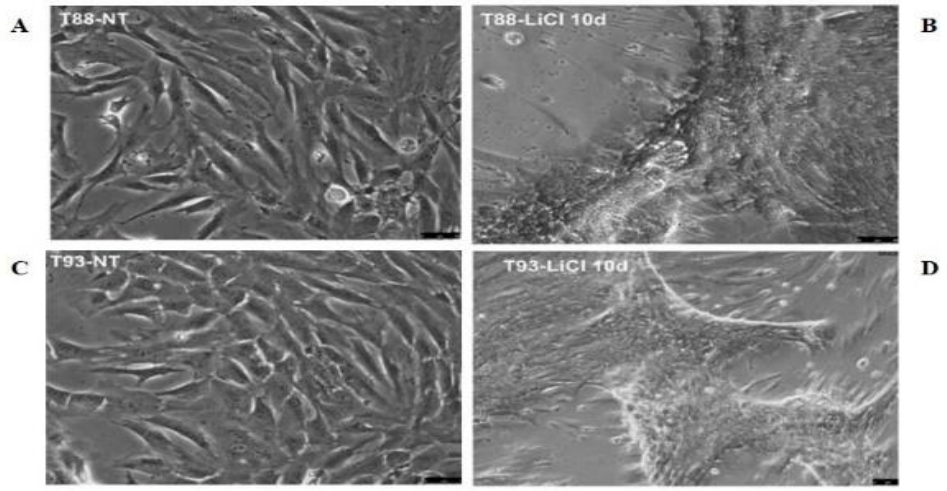
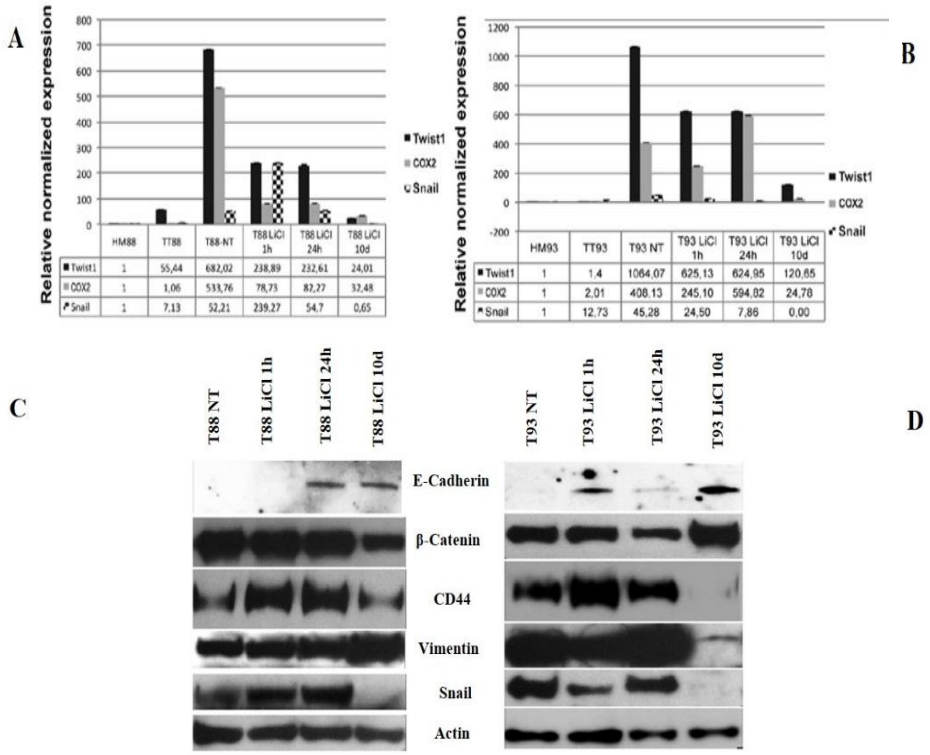


Figure 3.6. LiCl induced differentiation of T88 and T93 cell cultures. Images in bright field of T88 and T93 before treatment (A-C) and after 10 days of incubation with Lithium Chloride (B-D). Magnification x20 (Scale bar 50µm).

After LiCl treatment, we analysed the expression of EMT-TFs and of epithelial and mesenchymal markers, in T88 and T93 cells (Fig.3.7).
We observed that Twist1, Snail, COX2 and CD44 expression was down-regulated (Fig. 3.7A, B, C and D) and cells, originally negative for E-

cadherin, started to express this epithelial marker both at RNA (Fig. 3.7G and H) and protein level (Fig. 3.7C and D), suggesting a mesenchymal-to-epithelial reverting transition process. According to the previously described observation (Fig. 3.1), T88 untreated cells were more mesenchymal-like than the T93 cells, and did not express E-cadherin.
On the other hand, T93 untreated cells that showed more epithelial-like morphology, had low level of E-cadherin transcription (Fig. 3.7G and H).
When analysed by western blot assay, Snail and CD44 gave no signal after 10 days of LiCl incubation in T93 cells, while they strongly decreased in T88 cells (Fig. 3.7C and D). Interestingly, β-catenin and vimentin showed an opposite trend in response to GSK3β inhibition (Fig. 3.7C and D).
After 10 days of LiCl incubation, expression of Vimentin mRNA and protein strongly decreased in T93 cells (Fig. 3.7F and D) while they increased in T88 cells (Fig. 3.7E and C). On the other hand, in the same conditions, β-catenin was up-regulated in T93 cells and down-regulated in T88 cells (Fig. 3.7C and D).



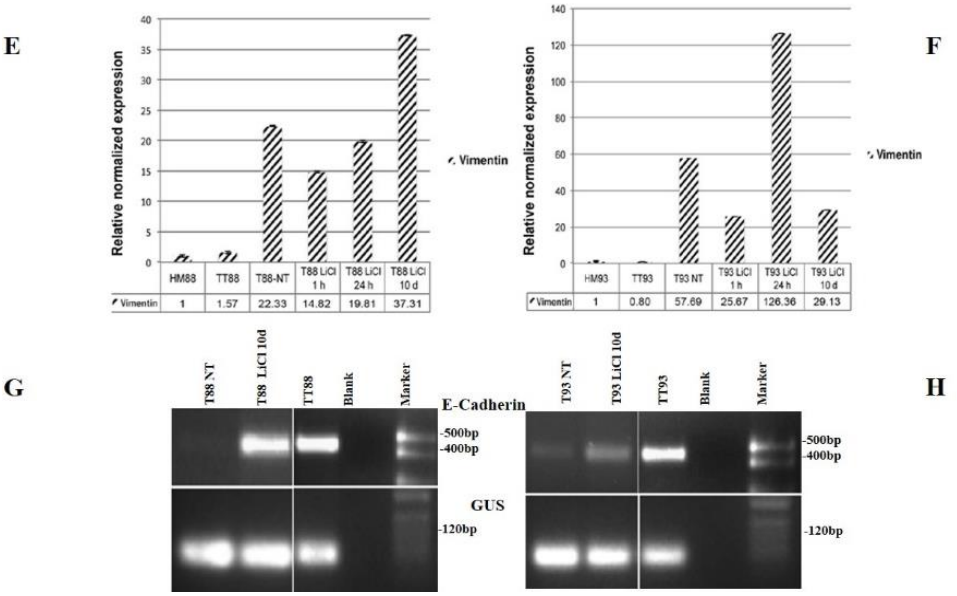
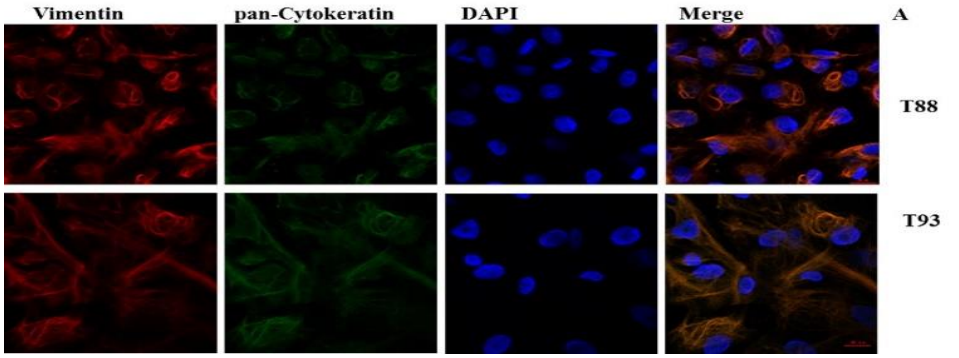
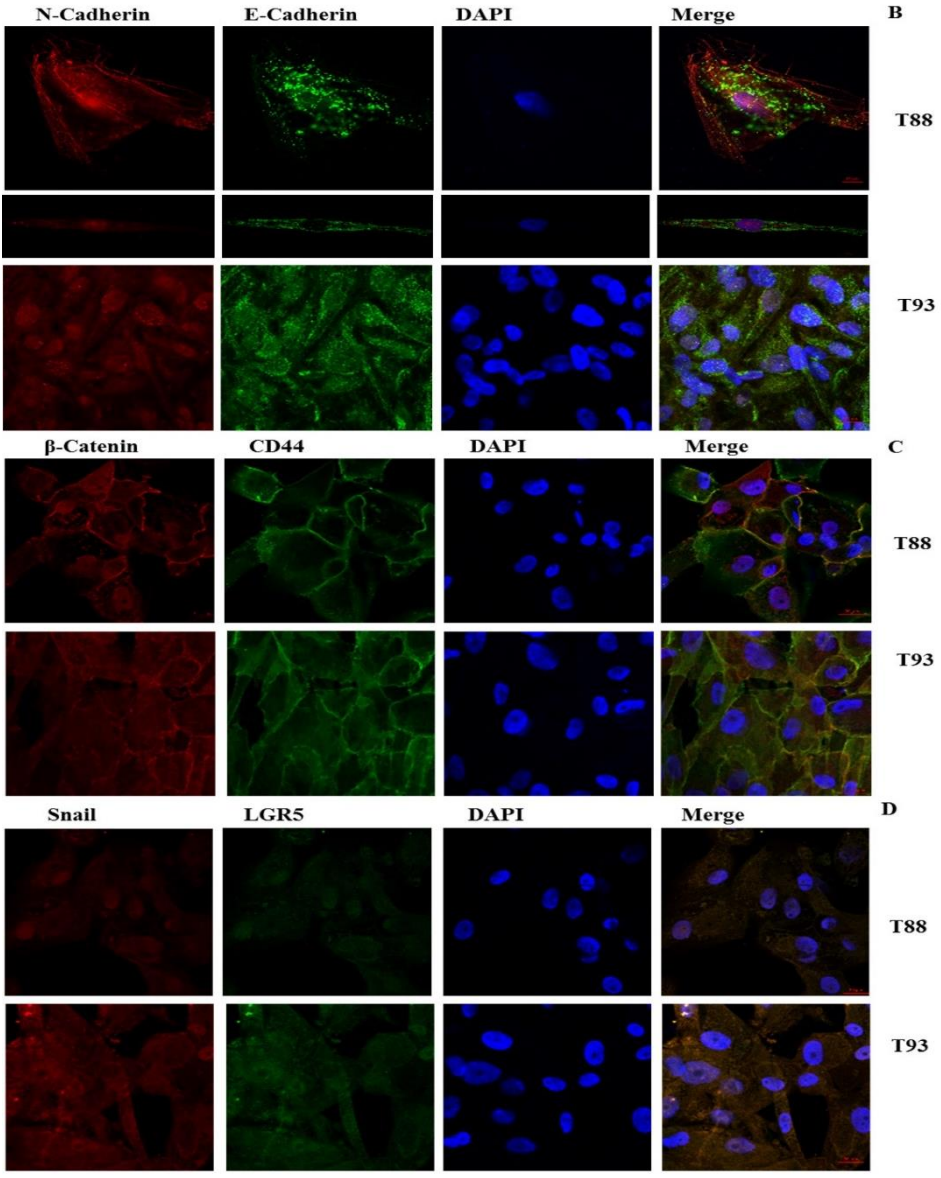


Figure 3.7. Effects of LiCl incubation on T88 and T93 cells. (A-B) Real-time RT- PCR analysis of Twist1, Snail and COX2 performed on HM, TT, untreated cells (T), and tumor cells after 1h, 24h and 10 days of LiCl incubation from patient (A) 88 and (B) 93. (C -D) Western blot assay of E- cadherin, β-Catenin, CD44, Vimentin, Snail and Actin performed on proteins extracted from untreated cells (T) and cells after 1h, 24h and 10 days of LiCl incubation of (C) T88 and (D) T93 cell cultures. (E-F) Real-time RT- PCR analysis of Vimentin performed on HM, TT, untreated T, and tumor cells after 1h, 24h and 10 days of LiCl incubation from patient (E) 88 and (F) 93. (G-H) RT- PCR analysis of E- cadherin performed on cDNA from HM, TT and untreated T from patient (G) 88 and (H) 93.

To confirm these observations and to investigate protein localization after GSK-3β inhibition, we performed immunofluorescence assay of cells incubated with LiCl (Figure 3.8)




N-Cadherin
E-Cadherin
DAPI
Merge
B
T88
T93
β-Catenin
CD44
DAPI
Merge
C
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Snail
LGR5
DAPI
Merge
D
T88
T93

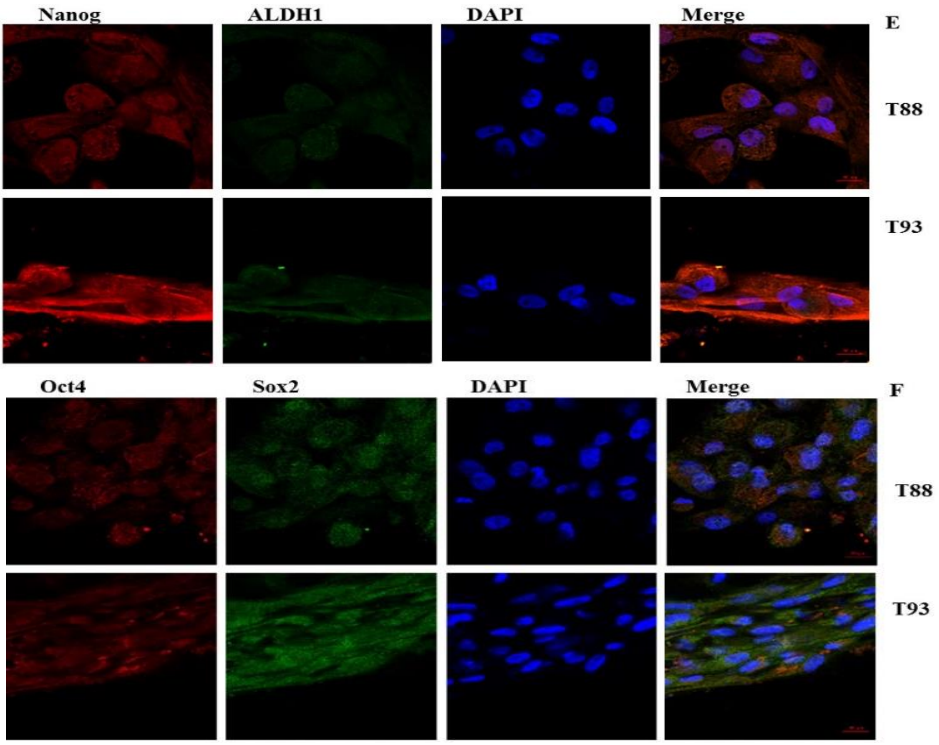


Figure 3.8. Expression of epithelial, mesenchymal and stemness markers in T88 and T93 cell cultures after 10 days of treatment with LiCl. Confocal microscopy images of immunofluorescence analysis on T88 and T93 cells performed with (A) Vimentin/ pan-Cytokeratin, (B) N-/ E-Cadherin, (C) β-Catenin/ CD44, (D) Snail/ LGR5, (E) Nanog/ALDH1 and (F) Oct4/ Sox2 antibodies.

As shown in Figure 3.8A, Vimentin and Cytokeratin co-localized as in untreated cells, however it was manifest a different cytoskeleton organization after treatment with LiCl. E-Cadherin was more expressed than in untreated cells, according to Western Blot and RT-PCR results, while N-Cadherin was down-regulated and its nuclear localization was lower than that observed in untreated cells (Fig. 3.8B). After 10 days of incubation with LiCl, CD44 expression was reduced, according to results obtained by Western Blot assay, and β-catenin was mainly localized at membrane level and less into the nuclei of these cells respect to the untreated cells (Fig. 3.8C). We also observed that the expression at nuclear level of the EMT-TF Snail (Fig. 3.8D), as well as all stemness markers analysed (LGR5, ALDH1, Nanog, Oct4 and Sox2) was down-regulated (Fig. 3.8D, E and F).

3.4 GSK-3β inhibition reduces migration in T88 and T93 cell cultures

Once analysed the effect of GSK-3β inhibition at molecular level, we decided to investigate cell migration capability in response to LiCl treatment. To this aim, we performed a wound healing assay on T88 and T93 cell cultures. After 24h, untreated cells of both cultures began to migrate and the wound was completely healed after 72h (Fig. 3.9A and C). On the contrary, T88 and T93 cells treated with LiCl were completely unable to migrate (Fig. 3.9B and D).

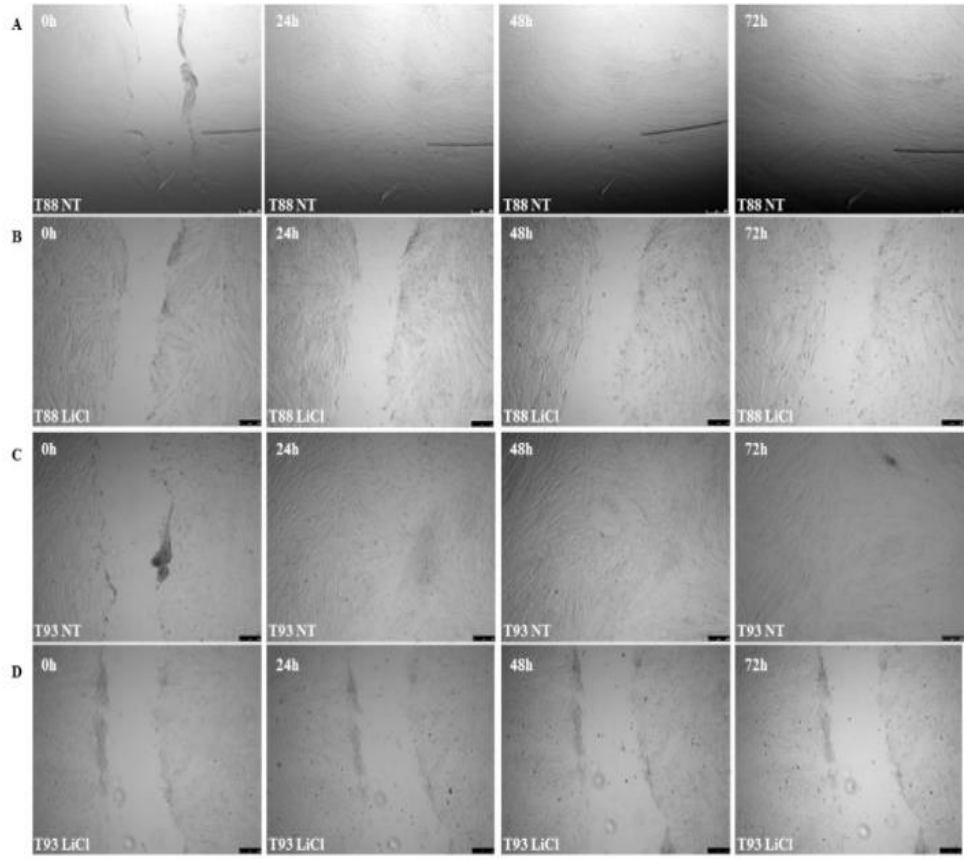


Figure 3.9. Wound healing assay on T88 and T93 cells. The wound healing was performed on (A-C) T88 and T93 untreated cells and on the same cells incubated with 30 mM Lithium Chloride. Image in bright field x4 magnification (Scale bar 250μm).

3.5 Isolation and molecular characterization of colorectal cancer stem-like cells from T88 and T93 cell cultures

T88 and T93 cells underwent EMT and they expressed not only epithelial and mesenchymal markers but also stemness markers, and this is in accordance with literature data reporting that EMT confers to the cells stem cell-like properties (Mani *et al.* 2008).

Primary mesenchymal colon cancer cells were cultured as spheres in low adhesion plates and serum-free medium. It is indeed reported that only stem cells and/or stem cell-like cells are able to survive and grow in these conditions (data not shown).

Cells were also placed in hanging drop culture and incubated under physiological conditions, in low serum concentration medium, until they form 3D spheroids (Fig. 3.10).

As show in Figure 3.10, after 24 hours, T88 and T93 cells aggregated to form spheroids in suspension

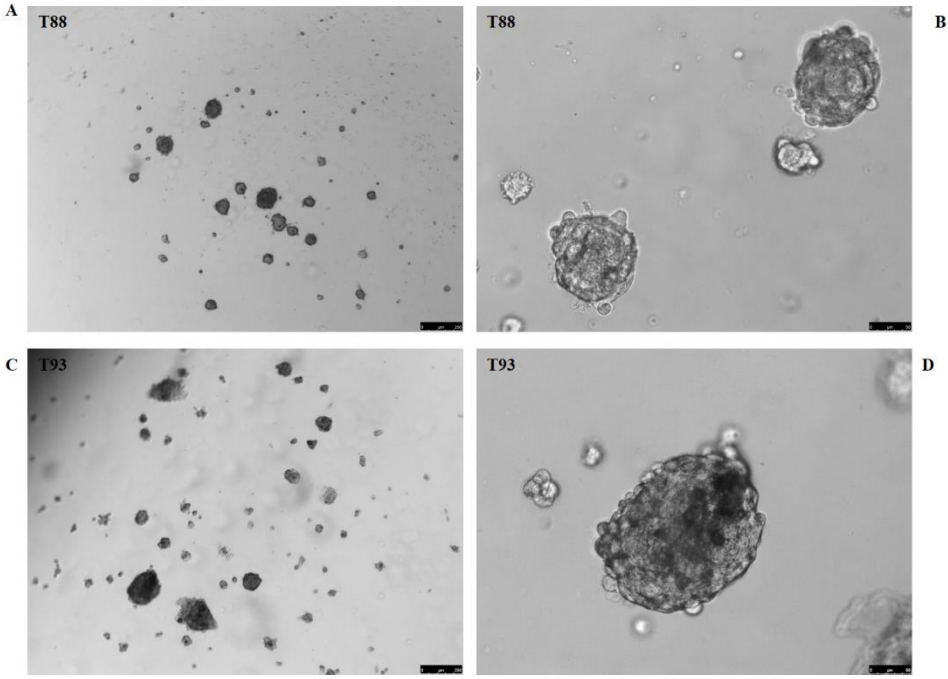
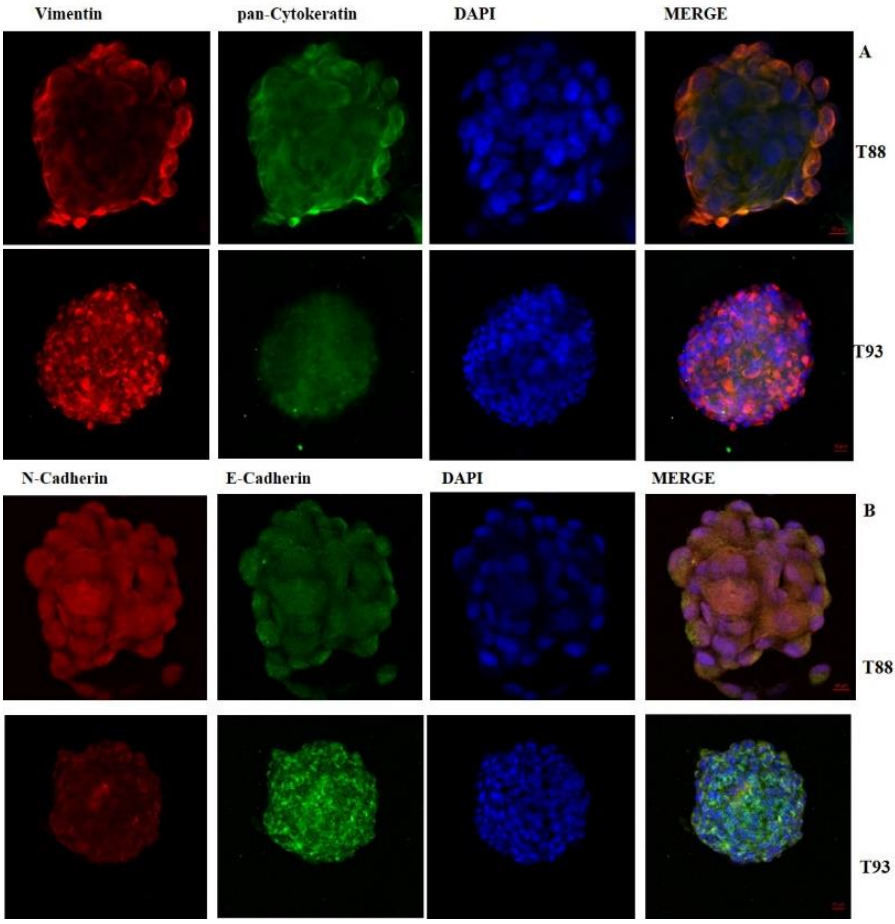
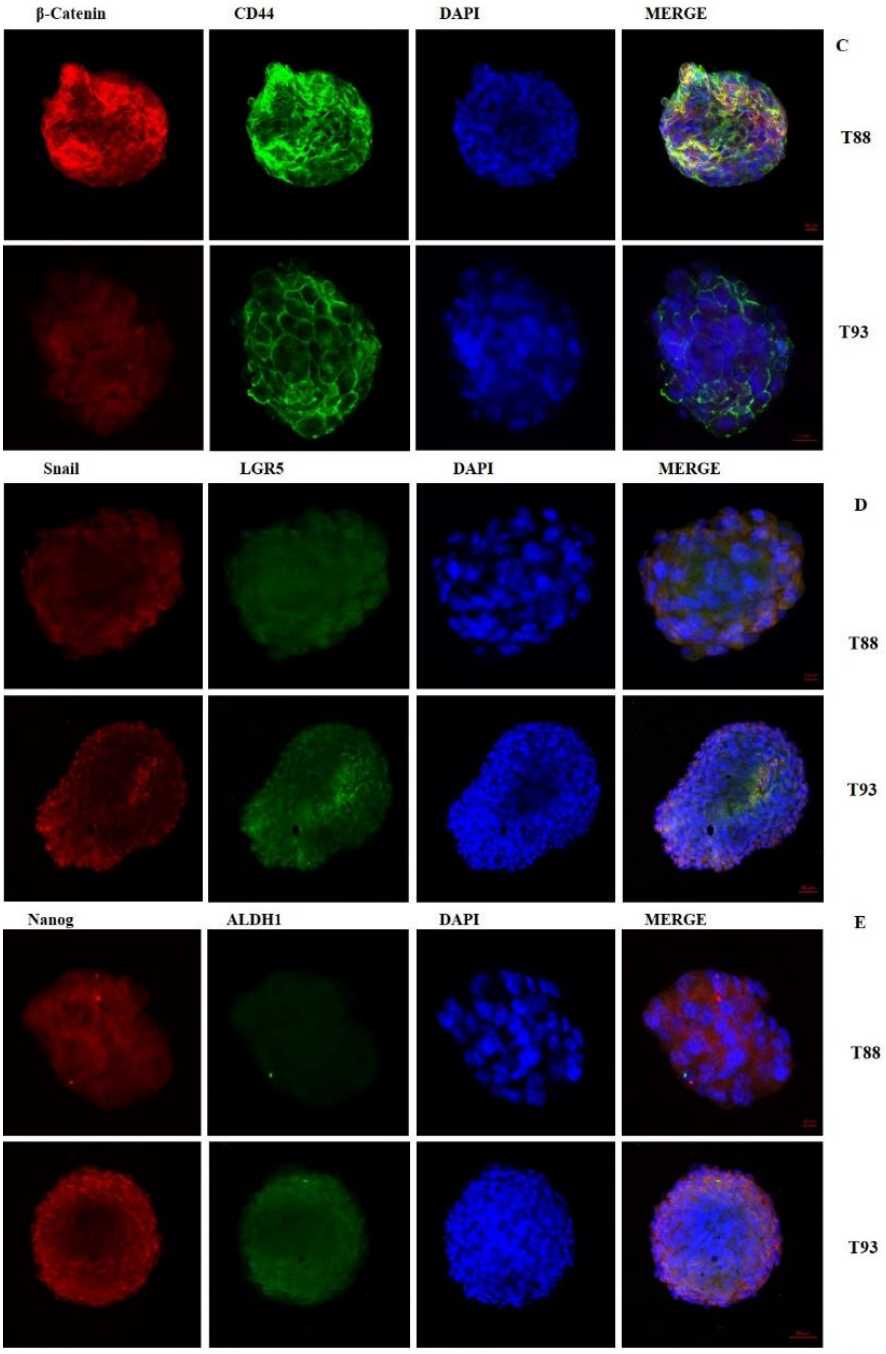


Figure 3.10. T88 and T93 cells were able to grow as spheres. Hanging drop assay performed on (A-B) T88 and (C-D) T93 cell cultures. Images in bright field with (A-C) x4 and (B-D) x20 magnification (Scale bar 250µm and 50µm).

Thus, we performed confocal immunofluorescence assay on cancer spheroids of the same epithelial, mesenchymal and stemness markers previously analysed on adherent cells (Fig. 3.11). First, we analysed cancer spheroids grown in suspension for six passages, passing cells every 10 days (data not shown). Later we analysed spheroids obtained in hanging drop assay, as previously described, and the results were quite similar.




β-Catenin
CD44
DAPI
MERGE
C
T88
T93
Snail
LGR5
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D
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Nanog
ALDH1
DAPI
MERGE
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T88
T93

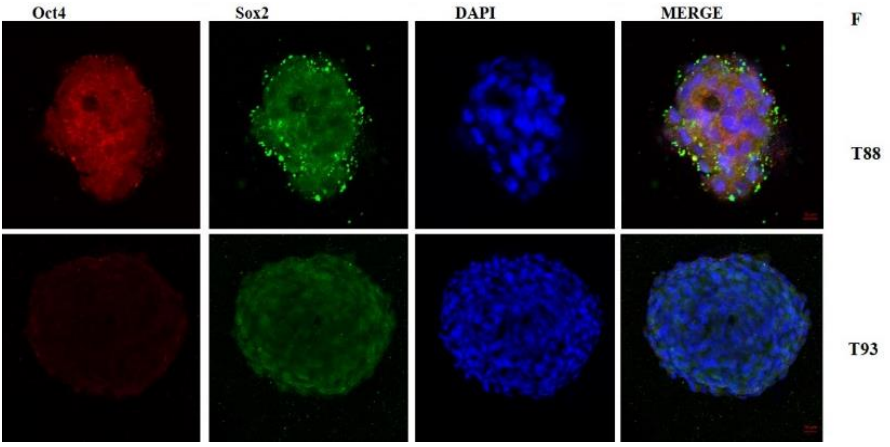


Figure 3.11. T88 and T93 cancer spheroids expressed epithelial, mesenchymal and stemness markers. Confocal microscopy images of immunofluorescence analysis on T88 and T93 cells performed with (A) Vimentin/ pan-Cytokeratin, (B) N-/ E-Cadherin, (C) β-Catenin/ CD44, (D) Snail/ LGR5, (E) Nanog/ALDH1 and (F) Oct4/ Sox2 and antibodies.

As shown in Figure 3.11, T88 and T93 cancer spheroids stored expression of the epithelial Cytokeratins and E-Cadherin, and mesenchymal Vimentin and N-Cadherin, markers; however, N-Cadherin did not show a high nuclear localization, as in T88 and T93 cells growth in adhesion (Fig. 3.11A and B). CD44 was mainly expressed at membrane level, like β-Catenin (Fig. 3.11C). Furthermore, cells in cancer spheroids showed a lower nuclear expression of the EMT-TF Snail, respect to original T88 and T93 cell cultures (Fig. 3.11D); the stemness markers Nanog, Oct4, Sox2, LGR5 and ALDH1 were expressed and they showed a turnover between nucleus and cytoplasm. Nevertheless, the expression of Oct4 was very low in T93 cancer spheroids, while T88 spheroids showed very low level of ALDH1 expression (Fig. 3.11E and F).

3.6 Inhibition of GSK-3β by LiCl affects stem cell-like properties of T88 and T93 cancer spheroids and alters cell plasticity

Since inhibition of GSK-3β reverted EMT program in T88 and T93 adherent cell cultures, we also evaluated the effect of LiCl on cells ability to grow as cancer spheres in serum-free medium.

Thus, we performed hanging drop assay adding this drug to the cell culture medium. As showed in Figure 3.12, after 72 hours of LiCl incubation, T88 cells formed 3D structures that lost the spherical shape, while T93 cells completely lost their ability of sphere formation generating only cellular aggregates.

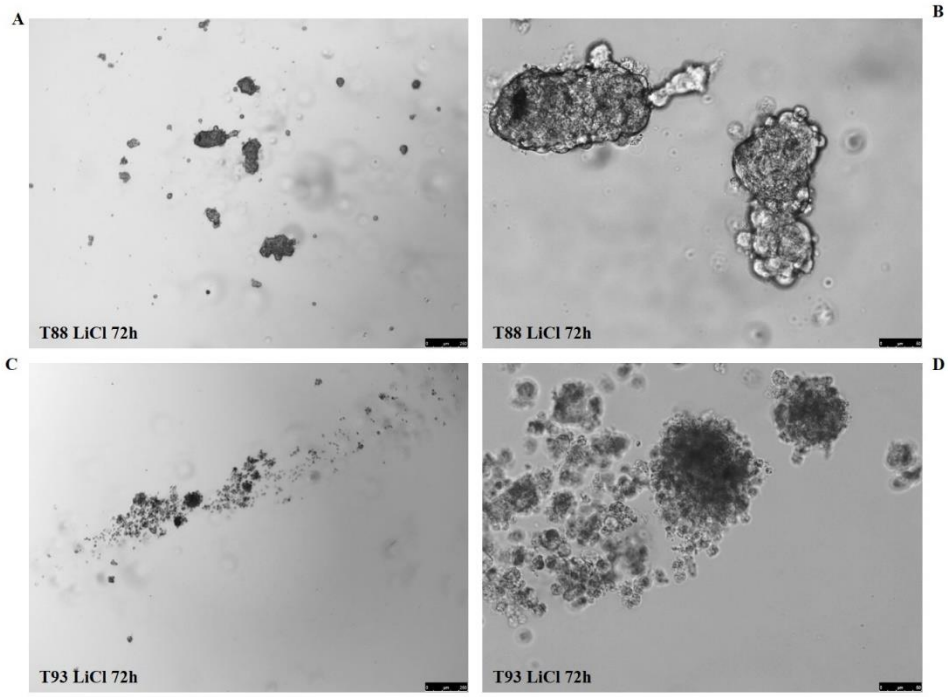
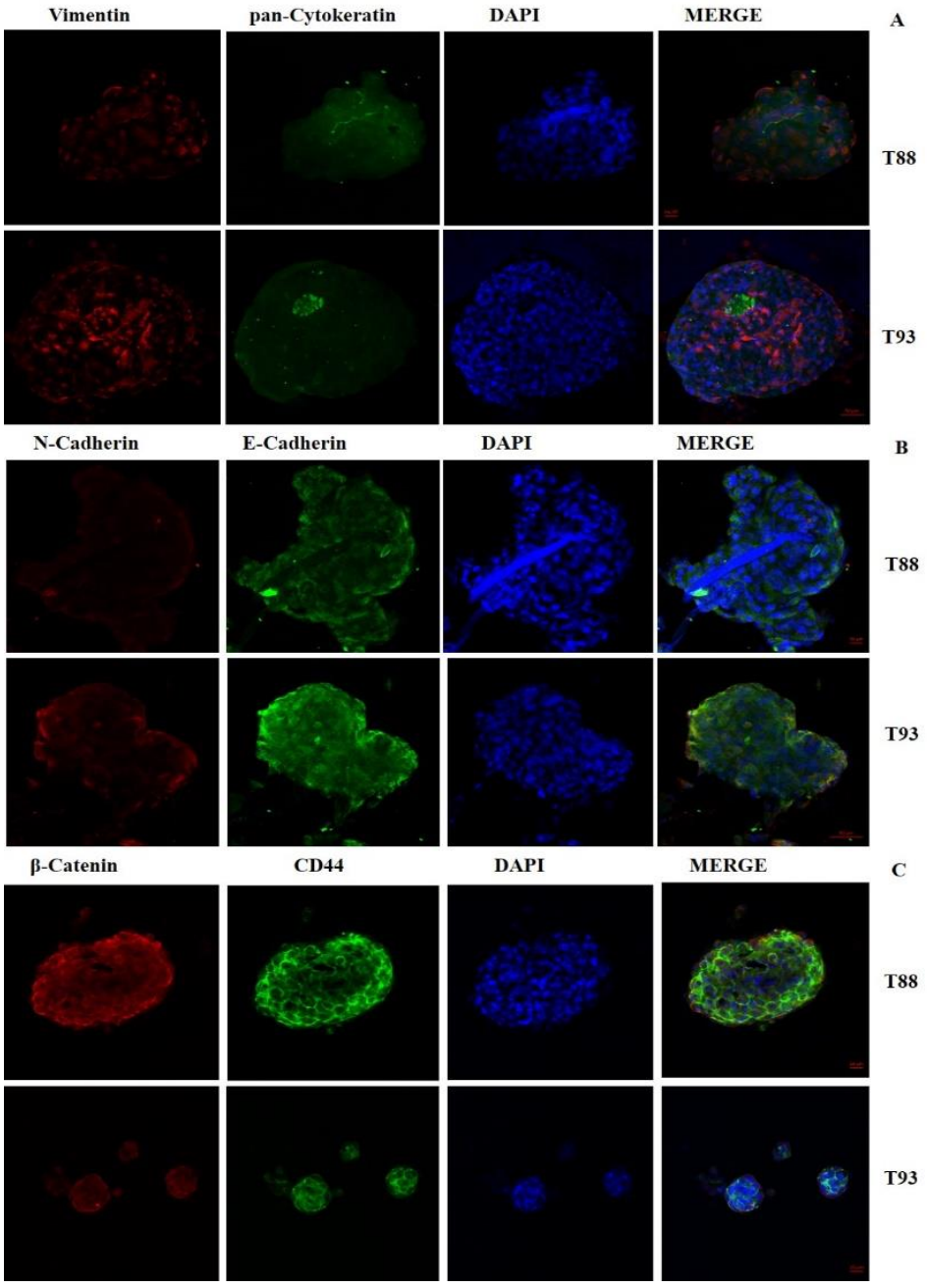


Figure 3.12. T88 and T93 cells ability to form spheroids was affected by LiCl. Hanging drop assay performed on (A-B) T88 and (C-D) T93 cell cultures treated with 30mM LiCl for 72 hours. Images in bright field with (A-C) x4 and (B-D) x20 magnification (Scale bar 250µm and 50µm).

To clarify the molecular basis of the GSK-3β inhibition on the ability of T88 and T93 cells to grow as spheres, we performed immunofluorescence assay of epithelial, mesenchymal and stemness markers in cancer spheroids obtained in hanging drop assay from cells treated with LiCl (Fig. 3.13).


Vimentin
pan-Cytokeratin
DAPI
MERGE
A
T88
T93
N-Cadherin
E-Cadherin
DAPI
MERGE
B
T88
T93
β-Catenin
CD44
DAPI
MERGE
C
T88
T93

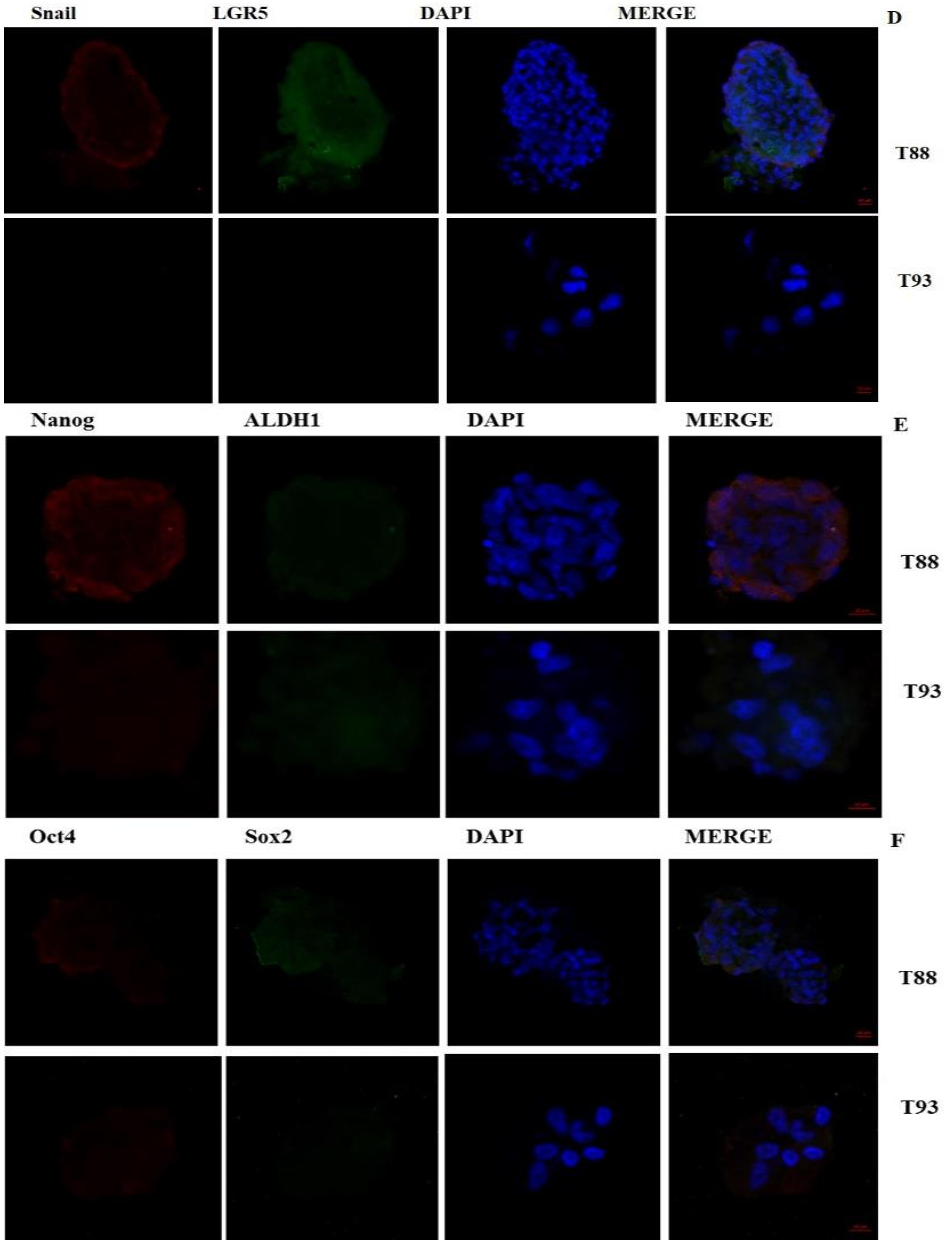


Figure 3.13. LiCl effect on the expression of epithelial, mesenchymal and stemness markers in spheroids derived from T88 and T93 cell cultures. Confocal microscopy images of immunofluorescence analysis of T88 and T93 cells performed with (A) Vimentin/ pan-Cytokeratin, (B) N-/ E-Cadherin, (C) β-Catenin/ CD44, (D) Snail/ LGR5, (E) Nanog/ALDH1, and (F) Oct4/ Sox2 antibodies.

As shown in Figure 3.13, inhibition of GSK-3β by LiCl induced a downregulation of the expression of Vimentin and N-Cadherin mesenchymal markers (Fig. 3.13A and B) and of the EMT-TF Snail (Fig. 3.13D). T88 and T93 cancer spheroids treated with LiCl stored the expression of E-Cadherin (Fig. 3.13B), however the hybridization signal of pan-Cytokeratin antibody was very low (Fig. 3.13A). Expression of CD44 and β-Catenin proteins seemed to be quite similar in T88 and T93 spheroids after incubation with LiCl (Fig. 3.13C); while the expression of specific stem cell markers, such as Nanog, Oct4, Sox2, LGR5, and ALDH1 strongly decreased in response to GSK-3β inhibition and this effect was predominant in T93 cancer spheroids (Fig. 3.13D, E and F).

To further investigate cell plasticity of T88 and T93 cell cultures, we disaggregated spheroids obtained by hanging drop assay, grown with or without LiCl; and cultivated them in adhesion and in the same conditions used for the original cell cultures (DMEM-F12 1:1 medium containing 10% FBS) (Fig. 3.14). These cells were again able to grow in adhesion; however, the number of cells attached to the plate was significantly lower when spheroids were treated with LiCl (Fig. 3.14B and D).

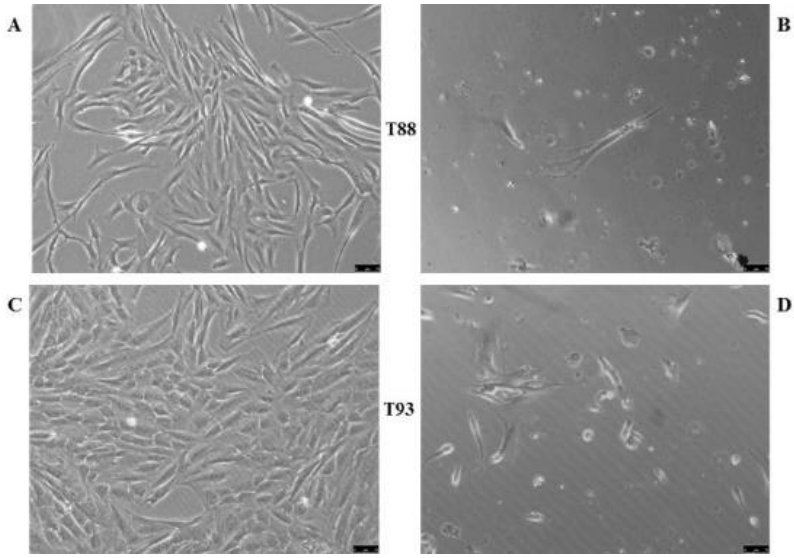


Figure 3.14. T88 and T93 cell plasticity was affected by LiCl. T88 (A-B) and T93 (C-D) cells derived from disaggregation of cancer spheroids, which were treated with 30mM LiCl for 72 hours (B-D) or untreated (A-C). Images in bright field with x10 magnification (Scale bar 75μm).

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Chapter 4

Discussion and conclusion

4.1 Discussion

We have performed an experimental system of primary mesenchymal colorectal cancer cell cultures, according to the hypothesis that EMT could be responsible for acquisition of cancer cell resistance to therapy. We set up a protocol to isolate primary colorectal cancer cell cultures from tumor tissues of patients affected by CRC. For molecular investigations, we chose two of these cultures because of their different morphologies; indeed T88 looked more mesenchymal-like than T93 cells, which showed an epithelial-like phenotype. First, we validated the cancer status of our cell cultures by performing MSI assay and karyotype analysis. We found that T93 cells had a CIN phenotype showing, in 95 % of the analysed karyotype, a translocation between the q arm of chromosome 20 and the q arm of chromosome 22, while T88 cells were characterized by a MSI-H phenotype with instability at mononucleotide markers NR21, NR24 and BAT40.

By using molecular biology techniques, we demonstrated that T88 and T93 cells expressed together epithelial (Cytokeratins and E-Cadherin) and mesenchymal (Vimentin and N-Cadherin) markers, and high levels of EMT-TFs Twist and Snail. Since T88 and T93 cell cultures were isolated from adenocarcinomas, which are epithelial tumors, the expression of mesenchymal markers and EMT-TFs suggested that these cells were epithelial cells undergone EMT.

In addition we demonstrated that T88 and T93 cells expressed stemness markers, such as Nanog, Oct4, Sox2, ALDH1 and LGR5. These results were in accordance with literature data indicating that EMT program induces the expression of stem cell specific genes, and might represent a source of cancer stem-like cells (Reya *et al.* 2001).

EMT and its reverting process MET, are physiological process occurring during embryonic development and tissue remodelling, furthermore, it has been suggested that EMT is involved in cancer progression and metastasis in several tumours, including CRCs (Loboda *et al.* 2011).

Since the multifunctional kinase GSK-3β plays an important role in cross talk between different pathways involved in CRCs development, we decided to study its role during EMT of CRC cells. To realize this aim, we incubated T88 and T93 cells with Lithium Chloride (LiCl). LiCl is the most studied among GSK-3β inhibitors and exerts its action by competing with Mg2+, a co-factor of this enzyme, and by activating Ser9 inhibitory phosphorylation of GSK-3β.

After 10 days of incubation with LiCl, the expression of the EMT-TFs, Twist1 and Snail, and mesenchymal markers was down-regulated and the

transcription of E-Cadherin was increased, suggesting that the inhibition of GSK-3β induced a reversion of EMT program, and cells underwent MET. Interestingly COX2 that was overexpressed in both cell cultures, was also highly down-regulated after treatment with LiCl. According to these results, literature data indicate a strong association between COX2 expression and cancer progression and metastasis (Elzagheid *et al.* 2013).
Furthermore, we performed wound healing assay and demonstrated that LiCl affected migration of T88 and T93 cells, which is a crucial feature in metastases development.
Cancer stem cells and cancer stem-like cells play a key role in cancer progression and metastases, and they are probably responsible for both relapse and resistance to therapy (Gangemi *et al.* 2009, Fanali *et al.* 2014).
It has been suggested that cancer cells, which are more undifferentiated and display stem cell like properties, are able to grow as spheres in suspension in a specific culture medium. Three-dimensional (3D) *in vitro* models have been used in cancer research as an intermediate model between *in vitro* cancer cell line cultures and *in vivo* tumor. Spherical cancer models represent major 3D *in vitro* models that have been described over the past 4 decades. These models have gained popularity in cancer stem cell research using tumorospheres. They contribute to chemoresistance, radioresistance, tumorigenicity, invasion and migration studies. Several methods to isolate CSCs have been well described in literature, one of these is hanging drop assay (Weiswald *et al.* 2015). We performed hanging drop assay of T88 and T93 cell cultures showing that these cells were able to aggregate and form spheroids in suspension. We characterized these cancer spheroids at molecular level, by performing immunofluorescence analysis, and found that they stored the expression of both mesenchymal and epithelial markers, however the EMT-TF Snail showed a lower nuclear localization respect to the original T88 and T93 cell cultures grown in adhesion.
We analysed the expression of a panel of stem cell biomarkers (Nanog, Oct4, Sox2, ALDH1 and LGR5), and demonstrated that T88 and T93 cancer spheroids expressed all these markers, with little differences between these two cultures.
Furthermore, we investigated the effect of GSK-3β inhibition on cancer spheroids formation and demonstrated that LiCl induced T88 and T93 cells to lose the ability to form well-defined spheroids in suspension, especially T93 cells. According to this observation, after 72 hours of treatment with LiCl, the expression of stemness and mesenchymal markers was strongly reduced and this effect was predominant in T93 cancer spheroids.

Thus, we concluded that epithelial-mesenchymal primary colon cancer cells formed spherical aggregates in hanging drop assays indicating a dedifferentiated state that correlates with expression of undifferentiation/differentiation markers. LiCl incubation altered dynamic of spheres formation in hanging drop assays indicating differentiated state that again correlates with expression of undifferentiation/differentiation markers, specifically inducing downregulation of all mesenchymal and stem cell biomarker analysed.

Since cellular plasticity is a crucial feature of cancer cells in metastasis development, we decided to test the ability of T88 and T93 cell cultures to switch from one phenotype to another. To this aim, we cultivated the cells derived from disaggregation of cancer spheroids grown for 72 hours with or without LiCl. These cells were again able to grow in adhesion like the original cultures. However, this ability was significantly affected in cells derived from spheroids treated with LiCl.

As stated in the Introduction, GSK-3β can act as pro-apoptotic factor negatively regulating Wnt signaling, by controlling the degradation of β-catenin. On the other hand, it positively regulates NF−κB pathway by mediating the degradation of IκB, a central inhibitor of NF−κB, so inducing an anti-apoptotic response (Beurel and Jope 2006). In colon and pancreatic cancer cells, GSK-3β activates a proliferative signal by activation of NF−κB, and inactivation of GSK-3β inhibits NF−κB activity (Shakoori *et al.* 2005, Shakoori *et al.* 2007). Interestingly, Snail, CD44, G3BP2, and YAP1 are targets of Wnt5A, a gene involved in invasion and metastasis of many cancers that is regulated by NF−κB signaling pathway (Du and Geller 2010, Katoh and Katoh 2009). E- cadherin and its transcriptional repressor Snail orchestrate a functional cross regulation between Wnt and NF−κB pathways during EMT. Expression of Snail promotes E-cadherin downregulation destroying epithelial organization and inducing mesenchymal phenotype of epithelial cells, that takes place together with upregulation of mesenchymal genes expression. E- cadherin and other cell adhesion components directly bind both β-catenin and NF−κB, sequestering them into adherens junctions and therefore preventing the transcription of target genes (Conacci-Sorrell *et al.* 2002, Solanas *et al.* 2008). As recently described (Benoit *et al.* 2014), we suggest that GSK-3β inhibition could represent a good strategy targeting the cross regulation between these two pathways and may be a promising direction for future cancer therapy that needs to be better elucidated.

Specifically, LiCl, a drug already used in clinical practices for treatment of bipolar disorders, could represent an alternative therapy in colon cancer care and/or able to sensitize cancer cells to chemo-radio-therapy, acting through downregulation of EMT-TFs and specific stem cell biomarkers.
Cell culture models obviously do not mimic the complex interaction that are found in the intestinal mucosa and a functional change in the cell cultures cannot tell us whether there will be the same effect *in vitro*. Nevertheless, cell cultures are used in an attempt to make complex systems simpler by isolating certain functions and investigate them in detail. From this point of view, we speculated that our cell culture could represent an interesting model to further investigate the molecular biology of mesenchymal CRC cells, clinical relevance of EMT in human CRC and the molecular basis of pharmacological resistance and metastasis.

4.2 Conclusion

We set up a protocol to isolate primary mesenchymal colorectal cancer cell cultures deriving from an EMT process of epithelial cancer cells. To our knowledge, there are no data in literature about primary colorectal cancer cells isolated from CRC patients, expressing both mesenchymal and epithelial markers and high level of EMT-TFs. We isolated primary cell cultures from different patients affected by CRCs; specifically we chose to analyze two of these cell cultures, named T88 and T93. T88 cells, that looked more mesenchymal-like, were characterized by a MSI-H phenotype, while T93 cells, that showed a more epithelial morphology, had a CIN phenotype. Molecular characterization of these cell cultures showed that they expressed both mesenchymal and epithelial markers, and genes specific of stem cells. This is in accordance with the features of EMT program that induces not only a mesenchymal phenotype but also stem cell like properties (Mani *et al.* 2008).
We decided to study the effect of the inhibition of GSK-3β, by treating our cell cultures with Lithium Chloride (LiCl), since this kinase plays a crucial role in the cross talk between several molecular pathways involved in CRCs development. We demonstrated that LiCl was able to induce mesenchymal to epithelial reverting transition (MET) in T88 and T93 cells, and down-regulated the expression of stem cell markers.
EMT confers to the epithelial cells migratory and invasive properties, which are crucial features in metastatic process. T88 and T93 cancer cells showed high migration ability that, however, was significantly affected by LiCl.

In accordance with the expression of stem cell genes, T88 and T93 cell cultures were able to grow in suspension as spheres in a conditioned medium without serum, but they lost the ability to form well-defined spheroids in presence of LiCl. These cancer spheres expressed epithelial, mesenchymal and stem cell markers; however, treatment with LiCl induced a strong downregulation of the EMT-TF Snail, mesenchymal and stemness genes.

Our study suggested that EMT could represent a common mechanism belonging to different CRC types, at molecular level. Indeed the inhibition of GSK-3β induced MET in both cell cultures; despite they were characterized by different pathways of genomic instability. We speculated that our cell cultures could be an interesting *in vitro* model to investigate the clinical relevance of EMT and the molecular basis of pharmacological resistance and metastasis, in human CRCs (Costabile *et al.* 2015).

Cellular plasticity plays an important role in cancer development and progression; cancer cells can switch from one phenotype to another and acquire resistance to therapy (Vicente-Dueñas *et al.* 2008, Doherty *et al.* 2016).

We proposed that LiCl might be able to sensitize cancer cells to therapy, by downregulating EMT-TFs and stemness genes, and affecting cell plasticity and migration. Thus, GSK-3β and LiCl could be an eligible target and a potential drug to develop therapy for CRCs care, respectively.

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